Heat shock proteins (Hsp) are ubiquitous intracellular proteins that can be released in various forms of cellular stress. Some Hsp, such as Hsp60, have been shown to stimulate directly T cell–mediated immune responses in vitro. Here, it is demonstrated that Hsp60 is released from the kidneys and excreted into the urine of mice with nephrotoxic nephritis (NTN), a model of rapidly progressive glomerulonephritis. For examining the functional relevance of Hsp60 release, this protein was injected into mice with subnephritogenic NTN, in which only transient proteinuria and minimal organ damage occur that do not progress to terminal kidney failure. Injection of Hsp60 strikingly aggravated disease, as evidenced by global glomerular necrosis, tubulointerstitial damage, and complete anuria after 10 to 12 d. This effect was mediated neither by endotoxin contaminations of Hsp60 nor by autologous antibodies. It was strictly T cell dependent but not associated with a systemic Th1/Th2 shift. Thus, Hsp60 is an endogenous mediator stimulating immune effector mechanisms that contribute to the progression of NTN. These findings demonstrate in vivo that Hsp60 fulfills criteria of immunologic danger signals and suggest that such signals may be involved in immune-mediated kidney disease.

The induction of immunity usually requires antigen and non–antigen-specific stimulatory signals (1), such as adjuvants or pathogen-associated molecular patterns (PAMP), e.g., bacterial LPS or hypomethylated DNA (1,2). The danger hypothesis (3) postulates endogenous immune stimulators that are released during infections by intrinsic cells of the body and that alert the immune system on the need to induce immunogenic responses. This may be important in infections with pathogens that do not express PAMP (3). Danger signals are suspected to play important roles also in transplant and tumor rejection and in autoimmunity (3). Recently, the danger hypothesis has gained substantial support by the molecular identification of danger signals: Purified uric acid was shown to activate antigen-presenting cells (APC) and displayed adjuvant activity in vaccination experiments (4). In that study, a high molecular weight danger signal was also detected, suggesting the existence of additional danger signals.

Heat shock proteins (Hsp) have long been suspected to act as danger signals (3,5–7). Initially described as a family of intracellular proteins essential for various vital cell functions (8,9), Hsp have later been demonstrated to stimulate potent immune responses. Although some biologic effects observed are now suspected to have been due to endotoxin contaminations of recombinant Hsp preparations, there is evidence that Hsp can bind to PAMP receptors of APC and stimulate T cell activation (5,6,9–13). Hsp are attractive as danger signals because they are evolutionarily highly conserved, constitutively present in virtually all body cells, and upregulated and released in response to cellular stress. In infections, this release could support induction of immunity. Hsp release, however, is not specific to infections but also occurs in response to physical stimuli, such as heat or irradiation, and in noninfectious inflammatory diseases (9). Under these conditions, Hsp release from injured tissue may aggravate autoimmune responses. In support of this hypothesis, injection of Hsp70 was shown recently to convert T cell tolerance into immune-mediated diabetes (7). In vitro, Hsp60 has been shown to be particularly efficient at T cell stimulation, presumably through a PAMP receptor (14–16), and thus has been proposed to represent a danger signal (5,6,10,11).

The effect of danger signals on models of kidney disease in vivo is unclear. Therefore, we studied the role of Hsp60 in nephrotoxic nephritis (NTN), a murine model of necrotic glomerulonephritis (GN) induced by injection of sheep antiserum against glomerular antigens nephrotoxic sheep serum (NSS).
Sheep Ig is deposited in the kidney and is presented by intrinsic renal cells to CD4 T cells, which are essential disease mediators (17,18). Kidney damage is characterized by glomerular leukocyte infiltration, mesangial damage, and crescent formation. Depending on the extent of the initial damage, either glomerular repair ensues or glomerular necrosis, tubulointerstitial damage, and progression to terminal kidney failure. Kidney disease models related to NTN were shown to be aggravated by PAMP such as LPS (19) or CpG-DNA (20). As Hsp60 is also thought to stimulate PAMP receptors, we investigated its role in NTN.

