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Immunoproteasomes Are Essential for Clearance of *Listeria monocytogenes* in Nonlymphoid Tissues but Not for Induction of Bacteria-Specific CD8+ T Cells

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Microbial infections induce the replacement of constitutive proteasomes by immunoproteasomes (I-proteasomes). I-proteasomes support efficient generation of MHC class I epitopes and influence immunodominance hierarchies of CD8+ T cells. Recently, the function of I-proteasomes in antimicrobial responses was challenged by showing that the lack of I-proteasomes has no effect on induction and function of lymphocytic choriomeningitis virus-specific CD8+ T cells. Here, we show that infection with *Listeria monocytogenes* rapidly induces I-proteasomes in nonlymphoid tissues, which leads to enhanced generation of protection relevant CD8+ T cell epitopes. I-proteasome-deficient mice (β5i−/− mice) exhibited normal frequencies of *L. monocytogenes*-specific CD8+ T cells. However, clearance of *L. monocytogenes* in liver but not spleen was significantly impaired in I-proteasome-deficient mice. In summary, our studies demonstrate that induction of I-proteasomes is required for CD8+ T cell-mediated elimination of *L. monocytogenes* from nonlymphoid but not lymphoid tissues. *The Journal of Immunology*, 2006, 177: 6238–6244.

The majority of MHC class I epitopes are generated by proteasomes, which appear in two major forms, either as constitutive proteasomes or as IFN-γ-inducible immunoproteasomes (I-proteasomes) (1, 2). According to current concepts, MHC class I epitope generation is strongly enhanced in the presence of I-proteasomes, which is thought to be beneficial for triggering an effective antimicrobial CD8+ T cell-mediated immune response (3). Although I-proteasomes are constitutively expressed in lymphoid tissues such as spleen and thymus, they can be induced in nonlymphoid tissues as a consequence of infection (4–7).

IFN-γ-dependent replacement of the constitutive catalytic subunits β1 (Y), β2 (Z), and β5 (X) by the corresponding immunoproteasome subunits β1i (Lmp2), β2i (Mec1i), and β5i (Lmp7) has been shown to augment the liberation of viral epitopes and to strongly enhance antiviral CD8+ T cell immune response in vitro (8–11). However, the biological relevance of I-proteasomes in vivo still remains controversial. Studies with β5i-deficient mice, which are virtually I-proteasome deficient, revealed normal numbers of CD8+ T cells despite reduced surface expression of H-2Db and H-2Kb MHC class I alleles (12). Virus infection of β1i-deficient mice suggested that I-proteasomes shape the immunodominance hierarchies of CD8+ T cell responses against influenza virus by altering Ag processing and therefore the T cell repertoire (13). Thus, none of the I-proteasome-deficient mice reflected the pronounced effects during antiviral immune responses that were expected from in vitro experiments. The relevance of I-proteasomes was further challenged in recent report demonstrating that I-proteasome-deficient mice infected with lymphochorial meningitis virus (LCMV) mounted normal CD8+ T cell responses and cleared LCMV with similar kinetics to wild-type (wt) mice (14). These data even suggested that, in LCMV infection, I-proteasomes are not required for CD8+ T cell-mediated protection.

To determine whether this phenomenon is unique to viral infections where protection is mediated by recognition of dominant CD8+ T cell epitopes, we further addressed the importance of I-proteasomes in the bacterial infection model of *Listeria monocytogenes*.

After i.v. infection, *L. monocytogenes* accumulates predominantly in the liver where the bacteria intracellularly replicate in parenchymal cells. By spreading directly from cell to cell, *L. monocytogenes* can avoid being exposed to professional phagocytes capable of ingesting and destroying it. Decline and sterile elimination has been attributed to stimulation of bacteria-specific CD8+ T lymphocytes, which are crucial for protection against a secondary infection with an otherwise lethal dose of *L. monocytogenes* (15, 16). In murine listeriosis, hepatocytes are the major sites of intracellular bacterial replication in the liver (17–19). Infiltrating leukocytes are attracted to the site of infection and lyse infected hepatocytes, thereby releasing *L. monocytogenes* for ingestion and destruction by macrophages and neutrophils (17, 20). Although it has been demonstrated that *L. monocytogenes*-infected hepatocytes are destroyed by CD8+ T cells in a classical MHC class Ia-restricted mechanism (21), some reports, however, have cast doubt that parenchymal tissue cells can serve as fully functional target cells.
Consequently, our aim was to analyze and to compare proteasomal processing of the H-2 K\(^b\)-restricted listeriolysin O\(\)\(\text{296-304}\) (LLO\(\text{296-304}\)) peptide in lymphoid and nonlymphoid tissues. We have used cytokine- and I-proteasome mutant mice to determine the requirements for efficient generation of the bacterial LLO\(\text{296-304}\) T cell epitope and correlated the abundance of this epitope with the proteasome structure and bacterial clearance in various tissues.

