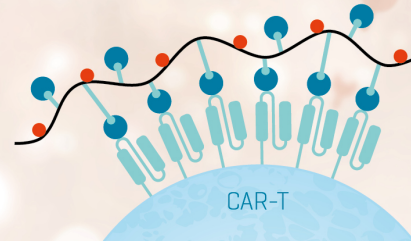


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# Regulation of Immunoproteasome Subunit Expression In Vivo Following Pathogenic Fungal Infection<sup>1</sup>

Lance F. Barton,\* Miguel Cruz,\*<sup>§</sup> Reshma Rangwala,\* George S. Deepe, Jr.,<sup>‡</sup> and John J. Monaco<sup>2\*†</sup>

The proteasome catalytic  $\beta$  subunits LMP2, LMP7, and MECL-1 and two proteasome activator proteins, PA28  $\alpha$  and  $\beta$ , are induced following exposure to IFN- $\gamma$  in vitro. Induction of these immunosubunits and the PA28  $\alpha/\beta$  hetero-oligomer alters proteasome catalytic functions and specificity and enhances production of certain MHC class I epitopes. We sought to determine whether and to what extent proteasome subunit composition is regulated in vivo and to elucidate the mechanisms of such regulation. We analyzed basal expression levels of these inducible genes in normal, IFN- $\gamma$ -deficient, and Stat-1-deficient mice. Mice of all three genotypes display constitutive expression of the immunosubunits and PA28, demonstrating that basal expression in vivo is independent of endogenous IFN- $\gamma$  production. However, basal expression levels are reduced in Stat-1<sup>-/-</sup> mice, demonstrating a role for Stat-1 independent of IFN- $\gamma$  signaling. To demonstrate that IFN- $\gamma$  can induce these genes in vivo, mice were infected with *Histoplasma capsulatum*. Elevated expression of these genes followed the same time course as IFN- $\gamma$  expression in infected mice. IFN- $\gamma$ -deficient mice did not display elevated protein expression following infection, suggesting that other inflammatory cytokines produced in infected mice are unable to influence proteasome expression. Cytokines other than IFN- $\gamma$  also failed to influence proteasome gene expression in vitro in cell lines that had no basal expression of LMP2, LMP7, or MECL-1. Thus, both in vitro and in vivo data demonstrate that IFN- $\gamma$  is essential for up-regulation, but not constitutive expression, of immunoproteasome subunits in mice. *The Journal of Immunology*, 2002, 169: 3046–3052.

The proteasome is a large multicatalytic proteinase responsible for degrading intracellular proteins in eukaryotes. This barrel-shaped enzyme is composed of 28 subunits assembled into four rings of seven subunits in an  $\alpha_7\beta_7\beta_7\alpha_7$  arrangement (1, 2). Subunits  $\beta_1$ ,  $\beta_2$ , and  $\beta_5$  contain active site threonine residues that enable them to function as N-terminal nucleophile hydrolases (3, 4). The three corresponding catalytic activities have been called peptidyl glutamyl-peptide hydrolyzing-like, tryptic-like, and chymotryptic-like, respectively, based on their activity against certain fluorogenic peptide substrates in vitro. (4, 5). These catalytic sites of the proteasome are only accessible via the core of the molecule, making the enzyme a tightly regulated, self-compartmentalized protease. Proteasomes account for up to 1% of the total cellular protein content and are responsible for performing numerous essential functions inside the cytosol and nucleus of a cell (6, 7). Proteasomes play a role in removing abnormal or improperly assembled proteins, regulating levels of transcription factors and signal transduction factors, as well as regulating cell cycle progression (1, 8–10). Furthermore, the proteasome is responsible for generating a large portion of the peptide epitopes to bind MHC class I molecules for Ag presentation to CD8<sup>+</sup> T cells.

The three ubiquitous catalytic  $\beta$  subunits,  $\delta$ , X, and Z, are expressed constitutively, while the other three, LMP2, LMP7, and MECL-1, are IFN- $\gamma$ -inducible immunosubunits or  $\beta_i$  (2, 11–15). In cells stimulated with IFN- $\gamma$ , LMP2 is incorporated into newly synthesized proteasomes as  $\beta_{1i}$  (in place of  $\delta$ ), LMP7 as  $\beta_{5i}$  (in place of X), and MECL-1 as  $\beta_{2i}$  (in place of Z) to produce so-called immunoproteasomes (16, 17). It is believed that immunoproteasomes have different substrate affinities and catalytic activities that both broaden and enhance the generation of MHC class I epitopes generated by proteasomes (18–22). IFN- $\gamma$  also induces the expression of MHC class I and II molecules, the TAP transporter proteins, and the PA28  $\alpha/\beta$  11S proteasome activator proteins, all of which may enhance immune function (2, 23, 24).

