

# Immunoproteasome subunit deficiency has no influence on the canonical pathway of NF- $\kappa$ B activation

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## ARTICLE INFO

### Keywords:

NF- $\kappa$ B

Immunoproteasome

LMP2

LMP7

Inflammation

## ABSTRACT

Activation of the pro-inflammatory transcription factor NF- $\kappa$ B requires signal-induced proteasomal degradation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B) in order to allow nuclear translocation. Most cell types are capable of expressing two types of 20S proteasome core particles, the constitutive proteasome and immunoproteasome. Inducible under inflammatory conditions, the immunoproteasome is mainly characterized through an altered cleavage specificity compared to the constitutive proteasome. However, the question whether immunoproteasome subunits affect NF- $\kappa$ B signal transduction differently from constitutive subunits is still up for debate. To study the effect of immunoproteasomes on LPS- or TNF- $\alpha$ -induced NF- $\kappa$ B activation, we used IFN- $\gamma$  stimulated peritoneal macrophages and mouse embryonic fibroblasts derived from mice deficient for the immunoproteasome subunits low molecular mass polypeptide (LMP) 2, or LMP7 and multicatalytic endopeptidase complex-like 1 (MECL-1). Along the canonical signaling pathway of NF- $\kappa$ B activation no differences in the extent and kinetic of I $\kappa$ B degradation were observed. Neither the nuclear translocation and DNA binding of NF- $\kappa$ B nor the production of the NF- $\kappa$ B dependent cytokines TNF- $\alpha$ , IL-6, and IL-10 differed between immunoproteasome deficient and proficient cells. Hence, we conclude that immunoproteasome subunits have no specialized function for canonical NF- $\kappa$ B activation.

## 1. Introduction

Nuclear factor kappa B (NF- $\kappa$ B) is a central pro-inflammatory transcription factor, which is ubiquitously expressed and a master switch between initiation and maintenance of inflammation. As such, NF- $\kappa$ B controls expression of a large number of immunoregulatory proteins like cytokines, chemokines and their receptors, as well as regulators of apoptosis and proliferation (Pahl, 1999). In unstimulated cells, NF- $\kappa$ B is sequestered in the cytoplasm as it is bound to inhibitors of  $\kappa$ B (I $\kappa$ Bs) (Hinz et al., 2012). Extensive signal transduction cascades integrate extracellular and/or intracellular signals ultimately leading to phosphorylation, polyubiquitylation, and proteasomal degradation of I $\kappa$ Bs (Hayden and Ghosh, 2008). Free NF- $\kappa$ B then migrates into the nucleus where it binds to consensus sites in promoters of numerous genes (Brasier, 2006).

NF- $\kappa$ B signaling can be subdivided into two distinct pathways, i.e. the canonical and noncanonical/alternative pathway. The canonical pathway can be activated by a variety of signals including cytokines, pathogens, stress signals, and radiation. Generally, receptor proximal signaling adapter molecules initiate a signaling cascade ultimately leading to a key step in the canonical NF- $\kappa$ B signaling pathway, the activation of the inhibitor  $\kappa$ B kinase (IKK) complex (Hayden and Ghosh, 2008). Once activated, the IKK complex phosphorylates I $\kappa$ B $\alpha$ , which is subsequently modified with ubiquitin by the SCF- $\beta$ -TrCP complex and degraded by the proteasome (Kanarek and Ben-Neriah, 2012).

Since degradation of I $\kappa$ B $\alpha$  is an essential step in NF- $\kappa$ B activation, the proteasome complex is a key player in NF- $\kappa$ B signaling. Structurally, the 20S proteasome core particle consists of four stacked rings, each consisting of seven subunits. While the outer rings contain catalytically inactive alpha subunits, the inner rings contain seven different beta subunits, three of which display N-terminal threonine protease activity ( $\beta$ 1,  $\beta$ 2,  $\beta$ 5) (Tanaka, 1998). Cells of hematopoietic origin and cells stimulated with IFN- $\gamma$  or, to a lesser extent, with type I interferons express an additional

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set of catalytically active proteasome subunits: low molecular mass polypeptide 2 (LMP2 or  $\beta$ 1i), multicatalytic endopeptidase complex-like 1 (MECL-1, or  $\beta$ 2i), and LMP7 ( $\beta$ 5i) (Barton et al., 2002; Khan et al., 2001; Shin et al., 2006). During *de novo* synthesis of proteasomes, these subunits are incorporated and form the immunoproteasome, whereas incorporation of constitutive subunits is proportionally reduced (Griffin et al., 1998). Compared to the constitutive proteasome, the immunoproteasome is mainly characterized by an altered cleavage specificity (Driscoll et al., 1993; Gaczynska et al., 1994; Van Kaer et al., 1994). Overall, immunoproteasomes produce more peptides with a hydrophobic C-terminus, which are well suitable for the presentation on MHC class I molecules (Strehl et al., 2005; Toes et al., 2001).