Materials and Methods
Mice, Reagents, NTN Model, and Overload Proteinuria Model
Female 6- to 8-wk-old C57/BL6 mice in specific pathogen-free condition were obtained from Bomholtgard (Ny, Denmark). All experiments were performed in accordance with local animal ethics procedures. Reagents were from Sigma-Aldrich (Steinheim, Germany), if not specified otherwise. Recombinant human Hsp60 with low endotoxin content was from Stressgen Biomol (Hamburg, Germany) or Lake Diagnostics Aps (Aarhus, Denmark). Only preparations that contained <1 EU endotoxin/mg Hsp60 as determined by limulus amebocyte lysate assay were used (21). NTN was induced by injection of NSS as described (22–24). A dose–response curve identified the amount of NSS required for induction of proteinuria and terminal kidney failure (Table 1). Injection of 4 μl/g body wt NSS led to proteinuria in most of the recipients but never to terminal kidney failure; 6 μl led to terminal kidney failure in all recipients (23–25) (data not shown). In the present study, 4 μl/g body wt NSS was used for determining aggravation of proteinuria, and 5 μl was used for aggravation of histologic damage. In control mice, irrelevant sheep Ig was injected. T cell depletion was performed by injection of anti-CD4 mAb (clone T24) purified from culture supernatant. Overload proteinuria was induced by daily intravenous injection of increasing amounts of BSA (2, 4, 6, 8, and 10 mg) as described (26). All animal studies were approved by Institutional and Government Review Boards.

Determination of Hsp60 Concentration by Western Blot
Tissue incubation supernatants, urine samples, and recombinant Hsp60 were separated by electrophoresis on a polyacrylamide gel and blotted onto nitrocellulose membrane. Hsp60 was revealed using an anti-Hsp60 monoclonal antibody (clone LK-1, isotype IgG1; Stressgen Biomol, Hamburg, Germany). A band at 60 kD was analyzed by densitometry on a Gel Doc system (Bio Rad, Munich, Germany) using Quantity One 4.2 software. Hsp60 concentration was determined by linear regression of logarithmic density values. Spike recovery assays demonstrated that this technique allowed detection of Hsp60 concentrations >5 μg/ml. A commercial ELISA for Hsp60 (Stressgen Biomol) allowed detection of Hsp60 neither in murine urine nor in serum. According to the manufacturer, the latter was noted also by other groups and was interpreted as due to soluble factors inhibiting the test.

Flow Cytometry
After treatment with FcBlock (Pharmingen, Heidelberg, Germany), cells were stained for 15 min on ice with fluorochrome-labeled mAbs specific for CD3 (clone 2c11), CD4 (clone GK1.5), CD8 (clone 53.6.7), B220 (clone RA36B2), NKH1–1 cells (clone PK136), CD11b (clone Mac-1), and CD11c (clone HL3; Pharmingen). Dead cells were excluded from analysis.

Table 1. Titration of NSSa

<table>
<thead>
<tr>
<th>Proteinuria Day 21</th>
<th>Proteinuria Day 14</th>
<th>Proteinuria Day 7</th>
<th>Proteinuria per Mouse (μl/g)</th>
<th>No. of Mice</th>
<th>Survival per Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>No damage visible</td>
<td>No damage visible</td>
<td>No damage visible</td>
<td>0</td>
<td>All</td>
<td>0/7 (All)</td>
</tr>
<tr>
<td>Protein casts, no clear damage visible</td>
<td>Protein casts, no clear damage visible</td>
<td>Protein casts, no clear damage visible</td>
<td>2</td>
<td>ND</td>
<td>0/2 (All)</td>
</tr>
<tr>
<td>Some mesangial damage, little tubulointerstitial damage</td>
<td>Some mesangial damage, little tubulointerstitial damage</td>
<td>Some mesangial damage, little tubulointerstitial damage</td>
<td>4</td>
<td>All</td>
<td>0/3 (All)</td>
</tr>
<tr>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in five mice</td>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in all mice</td>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in all mice</td>
<td>5.5</td>
<td>All</td>
<td>0/2 (All)</td>
</tr>
<tr>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in one mouse and slight tubulointerstitial damage in two mice</td>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in all mice</td>
<td>Glomerular necrosis, severe tubular atrophy, and leukocyte infiltration in all mice</td>
<td>6</td>
<td>All</td>
<td>0/3 (All)</td>
</tr>
<tr>
<td>Severe damage</td>
<td>Severe damage</td>
<td>Severe damage</td>
<td>8</td>
<td>All</td>
<td>0/2 (All)</td>
</tr>
</tbody>
</table>

