from HCC patients generally paralleled the level of AFB_1 exposure, our results support the hypothesis that AFB_1 is a causative mutagen in liver cancer. Indeed, this hypothesis is strengthened by laboratory studies showing preferential (but not exclusive) induction of the codon 249 AGT mutation by AFB_1 in cultured Hep G2 cells (*3*). The predominance of this mutation could be, at least in part, a consequence of preferential induction by AFB_1, as a result of sequence context and inefficient repair of pre-mutagenic lesions (*8*). However, we cannot yet rule out a role for other carcinogens. For example, bulky heterocyclic amines in cooked foods and oxidants released by inflammatory leukocytes (9) possess the same specificity for G to T transversions and HBV infection is associated with inflammation. All of the liver specimens from Qidong and Thailand were from HBV-infected individuals. However, no correlation has been reported between the incidence of *p53* mutations in HCC and in HBV infection alone (*10*), but HBV infection and exposure to AFB_1 may be synergistic risk factors (*11*).

The abundance of *p53* codon 249 AGT mutations in nonmalignant specimens from Qidong suggests that cells containing these mutations have undergone early, albeit limited, expansion in the histologically normal tissue. Because there was no consistent relation between the mutation frequency in the nonmalignant tissue and the genotype of the tumor from the same patient (Tables 1 and 2), it appears that additional factors, for example, loss of the remaining wild-type allele, are required for the further expansion of mutant cells during tumor progression. The presence of this mutation may merely increase the probability that a cell will progress along the pathway of hepatocarcinogenesis. Two tumors with the *p53* codon 249 AGT genotype also had elevated frequencies of codon 249 AGC mutations, which indicates that the tumors have a multifocal composition. Both codon 249 AGT and AGC mutations result in the replacement of arginine by serine in the mutant protein, which suggests that *p53* codon 249 serine has acquired an oncogenic function.

The presence of elevated frequencies of codon 249 AGT mutations in the nonmalignant tissue of HCC patients from Qidong suggests that the mutagenic event occurred early in hepatocarcinogenesis. In contrast, *p53* mutations in HCCs from geographic areas with low exposure to AFB_1 could be late events. For example, *p53* mutations have been observed more frequently in large tumors and in advanced grades of malignancy in HCCs from Japan (*12*). Similarly, in other organs such as the colon (*13*) and the bladder (*14*), *p53* mutations are thought to occur late in tumorigenesis. However, the methods used in previous work may not have been sensitive enough to detect mutations at early stages of tumorigenesis, before substantial expansion of the mutant cells had occurred.

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Prevention of Lipopolysaccharide-Induced Lethal Toxicity by Tyrosine Kinase Inhibitors

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Septic shock results from excessive stimulation of the host immune system, especially macrophages, by lipopolysaccharide (LPS), or endotoxin, which resides on the outer membrane of bacteria. Protein tyrosine kinase inhibitors of the tyrphostin AG 126 family protect mice against LPS-induced lethal toxicity. The protection correlates with the ability of these agents to block LPS-induced production of tumor necrosis factor α (TNF-α) and nitric oxide in macrophages as well as LPS-induced production of TNF-α in vivo. Furthermore, this inhibitory effect correlated with the potency of AG 126 to block LPS-induced tyrosine phosphorylation of a $p42^{MAPK}$ protein substrate in the murine macrophage.

Septic shock is a major cause of death among patients in intensive care units and ranks 13th in the causes of deaths overall in the United States (*1*). Septic shock results from a systemic infection by Gram-negative bacteria that causes hypotension and multiorgan dysfunction. Except for surgical and supportive care, no specific therapy is known, although very limited success has been reported recently through the use of antibodies against endotoxin (*2*).

Sepsis and septic shock result primarily, if not exclusively, from excessive stimulation of the host immune system, especially macrophages, by the complex glycolipid LPS (endotoxin), which resides in the outer membrane of the bacteria (*3*). Lipopolysaccharide stimulates immunocytes, mainly macrophages, to generate tumor necrosis

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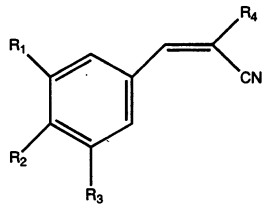
Table 1. Structure of tyrphostins.