It remains unclear whether any cytokines other than IFN- $\gamma$  can influence proteasome gene expression. TNF- $\alpha$  has been reported to increase mRNA expression of TAP-1 and MHC class I, although less efficiently than IFN- $\gamma$  (25), and it has been suggested that TNF- $\alpha$  can up-regulate the LMP2 gene, either alone or synergistically with IFN- $\gamma$  (26–28). However, TNF- $\alpha$  had no effect on the expression of these genes in other studies (17, 29), suggesting that its effects may be cell type or species specific or dependent on other variables. Moreover, there exists no evidence that any other cytokines, such as IL-1 $\beta$ , TGF- $\beta$ 1, or IL-4, significantly impact the expression of these IFN- $\gamma$ -inducible genes (25). IFN- $\gamma$  itself is well characterized, and much is known about its signaling pathway. IFN- $\gamma$  binds to its receptor at the cell surface, activating the phosphorylation of Stat-1 by Janus kinases. Phosphorylated Stat-1 forms homodimers and translocates to the nucleus, where it activates transcription of genes containing an IFN- $\gamma$ -activated sequence element (GAS).<sup>3</sup> The inducible subunits contain GAS elements in their promoters and are regulated this way by IFN- $\gamma$  (17,

\*Department of Molecular Genetics, Biochemistry, and Microbiology, <sup>†</sup>Howard Hughes Medical Institute, and <sup>‡</sup>Division of Infectious Diseases, Department of Medicine, University of Cincinnati, Cincinnati, OH 45267; and <sup>§</sup>Hospital de Especialidades, Centro Medico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Mexico City, Mexico

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<sup>2</sup> Address correspondence and reprint requests to Dr. John J. Monaco, Department of Molecular Genetics, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, OH 45267-0524. E-mail address: monacojj@ucmail.uc.edu

<sup>3</sup> Abbreviations used in this paper: GAS, IFN- $\gamma$ -activated sequence element; Hc, *Histoplasma capsulatum*; HEV, high endothelial venule; IRF-1, IFN- $\gamma$  regulatory factor 1; m, mouse.

25, 30–34). Functional activities of this signaling pathway have been dissected by the use of genetically targeted mice. Both IFN- $\gamma^{-/-}$  mice and Stat-1 $^{-/-}$  mice exhibit an impaired ability to clear viral and bacterial infections (35–39). Likewise, another transcription factor induced by IFN- $\gamma$  is the IFN- $\gamma$  regulatory factor 1 (IRF-1), which has been implicated in modulating expression of a variety of cytokines and immune-related genes. IRF-1 $^{-/-}$  mice also display an abnormal ability to clear viral infections and a reduced number of CD8 $^{+}$  T cells (40–42). Consistent with the known functions of IFN- $\gamma$ , IFN- $\gamma^{-/-}$  mice show reduced expression of MHC class II and defective NK cell and macrophage functions (36).

Previous experiments (with one exception, see *Discussion*) demonstrating IFN- $\gamma$  control of immunoproteasome expression have all been performed *in vitro* by treating cells in culture with high concentrations of cytokine. Moreover, we originally demonstrated immunoproteasome expression in a variety of untreated cell lines in culture (43), suggesting that constitutive expression, at least in certain tissues, may be cytokine independent, or that IFN- $\gamma$  is not the only signal capable of inducing immunosubunit expression. However, comparable experiments *in vivo* have not been reported. We addressed these issues using *Histoplasma capsulatum* (Hc) infection, which stimulates IFN- $\gamma$  production *in vivo* (44), as a model system to monitor immunosubunit expression in normal and in gene-targeted mice defective for the IFN- $\gamma$  signaling pathway. By examining mice deficient in IFN- $\gamma$  or Stat-1 we could dissect the IFN- $\gamma$  signaling pathway to determine what controls the constitutive expression of the immunosubunits *in vivo*. We found that Hc infection induces the expression of immunoproteasomes coordinately with IFN- $\gamma$  expression. Furthermore, the induction of immunoproteasome expression in Hc-infected mice is IFN- $\gamma$  dependent, and we found no evidence for other cytokine signals capable of affecting immunosubunit expression *in vitro* or *in vivo*. In contrast, constitutive expression of immunoproteasomes is only partially dependent on components of the IFN- $\gamma$  signaling pathway (i.e., Stat-1), but does not require IFN- $\gamma$  itself. We conclude that constitutive expression involves both Stat-1-dependent and Stat-1-independent mechanisms, independent of an IFN- $\gamma$  stimulus.

## Materials and Methods

### Cell culture

H6 mouse hepatoma cells were grown in RPMI 1640 supplemented with 10% FBS, 50  $\mu$ M 2-ME, and antibiotics. Murine high endothelial venule (HEV) cells were grown in RPMI supplemented with 20% FBS, 10 mM sodium bicarbonate, and antibiotics (45). To investigate gene expression in these cell lines, cells were exposed to one of the following recombinant cytokines for 72 h at 37°C: 10–100 U/ml mouse (m)IFN- $\gamma$  (Roche, Indianapolis, IN), 100 U/ml mL-1 $\beta$  (Genzyme, Cambridge, MA), 100 U/ml mTNF- $\alpha$  (Life Technologies, Gaithersburg, MD), 500 U/ml mL-4 (Life Technologies), 1000 U/ml mL-6 (Genzyme), 1–10 ng/ml human TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN), 1–4 ng/ml mGM-CSF (Genzyme), or 0.5–2 ng/ml mL-3 (R&D Systems).

### Mice

C57BL/6 (B6) mice were either bred at University of Cincinnati (Cincinnati, OH) or purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-Irfng<sup>tm1TS</sup> (IFN- $\gamma^{-/-}$ ) mice were purchased from The Jackson Laboratory, and 129S6/SvEv-stat<sup>tm1</sup> (Stat-1 $^{-/-}$ ) mice were purchased from Taconic Farms (Germantown, NY).