*Mice received injections of varying doses of nephrotoxic sheep serum (NSS). Proteinuria on days 7, 14, and 21, as well as histologic kidney damage on day 21, were assessed. Proteinuria was determined by Combur Test strips (+) is approximately 0.3 g/L, 2+ is approximately 1 g/L, 3+ is approximately 5 g/L, or Bradford assay (concentrations given). For histology, paraffin sections stained with periodic acid–Schiff were analyzed.
with propidium iodide. Flow cytometry was performed on a Becton Dickinson FACScalibur (Becton Dickinson, San Diego, CA).

**Ig Levels**

Serum levels of Ig specific for sheep Ig levels were assessed by ELISA (coating with 10 µg/ml sheep Ig [Serotec, Düsseldorf, Germany] overnight; blocking with 1% BSA; and serum diluted 1:100 in PBS, biotinylated rat anti-murine IgG1 and IgG2a [Pharmingen], streptavidin peroxidase [Boehringer Mannheim, Mannheim, Germany], and substrate 3,3’,5-triphenyltetrazolium [Roth, Karlsruhe, Germany] in DMSO). Total serum IgG1 and IgG2a levels against sheep Ig were determined using commercial ELISA kits (Pharmingen).

**Cytokine Production by Splenocytes**

Spleens were removed from mice, and single-cell suspensions were obtained. Splenocytes (2 × 10⁶ cells/well) were cultured in 100 µl of DMEM (10% FCS) for 72 h in the presence of 10 µg/ml sheep globulin or on plates that were coated with 10 µg/ml anti-CD3 (clone 2c-11). Concentrations of IFN-γ, IL-4, and IL5 in culture supernatants were measured by commercially available ELISA kits (Pharmingen). Flow cytometry ensured that the numbers of the major leukocyte populations in culture were comparable between experimental groups during culture (Table 2 and data not shown).

**Miscellaneous Determinations**

Proteinuria was determined by Bradford protein assay (Bio Rad), urine albumin excretion was determined by Albuwell M ELISA specific for murine albumin (Exocell, Philadelphia, PA), and leukocyturia was determined using Combur test strips (Roche, Mannheim, Germany). Determination of serum creatinine as well as the generation of paraffin sections of Methyl Carnoy’s fixed tissue and periodic acid-Schiff (PAS) staining were performed as described previously (25). For fibrinogen staining, paraffin sections were incubated with rabbit anti-human fibrinogen antiserum (cross-reactive with mouse; DAKO, Glostrup, Denmark), followed by biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA), ABC-Elite reagent (Vector), and 3,3’-diaminobenzidine with nickel chloride enhancement. Renal Ig deposits were detected using biotinylated horse anti-mouse IgG (Vector). Slides were counterstained with methyl green. Fibrinogen deposits were semiquantitatively scored by a blinded observer who assessed the stained area in at least 30 consecutive glomeruli per animal, using the following system: 0 = <5%, 1 = 5 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = >76% of glomerular area staining positively as described (24,27). Statistical significances of scored values were analyzed by the Mann-Whitney U test.