Tyrphostin	R_1	R_2	R_3	R_1
AG 126	OH	NO_2	H	CN
AG 1288	OH	OH	NO_2	CN
AG 702	NO_2	OH	H	COOH
AG 1290	OH	OH	NO_2	COOH

factor α (Cachectin; TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), prostanoids, leukotrienes (*4*), and nitric oxide (*5*). Many of the toxic effects of LPS, such as adult respiratory distress syndrome and vascular leak syndrome, can be mimicked by TNF-α and IL-1β, which synergize with each other (*6*). Indeed, mice in which the gene for the TNF-α receptor, TNFRp55, has been disrupted are resistant to endotoxic shock (*7*). Furthermore, pretreatment of mice with antibodies to TNF-α prevents death induced by the injection of endotoxin (*8*). Rabbits can be protected from septic shock by the injection of an IL-1 receptor antagonist shortly before the injection of *Escherichia coli* endotoxin (*9*).

Lipopolysaccharide induces protein tyrosine phosphorylation (*10*) in macrophages as well as the generation of eicosanoids (*11*), and the protein tyrosine kinase (PTK) inhibitors tyrphostins and herbimycin A inhibit these events (*10–11*). Furthermore, LPS induces in macrophages the ability to kill tumor cells, and these tumoricidal properties can be blocked by tyrphostins (*12*).

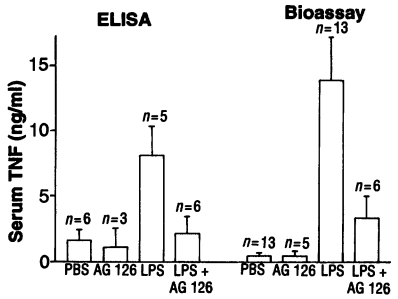


Fig. 1. Effect of AG 126 on serum TNF-α levels in LPS-treated mice. Female C57BL mice (6 to 8 weeks old) were injected [intraperitoneally (IP)] with AG 126 (400 μg per mouse) and injected 2 hours later with LPS (1.5 mg per mouse). After 2 hours, the mice were bled by orbital puncture, and the serum concentration of TNF-α was determined with an ELISA kit or by bioassay. The results are expressed as means ± SD of determinations.

Table 2. Inhibition of LPS-induced TNF-α production in murine peritoneal macrophages by tyrphostins. The assay was conducted as described (*22*).

AG	TNF-α (pg/ml)	
	Untreated	Treated with LPS
None	<5	46
126	<5	<5
1288	<5	<5
702	<5	38
1290	<5	17

In addition, TNF-α (*13*) and IL-1β (*14*) induce tyrosine phosphorylation in target cells, and the signaling events induced by these ligands are blocked by PTK inhibitors such as tyrphostins (*15*), herbimycin A (*12, 16*), and genistein (*11, 17*). These tyrosine phosphorylation events probably result from the activation of src-type PTKs such as Hck and Lyn (*18*). Some of the toxic manifestations of LPS may be mediated by NO (*19*). The production of NO is induced by LPS and inhibited by tyrphostins (*20*). The involvement of tyrosine phosphorylation events in the action of LPS and the cytokines produced suggested to us that septic shock might be blocked by tyrosine kinase inhibitors of the tyrphostin family. These agents can, in principle, simultaneously suppress both the LPS-induced production of TNF-α and IL-1β as well as their induced effects.

Table 3. Inhibition of TNF-α cytotoxicity by tyrphostins. TNF-α was applied to mouse A9 fibroblasts as described (*29*), and the protective effect of 10 μM tyrphostins is shown. The results are expressed as an average of three independent experiments with standard deviations in parentheses.

