

# Role of the proteasome in the downregulation of transcription factors NF $\kappa$ B and C/EBP in macrophages from tumor hosts

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**Abstract.** Macrophages from mice bearing advanced mammary tumors are critically impaired in their immune functions, exhibiting reduced expression at the mRNA and protein levels of the crucial transcription factors, nuclear factor  $\kappa$ B (NF $\kappa$ B) and CCAAT enhancer binding protein (C/EBP). We have previously shown that tumor-derived factors such as transforming growth factor  $\beta$  (TGF $\beta$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) modulate NF $\kappa$ B and C/EBP expression in macrophages. Transcriptional, post-transcriptional, translational and/or post-translational mechanisms may also play a role in altered levels of NF $\kappa$ B and C/EBP in macrophages from tumor hosts, contributing to impaired inflammatory response. One of the post-translational mechanisms that may tune down or recycle proteins in cells is the proteasomal pathway. Since upregulation of ubiquitin/proteasomal pathways has been described under cancer-induced cachexia, we examined the possible role of this proteolytic machinery in the decrease of NF $\kappa$ B and C/EBP proteins in macrophages from tumor hosts. Using MG-132 proteasome inhibitor to block the proteasome machinery in macrophages from normal and tumor-bearing animals we found that macrophages from tumor hosts display higher ubiquitination and proteolysis compared to those from normal mice and also that NF $\kappa$ B and C/EBP downregulation is reversed in these treated cells. Thus, proteasome degradation may contribute, at least in part, to NF $\kappa$ B and C/EBP impairment in macrophages from tumor-bearers.

## Introduction

Inflammation and immune suppression are two opposing responses of the immune system linked in different ways to

cancer: earlier stages of tumor development are associated with chronic inflammation, while established cancers induce immune suppression (1,2). Macrophages are key players of the inflammatory response and exhibit significant roles in the different stages of tumorigenesis. The release of mutation-inducing free radicals, such as nitrogen and oxygen intermediates as part of their cytotoxic/inflammatory response contributes to tumor initiation (3). Macrophages also play important roles in tumor progression by releasing factors that promote angiogenesis, invasion, extracellular matrix remodeling and metastasis (4). However, while tumor development is modulated by macrophages, the tumor likewise can shape macrophage behavior, with development of macrophages that exhibit immune suppressive traits, enabling tumor progression (5,6).

We have previously shown that peritoneal macrophages from mice with well-established mammary tumors are critically impaired in their immune functions (7-10). These cells exhibit decreased levels of inflammatory cytokines, important defects in the corresponding signaling pathways, increased apoptosis and less differentiation than their counterparts in normal mice (11). One of the most essential deficiencies found in these macrophages is a reduced function of nuclear factor  $\kappa$ B (NF $\kappa$ B) and CCAAT enhancer binding protein (C/EBP) transcription factors, likely due to their decreased expression (12). These two molecules are crucial in transcriptional regulation of inflammatory cytokine expression. Furthermore, NF $\kappa$ B, a transcription factor that is constitutively upregulated in tumor cells, is considered to be one of the fundamental molecular links between inflammation and cancer (13). Nevertheless, we demonstrated that in macrophages from tumor-bearing mice NF $\kappa$ B is down-regulated, providing additional evidence for the existence of tumor-induced immune suppression. Furthermore, we showed that this phenomenon is not only valid in macrophages from our D1-DMBA3 mouse mammary tumor-bearers, but we confirmed that it is a more general event present in macrophages from mice with other solid malignancies as well (11).

Among the different factors secreted by the DA-3 cells and other tumors that might modulate NF $\kappa$ B and C/EBP expression, we provided evidence that transforming growth factor  $\beta$  (TGF $\beta$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) are associated with a reduced expression of these transcription factors in

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macrophages. We have also demonstrated that NF $\kappa$ B and C/EBP are diminished at the mRNA and protein levels in macrophages from tumor hosts (11). Thus, transcriptional, post-transcriptional, translational and/or post-translational mechanisms might explain the decreased expression of NF $\kappa$ B and C/EBP in macrophages of tumor hosts, hence contributing to their impaired inflammatory response.

