Microenvironment and Immunology

Cancer Research

NF-κB, and not MYCN, Regulates MHC Class I and Endoplasmic Reticulum Aminopeptidases in Human Neuroblastoma Cells

Matteo Forloni[1], Sonia Albini[1], Maria Zaira Limongi[1], Loredana Cifaldi[1], Renata Boldrini[2], Maria Rita Nicotra[3], Giuseppe Giannini[4], Pier Giorgio Natali[5], Patrizio Giacomini[5], and Doriana Fruci[1]

Abstract

Neuroblastoma (NB) is the most common solid extracranial cancer of childhood. Amplification and overexpression of the MYCN oncogene characterize the most aggressive forms and are believed to severely downregulate MHC class I molecules by transcriptional inhibition of the p50 NF-κB subunit. In this study, we found that in human NB cell lines, high MYCN expression is not responsible for low MHC class I expression because neither transfection-mediated overexpression nor small interfering RNA suppression of MYCN affects MHC class I and p50 levels. Furthermore, we identified NF-κB as the immediate upstream regulator of MHC class I because the p65 NF-κB subunit binds MHC class I promoter in chromatin immunoprecipitation experiments, and MHC class I expression is enhanced by p65 transfection and reduced by (*a*) the chemical NF-κB inhibitor sulfasalazine, (*b*) a dominant-negative IKBα gene, and (*c*) p65 silencing. Moreover, we showed that the endoplasmic reticulum aminopeptidases ERAP1 and ERAP2, which generate MHC class I binding peptides, are regulated by NF-κB, contain functional NF-κB–binding elements in their promoters, and mimic MHC class I molecules in the expression pattern. Consistent with these findings, nuclear p65 was detected in NB cells that express MHC class I molecules in human NB specimens. Thus, the coordinated downregulation of MHC class I, ERAP1, and ERAP2 in aggressive NB cells is attributable to a low transcriptional availability of NF-κB, possibly due to an unknown suppressor other than MYCN. *Cancer Res; 70(3); 916–24. ©2010 AACR.*

Introduction

Neuroblastoma (NB) is the most common malignancy diagnosed in the first year of life. This tumor arises from neural crest cells and shows a wide range of clinical outcomes ranging from spontaneous regression to therapy-resistant progression. Patients bearing aggressive NB tumors have survival probabilities of <40% despite intensive chemoradiotherapy.

NB exhibits several genomic alterations, including MYCN gene amplification; chromosomal deletions at 1p, 3p, and 11q; and chromosomal gain at 17q (1, 2). *MYCN* amplification is consistently associated with high MYCN protein levels and is regarded as a hallmark of poor prognosis and treatment failure (3).

Authors' Affiliations: [1]Research Center and [2]Division of Pathology, Ospedale Bambino Gesù; [3]Molecular Biology and Pathology Institute, National Research Council; [4]Departments of Experimental Medicine and Pathology, University La Sapienza; [5]Laboratory of Immunology, Regina Elena Cancer Institute, Centro della Ricerca Sperimentale, Rome, Italy

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Doriana Fruci, Research Center, Ospedale Bambino Gesù, Piazza S. Onofrio 4, 00165 Rome, Italy. Phone: 39-06-68592657; Fax: 39-06-68592904; E-mail: fruci@opbg.net.

doi: 10.1158/0008-5472.CAN-09-2582

Like many other (proto)oncogene products (4), MYCN and the highly homologous nuclear oncogene c-MYC have been implicated in the downregulation of MHC class I (MHC I) molecules in man (5–7) and rat (8–10), but some aspects of this regulation remain controversial. In a rat NB cell line, high levels of MYCN, obtained by the gene transfection, have been shown to repress MHC I expression (8–10). However, such repression effect on MHC I expression has not been detected on the MYCN transfection in a human NB cell line (7).

In the B104 rat NB cell line, MYCN expression was shown to downregulate the expression of p50, a subunit shared by NF-κB and KBF1, two transcription factors of the NF-κB/Rel family (8–10). NF-κB is well known to transactivate MHC I heavy chains and their light chain β_2-microglobulin (β_2m) by binding to the *cis*-regulatory promoter element called enhancer A (11, 12). However, no direct evidence that MYCN impairs NF-κB/enhancer A interaction or MHC I expression has been reported in human cells.

Moreover, it is unknown whether MYCN is involved, besides MHC I and β_2m molecules, in the regulation of the MHC I antigen-processing functions that are required for MHC I expression.

In this respect, we have recently shown that malignant transformation causes losses, gains, and imbalances in the expression of human MHC I and two antigen-processing gene products: the endoplasmic reticulum (ER) aminopeptidases ERAP1 and ERAP2 (13, 14). The concerted peptide-trimming

activity of ERAP1 and ERAP2 optimizes precursor peptides for binding to MHC I molecules and shapes a normal T-cell repertoire (15). We showed that altered expression of these genes impairs, as expected, both peptide-trimming and MHC I expression in tumor tissues and cultured tumor cell lines (13, 14). Interestingly, MHC I and ERAP1 were coordinately expressed in normal and, to a lesser extent, neoplastic cells and tumor lesions (13, 14). This raises the possibility that MYCN affects the expression of these aminopeptidases and consequently represses MHC I expression.