The data provide conclusive evidence that infection with \(L.\ monopolis\) triggers expression of I-proteasomes exclusively in nonlymphoid tissues and enhances generation of protection-relevant T cell epitopes, essential for bacterial clearance in nonlymphoid tissues. In contrast to LCMV infection, protection against \(L.\ monopolis\) requires induction of I-proteasomes to allow efficient recognition of infected, parenchymal target cells by CD8\(^+\) T cells.

### Materials and Methods

#### Animals

Mice strains C57BL/6, TNFR\(^{-/-}\) (p55/p75), IFN-\(\gamma\)-R\(^{-/-}\), 129/OLA, and L129/IS\(^{-/-}\) were kept under specific pathogen-free conditions at the animal facilities of the Max-Planck Institute for Infection Biology (Berlin, Germany), and experiments were conducted according to the German animal protection law.

#### Proteasome purification from infected mice

20S proteasomes were isolated from liver, colon, spleen, and small intestine of WT C57BL/6 mice as well as of IFN-\(\gamma\)-R\(^{-/-}\), TNFR\(^{-/-}\), and B\(\beta\)\(5\)\(^{-/-}\) (L129 background) mice. 20S proteasomes were also prepared from isolated hepatocytes of wt C57BL/6 mice. At days 0 and 2 after i.v. infection with \(5 \times 10^6\) CFU of \(L.\ monopolis\), animals were sacrificed and organs were frozen in liquid nitrogen. For IFN-\(\gamma\)-R\(^{-/-}\) and wt mice, organs were also harvested at days 4 and 6, respectively. Further purification steps followed as described (6).

#### Two-dimensional gel electrophoresis (2-DE)

For resolution of 20S proteasomal subunits, nonequilibrium pH gel electrophoresis with carrier ampholytes was combined with SDS-PAGE as described by guest on April 29, 2017 http://www.jimmunol.org/Downloaded from (22). Individual spots were identified by MALDI-TOF-mass spectrometry (MS) (23). Each 2-DE was repeated three times.

#### In vitro peptide digest

To determine proteasome-mediated processing of a synthetic peptide, 3 \(\mu\)g of a 27-mer derived from LLO (LLO\(\text{291-317}\)) (AYISSVAYGRQV YLKLTSTNSHTSTKVA) was incubated at 37°C for 2–4 h with 1 \(\mu\)g of purified 20S proteasomes in 100 \(\mu\)l of buffer containing HEPESS/KOH (pH 7.8), 2 mM magnesium-aceatate, and 2 mM DTT. Reactions were stopped by adding trifluoroacetic acid to a final concentration of 0.1%.

#### Peptide identification, quantification, and statistical analysis

Digestion products were identified and quantified by liquid chromatogra TYPO-electrospray ionization-ion trap MS (LC-ESI-MS) according to Ref. 9. For statistical analysis, ion counts of each reaction were normalized to an internal standard, a peptide (YPHFMPTNLGPS), which was added in a 1:1000 dilution to the samples. Resulting data were analyzed using FCS Express 2 software.

### Results

#### Infection induces proteasome immunosubunits primarily in nonlymphoid tissues

The tissue-specific impact of \(L.\ monopolis\) infection on subunit composition of 20S proteasome was analyzed by 2-DE (Fig. 1, A and B) and immunoblot analysis (E and F). Bacteria induced strong expression of the immunosubunits \(\beta\)\(1\), \(\beta\)\(2\), and \(\beta\)\(5\) in the liver (Fig. 1A). During the time course of infection, the relative percentage of I-proteasome subunits in the liver increased \(\sim\)3-fold from basal 20% to \(\sim\)60% at day 6 postinfection (p.i.) (Fig. 1C). In contrast, small intestines exhibited constitutive high levels of I-proteasomes, which did not further increase after infection (Fig. 1, B and D). Differences in the induction of proteasomal immunosubunits upon infection are also shown by immunoblot analysis. Although expression of \(\beta\)\(1\) and \(\beta\)\(2\) after infection is strongly increased in colon (Fig. 1E), the constitutive expression of I-proteasome subunits in spleen was not further enhanced (F). Therefore, infection with \(L.\ monopolis\) induces strong I-proteasome up-regulation in nonlymphoid organs, e.g., liver and colon, but has only marginal influence on constitutive I-proteasome subunit expression of lymphoid tissues, e.g., small intestine and spleen.