There are two main protein degradation systems in cells, autophagy, which is a mechanism for bulk protein degradation in lysosomes (14,15) and the proteasomal pathway, which is a mechanism for degradation of individual proteins tagged with polyubiquitin in proteasomes (16). Proteasomes are large protein complexes, located in the nucleus or the cytoplasm inside all eukaryote cells. The main function of the proteasome is to degrade unnecessary or damaged (misfolded) proteins by proteolysis and also to modify or activate proteins. An example of the latter is the proteasomal activation of NF $\kappa$ B by processing p105 into p50 and p100 into p52 via internal proteolysis.

Proteins are generally tagged either for proteasomal degradation or modification by another small protein called ubiquitin. The tagging reaction is catalyzed by enzymes called ubiquitin ligases (17). Once a protein is tagged with a single ubiquitin molecule, this may be a signal to other ligases to attach additional ubiquitin molecules, resulting in a polyubiquitin chain. Depending on the type of ligation between ubiquitin molecules, the substrate will be targeted to destruction or modification (18). The overall system of ubiquitination and proteasomal degradation is known as the ubiquitin-proteasome system (UPS). UPS is essential for many cellular processes, including the cell cycle, regulation of gene expression and responses to oxidative stress.

Increased proteasome activity has been implicated in autoimmune syndromes and also in cancer (19,20). Enhanced proteasome activity results in the activation of NF $\kappa$ B. Due to its role in generating the activated form of NF $\kappa$ B, which contributes to anti-apoptotic and pro-inflammatory cytokine expression, proteasomal activity has been associated with inflammatory and autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Since upregulation of ubiquitin/proteasomal pathway has also been described under cancer-induced cachexia (21), we decided to examine the possible role of this proteolytic machinery in the decreased expression of NF $\kappa$ B and C/EBP proteins in macrophages from tumor hosts. Our results using MG-132 proteasome inhibitor to block the proteasome machinery in macrophages from normal and tumor-bearing animals suggest that macrophages from tumor hosts display higher ubiquitination and proteolysis than the corresponding cells from normal mice. Also, that proteasome degradation may contribute, at least in part, to NF $\kappa$ B and C/EBP impairment in macrophages from tumor bearers.

## Materials and methods

**Animals and tumors.** BALB/c mice of 10-14 weeks of age, maintained by brother-sister mating in our facilities, were used. The D1-DMBA-3 mammary adenocarcinoma and the DA-3 cell line isolated from the *in vivo* tumor were maintained and used as previously described (22). The Institutional

Animal Care and Use Committee approved the animal experiments.

**Macrophage collection and culture.** Age and gender matched normal mice and 4-week post implantation tumor-bearing mice were injected i.p. with 1.5 ml of 3% thioglycollate (Difco Laboratories, MD). On day 4, peritoneal elicited macrophages from normal animals (N-PEMs) and from tumor-bearers (T-PEMs) were obtained and cultured as previously described (12).

**Reagents.** RPMI-1640 containing 10% FCS, 2 mM L-glutamine with 100 U of penicillin and 100  $\mu$ g/ml of streptomycin (all from Hyclone, UT) was used as complete culture medium in all experiments. Dimethylsulfoxide (DMSO) and TGF- $\beta$  signaling inhibitor SB-431542 were purchased from Sigma-Aldrich (St. Louis, MO), whereas recombinant human TGF- $\beta_1$  was from Peprotech (Rocky Hill, NJ) and proteasome inhibitor MG-132 was from A.G. Scientific, Inc. (San Diego, CA).