**Results**

**Release of Hsp60 in Experimental Kidney Disease**

To investigate whether Hsp60 release occurred in NTN, we determined Hsp60 concentrations in the urine of mice 8 d after injection of 5 µl/g body wt NSS (for titration, see Table 1). Whereas low levels were found in the urine of healthy control mice (7.1 ± 2.4 µg/ml; n = 4), those in mice with NTN were much higher (80.4 ± 18.0 µg/ml; n = 5; P < 0.001; Figure 1A). Theoretically, this excretion could result from local production in the kidney or could simply be due to glomerular filtration of serum Hsp60, because NTN is characterized by high proteinuria (Figure 1B). The latter possibility was addressed by measuring excretion of murine albumin in the urine. This protein has a molecular weight comparable to that of Hsp60 and should be handled similarly during glomerular filtration. As expected, it was detectable in the urine of mice with NTN, whereas healthy mice showed no detectable albumin excretion (Figure 1C), demonstrating that serum proteins of approximately 60 kD are excreted in NTN. The albumin serum concentration is approximately 20 mg/ml (28). Western blot analysis established that the serum concentration of Hsp60 was <10 µg/ml (data not shown). Thus, although proteinuria as a result of NTN

**Table 2. Systemic immune response in NTN³**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Boiled Hsp60 + Sheep Ig</th>
<th>Hsp60 + Sheep Ig</th>
<th>Boiled Hsp60 + NSS</th>
<th>Hsp60 + NSS</th>
<th>Not Injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of spleen cells (×10⁻⁸)</td>
<td>1.87 ± 0.21</td>
<td>2.07 ± 0.30</td>
<td>2.21 ± 0.07</td>
<td>2.27 ± 0.06</td>
<td>2.25 ± 0.53</td>
</tr>
<tr>
<td>% CD4⁺ T cells</td>
<td>20.0 ± 0.95</td>
<td>20.8 ± 1.37</td>
<td>17.2 ± 2.08</td>
<td>20.5 ± 2.47</td>
<td>19.3 ± 1.28</td>
</tr>
<tr>
<td>% CD8⁺ T cells</td>
<td>12.1 ± 0.53</td>
<td>12.3 ± 0.05</td>
<td>10.4 ± 1.22</td>
<td>11.2 ± 0.41</td>
<td>12.1 ± 1.25</td>
</tr>
<tr>
<td>Splenic IFN-γ induced by sheep Ig (pg/ml)</td>
<td>145 ± 30.2</td>
<td>268 ± 62.1</td>
<td>238 ± 103</td>
<td>165 ± 64.5</td>
<td>104 ± 25.3</td>
</tr>
<tr>
<td>Splenic IL-4 induced by sheep Ig (pg/ml)</td>
<td>49.7 ± 65.0</td>
<td>26.7 ± 26.8</td>
<td>18.0 ± 24.3</td>
<td>26.3 ± 5.9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Splenic IL-5 induced by sheep Ig (pg/ml)</td>
<td>109 ± 7.6</td>
<td>106 ± 46.5</td>
<td>119 ± 12.2</td>
<td>65.7 ± 59</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Serum IgG1 (mg/ml)</td>
<td>92.3 ± 2.5</td>
<td>82.0 ± 6.0</td>
<td>91.3 ± 3.8</td>
<td>94.0 ± 4.6</td>
<td>81.0 ± 5.6</td>
</tr>
<tr>
<td>Serum IgG2a (mg/ml)</td>
<td>343 ± 10.4</td>
<td>306 ± 31.8</td>
<td>240 ± 120</td>
<td>216 ± 64.8</td>
<td>350 ± 42.5</td>
</tr>
<tr>
<td>Sheep Ig-specific IgG1 (OD450)</td>
<td>0.660 ± 0.163</td>
<td>0.608 ± 0.489</td>
<td>0.131 ± 0.059</td>
<td>0.216 ± 0.173</td>
<td>0.074 ± 0.070</td>
</tr>
<tr>
<td>Sheep Ig-specific IgG2a (OD450)</td>
<td>0.665 ± 0.381</td>
<td>0.223 ± 0.200</td>
<td>0.041 ± 0.019</td>
<td>0.022 ± 0.008</td>
<td>0.115 ± 0.110</td>
</tr>
</tbody>
</table>

