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*J Immunol* 2010; 184:4115-4122; Prepublished online 12 March 2010; doi: 10.4049/jimmunol.0903003
http://www.jimmunol.org/content/184/8/4115

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Unexpected Role for the Immunoproteasome Subunit LMP2 in Antiviral Humoral and Innate Immune Responses

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Proteasomes are multisubunit proteases that initiate degradation of many Ags presented by MHC class I molecules. Vertebrates express alternate forms of each of the three catalytic proteasome subunits: standard subunits, and immunosubunits, which are constitutively expressed by APCs and are induced in other cell types by exposure to cytokines. The assembly of mixed proteasomes containing standard subunits and immunosubunits is regulated in a tissue specific manner. In this study, we report that the presence of mixed proteasomes in immune cells in LMP2−/− mice compromises multiple components that contribute to the generation of antiviral Ab responses, including splenic B cell numbers, survival and function of adoptively transferred B cells, Th cell function, and dendritic cell secretion of IL-6, TNF-α, IL-1β, and type I IFNs. These defects did not result from compromised overall protein degradation, rather they were associated with altered NF-κB activity. These findings demonstrate an important role for immunoproteasomes in immune cell function beyond their contribution to Ag processing.


Materials and Methods

Mice and Viruses

C57BL/6 mice were purchased from Taconic (Germantown, NY) and CD45.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). LMP2−/− were originally obtained from Luc Van Kaer (Vanderbilt Medical Center, Nashville, TN), who backcrossed them 10 times, and then bred at Taconic. LMP2−/− mice were bred at the Cincinnati Children’s Hospital Medical Center. Mice were bred at the Cincinnati Children’s Hospital Medical Center. Mice were

Received for publication September 10, 2009. Accepted for publication February 7, 2010.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (R21 AI073384) and the Australian National Health and Medical Research Council (ID 433608). Address correspondence and reprint requests to Dr. Jonathan W. Yewdell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 33, Room 2E13, 33 North Drive, Bethesda, MD 20892. E-mail address: jywedd1@nih.gov

Abbreviations used in this paper: DC, dendritic cell; HAI, hemagglutination inhibition; IAV, influenza A virus; WT, wild type.

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roteasomes are abundant complex proteases that initiate degradation of full-length proteins and play a critical role in the generation of many MHC class I-associated peptides (1). Ag presentation is a nonessential proteasome function added during vertebrate evolution. Proteasomes are essential components of lower eukaryotes and archaeobacteria that function to initiate degradation of full-length proteins that are defective or deemed disposable by cellular control mechanisms. Although proteasomes typically generate peptides of 3–30 residues that are rapidly disposed of by endopeptidases and exopeptidases (2), there are several well-defined examples of proteasomes regulating protein function by liberating biologically active fragments. Proteasomal processing regulates NF-κB, Spt23p, and Mga2p transcription factors (3, 4).

Proteasomes consist of a four-ring structure with dyad symmetry of the form αββα (5). Fourteen distinct subunits assemble to generate seven subunit α and β rings. Only three of the 14 subunits (β1, β2, β5; also referred to as ΔZ, and X), are known to possess proteolytic activities. In vertebrates, each catalytic subunit is encoded by two genes. One set of genes is constitutively expressed by most cell types. The other set, termed immunosubunits (β1i, β2i, β5i; nominally LMP2, MECL-1, LMP7 in mice), are coordinately expressed in dendritic cells (DCs) and monocyte-derived cells, where they assemble to form immunoproteasomes (6, 7). Immunoproteasomes are also expressed by most cell types after exposure to interferons, where they supplement and eventually replace standard proteasomes. Immunoproteasomes are upregulated by viruses that induce high levels of interferons, such as hepatitis B virus (8, 9) and lymphocytic choriomeningitis virus (10).