TNF-α (ng/ml)	Percent of viable cells				
	None	AG 126	AG 1288	AG 702	AG 1290
0.0	100	100	100	100	100
0.2	41 (2.3)	67 (3.3)	67 (4.1)	41 (4.3)	42 (3.7)
0.5	20 (1.7)	38 (2.3)	46 (2.8)	23 (2.0)	18 (2.6)

Tyrphostins, which belong to different families (*15*), were screened for their ability to inhibit LPS-induced production of TNF-α by activated murine peritoneal macrophages in vitro (Table 1). Among the tyrphostins tested (Table 2), AG 126 and AG 1288 were the most potent. These tyrphostins have no effect on epidermal growth factor receptor, Her-2/neu receptor, or PDGFβR even at concentrations above 100 μM (*21*). These tyrphostins also inhibit the in vitro production of NO_2^- (an

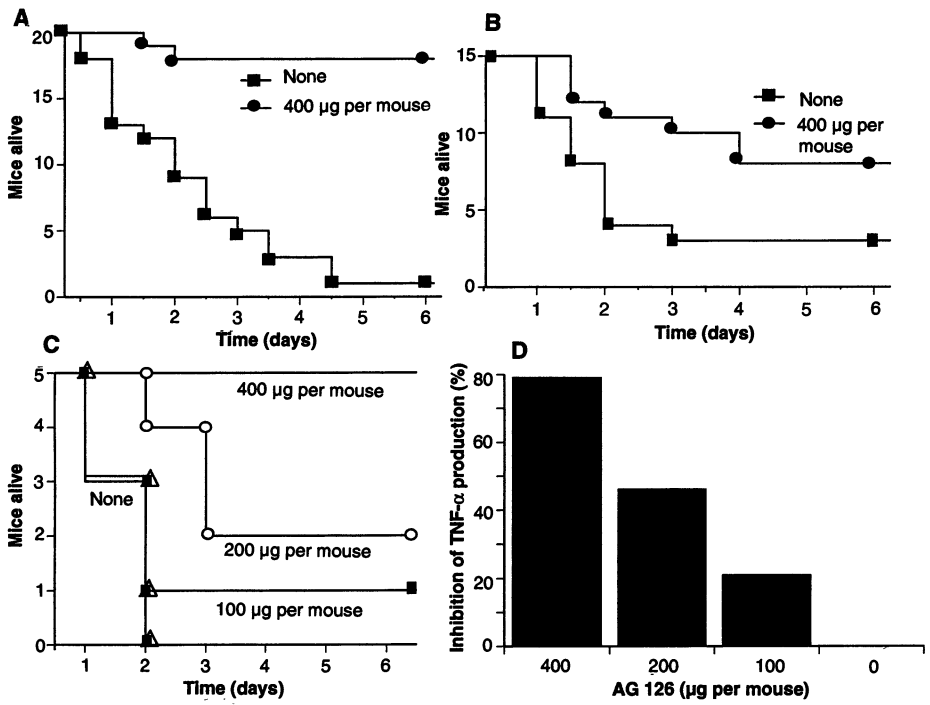


Fig. 2. Effect of tyrphostin AG 126 on LPS-induced lethal toxicity in mice. (**A**) Mice were injected (IP) with AG 126 or PBS/dimethyl sulfoxide control 2 hours before injection (IP) of LPS (1.5 mg per mouse). (**B**) Similar to (A), but AG 126 and LPS were administered simultaneously. The mortality of mice was monitored twice daily. (**C**) Similar to (A), but AG 126 was administered at different doses 2 hours before LPS injection. (**D**) The level of TNF-α in the injected mouse as a function of the dose of injected tyrphostin.

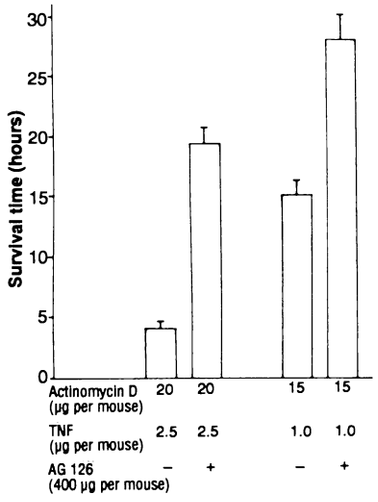


Fig. 3. Effect of tyrphostin AG 126 on TNF-α induced lethal toxicity in mice. Mice were injected (IP) with AG 126 and actinomycin D, 2 hours and 0.5 hour, respectively, before the injection (IP) of TNF-α. Each group contained five mice. The injection of actinomycin D alone caused death in two out of five animals after 120 hours and three out of five animals after 144 hours. Results are expressed as mean survival time (in hours) ± SD.

oxidative product of NO), confirming recent results (*22*). The tyrphostins AG 126 and AG 1288, but not AG 702 or AG 1290 (Tables 1 and 2), were also active in blocking TNF-α induced cytotoxicity in vitro (Table 3), as previously shown for nonselective PTK blockers (*12, 23*).