### Infections

B6 or IFN- $\gamma^{-/-}$  mice were sedated with isoflurane and infected intranasally with  $2 \times 10^6$  live Hc cells in HBSS (Life Technologies) (44). The mice were kept in specific pathogen-free barrier facilities for the indicated period of time. At the indicated time point, the mice were euthanized, and the indicated organs were removed, rinsed in HBSS, and immediately frozen in liquid nitrogen.

### Quantitative Western blotting

Frozen organs were pulverized in a frozen mortar and pestle on dry ice. The ground organ powder was then lysed in 0.5% Nonidet P-40 in TBS, pH 7.0, in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) for 30 min on ice. The total lysate was harvested following a 20-min spin at 8,000  $\times$  g to pellet the insoluble debris. Cultured cells were counted, pelleted, and lysed in the same manner. Either  $2 \times 10^6$  cell equivalents or 100  $\mu$ g total lysate was separated on an 11% SDS-PAGE gel and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were blocked with a 5% dry milk solution in TBS and 0.1% Tween 20 (TBST) and rinsed before incubating with the primary Ab. Abs against the proteasome  $\alpha$  subunit C9 (1/5,000), the  $\beta$  subunits LMP2 (1/3,000), LMP7 (1/3,000), MECL-1 (1/2,000), and  $\Delta$  (1/5,000) and the proteasome activators PA28 $\alpha$  (1/3,000) and PA28 $\beta$  (1/2,500) have been previously characterized (16, 46–48). The secondary Ab was a goat anti-rabbit IgG conjugated to alkaline phosphatase (1/10,000) from the Vistra developing kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The blots were developed using the Vistra ECF method and were scanned with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). All quantitation was completed with the ImageQuant software package (Molecular Dynamics).

### Flow cytometry

H6 and HEV cells ( $2 \times 10^6$ ) were resuspended in PBS/0.3% FBS for staining. The cells were labeled with primary Ab mAb 34-1-2 (anti-MHC class I) (49) for 60 min on ice (50). Cells were subsequently incubated with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) as a secondary Ab, fixed in 2% paraformaldehyde, and analyzed by FACScan flow cytometry (BD Biosciences, Mountain View, CA).

## Results

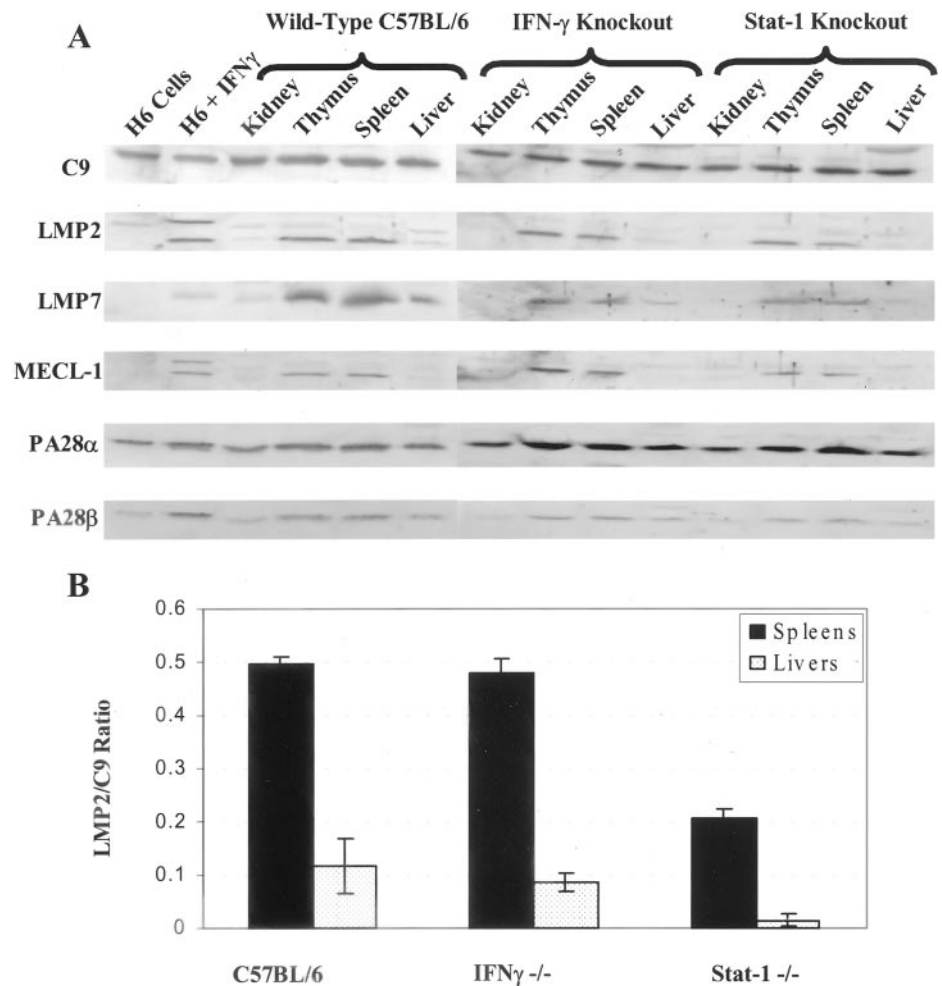
### Constitutive expression of IFN- $\gamma$ -inducible genes occurs independently of IFN- $\gamma$