Besides shaping the immunopeptidome presented on MHC class I molecules, several studies support a broader immunological role of the immunoproteasome (Groettrup et al., 2010). Mice deficient for any of the immunoproteasome subunits are protected from dextran sulphate sodium-induced colitis (Basler et al., 2010; Fitzpatrick et al., 2006; Schmidt et al., 2010). Moreover, inhibition of LMP7 has been proven effective for the treatment of autoimmune conditions in different mouse models (Basler et al., 2015) and attenuates LCMV-induced meningitis (Mundt et al., 2016). However, inhibition of the chymotrypsin-like activity in hematopoietic cells rather than the catalytic specificity of LMP7 seems to be the underlying mechanism in these models (Basler et al., 2014). Nevertheless, the mechanism by which LMP7 deficiency influences immune regulatory mechanisms is still not fully understood. Ultimately depending on the proteasome for activation, NF- $\kappa$ B is an important transcription factor in inflammatory conditions. Still, previous studies concerned with the effect of immunoproteasome subunits on NF- $\kappa$ B activation yielded contradictory results (Hayashi and Faustman, 2000, 1999; Hensley et al., 2010; Kessler et al., 2000; Maldonado et al., 2013; Visekruna et al., 2006).

In this study, we have revisited the influence of LMP2 knockout and LMP7/MECL-1 double knockout (L7M) on NF- $\kappa$ B activation along the canonical signaling pathway. Peritoneal macrophages and mouse embryonic fibroblasts (MEFs) derived from knockout mice were stimulated with LPS or TNF- $\alpha$  and I $\kappa$ B $\alpha$  degradation, cytokine secretion, and nuclear translocation of NF- $\kappa$ B were assessed. We found no influence of immunoproteasome subunit deficiency on any of these parameters and therefore propose NF- $\kappa$ B signaling to be independent of proteasome subunit composition.

## 2. Material and methods

### 2.1. Mice, cell lines and cytokines

C57BL/6 mice (H-2b) were originally purchased from Charles River. LMP2 (Van Kaer et al., 1994), LMP7 (Fehling et al., 1994), and MECL-1 (Basler et al., 2006) gene-targeted mice were provided by J. Monaco (Cincinnati, OH, USA). LMP7<sup>-/-</sup>/MECL-1<sup>-/-</sup> double deficient mice (L7M<sup>-/-</sup>) were generated by crossing the F1 generation of LMP7<sup>-/-</sup> x MECL-1<sup>-/-</sup> mice. Mice were kept in a specific pathogen-free facility and used at 8–12 weeks of age. Animal experiments were approved by the review board of Regierungspräsidium Freiburg. Peritoneal macrophages and primary mouse embryonic fibroblasts (MEFs) were cultured in DMEM with GlutaMAX supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. Media and supplements were purchased from Invitrogen–Life Technologies. Recombinant murine IFN- $\gamma$  and TNF- $\alpha$  was purchased from Peprotech and used at 200 U/ml and 100 U/ml, respectively.

### 2.2. Generation of peritoneal macrophages and mouse embryonic fibroblasts (MEFs)

Peritoneal macrophages were generated by i.p. injection of 0.5 ml 3% thioglycolate broth. After 4 days, cells were washed out of the abdominal cavity by peritoneal lavage using PBS. Cells were plated over night and adherent cells were further cultured in the presence of IFN- $\gamma$  for 2 days or left untreated.

MEFs were prepared from embryos on day 14 of gestation. After removal of head and liver, embryos were finely minced and digested in trypsin/EDTA solution (Invitrogen–Life Technologies) for 15 min at 37 °C. Trypsin was inactivated with culture medium and removed by centrifugation. Cells were passed through a 100  $\mu$ m filter and plated for two days at 37 °C and 5% CO<sub>2</sub>. Aliquots of cells were stored at –150 °C and a different batch of cells was used for each replication of an experiment.

### 2.3. Stimulation of cells

Peritoneal macrophages or MEFs were seeded into 12-well plates and stimulated with 200 U/ml IFN- $\gamma$  for two days to induce immunoproteasome expression. Control cells were left untreated. Cells were then stimulated with 100 U/ml TNF- $\alpha$  or 200 ng/ml LPS and harvested after different time points. Cells were washed with cold PBS and used for SDS-PAGE and western blot, EMSA, or TransAM NF- $\kappa$ B ELISA. Cells used for expression analysis of immunoproteasome subunits were directly harvested after IFN- $\gamma$  treatment. The protein concentration of samples was determined using DC protein assay (Bio-Rad) according to the manufacturer's instruction.

To analyze cytokine secretion peritoneal macrophages stimulated with 200 U/ml IFN- $\gamma$  for 2 days were seeded into 96-well plates. Two hours after seeding, cells were stimulated with 200 ng/ml LPS or left untreated. Supernatants of triplicates were collected 24 h later and analyzed using IL-6, TNF- $\alpha$ , or IL-10 ELISA according to the manufacturer's instruction (ebiosciences).