³NTN, nephrotic nephritis. Groups of three mice received injections of 5 µl of NSS or 5 µl of irrelevant sheep serum and 50 µg of Hsp60 or 50 µg of boiled Hsp60. On day 10, parameters of systemic immune responses were determined. Results are given as mean ± SD.
theoretically allowed filtration of Hsp60, there was little of this protein available in the serum for such filtration as compared to albumin, arguing against a significant contribution of filtration to Hsp60 urinary excretion. This conclusion was supported by observations in overload proteinuria, a noninflammatory model of proteinuria (Figure 1B) (26). In this model, excretion of murine albumin was significantly higher than in healthy mice (5.9 ± 1.9 versus 0.2 ± 0.1 g/L; n = 4; P < 0.001), albeit not as high as in NTN (Figure 1C). In contrast, Hsp60 excretion in overload proteinuria was not significantly increased (13.3 ± 7.8 versus 7.1 ± 2.4 μg/ml), as opposed to the impressive increase in NTN (Figure 1A). Thus, 60-kD proteins with high serum concentrations such as albumin are excreted into the urine in both noninflammatory and inflammatory renal damage. In contrast, Hsp60 is present in low concentrations in the serum and is selectively excreted into the urine in inflammatory renal disease. These findings suggest that the high urinary excretion of Hsp60 in NTN was mostly due to local release from the kidney.

To substantiate this conclusion, we designed an assay to quantify release of Hsp60 into the extracellular compartment of the diseased kidney. This was not possible by immunohistochemistry because of the high constitutive intracellular content of Hsp60 in most kidney cells, in particular in tubular epithelial cells (29–31). Instead, we cultured whole murine kidneys 8 d after injection of NSS for 2 h at 4°C. This approach was chosen because homogenization of the kidney would lead to cellular destruction and to release of intracellular Hsp60. We speculated that the incubation of an explanted kidney for a short time would allow extracellular Hsp60 to diffuse from the organ into the medium. Hsp60 concentration in culture supernatant and urine was determined by Western blot analysis. Shown are means and SD of groups of four to five animals. Similar results were seen in a separate experiment in groups of three to four mice.

Figure 1. Release of heat shock protein 60 (Hsp60) in nephrotoxic nephritis (NTN). (A through C) Urine samples from groups of healthy mice (■) 8 d after induction of NTN (●) or protein overload (▲) were assayed for concentrations of Hsp60 (A), total protein (B), and murine albumin (C). (D through E) Kidneys and livers from mice before (●) or 8 d after (■) NTN induction (D) and kidneys 2 d after ligation of the left ureter (●) and the contralateral kidney (■; E) were explanted, decapsulated, and incubated as whole organs in 2 ml of Hanks buffer for 2 h at 4°C to allow extracellular Hsp60 to diffuse from the organ into the medium. Hsp60 concentration in culture supernatant and urine was determined by Western blot analysis. Shown are means and SD of groups of four to five animals.
Thus, Hsp60 release occurred also in non–immune-mediated renal disease.

Hsp60 Severely Aggravates NTN

To investigate the consequences of Hsp60 release in NTN, we decided to co-inject recombinant Hsp60 in this disease model. To be able to detect aggravation of NTN, we used a low dose of NSS (4 μl/g body wt), which caused minor proteinuria and leukocyturia (Table 1). A single co-injection of Hsp60 6 h after NSS aggravated proteinuria and leukocyturia, first detectable 4 to 5 d after disease induction and very marked on day 12 (Figure 2, A and B), when kidneys were taken for histologic examination. On that day, histologic signs of nephritis were manifest only in mice that received a co-injection of NSS and Hsp60 (data not shown). Quantification of glomerular fibrinogen deposition by immunohistochemistry has been used as a parameter for damage in NTN (27). However, significant increase of this parameter was not seen under these conditions (Figure 2C). To render histologic changes more evident, we performed a series of experiments using a slightly higher dose of NSS (5 μl/g body wt). This caused proteinuria in most animals from day 3 on, which was increased in the group that received co-injections of Hsp60 on day 7 (Figure 2D). From then on, animals that received a co-injection began to show signs of wasting. On day 12, some animals had succumbed to disease. The surviving mice showed elevated serum creatinine levels (Figure 2E) and extremely high proteinuria or were anuric. Histology revealed pronounced signs of kidney damage in these animals: Severe global glomerular necrosis, tubulointerstitial damage, multiple PAS-positive protein casts in atrophic, and dilated tubuli were apparent (Figure 3). In contrast, animals that received a injection of NSS alone showed only segmental glomerular necrosis, focal PAS-positive protein casts, tubular atrophy and dilation, and no extracapillary proliferation (Figure 3). Sections of mice that received an injection of Hsp60 alone were indistinguishable from those of control animals. The glomeruli of mice that received a co-injection showed significantly higher fibrinogen deposits than those of mice that were given NSS alone (Figures 2F and 3), further illustrating aggravation of disease by Hsp60.