Most mouse tissues and their derived cell lines express low levels of the inducible proteasome subunits. To determine whether this basal immunosubunit expression is dependent on a low level constant IFN- $\gamma$  signal or is a result of true constitutive expression without a dependence on any IFN- $\gamma$  signal (or some combination of the two), 8-wk-old mice from three genetically defined mouse strains were analyzed. C57BL/6 mice were used as a control strain, while IFN- $\gamma$ -deficient and Stat-1-deficient mice were used to examine the role of the IFN- $\gamma$  signaling pathway in the expression of these genes. Kidney, thymus, spleen, and liver lysates from all three strains were examined by Western blot analysis for the expression of LMP2, LMP7, MECL-1, PA28 $\alpha$ , and PA28 $\beta$  (Fig. 1A). The proteasome  $\alpha$  subunit, C9, which is present in all proteasome particles, was used to control for total proteasome expression levels. Both IFN- $\gamma^{-/-}$  mice and Stat-1 $^{-/-}$  mice demonstrate immunosubunit expression, demonstrating that constitutive expression does not require an IFN- $\gamma$  stimulus. However, quantitation of the Western blots in Fig. 1 demonstrates a partial dependence of constitutive expression on Stat-1. The expression levels of both LMP2 and LMP7 (normalized to C9) in IFN- $\gamma^{-/-}$  mice were equivalent to those seen in wild-type B6 mice, while both immunosubunits were expressed at markedly (2- to 3-fold) reduced levels in Stat-1 $^{-/-}$  mice compared with control animals (Fig. 1B).

### Hc induces LMP2 and LMP7 expression *in vivo*

*In vitro*, the inducible subunits are up-regulated following exposure to IFN- $\gamma$  (10, 17, 51). These studies were all performed in cell culture where soluble IFN levels are generally high. Thus, it is unclear whether these genes are controlled by IFN- $\gamma$  *in vivo* in the same manner. To address this question, we needed a system that would generate high levels of IFN- $\gamma$  *in vivo*. IL-12 is known to be a potent inducer of IFN- $\gamma$  secretion and has been shown to increase serum IFN- $\gamma$  concentrations when administered to mice (52). However, we were unable to induce significantly higher levels of

**FIGURE 1.** Western blot analysis of tissue-specific proteasome subunit expression in C57BL/6, IFN- $\gamma^{-/-}$ , and Stat-1 $^{-/-}$  mice. **A**, Each lane was loaded with 100  $\mu$ g of the corresponding 0.5% Nonidet P-40 organ lysate, electrophoresed on an 11% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane. Primary Abs used for protein detection are specific for the proteasome  $\alpha$  subunit C9; the  $\beta$  subunits LMP2, LMP7, and MECL-1; and the activators PA28 $\alpha$  and PA28 $\beta$ . H6 mouse hepatoma cells before or after exposure to IFN- $\gamma$  were used as negative and positive controls for immunosubunit expression, respectively. The faint band between the m.w. of the precursor protein (upper band only present in IFN- $\gamma$ -treated cells) and the mature protein (lower band) visible in the anti-LMP2 and anti-MECL-1 blots is nonspecific background. The blots were developed using an alkaline phosphatase detection kit and were scanned with a Storm PhosphorImager. RNA expression levels were also confirmed by Northern analysis (data not shown). At least three independent animals were tested for each tissue/Ab combination, and representative blots are shown. **B**, The Western blots were quantitated using ImageQuant software. The LMP2 and LMP7 values were normalized against C9 expression. All experiments and quantitations were performed in triplicate. The LMP2/C9 ratio is displayed  $\pm$  SE. LMP7/C9 ratios (not shown) were essentially identical to LMP2/C9 ratios.



the immunosubunits in 6- to 8-wk-old C57BL/6 mice with single i.p. injections of 50 ng rIL-12 or 100  $\mu$ g *Escherichia coli* LPS (Fig. 2).

Intracellular pathogens, particularly viral infections, are known to be strong signals for IFN production. Hc is an intracellular pathogenic fungus that survives inside macrophages (44, 53, 54), and it has also been demonstrated that IFN- $\gamma$  production in mice infected with Hc is essential for clearance of infection and survival (55). We therefore examined immunosubunit expression in mice infected with  $2 \times 10^6$  live Hc cells and sacrificed 7–12 days later, during the peak of IFN- $\gamma$  production (44). Elevated expression of LMP2, LMP7, and MECL-1 is evident in these animals (Fig. 2 and data not shown). The ratio of LMP2/ $\delta$  was used to quantitate the incorporation of LMP2 into proteasomes (these two subunits occupy the same position in the proteasome  $\beta$  ring in a mutually exclusive manner). In the livers of infected mice the ratio was raised from the basal level observed in naive mice of  $\sim 0.1$  to a ratio of  $>2.0$  in infected animals (Fig. 2). In other words, LMP2 is incorporated into  $<10\%$  of liver proteasomes in normal animals, but into almost 70% of liver proteasomes in infected animals. Therefore, naturally occurring infections, which induce IFN- $\gamma$ , can dramatically induce immunoproteasome expression in vivo.