### 2.4. SDS PAGE and western blot

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% (v/v) Triton X-100, 0.5% (v/v) sodiumdesoxycholate, 0.1% (w/v) SDS) including protease inhibitors (cOmplete EDTA-free, Roche) for 30 min on ice. Lysates were centrifuged at 14,000 rpm for 15 min and supernatants were boiled with SDS sample buffer for 5 min at 95 °C. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Whatman). After blocking in Roti-Block solution (Roth) for 1 h at room temperature, membranes were incubated with primary antibodies at 4 °C over night. Membranes were washed and incubated with appropriate peroxidase-conjugated secondary antibodies (Dako) for 2 h at room temperature. Membranes were washed again and proteins were visualized with enhanced chemiluminescence. Primary antibodies used were: anti-LMP7 and anti-LMP2 (Kremer et al., 2010), anti- $\beta$ 5 (D1H6B, Cell Signaling Technology), anti- $\beta$ 1 (clone E1K90, Cell Signaling Technology), anti- $\alpha$ 1 (clone IB5, K. Scherrer, Paris, France), anti-I $\kappa$ B $\alpha$  (clone L35A5, Cell Signaling Technology), anti- $\alpha$ -tubulin (clone AA13, Sigma).

### 2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts prepared from MEFs were used for EMSA. MEFs were lysed in hypotonic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA) including protease inhibitors (cOmplete EDTA-free, Roche) and phosphatase inhibitors (PhosSTOP, Roche) for 10 min on ice. NP-40 was added to reach a final concentration of 0.2%. Lysates were vortexed and centrifuged at 13,000 rpm and

4°C for 20 s. Pellets containing the nuclear fraction were lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 10% (v/v) glycerol, 0.4 M NaCl, 1 mM EGTA, 0.1 mM EDTA) including phosphatase and protease inhibitors for 30 min on ice. Nuclear lysates were centrifuged at 14,000 rpm and 4°C for 14 min. The protein content of supernatants was determined with a DC protein assay (Bio-Rad) and further used for shift assays. To ensure equal loading of EMSA reactions, aliquots of nuclear lysates were boiled with SDS sample buffer for 5 min at 95°C and subjected to SDS-PAGE and western blot using anti-Lamin A/C antibody.

EMSA was performed using a <sup>32</sup>P-labeled double-stranded DNA probe containing the NF-κB binding site from the mouse H-2K promoter (5'-CAGGGCTGGGGATTCCCCATCTCCACAGG-3'). Complementary oligonucleotides containing 5' GATC overhangs were mixed in annealing buffer (50 mM Tris pH 8, 70 mM NaCl) at a concentration of 200 ng/ml, incubated at 90°C for 10 min and slowly cooled down to allow oligonucleotide annealing. Oligonucleotides were then radioactively labeled with dATP[α-<sup>32</sup>P] using DNA polymerase I Klenow fragment (NEB) according to the manufacturer's instructions. Labeled probes were purified using QIAquick nucleotide removal kit (Qiagen). Shift reactions (20 μl) contained 5 μg nuclear extracts, 0.5 μg BSA, 5 mM DTT, 0.1 μg/μl poly(dI-dC) (Affymetrix) and 0.5 ng probe in shift buffer (20 mM HEPES pH 7.9, 60 mM KCl, 4% Ficoll) and were incubated for 30 min at room temperature. The complexes were separated on a 5% native polyacrylamide gel in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.2). Gels were vacuum-dried onto whatman paper, exposed to phosphor screens, and the bands were visualized using a phosphorimager.

## 2.6. NF-κB ELISA

The DNA-binding activity of p65 in nuclear extracts prepared from stimulated MEFs was measured using a TransAM<sup>®</sup> NF-κB family ELISA kit (Active Motif) following the manufacturer's instruction. All samples were analyzed in duplicates using 10 μg protein per well.

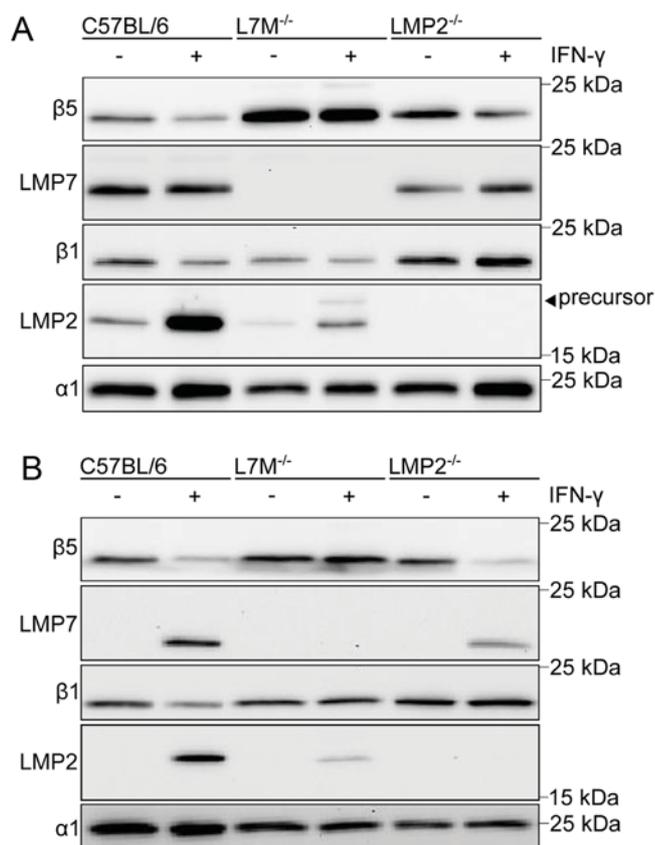
## 2.7. Statistical analysis

The unpaired two-tailed Student's *t* test and two-way ANOVA with Tukey correction were used for statistical analysis using GraphPad Prism software.