Mechanisms Involved in Hsp60-Mediated Aggravation of NTN

Some biologic effects of Hsp60 have been suggested to result from endotoxin contamination in certain commercial preparations (32,33). Furthermore, a rat NTN model has been reported to be affected by LPS (19). To exclude such effects in our systems, we determined the endotoxin content of all Hsp60

![Figure 2](image-url). Hsp60 severely aggravates NTN. (A through C) Groups of four mice received an intravenous injection of 4 μl/g body wt nephrotoxic sheep serum (NSS) or sheep Ig, followed by 50 μg of Hsp60 or PBS after 6 h. On day 12, proteinuria (A), leukocyturia (B), and glomerular fibrinogen deposits (C) were determined. (D through F) Groups of four mice received intravenous injections of 5 μl/g body wt NSS or sheep Ig, followed after 6 h by 50 μg of Hsp60, 50 μg of Hsp60 boiled for 2 h, or PBS. Proteinuria for day 7 is given (D) because one of the four animals in the NSS+Hsp60 group died on day 8 and two had become anuric by day 12, when serum was taken for creatinine determination (E) and kidneys were taken for immunohistologic assessment of fibrinogen (F) deposits. Similar results were obtained in a separate experiment in groups of three mice.
preparations by limulus amebocyte lysate assay and used only preparations with <1 EU endotoxin/mg Hsp60 (equivalent to 1 ng of the LPS used in the present study). These preparations were shown recently to improve T cell activation and effector functions independent of endotoxin contaminations (21). To control for an in vivo influence of such small endotoxin contamination, we took advantage of the heat stability of endotoxins and included in the experiments using 5 μl/g body wt NSS an experimental group that received co-injections of boiled Hsp60. Boiling denatures proteins and has been used to incapacitate various Hsp (7,11,13,34). In mice that received a co-injection of such boiled Hsp60, proteinuria (Figure 2D), creatinine levels (Figure 2E), leukocyturia (data not shown), fibrinogen deposits (Figure 2F), and histologic damage (Figure 3) were similar to those observed in mice that had received NSS alone, demonstrating that the aggravation of NTN was mediated by a heat-sensitive component of Hsp60. To investigate further the influence of potential endotoxin contaminations, we co-injected 1 ng of LPS with NSS. This dose was 20-fold higher than the LPS contamination detected in the 50-μg Hsp60 that was used in the co-injection experiments in this study. This dose of LPS did not aggravate proteinuria (Figure 4, A and B), serum creatinine (NSS: 20.5 ± 1.3 μM, NSS+LPS: 20.7 ± 1.8 μM; n = 4), glomerular fibrinogen deposits (Figure 4C), or histologic damage (data not shown). In summary, these controls indicate that endotoxin was not responsible for the Hsp60-mediated aggravation of NTN.