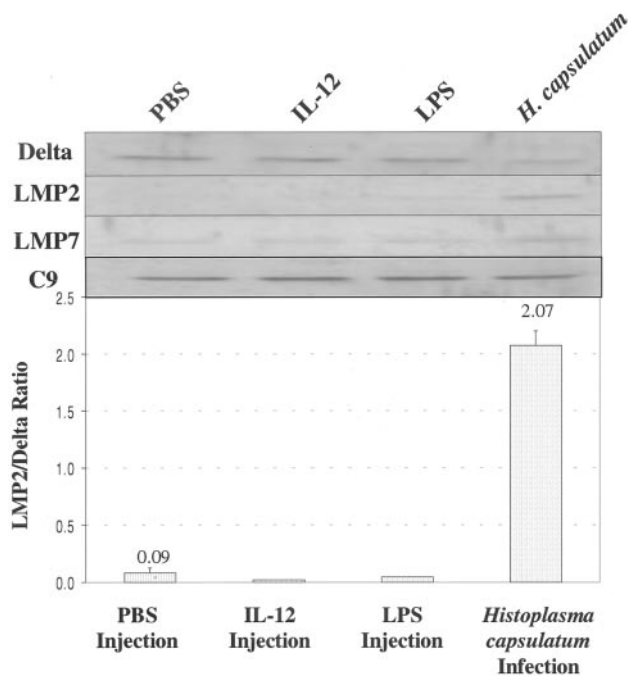
#### Immunosubunit expression follows the IFN- $\gamma$ expression time course in Hc-infected animals

Hc infection causes a series of interwoven complex cellular and physiological events, including the production and release of numerous cytokines and chemokines in addition to IFN- $\gamma$  (44, 54). To address the correlation between immunoproteasome expression

and IFN- $\gamma$  production, mice were infected with Hc and sacrificed over a time course that correlates with the rise and fall of IFN- $\gamma$  production. Following infection with  $2 \times 10^6$  organisms, IFN- $\gamma$  and IL-12 mRNA expression in the lung is enhanced by about day 3 and continues to increase from days 5–10. Peak expression usually occurs between 7 and 10 days, and mRNA levels have already declined by day 14 (44). Therefore, infected animals were sacrificed and examined on days 4, 7, 10, 13, and 19. Lungs, livers, and spleens were harvested from each animal and analyzed by Western blot. This enables examination of the primary site of infection (lung), a secondary site of infection (liver), and an immune organ (spleen). Following infection, live Hc organisms can be cultured from all three organs (55).

Tissue lysates were examined for both LMP2 and  $\delta$ , so that an LMP2/ $\delta$  ratio could be calculated for each organ for each time point. Although these ratios do not necessarily reflect the actual concentration of the immunoproteasomes in the organ itself (because two different Abs are used), this technique is still valid for comparative purposes. Moreover, in other experiments (not shown) the ratios of these two subunits in purified proteasomes from normal spleen and liver, determined by Coomassie blue staining, correlated very closely with those observed here by Western blot. In the uninfected spleen the LMP2/ $\delta$  ratio was generally between 1 and 2 (i.e., 50–60% immunoproteasomes) in C57BL/6 mice (Fig. 3A). This ratio was essentially unchanged on day 4 after infection. However, by day 7 the ratio increased to a peak of nearly 5.0 and then gradually dropped from day 7 to day 19. However, the





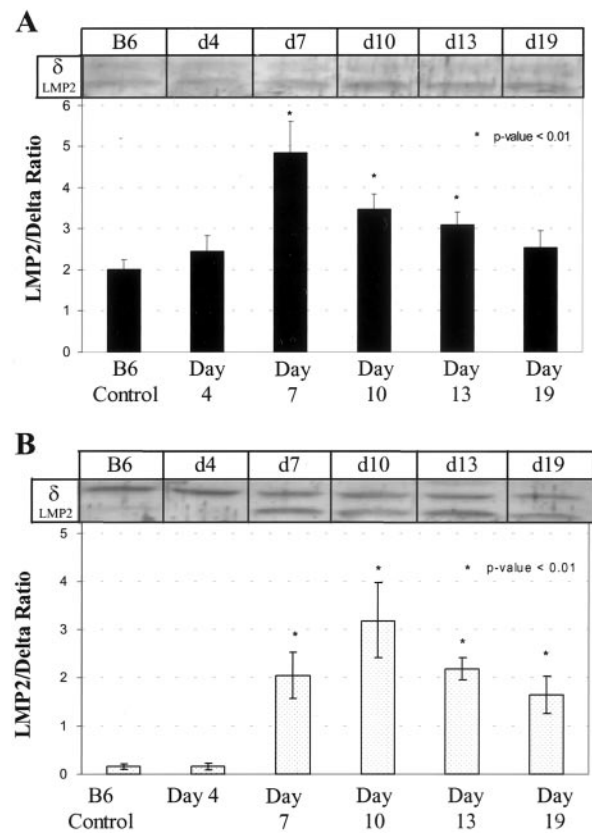
**FIGURE 2.** Quantitative Western blot analysis of mice treated to induce IFN- $\gamma$  expression in vivo. C57BL/6 mice were injected i.p. with either 50 ng rIL-12, 100  $\mu$ g *E. coli* O55:B5 LPS, or 100  $\mu$ l sterile PBS as a control. Mice injected with IL-12 received three sequential daily treatments and were harvested 48 h after the last injection. Mice injected with LPS were sacrificed 96 h after a single injection. Alternatively, mice were infected intranasally with  $2 \times 10^6$  Hc cells and sacrificed 7–14 days later. Lysates and Western blots for LPM2, LMP7, C9, and  $\delta$  expression were performed as described in *Materials and Methods*. All Abs were screened against heat-killed *Histoplasma* Nonidet P-40 lysates to verify that no cross-reactivity existed (data not shown). The bands were quantitated using ImageQuant software and the LMP2/ $\delta$  ratio was calculated as a measurement of immunoproteasome induction.

level of immunoproteasomes remained significantly elevated over control levels ( $p < 0.01$  by Student's *t* test) from days 7–13.