## 3. Results

### 3.1. Proteasome subunit composition in peritoneal macrophages and MEFs

As an approach for assessing the influence of immunoproteasome subunits on NF-κB activation two different types of primary cells were isolated from wild type, LMP7<sup>-/-</sup>/MECL-1<sup>-/-</sup> double knockout mice (L7M<sup>-/-</sup>), and LMP2<sup>-/-</sup> mice. Thioglycollate-elicited peritoneal macrophages (pMΦs) and mouse embryonic fibroblasts (MEFs) were chosen in order to include cell types of the hematopoietic and non-hematopoietic lineage, respectively. Isolated pMΦs were cultured in the presence of IFN-γ for two days to further upregulate immunoproteasome expression. The subunit composition was then analyzed with SDS-PAGE and western blot (Fig. 1A). Unstimulated wild type pMΦs strongly express LMP7, which appears not to be further upregulated after stimulation with IFN-γ. In contrast, expression of LMP2 is rather low in unstimulated cells and is strongly induced after stimulation. These results indicate that a proportion of proteasomes in unstimulated pMΦs has a mixed subunit composition consisting of LMP7 together with the constitutive subunits β1 and β2. This is in accordance with



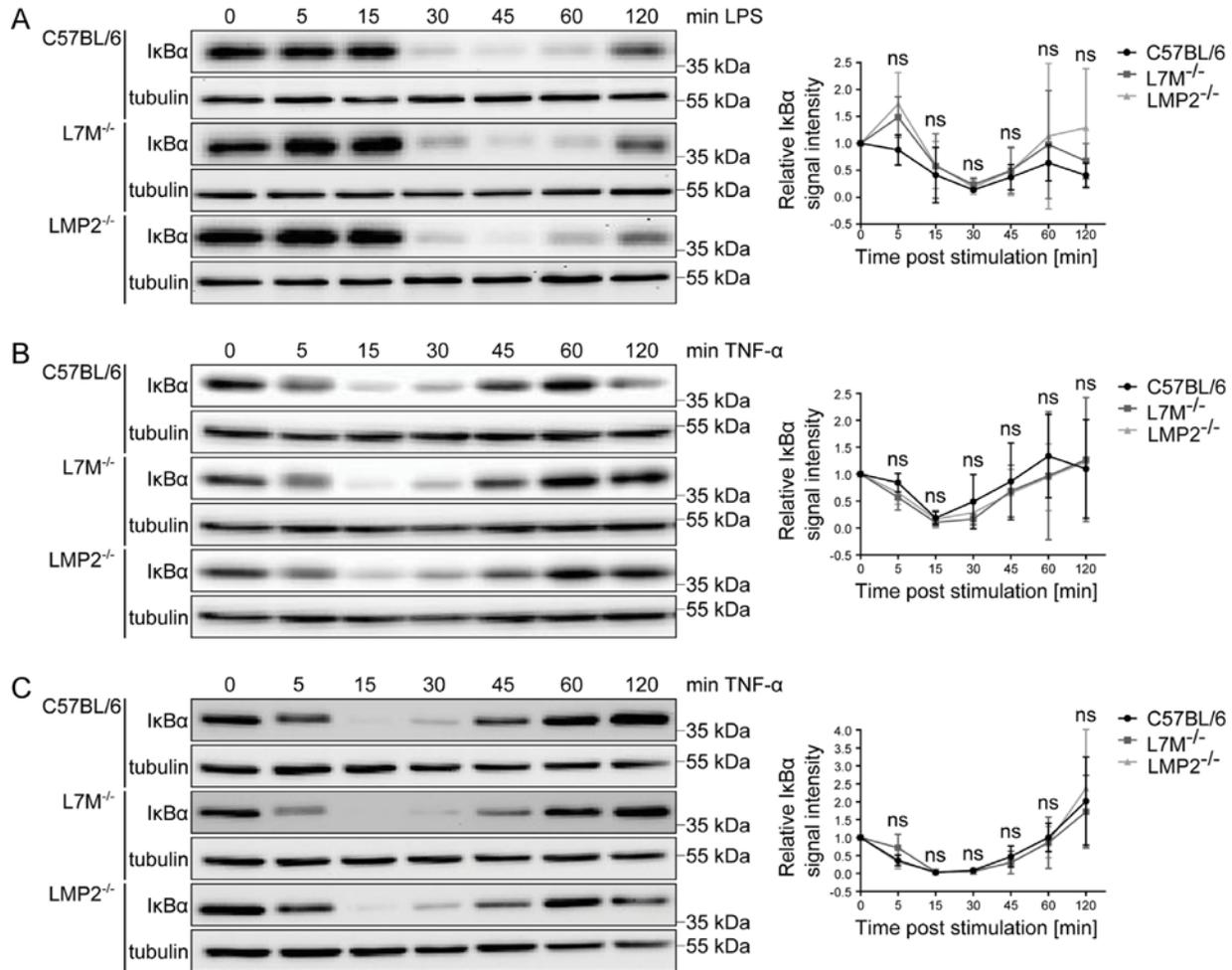
**Fig. 1.** Proteasome subunit composition in peritoneal macrophages (pMΦs) and mouse embryonic fibroblasts (MEFs). pMΦs (A) or MEFs (B) isolated from C57BL/6, LMP7<sup>-/-</sup>MECL-1<sup>-/-</sup> (L7M<sup>-/-</sup>), and LMP2<sup>-/-</sup> mice were cultured in the presence of 200 U/ml IFN-γ for 2 days or left untreated. Whole cell lysates were prepared and analyzed by western blotting. The catalytically inactive proteasome subunit α1 (iota) was used as loading control. One out of three independent experiments with similar outcome is shown.