We further evaluated the effect of AG 126 in attenuating the toxic effect of LPS in vivo. Endotoxin induced a rapid increase in serum concentration of TNF-α; the administration of AG 126 at 400 μg per mouse 2 hours before LPS treatment inhibits the increase in the concentration of TNF-α in serum (Fig. 1). Both a bioassay and a biochemical enzyme-linked immunosorbent assay (ELISA) were used to measure the amount of TNF-α in serum. The bioassay indicated the presence of greater amounts of TNF-α than did the ELISA, suggesting that the serum from LPS-treated mice may contain other cytotoxic factors that influence the bioassay. We investigated the effect of AG 126 on LPS-induced lethal toxicity in vivo. At a dose of 1.5 mg per mouse, LPS induced 95% lethality within 5 days. The administration of AG 126 2 hours before administration of LPS reduced the lethality to 10% (Fig. 2A). The animals were immobile and had diarrhea in both experimental groups during the first 36 hours. Thereafter, most of the animals that had been treated with AG 126 gradually recovered, and on the fifth day they appeared normal. There were no visible toxic manifestations in mice that were treated with AG 126 alone. The animals of both groups were followed for an additional 3 weeks; no life shortening or toxic effects were noted. Administration of AG 126 of up to 12 mg per mouse (30 times the 400 μg per mouse given in these experiments) did not show any toxicity as revealed by the appearance of the treated animals, hematological findings, or macroscopic pathological analysis. Simultaneous administration of AG 126 with LPS treatment was less effective in preventing lethal LPS toxicity than administration 2 hours before LPS treatment (Fig. 2B). Administration of AG 126 2 hours after LPS had essentially no protective effect on LPS-induced lethal toxicity. The protective effect of AG 126 against LPS-induced lethality was dose-dependent (Fig. 2C). A dose of 400 μg of AG 126 per mouse was the minimal dose that provided essentially full protection against LPS-induced mortality. The dosing of the protective effect of AG 126 correlates with its inhibition of TNF-α production in vivo (Fig. 2D). We also investigated the effect of AG 126 on lethal toxicity in mice induced by TNF-α. Because mice are relatively resistant to TNF-α when applied as a single agent, we sensitized them by pretreatment with actinomycin D (*24*). In mice treated with actinomycin D, AG 126 significantly delayed TNF-α induced lethal toxicity (Fig. 3). By itself, TNF-α is not effective in inducing lethality in mice. Lethality induced by LPS involves the synergistic effect of multiple effector molecules such as TNF-α, IL-1, interferon γ, and NO. Therefore, the prevention of LPS action results in more dramatic results. It is likely, however, that a different class of tyrphostins may prove more effective in blocking TNF-α toxicity than in blocking LPS toxicity (*25*). We examined the effect of AG 126 on LPS-induced tyrosine phosphorylation in murine macrophages. Tyrosine phosphorylation induced by LPS of a 42-kD protein in murine peritoneal macrophages was inhibited by pretreatment of the cells as before with protective concentrations of AG 126 (Fig. 4). This protein band was identified as $p42^{MAPK}$, confirming recent findings (*26*). The identity of the PTK or PTKs responsible for the tyrosine phosphorylation of specific macrophage proteins is still unknown, although recent studies suggest that LPS binds to CD14 and induces the activation of CD14-associated protein tyrosine kinase $p53/56^{Lyn}$ and $p58/64^{Hck}$ (*18*).