#### Infection alters proteasome-mediated generation of T cell epitopes

In previous investigations, we have shown that proteasomes cleave substrates in a tissue-specific manner including disease- and protection-relevant CD8\(^+\) T cell epitopes and that such in vitro processing experiments may reflect the in vivo situation (6). Here, we studied the influence of \(L.\ monopolis\) infection to generate the LLO\(\text{296-304}\) CTL epitope or potential epitope precursor peptides...
(25) from the LLO<sub>291–317</sub> polypeptide substrate by proteasomes purified from different tissues. Fig. 2A displays the main cleavage products related to epitope generation. To allow quantification of cleavage products, tissue-derived 20S proteasomes were normalized according to protein content, because adjustment based on the hydrolytic activity against fluorogenic peptide substrates strongly depends on the content of immunosubunits (26). Generation of the LLO<sub>296–304</sub> epitope and the LLO<sub>294–304</sub> precursor peptides by proteasomes derived from the liver and colon was increased 16-fold 2 days p.i. compared to naive controls (t = 0). In contrast, proteasomes derived from lymphoid tissues, e.g., spleen and small intestine, revealed consistently high epitope and precursor production during the course of infection (Fig. 2B). As previously demonstrated by Peters et al. (27), there is a linear correlation between the measured ion counts and the concentration of the LLO<sub>296–304</sub> epitope. Thus, generation of large quantities of the LLO epitope and precursor was strictly dependent on strong expression of I-proteasomes (see Fig. 1A).

**Induction and function of I-proteasomes by systemic IFN-γ cannot be compensated by other cytokines or bacterial components**

To study the effects of cytokines on induction and function of I-proteasomes, TNF-α receptor p55/p75 DKO (TNFR<sup>−/−</sup>) and IFN-γ receptor KO (IFN-γR<sub>−/−</sub>) mice were infected with L<sub>monocytogenes</sub>. In accordance with our biochemical and functional studies, infection-mediated up-regulation of immunosubunits was found in marginal zone macrophages, β2i expression was strongly induced after infection in hepatocytes and the liver and colon comparable with wt mice (data not shown). Hence, these proteasomes generated the LLO<sub>296–304</sub> epitope and precursors with comparable efficiency as proteasomes from wt mice (Fig. 3A). In contrast, infection of IFN-γR<sub>−/−</sub> mice did not enhance expression of immunosubunits and generation of the LLO<sub>296–304</sub> epitope, demonstrating IFN-γ-dependent induction of I-proteasomes in nonlymphoid tissues (Fig. 3B). To discriminate whether local or systemic IFN-γ is required for I-proteasome induction in these tissues, IFN-γ concentrations in serum and organs were measured at days 2 and 6 after infection (Fig. 3C). Despite marginal local IFN-γ concentrations and no detectable L. monocytogenes, strong induction of I-proteasomes was found in the colon. This suggests that systemic IFN-γ is sufficient to induce immunosubunits independently of the presence of bacteria.

**Infection triggers I-proteasome expression in epithelial cells and hepatocytes**

To visualize that infection primarily induces I-proteasomes in parenchymal or epithelial cells of nonlymphoid tissue, immunohistological analysis of I-proteasome subunit expression was performed in various tissues before and after infection with L. monocytogenes. In accordance with our biochemical and functional studies, infection-mediated up-regulation of the immunosubunit β2i was found in tissue sections of colon and liver, but not spleen. Although the spleen displayed constitutive high expression of the β2i immunosubunit in marginal zone macrophages, β2i expression was strongly induced after infection in hepatocytes and

**FIGURE 1.** Infection alters proteasome subunit composition in nonlymphoid organs. Purified 20S proteasomes from liver (A) and small intestine (S.I.) (B) of C57BL6 mice (wt) were analyzed at indicated time points p.i. by 2-DE and stained with Coomassie. Constitutive and corresponding I-proteasome subunits are indicated by arrows. Immunosubunit expression was evaluated densitometrically and identified by MALDI-TOF. Black bars (■) represent the percentage of the combined immunosubunits β1i, β2i, and β5i derived from different tissues. Western blots of purified proteasomes of colon (E) and spleen (F) are shown at indicated time points p.i. Loading of gels and blotting efficiency was controlled by Coomassie staining.