Results in the liver were very similar to those in the spleen. The LMP2/ $\delta$  ratio in livers of normal healthy mice was  $\sim 0.1$  (Fig. 3B). This ratio was largely unchanged on day 4 after infection and was elevated on day 7, but did not peak until day 10, when it reached a value of  $>3.0$ . In contrast to spleen, the LMP2/ $\delta$  ratio remained statistically significantly elevated over control levels through day 19, possibly due to the slower arrival of the infectious organism in the liver and its persistence there. In the lung basal and peak expression levels were similar to those in the liver, but the time course displayed a peak closer to day 7 (data not shown). Therefore, we concluded that not only does Hc infection induce immunoproteasome expression in vivo, but also the time course of elevated LMP2 protein expression closely mimics the kinetics of the IFN- $\gamma$  response in these animals.

#### *Hc* infection cannot induce LMP2 expression in IFN- $\gamma^{-/-}$ mice

The production of IFN- $\gamma$  is essential for mice to clear Hc infections. Mice depleted of IFN- $\gamma$  and infected with Hc survive no longer than 14 days (55). However, since peak expression of IFN- $\gamma$  and elevated expression of LMP2 both occur on day 7, it is possible to look at this early time point in IFN- $\gamma^{-/-}$  mice. Four IFN- $\gamma^{-/-}$  mice were each infected with  $2 \times 10^6$  Hc cells and sacrificed on day 7 following infection. Lungs, livers, and spleens were harvested from each mouse and analyzed by Western blot. Following quantitation, LMP2/ $\delta$  ratios were calculated for each

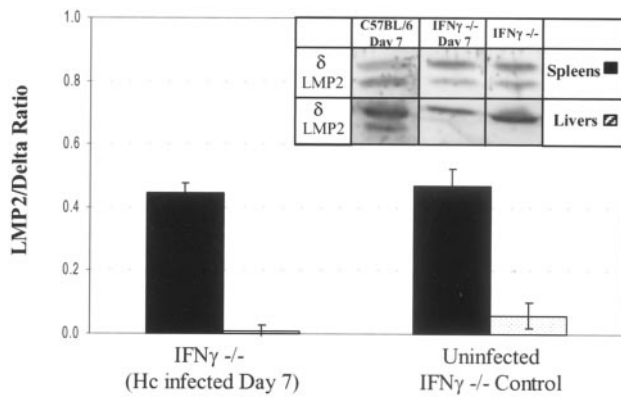


**FIGURE 3.** Immunoproteasome up-regulation in C57BL/6 mice infected with Hc. C57BL/6 mice were infected with  $2 \times 10^6$  Hc cells intranasally. Total spleen (A), liver (B) and lung (not shown) lysates were generated, and Western blots were performed. Data for each time point are representative of three independent mice. The inset shows a representative blot of the spleen lysates. Each band was quantified and normalized, and an LMP2/ $\delta$  ratio was calculated for each sample. Student's *t* test was used to verify statistical significance.

organ and compared with those in control animals. In all three organs of infected IFN- $\gamma^{-/-}$  mice, there was no detectable increase in LMP2 expression and no significant change in the LMP2/ $\delta$  ratio (Fig. 4).

#### *In vitro* control of immunoproteasome expression is driven solely by IFN- $\gamma$

Recent evidence has demonstrated TNF- $\alpha$ -mediated up-regulation of LMP2, LMP7, MECL-1, PA28 $\alpha$ , and TAP in human tumor cell lines (27). Other evidence in human cells indicates that not only can TNF- $\alpha$  up-regulate LMP7 expression, but it also acts synergistically with IFN- $\gamma$  (26). Since TNF- $\alpha$  and other proinflammatory cytokines, such as GM-CSF, are also important in Hc infections, it was necessary to investigate whether any other cytokines, particularly proinflammatory cytokines, might be playing a role in our in vivo model (54). Three cell lines that showed little or no constitutive expression of any of the immunosubunits (Fig. 5A) were chosen for this analysis. Each line was treated with a panel of cytokines, and immunoproteasome expression was analyzed by Western blot (Fig. 5B). Only IFN- $\gamma$  was able to induce the expression of LMP2, LMP7, or MECL-1 in any of the three cell lines. Not only was TNF- $\alpha$  unable to induce the expression of the inducible subunits alone, but attempts to show synergism with IFN- $\gamma$  were likewise unsuccessful (data not shown). TNF- $\alpha$  was able to up-regulate surface MHC class I expression in these cell lines, as analyzed by FACS analysis (Fig. 5C) (25), although it was not as