**Western blot analysis.** N-PEM and T-PEM ( $10^7$ ) were adhered to plastic tissue culture dishes in plain RPMI for 2 h at 37°C/5% CO $_2$ , washed and cultured in complete RPMI for the indicated amounts of time, whole cell extracts were obtained and Western blot analysis was performed as previously described (12). Rabbit  $\alpha$ -mouse polyclonal antibodies from Santa Cruz Biotechnologies were used as the primary antibodies for NF $\kappa$ B (p50, p65 and c-rel), C/EBP ( $\alpha$  and  $\beta$ ) and ubiquitin, whereas goat  $\alpha$ -rabbit IgG-HRP was used as the secondary antibody. Filters were stripped using Restore™ Plus Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) and rabbit  $\alpha$ -mouse actin polyclonal antibody (Sigma-Aldrich) was used to standardize the signals.

**Treatment of macrophages with TGF $\beta_1$  and TGF- $\beta$  signaling inhibitor SB-431542.** Solutions of recombinant TGF $\beta_1$  and of SB-431542 were freshly prepared before use. Adhered N-PEMs were cultured for 20 h in complete medium containing recombinant TGF $\beta_1$  (100 ng/ml), TGF $\beta$  signaling inhibitor SB-431542 (10  $\mu$ M) and both together. Cells were then lysed, whole cell extracts were obtained and Western blot analysis was performed to detect expression of NF $\kappa$ B.

**Treatment of macrophages with proteasome inhibitor MG-132.** Adhered N-PEMs and T-PEMs were cultured in complete medium containing freshly prepared MG-132 (25  $\mu$ M for 20 h) or similar volumes of DMSO. Cells were then lysed, whole cell extracts were obtained and Western blot analysis was performed to detect expression of NF $\kappa$ B, C/EBP and ubiquitin.

**Statistical analysis.** Paired t-test was used to analyze statistical significance and error bars represent SEM. Differences were considered statistically significant when P<0.05.

## Results

**Pre-treatment of macrophages with TGF $\beta$  signaling inhibitor partially restores TGF $\beta$ -induced downregulation of NF $\kappa$ Bp65.**