Immunoproteasome assembly is intricately coordinated; the generation of “mixed” proteasomes with standard subunits and immunosubunits is under tissue-specific control (11–14). The codependent incorporation of LMP2 and MECL1 suggests that mixed proteasomes with ΔMEC1 or LMP2/Z may be detrimental in certain cell types. Standard and immunoproteasomes generate an overlapping repertoire with differences in cleavage at both N- and COOH-termini (15), and there are numerous examples of defined peptides whose generation is enhanced by immunoproteasomes (16). Based on these findings, the favored hypothesis is that immunoproteasomes principally function to optimally generate peptides for immunosurveillance. There are, however, a few findings that undermine this hypothesis. A number of defined determinants are presented more efficiently following knockout of LMP2 or LMP7 (16). T cells lacking one or more of the immunosubunits demonstrate altered proliferation upon activation by cognate Ags (17) or mitogens (18) and differ in homeostatic proliferation (19). Based on these findings, it has been proposed that major functions of immunoproteasomes are unrelated to Ag processing (20, 21). To explore potential alternative functions of immunoproteasomes, we characterized antiviral innate and humoral immune responses in immunoproteasome-deficient mice.
maintained under specific pathogen-free conditions and all procedures involving mice were approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases. Influenza A virus (IAV; A/Puerto Rico/8/34) was propagated in 10-d-old embryonated chicken eggs. For all vaccinations, the virus was first inactivated by treating with paraformaldehyde (1:864 dilution) for 2 d at 4°C, and then ~100 hemagglutination units of inactivated IAV was injected i.p. At several time points after vaccination, mice were retro-orbitally bled, and RBCs were removed using serum-gel tubes (Sarstedt, Newton, NC). Serum was inactivated by incubating at 56°C for 30 min.

Hemagglutination inhibition titrations

Hemagglutination inhibition (HAI) titrations were performed in 96-well round polystyrene plates (Corning, Corning, NY). Serial 2-fold dilutions of sera in 100 μl PBS were mixed with four agglutinating doses of IAV in 100 μl of PBS. Next, 25 μl of a 2% (v/v) human RBC solution was added, and the reaction was allowed to proceed for 60 min at room temperature. The plates were held perpendicularly to the ground, and HAI was observed as the agglutinated RBCs formed streaks in each well. HAI titers are expressed as the reciprocal of the highest Ab dilution that was able to inhibit four agglutinating doses of virus in a total volume of 225 μl.

IAV ELISAs

Flat bottom microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated with ~75 hemagglutination units of IAV per well in a 0.1 M sodium carbonate coating solution (BD Biosciences, San Jose, CA) overnight at 4°C and then blocked with PBS containing 10% PBS for 2 h at room temperature. Mouse sera was then added and incubated for 2 h at room temperature. Next, rabbit (IgG) anti-mouse secondary Abs of different isotypes (Calbiochem, San Diego, CA) were added and incubated for 1 h at room temperature, and then HRP conjugated goat anti-rabbit IgG (Calbiochem) was added and incubated for 1 h at room temperature. Finally, tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and then 1 M H₃PO₄ was quickly added to stop the reaction. OD was read at 450 nm. In between each step of the anti-IAV ELISAs, the plates were washed with PBS-Tween (0.05%).

Cell phenotyping

Spleens and bone marrow was isolated, single cell suspensions were created, and ACK lysing buffer (BioWhittaker, Walkersville, MD) was added to lyse RBCs. The cells were then passed through a 70-μm nylon cell strainer (BD Falcon, San Jose, CA) and washed twice with balanced salt solution containing 0.1% BSA. Cells were incubated with different combinations of fluorescently labeled Abs (eBiosciences, San Diego, CA and BD Pharmingen, San Jose, CA) and samples were analyzed using an LSR II machine (BD Biosciences).

