Priming of *Leishmania*-Reactive CD8$^+$ T cells *In Vivo* Does Not Require LMP7-Containing Immunoproteasomes

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TO THE EDITOR

In murine cutaneous leishmaniasis, control of infection is associated with both IFN-γ–producing CD4$^+$ Th1 and CD8$^+$ Tc1 cells. Secreted IFN-γ promotes host defense by inducing nitric oxide release from activated macrophages (MΦ). The key player for the induction of Th1/Tc1-dependent immunity in leishmaniasis is IL-12 from *Leishmania major*-infected dendritic cells (DC), but not from Mϕ (von Stebut *et al.*, 1998). Only *Leishmania*-infected DCs were capable of restimulating *L. major*-specific CD8$^+$ T cells to induce IFN-γ secretion (Belkaid *et al.*, 2002). Major histocompatibility complex (MHC) class I$^{-/-}$ mice or those depleted from CD8$^+$ T cells did not heal (Belkaid *et al.*, 2002). Thus, DC-mediated induction of *L. major*-specific CD8$^+$ T cells is absolutely required for primary immunity in murine leishmaniasis.

In general, priming of CD8$^+$ T cells is induced by presentation of peptides degraded by the proteasome and bound to MHC class I (Yewdell and Bennink, 1999; Kloetzel, 2001). The 20S core complex of the proteasome is composed of 14 nonidentical subunits, which form four stacked rings of seven different subunits each. The hydrolyzing activity is conferred by three of these seven β subunits in the two inner rings β1, β2, and β5 (Groll *et al.*, 1997). There are two different types of proteasomes: the constitutive form and the IFN-γ–inducible immunoproteasome. IFN-γ induces the synthesis of immunosubunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) all of which replace the constitutive β1, β2, and β5 subunits in the 20S core proteasome (Frentzel *et al.*, 1994). The β5i/LMP7 subunit is essential for an accelerated upregulation of the functional immunoproteasome catalytic core complex (Heink *et al.*, 2005). Constitutive proteasomes and immunoproteasomes have distinct proteolytic activities (Boes *et al.*, 1994; Eleuteri *et al.*, 1997), and the immunoproteasome can enhance the presentation of some epitopes (Schwarz *et al.*, 2000), but abrogate the presentation of others (Morel *et al.*, 2000).

Previously, it has been shown (Bertholet *et al.*, 2006) that *L. major* antigens are presented to CD8$^+$ T cells in a transporter associated with antigen processing-independent pathway. *In vitro,* antigen presentation was also not influenced by constitutive proteasomes (Bertholet *et al.*, 2006). Thus, it remains unclear how parasite antigen processing in the MHC class I pathway in DC is regulated. The Th1/Tc1-dominated immunity of resistant mouse strains (e.g., C57BL/6) as well as humans is characterized by high levels of IFN-γ. Confirmingly, we detected immunoproteasome subunit expression during *L. major* infections by western blot of infected DC *in vitro* and in draining lymph nodes (dLN) of infected C57BL/6 mice *in vivo* (Figure 1a and b) (Tenzer *et al.*, 2004). This prompted us to investigate the role of the IFN-γ–induced immunoproteasome in *L. major* infections *in vivo* using LMP7-deficient mice.

Natural transmission of *L. major* infections was mimicked experimentally by intradermal infection with ~1,000 metacyclic *L. major* promastigotes (Belkaid *et al.*, 2000). Both, LMP7$^{-/-}$ as well as wild-type mice developed comparable disease progression with regard to lesion sizes (Figure 1c). Lesion volumes in week 6 and 9 correlated with parasite burdens (Figure 1d), and the parasite numbers in infected ears did not differ between the strains. Similar levels of antigen-specific IFN-γ were secreted by dLN cells from both strains at different time points (Figure 1e). IL-10 was detected only at low levels.

Next, to investigate the ability of DC from C57BL/6 or LMP7-deficient mice to efficiently restimulate Tc1 cells, we cocultured infected DC from both mouse strains with isolated syngeneic CD8$^+$ T cells for 48 hours (Figure 2; Supplementary Figure S1 online) and determined secretion of IFN-γ. Interestingly, similar IFN-γ levels of *L. major*-specific CD8$^+$ T cells were found and additionally, T-cell proliferation did not differ significantly (Figure 2). Despite the fact that background T-cell proliferation was higher when LMP7$^{-/-}$ DC were used, a significantly increased antigen-specific T-cell proliferation was observed upon coculture with infected bone marrow–derived DC of either mouse strain. Thus, restimulation of *L. major*-specific CD8$^+$ T cells by DC is independent of the immunoproteasome.