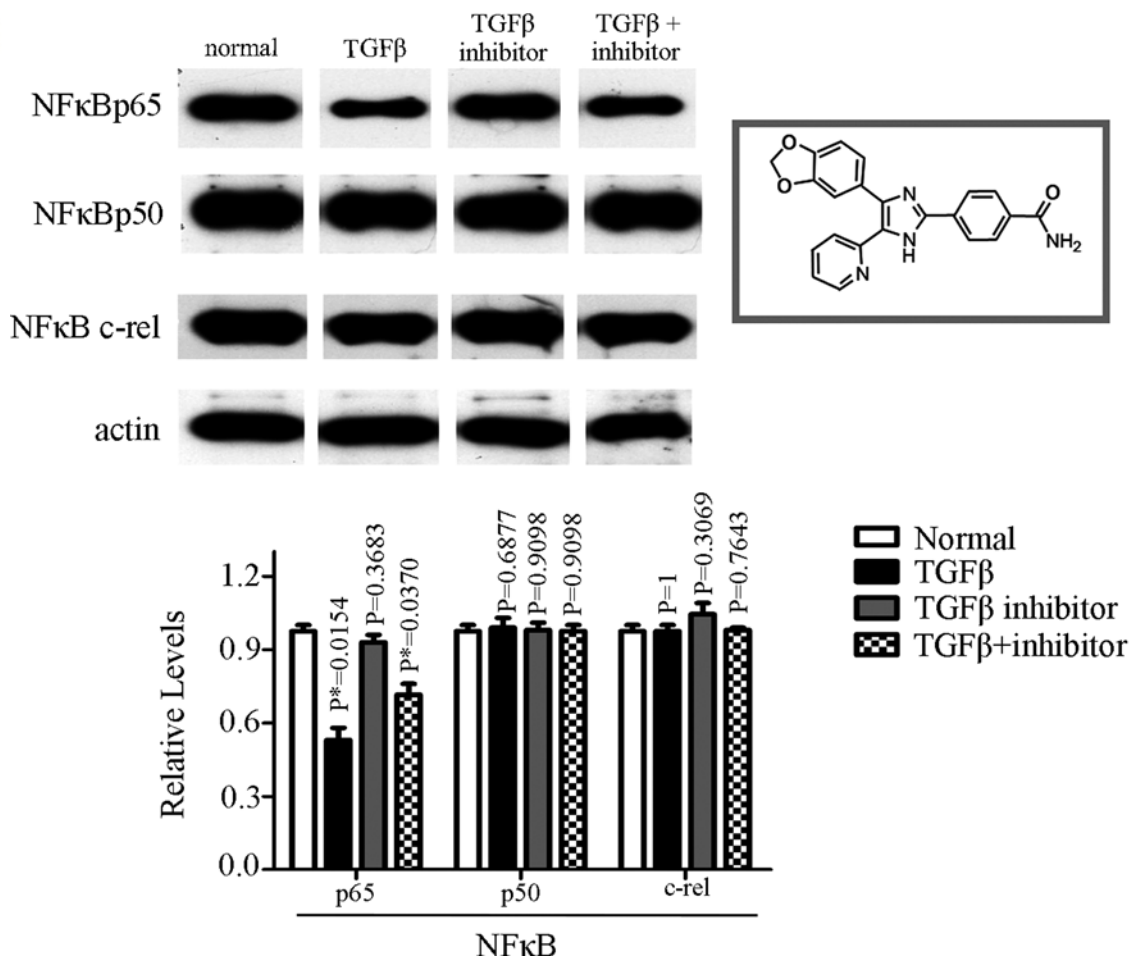


Figure 1. Inhibition of TGF- $\beta$  signaling partially reverses TGF- $\beta$ -induced NF $\kappa$ Bp65 downregulation. Macrophages from normal mice ( $10 \times 10^6$ ) were incubated for 20 h in complete medium containing recombinant TGF $\beta_1$  (100 ng/ml), TGF $\beta_1$  signaling inhibitor SB-431542 (10  $\mu$ M; chemical structure shown) and both together. Cells were then lysed, whole cell extracts were obtained and Western blot analysis was performed to detect expression of NF $\kappa$ B proteins. Values in the densitometry were normalized with  $\beta$ -actin and represent mean  $\pm$  SE from three separate experiments (untreated macrophages or controls were arbitrarily assigned a value of 1.0 in all the cases for comparison with treated samples).

Our previous studies demonstrated that *ex vivo* culture of N-PEMs with increasing concentrations of recombinant TGF $\beta_1$  resulted in NF $\kappa$ Bp65 protein downregulation (11). We now examined whether this decreased expression of NF $\kappa$ Bp65 could be reversed upon inhibition of TGF $\beta$  signaling. To do this, we used the small molecule SB-431542 which blocks signaling downstream from TGF $\beta$  type I receptor (23). As seen in Fig. 1, treatment of N-PEMs with recombinant TGF $\beta_1$  specifically downregulates NF $\kappa$ Bp65 and simultaneous culture of N-PEMs with TGF $\beta$  and the SB-431542 TGF $\beta$  signaling inhibitor partially blocks TGF $\beta$  signaling, resulting in a moderate reversion of NF $\kappa$ Bp65 downregulation. As expected, SB-431542 by itself did not modulate NF $\kappa$ B expression. Importantly, viability was not affected by treatment with SB-431542, as assessed by trypan blue exclusion (data not shown).

*Increased ubiquitination and proteolysis takes place in macrophages from tumor bearers.* As mentioned, upregulation of the ubiquitin-proteasomal pathway has been reported under conditions of cancer-induced cachexia. NF $\kappa$ Bp105 is normally processed to its active form NF $\kappa$ Bp50 by means of a physiological proteasomal proteolysis mechanism. However,

increased and sustained activity of the proteasomal machinery in macrophages from tumor bearers might also result in abnormal degradation of NF $\kappa$ B and other proteins. To verify if indeed there is enhanced ubiquitination and proteolysis in T-PEMs, we treated N-PEMs and T-PEMs with and without the proteasome inhibitor MG-132 and studied the resulting expression of ubiquitin in these cells. When comparing untreated N-PEMs with untreated T-PEMs, our results indicate (Fig. 2) that higher proteolysis is occurring in T-PEMs, as revealed by lesser amounts of polyubiquitin in T-PEMs, suggesting that it is being more rapidly degraded together with the ubiquitin-tagged proteins by the actively functioning proteasome complex in these cells. On the other hand, when comparing the two cell types upon treatment with the MG-132 proteasome inhibitor, increased amounts of accumulated polyubiquitin in T-PEMs suggest enhanced ubiquitination (ubiquitin accumulation) in T-PEMs, due to a block in proteolysis.