Lymphocyte Transfer Studies

Splenocytes were isolated as described above, and CD4⁺ T cells and B cells were purified by negative selection using commercially available kits (Miltenyi Biotec, Auburn, CA). For some experiments, APCs (CD11c⁺ and CD11b⁺) were removed from splenocytes preparations after staining the cells with anti-CD11c FITC and anti-CD11b FITC and then using a commercially available kit (Miltenyi Biotec) that selects FITC-negative cells. Mice were irradiated with ~800 rad; 6 h later, purified cell populations that were ~83–98% pure were injected i.v. through the tail vein; 1.5 × 10⁶ cells were injected for APC-depletion studies, and 8 × 10⁶ B cells and 3 × 10⁷ CD4⁺ T cells were injected for fractionated splenocytes studies. For all studies, equal numbers of C57BL/6 and LMP2⁻/⁻ cells were injected. Two hours later, mice were injected i.p. with ~100 hemagglutination units of inactivated IAV, and anti-IAV Abs were determined at several time points as described above. For experiments involving CD45.1 mice, we did not inject IAV, but instead isolated the spleens from these irradiated mice 7 d after transferring cells. For these experiments, single cell suspensions were prepared as described above and cells were stained using Abs against CD45.1 and CD45.2 (eBiosciences) and analyzed on an LSR II flow cytometer (BD Biosciences).

Lymphocyte proliferation assays

Proliferation assays were performed as described previously (18). CD4⁺ T cells and B cells were purified by negative selection as described above and plated in RPMI 1640 with 10% FCS in flat bottom polystyrene plates (Corning). For B cell assays, 1 × 10⁶ cells per well were plated; for CD4⁺
T cell assays, 2 × 10^3 cells per well were plated. Various amounts of Con A or LPS were added to CD4^+ T cells or B cells, respectively. The cells were pulsed with [3H]thymidine 2 d later and [3H] incorporation was determined 1 d after pulsing.

**DC experiments**

Bone marrow-derived DCs were cultured for 10 d in RPMI 1640 containing 20 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and then reseeded at 2.5 × 10^6 cells per well in round-bottom polystyrene plates (Corning). IAV was added to the cells, and supernatant was collected 18 h later. Cytokine levels in supernatants were measured by Luminox technology (Bio-Rad, Hercules, CA) and by an IFN-α ELISA that was performed using a previously described protocol (22).

**Immunoblots**

B cells were purified by negative or positive selection using magnetic beads. For some experiments, cells were treated with LPS and then removed from culture and resuspended in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM sodium pyrophosphate, 25 mM β-glycerolphosphate, 5 mM NEM, 1% NP-40) and incubated on ice for 30 min. Nucleoli and insoluble material were pelleted by centrifugation, and the resulting supernatants were mixed with SDS-PAGE buffer (Quality Biologicals, Gaithersburg, MD) and run on a 12% SDS-PAGE gel followed by blotting onto nitrocellulose. For polyubiquitin and immunoproteasome subunit staining, total cell lysates were prepared. Blots were probed with rabbit polyclonal anti-IAA (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-actin (Abcam, Cambridge, MA) Abs or with mouse monoclonal anti-polyubiquitin (clone FK2; BIOMOL, Plymouth Meeting, PA) Ab followed by infrared dye coupled secondary Abs. For immunoproteasome subunit expression studies, blots were probed with rabbit anti-LMP2 (Abcam), rabbit anti-LMP7 (Abcam), or rabbit anti-MECL1 (BIOMOL), as well as a GAPDH loading control Ab (Abcam). Blots were analyzed using an Odyssey imaging system (LI-COR, Lincoln, NE) or a STORM phosphorimager (GE Healthcare Bio-Sciences, Uppsala, Sweden).

**Proteasome purification**

Cells were homogenized in ice cold homogenization buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 25 mM KCl, 0.2 M sucrose, 0.5% NP-40, 100 μg/ml CHX, EDTA-free protease inhibitors [Roche, Basel, Switzerland], 10 U/ml RNase Out [Invitrogen, Carlsbad, CA], diethyl pyrocarbonate water), and the lysate was centrifuged at 20,000 × g for 10 min at 4°C. The cleared lysate was layered on a 15–50% sucrose gradient in the same buffer. After centrifugation at 35,000 rpm (Beckman, SW41 Ti) for 2.5 h at 4°C, the gradient was fractionated. Each fraction was pelleted to concentrate proteins and analyzed by immunoblotting as described above.