To investigate the priming of CD8$^+$ T cells in the absence of a functional immunoproteasome *in vivo,* dLN cells from both strains were harvested at 4 weeks post infection. Proliferation was analyzed using flow cytometry. In C57BL/6 cells, an increase of the antigen-specific proliferation of CD8$^+$ T cells compared with controls was observed (Figure 2c). Similar proliferation of CD8$^+$ T cells was detected from LMP7-deficient mice after antigen-specific restimulation. In addition, we observed a comparable frequency of IFN-γ–positive CD8 T cells in the dLN as well as ear lesions of C57BL/6 and LMP7$^{-/-}$ mice (Figure 2d and e). These data indicate that priming and restimulation of CD8$^+$ T cells in LMP7$^{-/-}$

Abbreviations: DC, dendritic cells; dLN, draining lymph nodes

mice is comparable to that in immunoproteasome-competent mice.

Normally, priming of CD8$^+$ T cells for effective immune responses toward antigens requires antigen processing in a proteasome/immunoproteasome-dependent manner (Cresswell, 2000; Yewdell and Bennink, 2001). In the present study, we found that the immunoproteasome is dispensable for MHC class I-restricted CD8$^+$ T-cell priming *in vivo*. Our findings are in line with a recent paper by Bertholet *et al.* (2006) reporting that antigen presentation of *L. major* peptides to