*Augmented proteasome activity in macrophages from tumor-bearers may account for NF $\kappa$ B and C/EBP downregulation in these cells.* We hypothesized that increased proteasomal activity in T-PEMs may contribute to the diminished levels

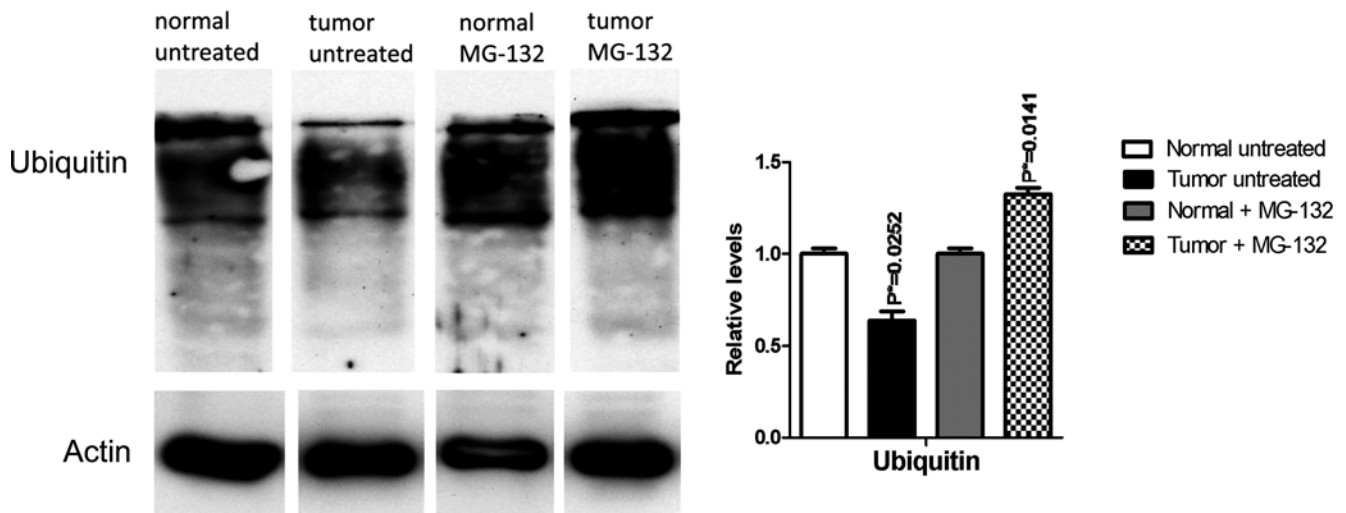


Figure 2. Increased ubiquitination and proteolysis occur in macrophages from tumor bearing mice. Macrophages from normal and 4-week tumor bearing mice ( $10 \times 10^6$ ) were incubated for 20 h in complete medium containing  $25 \mu\text{M}$  of the proteasome inhibitor MG-132 or similar volumes of DMSO. Cells were lysed, whole cell extracts were obtained and Western blot analysis was performed to detect expression of ubiquitin. Densitometry values were normalized with  $\beta$ -actin and represent mean  $\pm$  SE from three separate experiments (normal untreated and treated macrophages were arbitrarily assigned a value of 1.0 for comparison with tumor samples).