Previous studies have demonstrated that T cells are essential in NTN (17). Hsp60 has been shown to increase T cell proliferation and cytokine production in vitro (6,11). To evaluate whether the Hsp60-mediated aggravation of NTN was T cell dependent, we depleted T cells in mice using anti-Thy1-specific mAb and induced NTN on the following day. On that day, the
proportion of T cells in the blood was reduced from 35 to 40% of total CD3<sup>+</sup> lymphocytes to <1% (data not shown). This depletion did not significantly change proteinuria in mice that had received NSS without Hsp60 (Figure 3D). In contrast, the aggravation of NTN after co-injection of Hsp60 was completely abrogated in the absence of T cells, as determined by histologic damage (Figure 4C and data not shown) and proteinuria (Figure 4, A and B). T cell–depleted mice showed only sporadic glomerular damage and neither fibrinogen deposits (Figure 4C) nor tubulointerstitial damage (data not shown), regardless of whether Hsp60 was co-injected or not. The functionality of the Hsp60 preparation used in this experiment was demonstrated by aggravation of NTN in the positive control group that had received NSS, Hsp60, and rat Ig as a control for the anti-Thy1 antibody. Only in this group was proteinuria increased by day 7 (Figure 4A), and on day 12, two mice had died, one mouse was anuric, and one showed severe proteinuria (Figure 3D). Both surviving mice showed glomerular necrosis, tubular atrophy, mononuclear tubulointerstitial infiltration, and fibrinogen deposits (Figure 3C). Thus, T cells were essential for Hsp60-mediated aggravation of NTN. On the day of analysis, i.e., day 12, CD3<sup>+</sup> T cell numbers in the blood had risen to approximately half of that in control animals, indicating that T cells were required in the early induction phase of disease to mediate Hsp60-induced aggravation of disease.

A shift of the T cell response toward Th1 has been shown to be associated with crescentic GN (18,35). Hsp60 caused a Th1 shift in vitro (6,36). To analyze whether Hsp60 aggravated NTN in our model via a Th1 shift, we examined the ability of spleen cells to produce cytokines in response to sheep Ig in vitro. Significant changes were observed in the production neither of the Th1 cytokine IFN-γ nor of the Th2 cytokines IL-4 and IL-5 (Table 2). This could not be explained by changes in the cellular composition of splenic leukocyte populations, as the total cell numbers and the proportions of T cells (Table 2) and of B cells, T cells, NK cells, macrophages, and CD11b<sup>+</sup> and CD11b<sup>-</sup> dendritic cells (data not shown) were not different between experimental groups. A Th1-type response can also be demonstrated by a shift of from the serum Ig subclass IgG1 to IgG2a. We did not detect significant changes in total IgG1 concentrations between mice that received an injection of Hsp60 or not (Table 2). It is interesting that nephritic animals showed lowered total IgG2a levels as compared with controls that received an injection of control sheep serum (Table 2). When we examined Ig titers specific for sheep Ig, a decrease was seen for both subclasses (Table 2), arguing against an important role of the autologous phase in our model. This was supported by immunohistochemical staining of kidney sections for deposits of murine Ig. No such deposits were detected in the glomeruli and in the tubulointerstitium (Figure 3). Taken together, these findings do not support a Th1 shift or an aggravation of the autologous phase as underlying causes for the T cell–mediated aggravation of NTN induced by Hsp60.

Discussion
Recent studies have demonstrated that uric acid (4) and Hsp70 (7) can activate APC and boost resulting T cell responses in animal models. These findings are considered to represent the first in vivo identifications of immunologic danger signals. These signals have been defined as endogenous mediators released in harmful conditions, such as infections, that stimulate APC to induce immunogenic responses (3). Our present study provides in vivo evidence that also Hsp60 fulfills criteria of a danger signal. We demonstrated that Hsp60 was released from the kidney in NTN and aggravated this disease model. Experimental conditions that normally cause minor kidney damage and glomerular repair led to complete organ failure in the presence of Hsp60. These results demonstrate that this molecule can shift the balance between tolerance and immunity toward immunity. They are consistent with recent findings in a transgenic model of diabetes, in which Hsp70 converted T cell tolerance into autoimmunity (7). Our study extends this previous report to a nontransgenic situation in a different organ and by demonstrating release of the danger signal from the affected organ. Upregulation of various Hsp, including Hsp60, has been demonstrated by immunohistochemistry in kidney disease (29–31), but there has been little direct evidence for their release. Our findings do not prove that Hsp60 release from the kidney can directly aggravate NTN. This direct proof would require the ability to specifically block Hsp60 function in the kidney. Nevertheless, our findings support the idea of a positive feedback loop consisting of kidney disease–mediated release of Hsp60, immune-mediated aggravation of kidney disease, further release of Hsp60, and so on. Such vicious circles may represent general mechanisms relevant in the perpetuation of immune-mediated diseases.