**FIGURE 2.** Infection increases generation of the LLO T cell epitope in nonlymphoid tissues. A. The amino acid sequence of the 27-mer peptide substrate derived from LLO (LLO<sub>291–317</sub>) and selected cleavage products by proteasomes are shown. The LLO<sub>296–304</sub> epitope (■) and LLO<sub>294–304</sub> precursor (•••) are marked. B. In vitro digestion of the LLO substrate was performed with 208 proteasomes isolated from wt liver, colon, small intestine (S.I.), and spleen at indicated time points p.i. The amount of LLO epitope and precursor was measured by LC-ESI-MS. Normalized ion counts are measured by guest on April 29, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/ Downloaded from
colonic epithelial cells (Fig. 4A). Similar results were also obtained for the β1i and β5i subunits (our unpublished data). In addition, proteasomes isolated from purified hepatocytes of L. monocytogenes-infected mice, exhibited up-regulation of all three immunosubunits (Fig. 4B), reflecting the proteasome pattern of the total organ (see Fig. 1A). After infection, hepatocyte-derived proteasomes showed an increased substrate turnover (data not shown) and enhanced generation of the LLO296–304 epitope and its precursor (Fig. 4C). In summary, our findings demonstrate that in liver and colon predominantly nonlymphoid and nonmyeloid cell types contribute to the I-proteasome phenotype.

Reduced processing of LLO296–304 and impaired bacterial clearance in β5i−/− mice

As I-proteasomes are strongly induced in epithelial cells and hepatocytes, both permissive host cells of L. monocytogenes, we wondered whether this mechanism reflects the special needs of parenchymal cells to cope with L. monocytogenes infection. We thus infected β5i−/− mice that fail to express functional I-proteasomes with L. monocytogenes. As expected, proteasomes isolated from livers and spleens of β5i−/− mice revealed reduced LLO296–304 epitope cleavage compared with wt mice (Fig. 5A). We next wondered whether the reduced epitope cleavage in β5i−/− mice influences the LLO296–304-specific CD8+ T cell frequency. Despite clear effects on epitope generation, the frequency of LLO296–304-specific CD8+ T cells was similar between wt and β5i−/− mice. This demonstrates that priming and expansion of CD8+ T cells do not require I-proteasome function (Fig. 5B). Finally, bacterial counts revealed that control of L. monocytogenes was not impaired during early immune response; however, bacterial titers were 1000-fold higher in livers of β5i−/− mice at day 10 (Fig. 5C). Thus, our experiments suggest that the amount of epitopes required for efficient priming of CD8+ T cells does not require I-proteasomes, whereas lack of I-proteasomes seriously interferes with the recognition of infected, nonprofessional target cells by CD8+ T cells.

Discussion

We here report that I-proteasomes are essential for protection against L. monocytogenes in nonlymphoid tissues. Following infection, I-proteasomes are induced in nonlymphoid tissues to increase the generation of protection-relevant CD8+ T cell epitopes. This mechanism ensures efficient Ag presentation of infected, nonlymphoid target cells. In contrast, I-proteasomes seem to be of minor importance for the function of professional APCs, because no differences in CD8+ T cell priming or control of L. monocytogenes could be observed in lymphoid organs in the I-proteasome-deficient β5i−/− mice. Accordingly, these mice revealed impaired bacterial clearance in liver but not in spleen, emphasizing the importance of I-proteasome up-regulation in the recognition of infected, nonprofessional APCs.

For a variety of viral CD8+ T cell epitopes, it has been shown that efficient processing is directly linked with the function of I-proteasomes. In contrast, down-regulation of epitope generation in the presence of I-proteasomes has also been observed for subdominant viral or tumor epitopes (9, 28, 29). However, the in vivo significance of these alterations in epitope cleavage remained controversial: Although lack of I-proteasomes alters the overall cytotoxic T cell specificity as well as the immunodominance hierarchies and the repertoire of T cells (10, 13), it was shown that I-proteasomes have only marginal impact on protection against LCMV infection (14). This finding provoked the question whether the reported functions of I-proteasomes apply only in vitro, but are of no or little relevance in vivo. Another explanation could be that infection with LCMV is not suitable to decipher the role of I-proteasomes because efficient protection against viral pathogens is linked to the emergence of CTLs directed against a few immunodominant T cell epitopes.

We therefore analyzed the role of I-proteasomes during infection with L. monocytogenes, where antibacterial CD8+ T cells are crucial for protection. In contrast to LCMV, L. monocytogenes-specific CD8+ T cell responses in the H2b-background are not directed against few strong determinants rather than a variety of weak or intermediate epitopes (25). We hypothesized that protection against L. monocytogenes is only achieved if infected tissues are able to process and present bacterial proteins above a critical threshold, mediated by I-proteasomes.