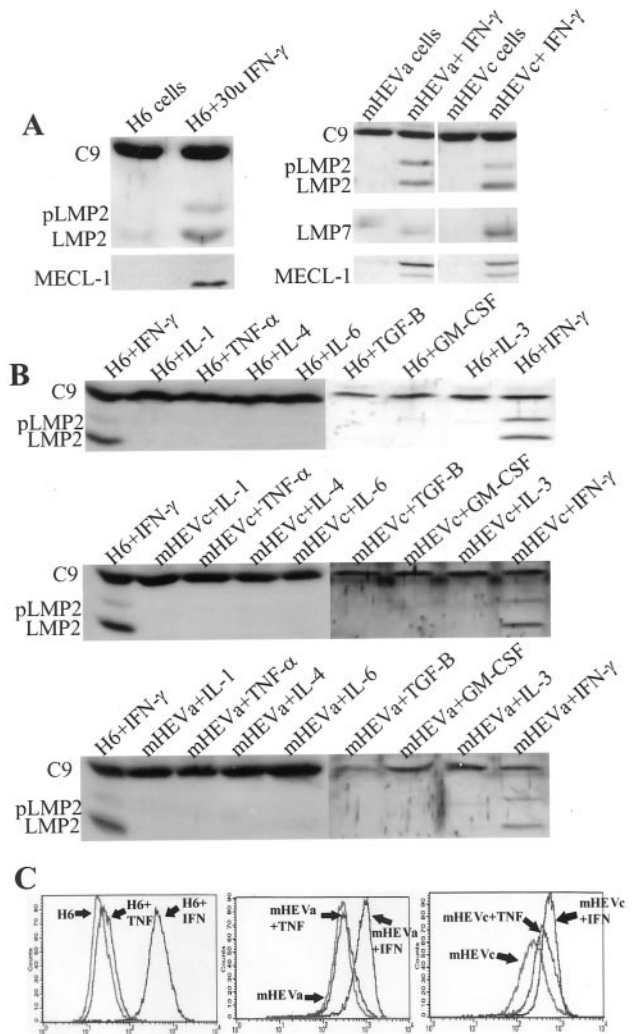
**FIGURE 4.** Hc cannot induce immunoproteasome expression in IFN- $\gamma$ <sup>-/-</sup> mice. C57BL/6 or IFN- $\gamma$ <sup>-/-</sup> mice were infected with  $2 \times 10^6$  Hc cells intranasally and sacrificed after 7 days. Spleens, livers, and lungs were harvested, and Nonidet P-40 lysates were generated for each organ. All lysates were analyzed by Western blot as described (see inset) and compared with lysates from uninfected IFN- $\gamma$ <sup>-/-</sup> mice. The blots were quantitated with ImageQuant software. LMP2/ $\delta$  ratios were calculated for each sample and are plotted  $\pm$ SE. As witnessed in Fig. 3, the LMP2/ $\delta$  ratio was increased to  $\sim$ 5.0 in C57BL/6 control spleens and to 2.0 in C57BL/6 control livers (not shown due to scale). Lungs displayed similar results (data not shown).

effective as IFN- $\gamma$ . Nonetheless, this experiment demonstrates that the cells are responsive to TNF, ruling out a trivial explanation for the lack of immunosubunit induction by this cytokine. We conclude that IFN- $\gamma$  is the only cytokine that can significantly modulate mouse immunoproteasome expression in vivo or in vitro.

## Discussion

In this study we examined constitutive and induced expression of the IFN- $\gamma$  inducible  $\beta$  subunits of the proteasome. C57BL/6 mice basally express immunoproteasomes, containing LMP2, LMP7, and MECL-1, as  $\sim$ 10% of the total proteasome content in the liver and  $\sim$ 50% in spleen. We sought to determine whether this basal expression is truly constitutive (i.e., independent of external signaling requirements), or is the result of constant, low level signaling by cytokines in vivo. We found that basal expression levels of the immunosubunits in IFN- $\gamma$ <sup>-/-</sup> mice are equivalent to those in wild-type animals. Taken together with our subsequent data indicating that IFN- $\gamma$  appears to be the major, if not the only, cytokine capable of significantly influencing the expression of these genes, these results support the existence of true, signal-independent constitutive expression. This conclusion is also consistent with the near ubiquitous basal expression seen previously in cultured cell lines (43). While such expression in vitro may have been the result of autocrine stimulation, its presence in a wide variety of tissue types, particularly in tissues that do not produce IFN- $\gamma$ , makes this possibility unlikely.

In light of normal expression levels in IFN- $\gamma$ <sup>-/-</sup> mice, we were surprised to find that basal expression levels are significantly (2- to 3-fold) reduced in Stat-1<sup>-/-</sup> animals, although this observation is consistent with a previous report of reduced LMP2 mRNA levels in these animals (56). These data may be explained by the recently published observation that unphosphorylated Stat-1 and IRF-1 complexes occupy GAS sequences in the LMP2 promoter (57). We conclude that constitutive expression of the immunosubunits is independent of IFN- $\gamma$  signaling (and, hence, independent of phosphorylated Stat-1), but at least partially dependent on nonphosphorylated Stat-1. The observation that at least some immunosubunit expression is still found in the Stat-1<sup>-/-</sup> animals further



**FIGURE 5.** Expression of Ag-processing genes following cytokine exposure in vitro. The H6 mouse hepatoma cell line, mouse axillary HEV (HEVa) cells, and mouse cervical HEV (HEVc) cells were used to examine induction of immunoproteasome expression in vitro. **A**, These cell lines were chosen because they do not express the immunosubunits constitutively, but do retain high inducibility in the presence of IFN- $\gamma$ . **B**, Cells were exposed to one of the following recombinant cytokines for 72 h and then harvested to make a Nonidet P-40 lysate: 10–100 U/ml mIFN- $\gamma$ , 100 U/ml mL-1 $\beta$ , 100 U/ml mTNF- $\alpha$ , 500 U/ml mL-4, 1000 U/ml mL-6, 1 ng/ml human TGF- $\beta$ 1, 1 ng/ml mGM-CSF, or 1 ng/ml mL-3. Western blotting was performed as described above on  $2 \times 10^6$  cell equivalents/lane. **C**, H6, HEVa, and HEVc cells were all exposed to either IFN- $\gamma$  or TNF- $\alpha$  as described above and then incubated with the MHC class I Ab 34-1-2. A secondary FITC-conjugated goat anti-mouse IgG Ab was used to visualize the MHC-Ab complexes. The samples were analyzed with a FACSscan (BD Biosciences).

indicates that Stat-1 enhances, but is not absolutely essential for, basal expression.