**Results**

**Mixed proteasomes inhibit B cell development**

Immunoproteasomes are constitutively expressed in professional APCs, such as DCs (23), and other cell types, such as T cells (24). Immunoblotting revealed that B cells purified from the spleen express immunoproteasomes (Fig. 1). To determine whether immunoproteasome expression is required for maintaining homeostatic levels of resting B cells, we enumerated B cells in spleens isolated from immunoproteasome-deficient and wild type (WT; C57BL/6) mice. Consistent with previous studies (25, 18), immunoproteasome-deficient mice had fewer CD4^+ T cells compared with WT mice (Fig. 2A), an observation originally attributed to aberrant thymic selection. Although we found that most strains of immunoproteasome-deficient mice posses normal numbers of B cells, LMP2^−/− mice had dramatically reduced numbers of splenic B cells compared with WT mice (Fig. 2A). Further analysis revealed that transitional type 1 (T1) and T2 B cells were underrepresented in the spleens of LMP2^−/− mice (Fig. 2B), yet normal numbers of pro B, pre B, and immature B cells were present in the bone marrow of these mice (Fig. 2C). The defect in mature B cells in LMP2^−/− mice is not simply caused by the absence of LMP2, because LMP2^−/− MECL1^−/− mice have normal numbers of B cells (Fig. 2A). Rather, this effect is likely due to the expression of the mixed proteasomes (11, 12). Immunoblotting of sucrose gradient fractions from LMP2^−/− B cell lysates revealed that these cells express ΔMECL1/LMP7 mixed proteasomes, as inferred from their sedimentation characteristics (Fig. 3). It is possible that other types of mixed proteasomes exist in these mice as well. Thus, LMP2^−/− mice provide a unique opportunity to study why vertebrates evolved stringent mechanisms to coordinate the assembly of immunoproteasomes.

**Overexpression of mixed proteasomes leads to impaired anti-IAV Ab responses**

To determine whether mixed proteasomes alter the generation of primary antiviral Ab responses, we immunized LMP2^−/− mice with inactivated IAV and determined anti-IAV serum titers by HAI. HAI titers were reduced more than 2-fold in LMP2^−/− mice at early and late time points after vaccination (Fig. 4A). A closer examination of the anti-IAV Ab response by ELISA revealed that LMP2^−/− mice were able to mount normal IgM responses but failed to isotype switch (Fig. 4B, 4C).

A logical explanation of why LMP2^−/− mice mount defective IAV Ab responses is that these mice possess reduced numbers of B cells. It is also possible that mixed proteasome expression alters the functions of mature B cells (and/or helper CD4^+ T cells). To characterize the contribution of B cells, CD4^+ T cells, and APCs to diminished Ab responses in LMP2^−/− mice, we transferred equal numbers of WT or LMP2^−/− splenic lymphocytes (i.e., splenocytes

![FIGURE 4.](http://www.jimmunol.org/)}

Mixed proteasomes negatively affect antiviral Ab responses. Following vaccination with IAV, Ab responses were determined by HAI titers (A) and by ELISA (B, C; n = 10–12 mice per group). Data are representative of four independent experiments. Shown is mean ± SEM. *p < 0.05.
depleted of APCs) into irradiated WT recipients before immunization with inactivated IAV. Transfer of WT lymphocytes into WT mice resulted in strong Ab responses that involved delayed but robust Ig class switching (Fig. 5A, 5C, 5E). Strikingly, mice receiving LMP2−/− lymphocytes mounted undetectable (by HAI) to barely detectable (by ELISA) Ab responses.

In the same experiment, we performed adoptive transfers in LMP2−/− mice. Mice receiving LMP2−/− lymphocytes again generated weak Ab responses, whereas mice receiving WT lymphocytes generated Ab responses that failed to properly class switch (wt → wt, LMP2−/− → LMP2−/−), responses were weaker than in nonmanipulated mice. This is likely due to limitations in lymphocytes homing to the proper anatomical regions as well as radiation-induced damage in lymphatic organs that interferes with the immune response.

CD4+ T cells are required for the generation of fully functional IAV Ab responses in mice (26, 27), although weak responses (with H chain class switching) can occur in the absence of CD4+ T cells (28, 29). To determine the extent to which intact immunoproteasome expression in B cells versus CD4+ T cells is required for optimal Ab responses, we repeated the adoptive transfer experiment using purified B and CD4+ T cells from WT and LMP2−/− mice in all permutations (Fig. 6). Transfer of B and CD4+ T cells from the same donor completely recapitulated the observations obtained with unfractionated lymphocyte transfers. WT B cells transferred with LMP2−/− CD4+ T cells were able to generate early IAV Ab responses in both WT and LMP2−/− recipients, but these responses were transient. By contrast, all other permutations resulted in weak responses.