data published by Guillaume et al., who found about 40% of proteasomes present in dendritic cells to have incorporated only LMP7 but not LMP2 or MECL-1 (Guillaume et al., 2010). After stimulation, the subunit composition shifts further towards immunoproteasomes. However, expression of MECL-1 and β2 was not tested and it is therefore not possible to discriminate the proportion of full immunoproteasomes and complexes containing LMP7 and LMP2 together with β2. In agreement with previous reports, L7M<sup>-/-</sup> pMΦs show a strongly reduced incorporation of LMP2 and an accumulation of the unprocessed LMP2 precursor (Griffin et al., 1998; De et al., 2003). Hence, L7M<sup>-/-</sup> pMΦ almost exclusively express constitutive proteasomes. In contrast, LMP2<sup>-/-</sup> cells show LMP7 expression levels similar to wild type cells.

Unlike pMΦs, MEFs do not express immunoproteasome subunits in the absence of IFN-γ stimulation (Fig. 1B). After IFN-γ stimulation, expression of LMP7 and LMP2 is induced, while the respective constitutive subunits β5 and β1 are downregulated. Similar to pMΦs, L7M<sup>-/-</sup> MEFs display strongly reduced incorporation of LMP2, whereas LMP2<sup>-/-</sup> MEFs show reduced expression of LMP7.

### 3.2. Degradation of IκBα is not altered in immunoproteasome-deficient cells

After having analyzed the proteasome composition in pMΦs and MEFs, stimulus-induced degradation of IκBα was investigated next. At this point, the proteasome is directly involved in the signaling cascade. Incomplete or delayed degradation of IκBα leads to



**Fig. 2.** Stimulus-induced degradation of IκBα in pMΦs and MEFs. pMΦs (A and B) or MEFs (C) isolated from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice were cultured in the presence of 200 U/ml IFN-γ for 2 days. Cells were then stimulated with 200 ng/ml LPS (A) or 100 U/ml TNF-α (B and C). Whole cell lysates were prepared at the indicated time points and subjected to western blot analysis. Tubulin was used as loading control. One out of at least three independent experiments with similar outcome is shown. The means of relative IκBα signal intensities from at least three independent experiments were calculated and statistically analyzed by two-way ANOVA with Tukey correction; ns (not significant) P > 0.05.

retention of NF-κB in the cytoplasm. Thus, any alteration affecting the extent or kinetic of IκBα degradation would imminently affect all downstream signaling events. pMΦs from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice were incubated in the presence of IFN-γ for two days to further upregulate immunoproteasome expression. Degradation of IκBα was induced by stimulating the cells with the TLR4 ligand LPS and monitored by SDS-PAGE and western blotting. No difference in IκBα degradation could be detected between cells derived from wild type C57BL/6 mice and either L7M<sup>-/-</sup> or LMP2<sup>-/-</sup> mice (Fig. 2A). The same result was obtained for pMΦs that were not pre-treated with IFN-γ in vitro (data not shown). In order not to rely only on a single stimulus, pMΦs were also stimulated with tumor necrosis factor (TNF)-α. Due to a different upstream signaling cascade, receptor binding of TNF-α triggers a faster response compared to LPS. Here, maximum IκBα degradation is already reached after 15 min (Fig. 2B). Nevertheless, immunoproteasome deficiency had no apparent influence on IκBα degradation.