of NF $\kappa$ B and C/EBP proteins in these cells. To test this idea we treated N-PEMs and T-PEMs with the proteasome inhibitor MG-132 and examined the expression of the different NF $\kappa$ B and C/EBP proteins in these two macrophage populations. As Fig. 3 shows, the reduced expression of NF $\kappa$ B (p50, p65 and c-rel) and C/EBP ( $\alpha$  and  $\beta$ ) observed in resting T-PEMs is significantly reversed in all cases upon their culture with MG-132, although the effect is especially observed for NF $\kappa$ B p65, c-rel and C/EBP $\alpha$ . This may suggest that enhanced proteasome-induced proteolysis plays an important role in NF $\kappa$ B and C/EBP deficiencies in T-PEMs, but that additional mechanisms may be also important in NF $\kappa$ Bp50 and C/EBP $\beta$  impairments in these cells. Interestingly, with the exception of NF $\kappa$ Bp50, there is increased NF $\kappa$ B and C/EBP protein expression in MG-132-treated N-PEMs as well, suggesting that a certain amount of proteolysis also occurs in macrophages from normal mice, although the proteasome effect is much more amplified in T-PEMs, likely accounting for a great deal of the transcription factor protein defect in these cells. There were no differences in NF $\kappa$ B and C/EBP expression in macrophages grown in complete media as compared to cells grown in DMSO. Cell viability was not affected by treatment with MG-132 (results not shown).

## Discussion

Proteolytic degradation of eukaryotic cell proteins by the UPS is the result of a highly complex and tightly controlled series of reactions that are central to the regulation of basic cellular features, such as development, differentiation, proliferation, cell cycling, gene expression, signal transduction, apoptosis and senescence, as well as antigen presentation, inflammation and stress response. This process is carried out by a complex cascade of enzymes and displays a high degree of specificity towards its numerous substrates. Non-proteolytic post-translational modifications of proteins represent another layer of regulation of cellular homeostasis of central importance. In

addition, the UPS functions as a quality control mechanism that selectively removes abnormal and damaged proteins, which would otherwise form toxic intracellular inclusions as seen in various neurodegenerative diseases. The UPS has been identified as the cell's major tool for extralysosomal cytosolic and nuclear protein degradation. The 26S proteasome, a large multicatalytic multisubunit protease complex, constitutes the central proteolytic machinery of the UPS and is responsible for the degradation and proteolytic processing of many different cell proteins. Cell proteins destined to undergo processing by the UPS must be targeted for recognition and subsequent degradation by the 26S proteasome (24) by covalent attachments of multiple monomers of the 76 amino acid, 8.5 kDa polypeptide ubiquitin, which is highly conserved and present in the cytoplasm of all eukaryotic cells. Proteins are rapidly degraded into small peptides in the proteasome and ubiquitin molecules are cleaved off the protein immediately prior to destruction and are recycled for further use.

The most prominent role of ubiquitin is labeling proteins for proteasomal degradation, although modification to modulate stability, function and intracellular localization of a wide variety of proteins synthesized as inactive precursors are also regulated by this molecule. A prerequisite for the selective degradation of intracellular proteins by the UPS is the attachment of a chain of ubiquitin monomers to the targeted protein. Polyubiquitinated proteins with the polymerization at ubiquitin lysine 48 are targeted for degradation by the proteolytic 26S proteasome, where proteins are cut progressively into small peptides of 6 to 12 amino acids that are subsequently released and rapidly hydrolysed to amino acids by cytosolic exopeptidases. The release of ubiquitin from the substrate protein makes ubiquitin available for recycling in the proteolytic pathway.

One would expect that aberrations in such a complex system may be implicated in the pathogenesis of many diseases. In view of this complexity, it is not surprising that the UPS can undergo substantial deregulation that contributes