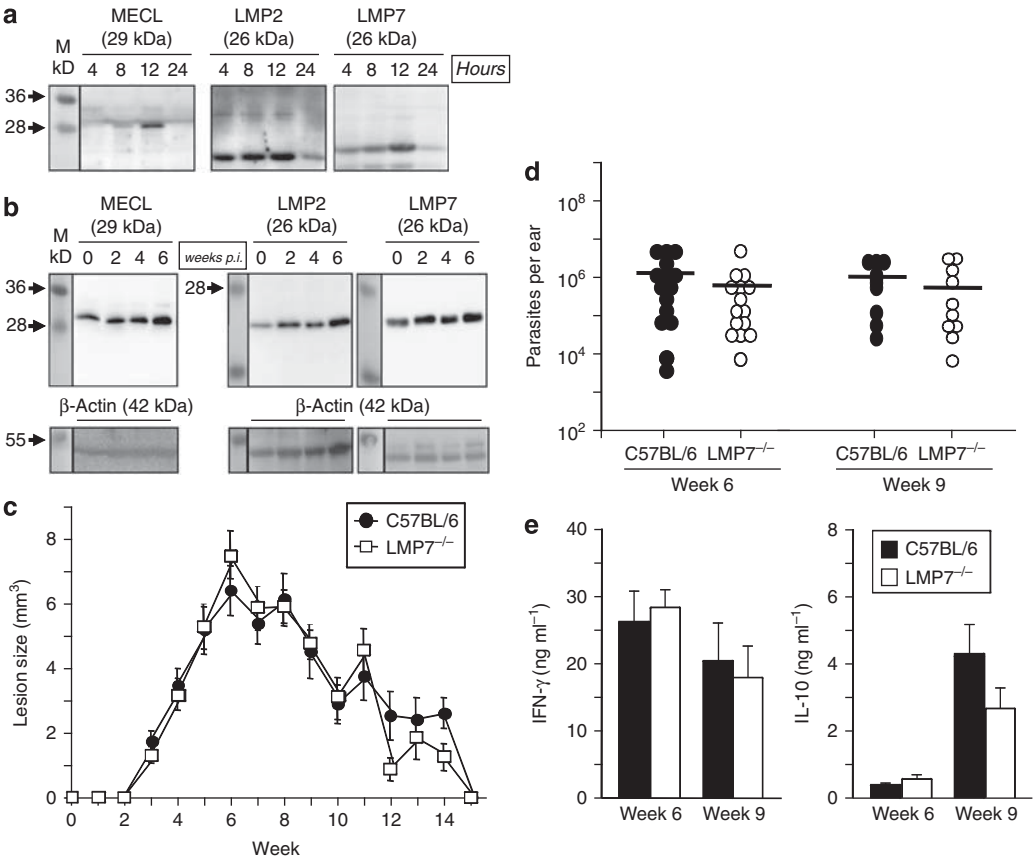
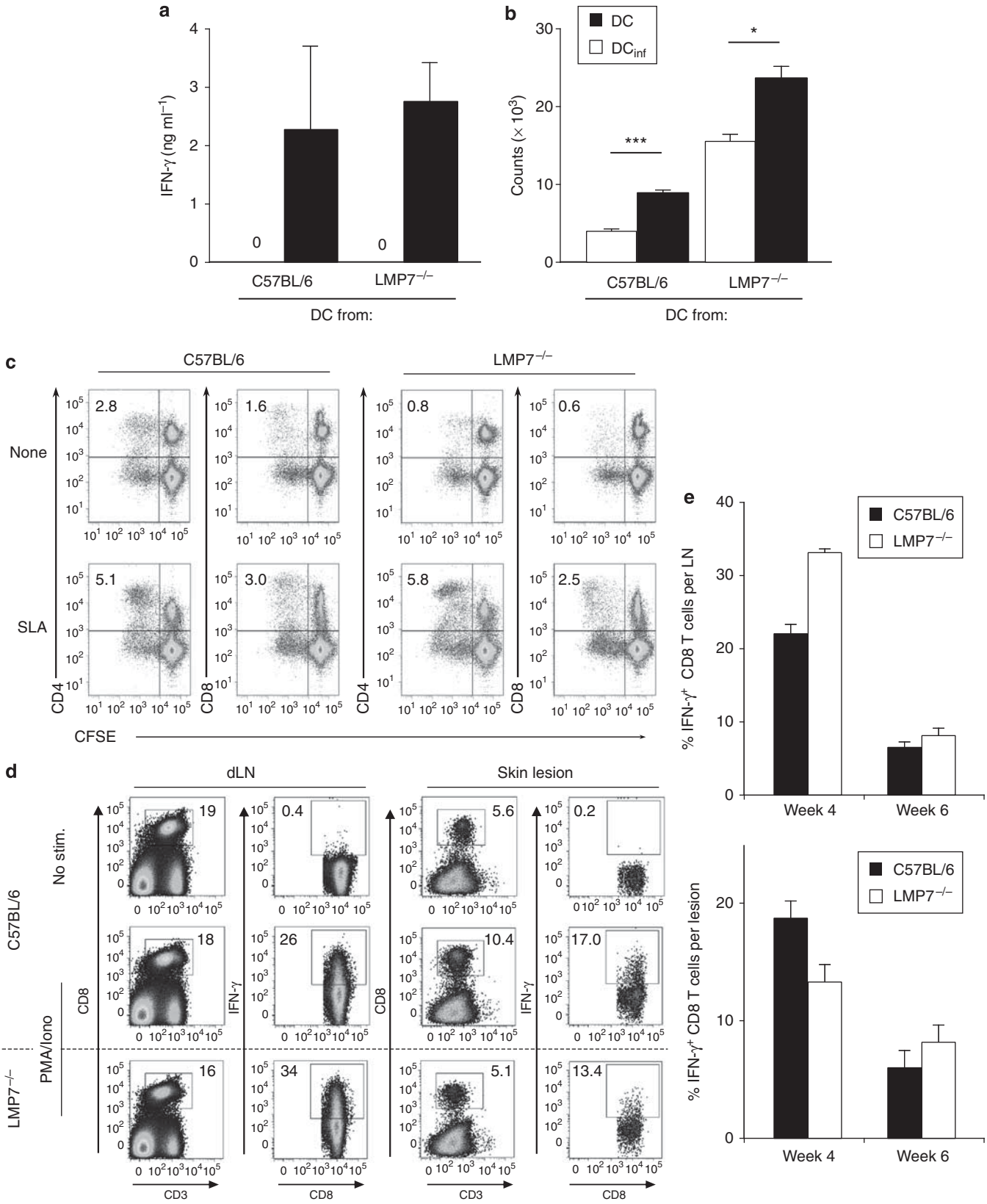


Figure 1. *Leishmania*-resistant LMP7$^{-/-}$ mice display no alterations in lesion sizes, parasite burdens, and cytokine profiles in *L. major* infections. Bone marrow-derived dendritic cells (2×10^5 cells ml^{-1}) infected with *L. major* amastigotes at a cell/parasite ratio of 1:5, (**a**) and lymph nodes of *L. major*-infected C57BL/6 mice (10^3 parasites) (**b**) were lysed at the time points indicated. Western blots for immunoproteasome subunits MECL (β2i), LMP2 (β1i), LMP7 (β5i), and β-actin as loading control were performed as described (Tenzer *et al.*, 2004). (**b**) Quantitative densitometry revealed an ~2-fold upregulation for all subunits within 6 weeks post infection. (**c**–**e**) LMP7$^{-/-}$, and wild-type C57BL/6 mice were infected intradermally with physiological low-dose inocula of *L. major* (10^3 metacyclic promastigotes) in both ears. (**c**) Lesion development was assessed weekly in three dimensions and expressed as mean ± SEM ($n \geqslant 5$, pooled data from two independent experiments). (**d**) Ears were harvested 6 and 9 weeks post infection, and the number of parasites was assessed using a limiting dilution assay. Dots represent numbers of parasites in individual ears and bars indicate means. (**e**) Lymph node cells were plated at 1×10^6 cells per 200 µl and restimulated with soluble *Leishmania* lysate. Cytokine release was determined after 48 hours by ELISA and expressed as mean ± SEM ($n \geqslant 9$, pooled data from two independent experiments). p.i., post infection.