The treatment of mice with the tyrphostin AG 126 reduced lethal toxicity induced by LPS. The protective effect of AG 126 correlates with its inhibition of TNF-α production, NO production, and protein phosphorylation. In this study, we have used a model in which LPS doses of LD_{95} were used. Under these conditions, AG 126 conferred nearly full protection when injected before LPS treatment and reduced protection when it was administrated later (Fig. 2). We used a single lethal dose of LPS in this study and examined the protective effect of tyrphostins. In the clinical situation, the development of septic shock followed by Gram-negative sepsis is a gradual process. Therefore, tyrphostins should be effective in preventing septic shock when administered at the onset of the clinical signs of sepsis or septic shock. Tyrphostins prevent the onset of LPS toxicity as well as the action of LPS-induced cytokines. Thus, PTK inhibitors such as AG 126 may be effective in preventing the effects of septic shock in Gram-negative infections. Other agents, such as steroids (*27*) or chlorpromazine (*28*), prevent LPS toxicity by mechanisms that are distinct from that mediated by tyrphostins. These agents were also shown to be effective by their application before lethal doses of LPS. It is possible that treatment of septic shock by a combination of these agents may be more effective than treatment by each agent alone.

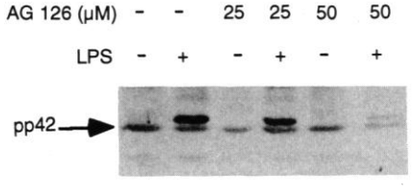


Fig. 4. Effect of tyrphostin AG 126 on the induction of protein tyrosine phosphorylation by LPS. $NaIO_4$-activated murine macrophages were obtained as described (Table 1). After isolation, they were incubated for 18 hours in the presence of murine recombinant IFN-γ (10 U/ml). Macrophage monolayers were incubated with AG 126 for 3 hours before the addition of LPS (1 μg/ml). After 15 min, the medium was aspirated and cell lysates were prepared essentially as described (*10*). Cells were washed with PBS containing 1 mM sodium orthovanadate (Na_3VO_4) and 5 mM EDTA and scraped into lysis buffer [1% Triton X-100, 20 mM tris-HCL (pH 8.0), 137 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 20 mM leupeptin, and aprotinin (0.15 U/ml)]. Lysates were diluted to 2 mg of protein per milliliter of buffer consisting of SDS (2.3%) and dithiothreitol (100 mM) and boiled for 2 min. The proteins were resolved by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After blocking with bovine serum albumin (5%), the proteins were probed overnight with monoclonal antibody to phosphotyrosine at 4°C and, after washing, they were incubated for 1 hour with horseradish peroxidase–conjugated $F(ab)_2$ of goat antibody to mouse immunoglobulin G and then rinsed. Bands were detected with an emmission chemiluminescence (ECL) system (Amersham).

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22. S. Tsunawaki and C. F. Nathan, *J. Biol. Chem.* **259**, 4305 (1984). Mice were injected intraperitoneally with 1.0 ml of $NaIO_4$. Macrophages were washed 3 to 4 days later from the peritoneal cavity with phosphate-buffered saline (PBS). After centrifugation at 170*g* for 10 min at 4°C, the cell pellet was resuspended in RPMI 164 containing heat-inactivated newborn calf serum (20%). Adherent macrophage monolayers were obtained by plating of the cells in 96-well microtiter plates at 4×10^5 cells per well and incubation for 2 hours at 37°C in 5% CO_2. Nonadherent cells were removed, and complete medium (RPMI 1640 containing heat-inactivated newborn calf serum) was added. After incubation of macrophage monolayers for 20 hours, tyrphostins were added at a final concentration of 20 μM followed 2 hours later by the addition of *E. coli* 055:B5 LPS (10 μg/ml). Endotoxin (Sigma) was prepared by phenol extraction. After incubation for 6 hours, supernatants were collected and the concentration of TNF-α was determined with an ELISA kit (Endogen). The results are expressed as the mean of two determinators; deviations from the mean did not exceed 8%.
23. Unstimulated macrophages possess basal activity of NO production, which was found to be between 20 and 35% of the maximally LPS-stimulated cells. This activity occurs because the macrophages probably became partially activated during preparation. Tyrphostins suppress this basal activity down to the same level at which they suppress the LPS-induced NO production. The level of basal NO_2^- production seems to depend on the method of macrophage preparation, whereas maximal LPS does not depend on the preparation.
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25. The tyrphostins AG 490 and AG 556 were found to be more active in inhibiting TNF-α cytotoxicity in vitro (two- to threefold). This finding suggests that different sets of PTKs mediate the effects of LPS and TNF-α and, therefore, that different families of tyrphostins will be effective against these two agents.
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29. Inhibition of TNF-α cytotoxicity by tyrphostins. Mouse A9 fibroblasts, a TNF-α–sensitive cell line, were plated in 96-well flat-bottom microtiter plates at 30,000 cells per 0.1 ml to establish a dense monolayer. After incubation for 24 hours at 37°C in a humidified atmosphere with 5% CO_2, cycloheximide was added to a final concentration of 50 μg/ml. Cells were treated with murine recombinant TNF-α [5×10^6 U/mg (Reportech, Rocky Hill, NJ) at 0.0, 0.2, and 0.5 ng/ml with and without 10 μM tyrphostin. After incubation for 18 hours, the supernatants were aspirated, the monolayers were washed twice with PBS, and 200 μl of neutral red solution (0.02%) was added. After incubation for 2 hours, cells were washed out and the dye that had been absorbed by the live cells was extracted upon the addition of 200 μl of Sorenson buffer containing 50% ethanol. The concentration of the dye was determined by an ELISA autoreader with a 550-nm filter.