These data indicate that expression of mixed proteasomes in multiple cell types leads to the reduction of anti-IAV responses in LMP2−/− mice. Notably, mixed proteasome expression in B cells appears to greatly affect Ab responses, because miniscule Ab responses were generated in WT mice when LMP2−/− B cells were cotransferred with WT CD4+ T cells.

**Mixed proteasome expression affects lymphocyte function**

To further characterize the deleterious effects of mixed proteasomes on B and CD4+ T cell function, we measured the ability of LMP2−/− cells to proliferate after activation with their respective mitogens, LPS or Con A (Fig. 7A, 7B). LMP2 knockout did not affect proliferation of either cell type, as measured by incorporation of [3H]thymidine. We previously reported that LMP2−/− CD8+ T cells are immunodominated in anti-IAV responses by WT CD8+ T cells in adoptive transfer experiments (17, 24). Based on this finding and our observation that naive LMP2−/− mice have dramatically decreased numbers of B cells (Fig. 2), we hypothesized that LMP2−/− cells have a survival disadvantage compared with WT cells. To test this idea, we transferred WT or LMP2−/− lymphocytes into irradiated CD45.1+ congenic mice and enumerated WT or LMP2−/− lymphocytes, which express CD45.2, present in spleens 7 d later. Approximately 3-fold as many WT lymphocytes were recovered under these conditions (Fig. 7C). These findings indicate that mixed

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**FIGURE 5.** LMP2−/− lymphocytes are defective. CD11c/CD11b depleted C57BL/6 or LMP2−/− spleenocytes were transferred into sublethally irradiated C57BL/6 or LMP2−/− recipient mice. The mice were then vaccinated with IAV and Ab titers were determined by HAI (A, B) and ELISA (C–F; n = 3 mice per group). Data are mean ± SEM.
proteasome expression does not have a gross effect on lymphocyte proliferative capacity, but may affect the ability of lymphocytes to home to, survive, or homeostatically proliferate in the spleen.

**Mixed proteasomes expression affects DC activation**

The adoptive transfer experiments described above implicated that mixed proteasomes expressed in radiation resistant cells contribute to impaired Ab responses, because after transfer of WT lymphocytes, LMP2−/− recipients generated weaker Ab responses compared with WT recipients. A trivial explanation for this finding is that WT lymphocytes were rejected by LMP2−/− recipients. We eliminated this possibility by showing that 7 d after transferring congenic WT CD45.1+ lymphocytes into irradiated mice, there were more transferred cells in LMP2−/− versus WT recipients (Fig. 8A), although this difference did not reach statistical significance. One explanation for the poor Ab response mounted by LMP2−/− recipients is that they exhibit defective innate immune responses, because early production of cytokines, such as IL-6 and type I IFNs, enhance Ab responses and promote isotype switching (30, 31). Could LMP2−/− mice simply possess lower numbers of innate immune cells? This was not the case, as equal numbers of DCs, macrophages, and neutrophils are present in spleens of wt and LMP2−/− mice (Fig. 8B). To examine the intriguing possibility that mixed proteasome expression alters cytokine production by innate immune cells, we infected WT and LMP2−/− bone marrow-derived DCs with IAV. Each of four cytokines measured after 18 h in culture, IFN-α, IL-6, IL-1β, and TNF-α, were present at significantly lower levels in supernatants from LMP2−/− versus WT DCs (Fig. 8C–F).