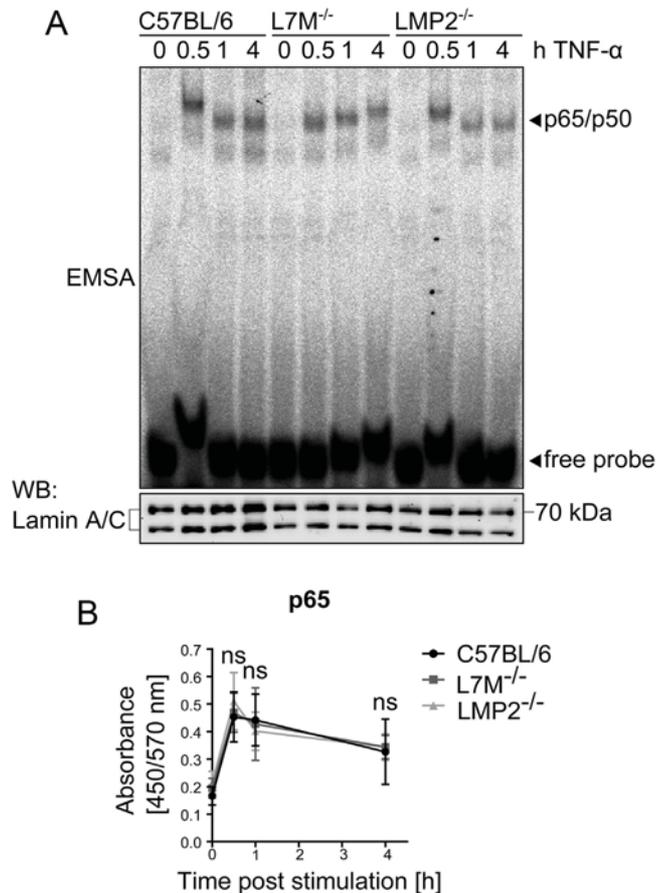
Similar to pMΦs, MEFs stimulated with TNF-α displayed a fast response, which is evidenced by the rapid degradation of IκBα (Fig. 2C). Although MEFs were pre-stimulated with IFN-γ to upregulate immunoproteasome expression, IκBα degradation was not influenced by either LMP7 and MECL-1 or LMP2 deficiency. Even though incorporation of immunoproteasome subunits alters the cleavage specificity and substrate binding pockets of the 20S core

particle (Huber et al., 2012; Toes et al., 2001) there seems to be no influence of these subunits on the degradation of IκBα.

### 3.3. Nuclear translocation of free NF-κB and transactivation of target genes is normal in immunoproteasome knockout cells

The final steps in NF-κB signaling are nuclear translocation of free NF-κB dimers and transactivation of target genes. To test whether the immunoproteasome has any influence on NF-κB signaling downstream of IκBα, active nuclear NF-κB was quantified using an electrophoretic mobility shift assay (EMSA). MEFs derived from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice were incubated in the presence of IFN-γ for two days followed by stimulation with TNF-α. Nuclear extracts were prepared after different time points and subjected to EMSA using an NF-κB binding site probe (Fig. 3A). As expected from the results obtained for IκBα degradation, no difference in the amount of active NF-κB could be detected between wild type and immunoproteasome knockout cell lines. Likewise, the amount of p65 bound to oligonucleotide containing an NF-κB consensus sequence as determined by ELISA did not differ in these nuclear extracts at any time point after stimulation (Fig. 3B).

Cytokines mediate important effector functions of the immune system. Hence, many genes encoding cytokines contain NF-κB responsive elements in their promoter region. As a measure for



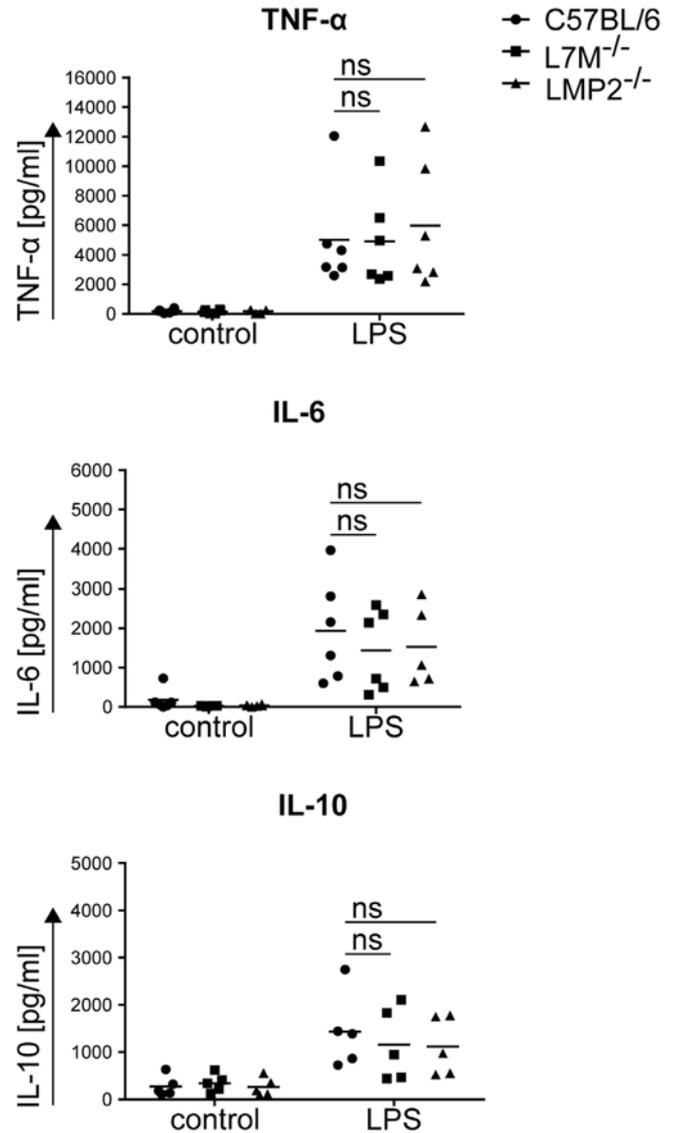
**Fig. 3.** Nuclear translocation of free NF- $\kappa$ B. MEFs isolated from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice were cultured in the presence of 200 U/ml IFN- $\gamma$  for 2 days. Cells were then stimulated with 100 U/ml TNF- $\alpha$  for the indicated time periods. Nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay (EMSA) using an NF- $\kappa$ B binding site probe (A) or TransAM<sup>®</sup> NF- $\kappa$ B ELISA (Active Motif) employing anti-p65 antibody (B). (A) As a loading control, nuclear extracts were also subjected to western blot (WB) analysis using an anti-Lamin A/C antibody. One out of three independent experiments with similar outcome is shown. (B) The graph shows pooled data from three independent experiments including mean; ns (not significant)  $P > 0.05$  (two-way ANOVA with Tukey correction).