The first observation was that, despite high local and systemic IFN-γ levels, infection with L. monocytogenes did not increase the constitutive high levels of I-proteasomes in spleen and small intestine in contrast to the nonlymphatic organs liver and colon. Our experiments revealed that the content of I-proteasome subunits never exceeded 60% in the analyzed organs. This is in accordance with previous observations that aimed to artificially maximize the content of I-proteasomes in cell lines (30, 31). Because some T cell epitopes are exclusively generated by constitutive proteasomes, this finding suggests that the mixture of constitutive and I-proteasomes guarantees production of a broad spectrum of potential T cell epitopes.
cell epitopes. This might explain why assembly of I-proteasomes above a certain threshold is of no use, even in professional APC.

Although constitutive expression of I-proteasomes in lymphoid tissues appears to be essential for selection, induction, and tolerance of CD8+ T cells (7), induction of I-proteasomes in nonlymphoid tissues seems to be advantageous only during infection and inflammation. This functional difference in I-proteasome expression is reflected by different signaling requirements: Induction by infection depends on IFN-γ, whereas constitutive expression does not.

It was unexpected that induction of I-proteasomes by *L. monocytogenes* was strictly IFN-γ dependent and could not be compensated by other inflammatory cytokines or bacterial components. Although TNF-α has been shown to up-regulate β5i in human cells, either alone or synergistically with IFN-γ (32, 33), no effects on expression of these genes were found in fungal infection of mice (34). Our experiments with cytokine receptor knockout (KO) mice demonstrate that lack of TNF-α signaling does not influence the induction of I-proteasome during *L. monocytogenes* infection. Furthermore, it has been shown that stimulation of TLRs leads to induction of immunosubunits in dendritic cells (35) and is involved in the control of *L. monocytogenes* infection (36). However, our studies suggest that stimulation of TLR by bacterial components cannot induce I-proteasomes in nonlymphoid tissues.

However, of central importance are the immunological consequences of I-proteasome induction by *L. monocytogenes*. Tissue-specific proteasome function was analyzed with respect to generation of CD8+ T cell epitopes, T cell frequencies, and antibacterial protection. *L. monocytogenes* infection significantly generation of the LLO296–304 CD8+ T cell epitope in colon and liver, directly linked to strong induction of I-proteasomes in nonlymphoid tissues. In contrast, generation of T cell epitopes in lymphoid tissues was not further increased by infection, despite increased levels of IFN-γ. Similar results were also obtained for T cell epitopes of viral (MCMVpp89) and murine (hsp60) origin (data not shown). Remarkably, induction of I-proteasomes in nonlymphoid organs, enabled purified hepatocytes to process CD8+ T cell epitopes as efficiently as professional APC from lymphoid tissues. Consequently, our data support the concept that during infection hepatocytes become effective APCs (37). Two mechanisms for enhanced epitope generation could explain this effect: enhanced substrate turnover or altered cleavage site preference.

The biological significance of I-proteasome induction for antilisterial immunity in nonlymphoid tissues was demonstrated in β5i-deficient mice, which lack functional I-proteasomes (1). Interestingly, the frequencies of LLO296–304-specific CD8+ T cells did not differ between β5i+/− and wt mice. This is consistent with the finding in the LCMV model that neither the induction of antiviral CD8+ T cell responses nor the immunodominance hierarchy is altered in these mice (14). In contrast, failure of I-proteasome induction resulted in drastically impaired clearance of *L. monocytogenes* in the liver at late-stage immune response. Because early resistance was not impaired, our findings suggest that deficiency of I-proteasomes primarily affects adaptive immunity.

Combining the results from viral and bacterial infections, we propose that IFN-γ-induced I-proteasomes are not essential for priming of CD8+ T cells nor for recognition of infected professional APCs. Lymphoid cells are equipped with numerous co-stimulatory molecules and an efficient MHC class I presentation machinery, which allows sufficient peptide loading even in the absence of I-proteasomes. In contrast, parenchymal cells of nonlymphoid organs are not designed to interact with the immune system. If, however, such cells are infected, rapid elimination of the pathogen is required to avoid tissue damage. Within a short time, I-proteasome subunits are up-regulated and enable parenchymal cells to enhance MHC class I processing to temporarily act as targets for CTLs. In summary, up-regulation of I-proteasomes is essential to exceed a threshold of Ag presentation, which is necessary for immune recognition of tissue cells infected with pathogens that lack strong and dominant T cell epitopes.

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Disclosures
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