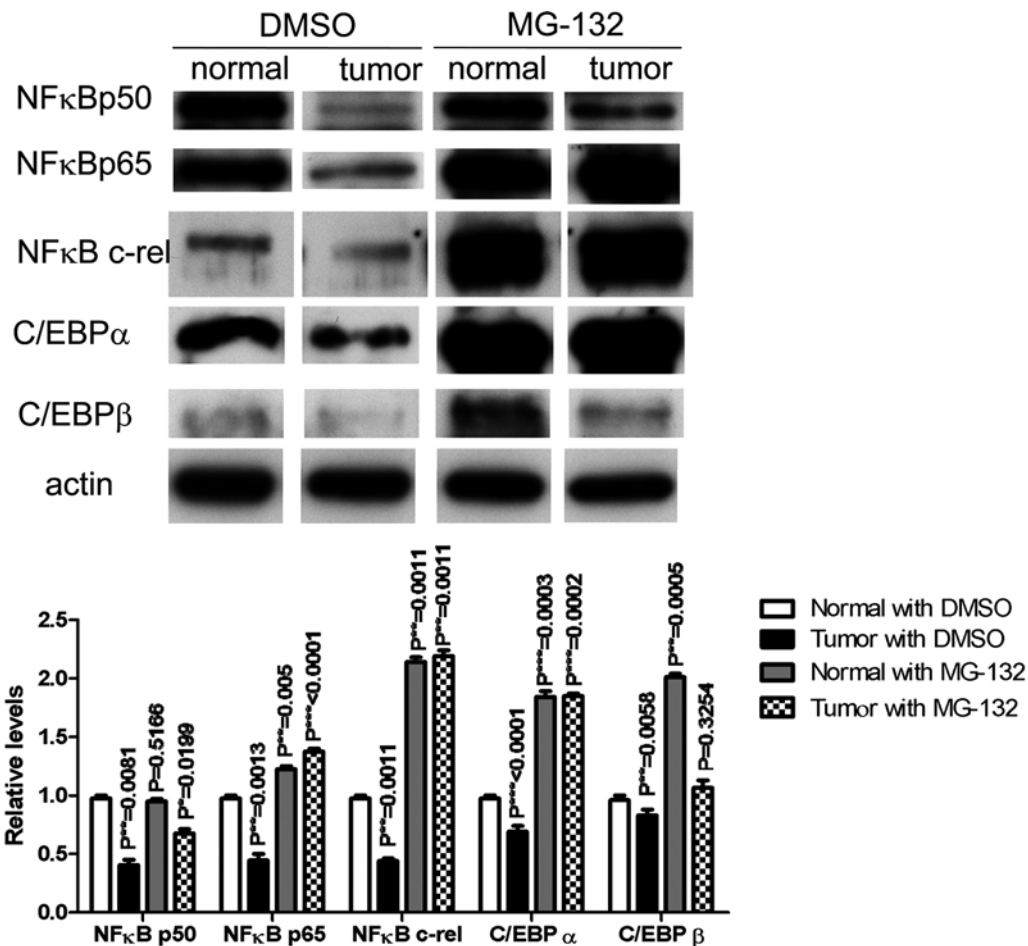


Figure 3. Enhanced proteasomal-induced proteolysis may account for NFκB and C/EBP deficit in macrophages from tumor hosts. Macrophages from normal and 4-week tumor-bearing mice ( $10 \times 10^6$ ) were incubated for 20 h in complete medium containing  $25 \mu\text{M}$  of the proteasome inhibitor MG-132 or similar volumes of DMSO. Cells were lysed, whole cell extracts were obtained and Western blot analysis was performed to detect expression of NFκB and C/EBP. Densitometry values were normalized with  $\beta$ -actin and represent mean  $\pm$  SE from three separate experiments (normal DMSO-treated macrophages were arbitrarily assigned a value of 1.0 for comparison with other samples).

to the pathogenesis of various human diseases, such as cancer, neurodegenerative, autoimmune, genetic and metabolic disorders. Importantly, the findings that inhibition of proteasome activity induces apoptosis selectively in cancer cells and interferes with essential functions of immune cells (24) have led to the exploitation of the UPS as a molecular target for cancer therapy and immune modulation. On the other hand, proteasome inhibitors represent a powerful tool for dissecting the role of the UPS in cellular physiology and also have practical applications in cancer therapy (25). They have already played an essential role in advancing our understanding of importance of the proteasome in biology.