**Mixed proteasomes alter NF-κB activation**

How might mixed proteasomes modify B cell and DC function? Proteasomes are responsible for most protein degradation, and compromising proteasome function has potent effects on cell physiology (32). Activated B cells are particularly sensitive to limitations in proteasome function, presumably owing to increased numbers of DRiPs associated with the massive increase in Ig synthesis (33, 34). To examine the effect of mixed proteasome expression on overall proteasome function, we measured levels of polyubiquitylated proteins, which are known to accumulate when proteasome degradation is limiting. As seen in Fig. 9, there was no significant difference in the levels of polyubiquitylated proteins in activated B cells in LMP2−/− versus WT mice. Proteasomes also play a critical role in regulating transcription factors, such as NF-κB, Spt23p, and Mga2p (3, 4, 35). Following IκB degradation by standard proteasomes, NF-κB translocates to the nucleus, rapidly activating subsets of genes, many of which are involved in immune activation. Because both lymphocyte survival and DC activation require NF-κB activation, we examined the effect of mixed proteasome expression on NF-κB activation. We stimulated purified LMP2−/− or WT B cells with LPS and measured IκBα degradation by immunoblotting. Consistent with the hypothesis that mixed proteasomes impair NF-κB activation, IκBα degradation in...
LMP2<sup>−/−</sup> cells was delayed and less complete than in WT cells (p < 0.05 at 60 min following LPS; Fig. 10).

Our findings indicate that mixed proteasomes present in LMP2<sup>−/−</sup> mice compromise virtually every parameter of the immune response against IAV, potentially through dysregulation of signaling pathways, such as the NF-κB pathway. The highly coordinated assembly of immunoproteasomes might be necessary to prevent immunologic chaos that can result from altered signaling pathways.

Discussion
It has long been known that splenic cells express immunoproteasomes in preference to standard proteasomes (6, 7). Inasmuch as B cells constitute a large fraction of splenocytes, it is hardly surprising that B cells—like T cells, DCs, and monocytes—preferentially express immunoproteasomes. Because B and T cells probably play limited roles in activating afferent CD8<sup>+</sup> T cells, it is logical that the major function of immunoproteasomes in lymphocytes is unrelated to generation of class I peptide ligands.
One of the mysterious features of immunoproteasomes is that their composition is limited to a small subset of the possible permutations of immunosubunits and standard subunits in some cell types. One possibility is that certain types of mixed proteasomes compromise cellular function. In this study, we show that mixed proteasomes expressed in LMP2<sup>−/−</sup> mice decrease lymphocyte survival and reduce cytokine production by DCs. These defects are quantified using an Odyssey imaging system.

The involvement of immunoproteasomes in NF-κB activation is controversial (36, 37). Our studies suggest that mixed proteasomes are associated with impaired NF-κB signaling in LMP2<sup>−/−</sup> cells.

The dysfunction of immunoproteasomes in NF-κB activation is not limited to the LMP2<sup>−/−</sup> mice. It is entirely possible, even likely, that other signaling components are impaired in cells that express mixed proteasomes. Consistent with this possibility, it was recently reported that cardiac LMP2 expression is required for the activation of the protein kinase AKT (38).

Several recent studies suggest that immunoproteasomes are particularly adept at degrading damaged proteins (39–41). Of particular relevance is the finding that oxidized proteins are increased in the liver and brain of LMP2<sup>−/−</sup> mice (39). Resting lymphocytes are nearly inert metabolically and are unlikely to challenge proteosomal capacity. After activation, however, they divide at a rate unparalleled among vertebrate cells. Although it is possible that the defect in LMP2<sup>−/−</sup> cells is associated with a decreased ability to degrade damaged proteins, we do not find altered levels of ubiquitylated proteins in stimulated LMP2<sup>−/−</sup> cells.

Our findings demonstrate that LMP2<sup>−/−</sup> mice display severe defects in B cell and DC function, limiting their use in studying the contribution of immunoproteasomes to Ag processing. The myriad dysfunctions associated with the expression of mixed proteasomes provide a ready explanation for their low abundance in some types of WT cells. It will be of great interest in future studies to delineate the unique function performed by immunoproteasomes in cells of immune lineage.

**Acknowledgments**

We thank the staff of the Comparative Medical Branch of the National Institute of Allergy and Infectious Diseases for assistance with animal studies, Luc Van Kaer for LMP2<sup>−/−</sup> mice, Glennys Reynoso for technical assistance, and Dietrich Conze for expert NFXB advice.

**Disclosures**

The authors have no financial conflicts of interest.

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