Figure 2. *L. major*-infected LMP7$^{-/-}$ dendritic cells (DC) show a restimulatory capacity of antigen-specific CD8$^+$ T cells comparable to wild-type DC. (**a**) DC from LMP7$^{-/-}$ and C57BL/6 mice were infected overnight with amastigotes of *L. major* (multiplicity of infection 10:1). Cells were harvested after 18 hours and infection rates determined. CD8$^+$ T cells from *L. major*-infected C57BL/6 mice were added to DC at 5×10^5 per well (T cell/DC ratio 5:1). IFN-γ secretion was determined after 48 h. (**b**) CD8 T-cell proliferation was determined after addition of ^{3}H-thymidine for the last 18 hours of the 48-hour coculture. (**c**) Draining lymph node (dLN) cells of infected C57BL/6 or LMP7$^{-/-}$ mice were harvested after 4 weeks, labeled with 5-(and-6)-carboxyfluorescein diacetate (CFSE), plated at 1×10^6 cells per 200 µl, and restimulated with medium or with *Leishmania* antigen (soluble *Leishmania* lysate (SLA), 25 µg ml^{-1}). Proliferation of CD4$^+$ and CD8$^+$ T cells was determined by flow cytometry on day 6. One representative experiment of two with ⩾3 mice per group is shown. All data are expressed as mean ± SEM ($n = 3$, $^*P \leqslant 0.05$, $^{***}P \leqslant 0.002$). (**d**, **e**) Draining lymph node (dLN) and inflammatory ear cells were harvested at week 4 and 6 post infection, plated at 5×10^6 cells per 200 µl, and stimulated for 4–6 hours using phorbol 12-myristate 13-acetate (PMA) (50 ng ml^{-1}), ionomycin (Iono, 500 ng ml^{-1}), and Brefeldin A (1 µg ml^{-1}). Cells were then stained and analyzed by flow cytometry. T cells were identified by CD3/CD8 staining, and the percentage of IFN-γ$^+$ cells was calculated. (**d**) One of the several independent stainings in week 4 is depicted. (**e**) The frequency of IFN-γ–producing CD8 T cells was expressed as mean ± SEM ($n = 5$). Stim., stimulation.


a
IFN-γ (ng ml^{-1})
0
1
2
3
4
0
0
C57BL/6
LMP7$^{-/-}$
DC from:
b
DC
DC$_{inf}$
Counts (×10^3)
0
10
20
30
***
*
C57BL/6
LMP7$^{-/-}$
DC from:
c
C57BL/6
LMP7$^{-/-}$
None
SLA
2.8
1.6
0.8
0.6
5.1
3.0
5.8
2.5
CD4
CD8
CFSE
d
dLN
Skin lesion
C57BL/6
LMP7$^{-/-}$
No stim.
PMA/Iono
19
0.4
5.6
0.2
18
26
10.4
17.0
16
34
5.1
13.4
CD8
IFN-γ
CD3
CD8
e
% IFN-γ$^+$ CD8 T cells per LN
% IFN-γ$^+$ CD8 T cells per lesion
C57BL/6
LMP7$^{-/-}$
Week 4
Week 6

CD8$^+$ T cells is proteasome/transporter associated with antigen processing-independent (Bertholet *et al.*, 2006). Considering the distinct proteolytic activities of the immunoproteasome and the constitutively expressed proteasome (Boes *et al.*, 1994; Eleuteri *et al.*, 1997), presentation of *L. major* antigens to CD8$^+$ T cells appears to be independent of these two major nonlysosomal protein degradation machineries, implying the involvement of alternative cytosolic pathways of cross-presentation emcompassing protease candidates, such as tripeptidyl peptidase II and nardilysin.

In summary, our data indicate that despite the fact that CD8 responses are highly effective in protecting against natural *L. major* infections, it remains unclear how parasite epitopes are generated in infected DC. Interestingly, recent data suggest that priming of *Leishmania*-specific CD4$^+$ T cells is mediated by Langerin^{neg} dermal DCs, whereas Langerin$^+$ dermal DCs promote early CD8$^+$ T-cell priming (Brewig *et al.*, 2009). A detailed understanding of the pathways utilized for processing of *L. major*-derived CTL-epitopes in the MHC class I pathway as well as knowing which DC subset to target during vaccination will be helpful for the development of new vaccine candidates against this important human pathogen.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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