transactivation, secretion of the pro-inflammatory cytokines TNF- $\alpha$  and IL-6 as well as the anti-inflammatory cytokine IL-10 by IFN- $\gamma$  – pre-conditioned and LPS-stimulated pM $\Phi$ s was quantified using ELISA (Fig. 4). Concentrations of none of the cytokines differed in a statistically significant way between supernatants of pM $\Phi$ s derived from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice. Thus, NF- $\kappa$ B mediated transactivation is not influenced by immunoproteasome deficiency.

Overall, I $\kappa$ B degradation and signaling events further downstream of this step are not influenced by the immunoproteasome subunits LMP7, MECL-1, and LMP2. Collectively, these data strongly support a model of NF- $\kappa$ B activation being independent of the 20S proteasome subunit composition.

#### 4. Discussion

Two decades of immunoproteasome research solidified the hypothesis of immunoproteasome particles shaping the immunopeptidome presented on MHC class I molecules (Basler et al., 2011; Basler et al., 2006; Basler et al., 2004; Kincaid et al., 2012; Mishto et al., 2014; Morel et al., 2000; Osterloh et al., 2006; Sijts et al., 2000; Toes et al., 2001). Moreover, it became evident that the immunoproteasome has, apart from antigen processing,



**Fig. 4.** Transactivation of NF- $\kappa$ B target genes. pM $\Phi$ s isolated from C57BL/6, L7M<sup>-/-</sup>, and LMP2<sup>-/-</sup> mice were cultured in the presence of 200 U/ml IFN- $\gamma$  for 2 days. Cells were then stimulated with 200 ng/ml LPS for 20 h or left untreated (control). Supernatants were collected and concentrations of cytokines TNF- $\alpha$ , IL-6, and IL-10 were determined by ELISA in triplicates. Graphs show pooled data from at least five independent experiments including mean; ns (not significant)  $P > 0.05$  (unpaired two-tailed Student's  $t$  test).

additional immunological functions. Immunoproteasome deficiency or inhibition affects T cell survival, expansion, and differentiation (Basler et al., 2004; Chen et al., 2001; Kalim et al., 2012; Moebius et al., 2010; Muchamuel et al., 2009; Zaiss et al., 2008), cytokine production (Basler et al., 2011; Basler et al., 2010; Basler et al., 2014; Muchamuel et al., 2009), and progression of autoimmune conditions (Basler et al., 2015). Moreover, mutations in LMP7 and LMP2 in humans cause complex autoimmune and inflammatory phenotypes (Brehm and Kruger, 2015; Brehm et al., 2015). The impact of the immunoproteasome on various immunological aspects rendered this complex an emerging pharmacological target for various diseases and raised an even higher interest in understanding its exact cellular function in autoimmunity and inflammation. Since NF- $\kappa$ B is a master switch in the initiation and maintenance of inflammation, it is of particular interest to clarify whether altered NF- $\kappa$ B signal transduction is the underlying mechanism of the observed phenomena.

In our study, the expression of different proteasome subunits was first determined by SDS-PAGE and western blotting. After stimulation with IFN- $\gamma$ , both pM $\Phi$ s and MEFs expressed immunoproteasomes. Still, expression of constitutive subunits was not completely downregulated indicating a mix of constitutive and immunoproteasomes present in the cells. Due to the assembly defect in L7M $^{-/-}$  cells the particles present are almost exclusively composed of constitutive subunits. In contrast, LMP2 $^{-/-}$  cells seem to express LMP7 but due to the defect in MECL-1 incorporation most likely contain constitutive proteasomes as well as mixed particles containing  $\beta$ 1,  $\beta$ 2, and LMP7.