Hsp60-mediated aggravation of NTN involved immune effector mechanisms, because it was strictly T cell dependent. CD4 T cells have been shown to be essential in the progression of crescentic GN to terminal kidney failure (17). Recent studies suggest APC as likely intermediates between Hsp60 and T cells, as they seemed to express an Hsp60 receptor whose stimulation led to APC activation (10,14–16). In vitro, APC activated under the influence of Hsp60 were shown to induce CD4 T cells to produce increased amounts of inflammatory cytokines such as IFN-γ and IL-12 (5,6,11), which have been shown to aggravate crescentic GN (18). An involvement of APC in our findings, however, remains to be shown. A role for CD8 T cells is less likely, as these cells were irrelevant in crescentic GN (35). Furthermore, Hsp60, unlike Hsp70 and gp96, does not seem to introduce chaperoned peptides into the cross-presentation pathway, which could recruit CD8 T cells as effectors. T cell responses against Hsp70 have been shown to be important in CdCl<sub>2</sub>-induced interstitial nephritis (37), and T cells specific for Hsp60 have been shown to contribute to colitis in immunocompromised mice (38). However, if this played a role in the present study, then it would be difficult to explain why pathology was confined to the kidney and why systemic administration of Hsp60 alone did not cause any pathology. An increase in the autologous Ig response was not observed, consistent with previous observations in crescentic GN (35). This response was even diminished, suggesting that humoral immune responses were not accountable for the Hsp60-mediated effects. Likewise, we did not detect an Hsp60-induced shift of the immune response against sheep Ig toward Th1, which has been shown to
aggravate crescentic GN (18,35) and which is a well-documented effect of Hsp60 (6,36). It is interesting that we noted in nephritogenic animals, regardless of Hsp60, a decrease in total Ig2a serum levels (Table 2) and an increase in splenic production of IL-4 and IL-5 induced by anti-CD3 antibody (data not shown), which stimulates all T cells, including those not specific for the nephritogenic sheep Ig antigen. These findings argue against a systemic Th1 shift and suggest that the NSS used in the present study (22) induced immune reactions different from those in studies on crescentic GN (35). In summary, these results do not support a systemic or sheep Ig–specific Th1 shift or an aggravation of the autologous phase as crucial mechanisms in Hsp60-mediated aggravation of NTN.

The target cell stimulated by Hsp60 in our system remains to be identified. Addressing this question would be possible by targeting the elusive Hsp60 receptor. As candidate receptors, the Toll-like receptors TLR 2 (14,16), TLR 4 (14,15), and CD14 (10) have been proposed. These molecules have also been shown to mediate APC activation by pathogen-derived endotoxins (32). Therefore, we performed extensive controls to exclude that endotoxin contaminations in the recombinant Hsp60 preparations used were responsible for our observations. These controls do not strictly rule out the notion that Hsp60 may bind small amounts of endotoxins and increase their ability to stimulate PAMP receptors (32), but even if Hsp60 were not sufficient on its own, it would still be an essential component of a danger signal. Strong evidence for endotoxin-independent effects are transgenic mice expressing the Hsp gp96, in which ubiquitous APC activation and a lupus-like phenotype were observed (39). In such transgenic animals, endotoxin contaminations could be excluded. Finally, direct stimulation of T cells by Hsp60, as recently demonstrated in vitro (16), is unlikely to explain the present findings, because such stimulation was reported to attenuate the resulting T cell response.

Our findings may have diagnostic and therapeutic implications. The presence of Hsp60 in the urine of animals with NTN raises the possibility of using such molecules as noninvasive markers for immune-mediated kidney disease. Blocking of Hsp60 function is not likely to represent a feasible therapeutic approach, because of the numerous vital housekeeping functions of Hsp (8). Even if specific blockade of Hsp60 or its receptor were possible, then other Hsp, such as Hsp70 or gp96, may possess overlapping functionality, so incapacitation of only one such protein may be compensated by others. However, components of common Hsp signaling pathways may represent promising targets for therapeutic strategies to slow progression of immune-mediated kidney disease.

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