If IFN- $\gamma$  plays no role in constitutive immunosubunit expression in vivo, does it play any role in immunosubunit expression in vivo? With only one recent exception, published during the preparation of this manuscript (58), previous studies demonstrating induction of immunosubunit expression by IFN- $\gamma$  were performed exclusively on immortalized cell lines in vitro. To investigate more physiological IFN- $\gamma$  effects, we chose to examine immunosubunit expression in mice infected with Hc, a dimorphic fungus endemic to the Ohio River valley that survives inside host macrophages

following lung infection (54). We found that Hc infection dramatically enhances immunoproteasome expression in C57BL/6 mice (Fig. 2). Moreover, the kinetics of enhanced immunosubunit expression in vivo correlated well with IFN- $\gamma$  production in the infected animals, peaking on day 7 in lung and spleen and on day 10 in liver, and then dropping back to almost basal levels by day 19 following infection.

These results demonstrate that immunoproteasome levels can be dramatically affected by infection in vivo and further suggest that IFN- $\gamma$  production is directly responsible for the up-regulation of immunoproteasome expression in these animals. However, Hc infection does induce the expression of numerous other proinflammatory cytokines, such as TNF- $\alpha$  and GM-CSF, with similar kinetics (54). We therefore performed a similar set of experiments in IFN- $\gamma^{-/-}$  mice, which rapidly succumb to the normally sublethal infection, usually within 10 days. Following Hc infection, IFN- $\gamma^{-/-}$  mice do not up-regulate immunoproteasome expression in the spleen, liver, or lung (Fig. 4), demonstrating that IFN- $\gamma$  production is essential for the up-regulation of immunoproteasome expression in vivo. Moreover, the results suggest that no other cytokines produced during Hc infection are capable of significantly altering immunoproteasome expression in vivo.

To further examine this issue, we tested the ability of other proinflammatory cytokines to induce the expression of immunoproteasomes in vitro. We examined cytokines known to be induced by Hc infection as well as cytokines previously implicated in regulating MHC class I-related gene expression. We found that none of the cytokines tested (except IFN- $\gamma$ ) was able to induce immunoproteasome expression in any of three cell lines (Fig. 5B). We conclude that in vivo, following infection with Hc, IFN- $\gamma$  is the sole mediator of the observed induced expression of LMP2, LMP7, and MECL-1.

It is also important to discuss one other interesting finding of these experiments. Immunosubunits cannot exchange with constitutive subunits in preformed proteasomes, but, rather, require de novo synthesis of new proteasomes (10, 14, 15). The previously reported half-life of liver proteasomes in vivo is  $\sim 2$  wk (6). Following Hc infection, the concentration of immunoproteasomes rapidly increased within 1 wk and decreased thereafter. This finding suggests that the turnover of proteasomes following Hc infection is significantly faster than the previously reported half-life. Khan et al. (58) also reported similar rapid kinetics of immunoproteasome turnover in vivo following LCMV and *Listeria* infection. It seems unlikely that this effect is solely the result of IFN- $\gamma$  stimulation, since we did not observe enhanced proteasome turnover in mouse cell lines treated with IFN- $\gamma$ , and we observed similar half-lives for constitutive proteasomes and immunoproteasomes (59). Interestingly, proteasome half-life measurements for a mouse liver (hepatoma) cell line in vitro are on the order of 2 days (59), although this may be more closely related to the faster proliferation rate of cell lines in vitro compared with normal tissue in vivo. It is possible that some aspect of the infection process, perhaps exposure to a cytokine other than IFN- $\gamma$ , is responsible for accelerated proteasome turnover rates. Alternatively, normal turnover rates in mouse liver (which have never been measured directly) may be significantly faster than those reported for rat liver proteasomes.

In summary, the results of this study demonstrate that constitutive expression of immunoproteasomes in vivo is IFN- $\gamma$  independent and uses both Stat-1-dependent and Stat-1-independent mechanisms. However, immunoproteasome expression is dramatically up-regulated in vivo following infection, and this expression is completely IFN- $\gamma$  dependent. These results are largely in agreement with the proteasome regulation observed during viral and

bacterial infection in reports published during the preparation of this manuscript (58). We demonstrate similar kinetics and a dependence upon IFN- $\gamma$ . Our fungal infection model system is a Th1-mediated immune response rather than a CTL-driven response, which may account for the (relatively minor) quantitative differences in induction between these two studies.

Finally, we have demonstrated that the replacement of constitutive proteasomes with immunoproteasomes occurs outside of the primary site of infection (lung). The magnitude of this replacement is considerable; the liver proteasome pool changes from  $\sim 10\%$  immunoproteasomes/90% constitutive proteasomes to  $\sim 70\%$  immunoproteasomes/30% constitutive proteasomes. This dramatic effect on proteasome subunit composition may profoundly influence the quality and quantity of peptide epitopes presented to T cells during infection.

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