One of the best-studied signal transduction pathways ultimately depending on the proteasome is the NF- $\kappa$ B pathway. Here, proteasomes are required for the processing of NF- $\kappa$ B precursors as well as for the signal-induced degradation of I $\kappa$ B $\alpha$ . The literature about the role of the immunoproteasome in NF- $\kappa$ B activation is quite controversial. An early study performed with splenocytes from BALB/c, NOD, and LMP2 $^{-/-}$  mice by Hayashi and Faustman suggested that spleen cells from NOD mice lack LMP2 and that LMP2 is required for the processing of the NF- $\kappa$ B p50 subunit from the p105 precursor and for the degradation of I $\kappa$ B (Hayashi and Faustman, 1999). The latter notion was subsequently confirmed by the same authors in the LMP2/LMP7 double deficient human lymphoblastoid cell line T2 when this mutant was compared to the LMP2/LMP7 proficient parental cell line T1 (Hayashi and Faustman, 2000). However, two independent groups could not confirm a lower expression of LMP2 mRNA or protein in NOD as compared to BALB/c splenocytes. (Kessler et al., 2000; Runnels et al., 2000). Moreover, a reconstitution of T2 cells with LMP2 and/or LMP7 did not change the quantity of the mature p50 subunit of NF- $\kappa$ B (Runnels et al., 2000). The follow-up study by Hayashi and Faustman also found reduced I $\kappa$ B $\alpha$  degradation in LMP2 $^{-/-}$  lymphocytes upon stimulation with TNF- $\alpha$  (Hayashi and Faustman, 2000). This finding could be confirmed with LPS-stimulated B cells derived from LMP2 $^{-/-}$  mice by another group, although the observed effect was rather minor (Hensley et al., 2010). In a study investigating human colitis, Visekruna et al. showed enhanced processing of p105 to p50 in cytosolic extracts from the colonic mucosa of patients with Crohn's disease (CD), which expressed higher levels of immunoproteasome, as compared to mucosal extracts from patients with ulcerative colitis (UC) containing less immunoproteasome (Visekruna et al., 2006). However, this finding was merely correlative and it could not be ruled out that other factors that differ in the CD and UC extracts accounted for different p105 processing rates. The same study also found enhanced *in vitro* degradation of I $\kappa$ B $\alpha$  in the presence of purified 20S immunoproteasomes compared to constitutive proteasomes. While free I $\kappa$ B $\alpha$  can indeed be degraded in a ubiquitin-independent fashion (Mathes et al., 2008) this degradation pathway might not be representative for the canonical ubiquitin-dependent degradation of NF- $\kappa$ B-bound I $\kappa$ B $\alpha$  via the 26S proteasome. In contrast, a recent report by Maldonado and colleagues found that activation of the canonical pathway was not affected in LMP2 $^{-/-}$  and L7M $^{-/-}$  cells while the alternative pathway seemed to be "aberrant" in LMP2 $^{-/-}$  cells (Maldonado et al., 2013). Furthermore, it has been proposed that the immunoproteasome has a higher capacity to clear ubiquitylated proteins accumulating after stimulation with IFN- $\gamma$  (Seifert et al., 2010). However, this finding too could not be reproduced by others (Nathan et al., 2013; Kincaid et al., 2012). Unlike in the previous studies, we used two different types of primary cells directly derived from knockout mice. By choosing pM $\Phi$ s and MEFs we included cells from hematopoietic and non-hematopoietic origin, respectively, and our experiments could not be complicated by potential differences in the distribution of lymphocyte subpopulations that occur in immunoproteasome knockout mice (Basler et al., 2006; Van Kaer et al., 1994). Thus, the avoidance of highly

specialized cell types, total lymphocytes, and genetically modified cell lines further strengthens our data set.

In conclusion, the results presented in our study clearly argue against an influence of immunoproteasomes on the canonical pathway of NF- $\kappa$ B activation at least in MEFs and pM $\Phi$ s. Two different types of primary cells prepared from LMP2 $^{-/-}$ , L7M $^{-/-}$ , and wild type mice displayed no differences in the extent and kinetic of I $\kappa$ B $\alpha$  degradation when stimulated with LPS or TNF- $\alpha$ . Consistent with this finding, the amount of active NF- $\kappa$ B in the nucleus of knockout cells as well as the transactivation activity was normal. Although generation of p50 from the p105 precursor was not analyzed here, our results do not indicate a deficit in matured NF- $\kappa$ B subunits in the knockout cells. A study conducted with small molecule inhibitors targeting LMP2 or LMP7 further supports the conception of NF- $\kappa$ B activation being independent of proteasome composition. Inhibition of LMP2, LMP7, or even both had no influence on I $\kappa$ B $\alpha$  degradation in cells stimulated with TNF- $\alpha$  (Jang et al., 2012). Given that neither genetic deletion nor chemical inhibition of immunoproteasome subunits affects canonical NF- $\kappa$ B activation, the elucidation of alternative mechanisms how the immunoproteasome influences cytokine production, T helper cell differentiation, and autoimmunity requires substantial research efforts in the future.

#### Conflict of interest

The authors declare no conflicts of interest.

#### Funding

This work was supported by grants BA4199/2-1 and GR1517/14-1 from the German Research Foundation (DFG). A.B. was a member of the DFG Research Training Group 1331. The funders had no role in the study design, nor in the collection, analysis, and interpretation of the data, writing of the report, and decision to submit the article for publication.

#### Acknowledgements

We thank M. Vincendeau for help with EMSAs and G. Schmidtke for help with radioactive work.

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