We have previously shown that macrophages from tumor-bearing mice have impaired gene and protein expression of NFκB and C/EBP and that tumor-secreted TGF- $\beta$  is associated with these deficiencies (11,12). Among other mechanisms that might explain these defects, we hypothesized that enhanced proteasomal degradation in macrophages from tumor hosts may contribute to this effect. To provide additional evidence of the role of TGF- $\beta$  in abating NFκB protein expression, we treated macrophages from normal mice with recombinant TGF- $\beta_1$  and the TGF- $\beta$  signaling inhibitor SB-431542, and measured NFκB proteins in normal macrophages.

Small molecules, such as SB-431542, are unlikely to completely block signaling (26), thus it was not surprising to realize that the simultaneous culture with TGF- $\beta_1$  together with SB-431542 could only partially reverse NFκBp65 down-regulation. Recent studies of the Smad family proteins, which are the key signal transducers of the TGF- $\beta$  family ligands, have revealed the ability of Smads to interact with various components of the 26S proteasome system (27). In breast cancer cell lines, TGF- $\beta$  increases the activity of the proteasome (28). We speculate that TGF- $\beta$ -induced modulation of proteasomal function may in part mediate the immunosuppressive effects of TGF- $\beta$  in macrophages by accelerating degradation of inflammatory mediators. Further studies to elucidate the role of TGF- $\beta$  on proteasome function in macrophages are required to assess the potential involvement of the proteasome in immune regulation by TGF- $\beta$ , particularly as it regards to increased NFκB and C/EBP proteolysis.

To examine the consequences of inhibiting proteasome activity in macrophages from tumor hosts, we used MG-132, a peptide aldehyde proteasome inhibitor. Our results show that upon blocking proteasome activation in macrophages from tumor-bearing animals, excess ubiquitination and proteolysis were inhibited and the impaired NFκB and C/EBP protein

expression was restored in the dysfunctional cells. Importantly, however, even after proteasome inhibition, NF $\kappa$ Bp50 and C/EBP $\beta$  remain downregulated in macrophages from tumor-bearers, suggesting the existence of additional mechanisms of transcriptional, post-transcriptional or even translational nature, accounting for the deficit of these two proteins in macrophages from tumor-bearing mice. Interestingly, a less pronounced effect is also observed in macrophages from normal mice, which exhibit increased protein expression upon proteasome inhibition in all the cases except with NF $\kappa$ Bp50. A certain level of NF $\kappa$ B and C/EBP proteolysis may normally exist as a homeostatic mechanism for breakdown and recycling of intracellular proteins in macrophages. However, our results suggest that this process is remarkably accelerated in macrophages from tumor hosts, probably due to the presence of tumor-derived factors such as TGF $\beta$ .

The proteasome is also critical to the regulation of LPS-induced signaling in macrophages, controlling functions such as TLR signaling and thus NF $\kappa$ B activation. Hence, proteasome inhibition results in a conversion to an anti-inflammatory phenotype in LPS-activated macrophages (29). If this is the case, our data implicate that proteasome inhibition in resting macrophages, particularly the ones from tumor-bearers, results in an opposite phenotype to that reported with proteasome inhibition in LPS-activated macrophages, i.e., resting macrophages, especially from tumor-bearers, exhibit enhanced UPS activity and increased proteolysis of several proteins such as the pro-inflammatory NF $\kappa$ B and C/EBP, thus, proteasome inhibition in resting macrophages results in a pro-inflammatory phenotype.

Macrophages infected by *Leishmania* have STAT1 degraded by means of the UPS (30). Similar to that observed with parasites, tumors may subvert the microbicidal or inflammatory functions of macrophages to promote their own survival and propagation. We have shown that not only NF $\kappa$ B and C/EBP but also several other inflammatory cytokines and signaling intermediates such as STAT1, MAK kinases and IRAK1 are impaired in macrophages from tumor hosts (11). It will be important to examine whether the abnormal increase in ubiquitination and proteolysis that we have shown here in macrophages from tumor hosts might explain the deficit in some of these other proteins as well. The use of proteasome inhibitors will then be extremely useful to reverse immune suppression in dysfunctional macrophages from tumor hosts.

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