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Interaction of MHC Class I Molecules with the Transporter Associated with Antigen Processing

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The transporter associated with antigen processing (TAP) delivers cytosolic peptides into the endoplasmic reticulum (ER) where they bind to nascent class I histocompatibility molecules. Class I–peptide complexes are then displayed at the cell surface for recognition by cytotoxic T lymphocytes. Immunoprecipitation of either TAP or class I molecules revealed an association between the transporter and diverse class I products. TAP bound preferentially to heterodimers of the class I heavy chain and β_2-microglobulin, and the complex subsequently dissociated in parallel with transport of class I molecules from the ER to the Golgi apparatus. The TAP–class I complexes could also be dissociated in vitro by the addition of class I–binding peptides. The association of class I molecules with TAP likely promotes efficient capture of peptides before their exposure to the lumen of the ER.

Class I molecules of the major histocompatibility complex (MHC) consist of a polymorphic transmembrane heavy chain (~45 kD), a soluble subunit termed β_2-microglobulin (β_2M, ~12 kD), and a peptide ligand of eight to ten residues (*1–3*). Peptide ligands are derived predominantly from proteins degraded in the cytosol (*4*), and they must be transported into the lumen of the ER and possibly the cis-Golgi for binding to newly synthesized class I molecules (*5, 6*). Peptide transport is accomplished by the TAP transporter, a heterodimer consisting of the subunits TAP1 and TAP2 (*7, 8*). TAP1 and TAP2 are encoded by genes located in the MHC. Each consists of a transmembrane segment that spans the membrane six to eight times and a proposed cytosolic domain containing an adenosine triphosphate (ATP)–binding cassette (*9*). The TAP1 protein is located on the membranes of the ER and cis-Golgi (*10*). Direct assays of TAP-mediated peptide transport into the ER with the use of either intact microsomes or permeabilized cells reveal that peptides ranging in length from about 8 to 15 residues are transported and some sequence preferences have been noted (*11–13*). Unless bound to a specific class I molecule, peptide ligands for class I are not detectable in cells, which suggests that they may be degraded rapidly in the cytosol and probably also in the ER (*3, 13*). This raises the question of how newly synthesized class I molecules can be efficiently loaded with peptide in the face of other processes competing for peptide, such as rapid degradation, dilution after transport into the ER lumen, and binding to other ER proteins such as the molecular chaperone, BiP (*14*). Presumably, these problems would be minimized if class I molecules and TAP were associated.

Initially, an association between TAP and class I was sought by immunoprecipitating one of these proteins from digitonin-solubilized cells and then testing for the presence of the other protein in the immunoprecipitate by immunoblotting. When lysates from mouse EL4 (*b* haplotype),

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