In this report, we show that not only MHC I but also ERAP1 and ERAP2 are under the control of NF-κB through enhancer A in human NB cells. However, none of these genes is under the direct control of MYCN, and the low availability of transcriptionally active NF-κB by itself explains the low expression levels of ERAP1, ERAP2, and MHC I in NB cell lines regardless of the levels of MYCN expression.

Materials and Methods

Tumor cell lines and reagents. Human NB cell lines, obtained from the American Type Culture Collection, grown in RPMI 1640 with 10% FCS, were characterized by morphology and HLA class I typing. SH-EP MYCN-ER and Tet-21/N cell lines were kindly provided by Drs. B. Berwanger (Children's Hospital, Medical Center, Marburg, Germany; ref. 16) and M. Schwab (Johann Wolfgang Goethe-University, Frankfurt, Germany; ref. 17), respectively. 4-Hydroxytamoxifen (4-OHT) and doxycycline, both from Sigma-Aldrich, were used at 200 nmol/L and 10 ng/mL, respectively. Recombinant human tumor necrosis factor α (TNFα; PeproTech) and IFNγ (R&D Systems) were used at 50 ng/mL and 500 units/mL, respectively. Sulfasalazine and MYCN small interfering RNAs (siRNA) were from Sigma-Aldrich. The p65 siRNAs were from Cell Signaling Technology.

DNA constructs and transfections. Mutant IκBα (18) and NF-κB p65 (kindly provided by Drs. M. Cippitelli, University of Rome Sapienza, Rome, Italy, and M. Levrero, Rome Oncogenomic Center, Regina Elena Institute, Rome, Italy, respectively) and the corresponding empty vectors were transfected using Lipofectamine 2000 (Invitrogen). For p65 and MYCN silencing, cells were transfected with 100 nmol/L siRNAs or scrambled siRNA using Lipofectamine 2000.

Real-time PCR. Total RNA was extracted with Trizol reagent (Invitrogen) and retrotranscribed with SuperScript II (Invitrogen). Reverse transcription and real-time PCR were performed as described (19). 18S RNA was used for normalization.

Antibodies. The A-LAP and 3F5 antibodies were raised against ERAP1 and ERAP2, respectively (15, 20). MHC I was detected by Western blotting using R5996-4 (21) and by flow cytometry using W6/32 (22) and the HLA-A locus-specific TU155 (23). The antibodies to MYCN, p50, p65, and proliferating cell nuclear antigen (PCNA) were from Santa Cruz Biotechnology.

Immunoblotting. Whole-cell and nuclear extracts obtained as previously described (13, 24) were quantified by bicinchoninic acid assay (Pierce), resolved on 10% SDS-PAGE, and electroblotted. Filters were probed with primary antibodies followed by secondary peroxidase-coupled antibodies. ERp57 and PCNA were used as loading control of whole-cell and nuclear extracts, respectively.

Immunofluorescence and immunohistochemistry. MHC I surface expression was determined by flow cytometry with the indicated antibodies on a FACSCalibur (Becton Dickinson). For detection of NF-κB nuclear translocation, cells were grown on polystyrene glass slides, fixed in cold methanol-acetone (2:1), blocked in 1% bovine serum albumin and 0.5% goat serum, and stained with antibody to p65. The cells were then washed with PBS, incubated with FITC-conjugated goat anti-rabbit IgG (Vector), and counterstained with the nuclear dye DRAQ5 (Biostatus). The cells were analyzed in a confocal microscope (Olympus FluoView FV1000).

Primary NB lesions were obtained on informed parental consent and snap frozen in liquid nitrogen. Cryostat sections (4 μm thick) were fixed either in cold absolute acetone and stained by W6/32 to MHC I, or in acetone-methanol (1:1, v/v) and stained by NF-κB p65 antibody. Staining was revealed by a supersensitive immunohistochemistry kit (BioGenex), as described (14).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed as described (25) using the NF-κB p65 monoclonal antibody. The immunoprecipitated DNA was amplified by using specific primers. Primer sequences are listed in Supplementary Data.

Statistical analysis. Raw data were normalized as the percent of the highest value obtained for each assay. Regression and significance were analyzed by the StatView software. A P value of <0.05 was considered statistically significant.

Results

MYCN, MHC I, ERAP1, ERAP2, and NF-κB expression in NB cell lines. Because MYCN is inversely correlated with MHC I (8–10), and MHC I is positively correlated with ERAP1 expression in human cell lines of various lineages (13, 14), we looked for expression patterns of MYCN, MHC I heavy chain, ERAP1, and ERAP2 by Western blotting (Fig. 1A) and of cell surface MHC I by flow cytometry (Fig. 1B) in a panel of human NB cell lines. Representative regression plots of normalized densitometric values of Western blotting and mean fluorescence intensity values of flow cytometry are shown in Supplementary Fig. S1A.

It was evident (Fig. 1A) that MHC I heavy chain and ERAP1 are expressed at high levels in the ACN, SH-EP, and SK-N-AS cell lines and at low levels in the IMR-32, KCNR, LA-N-5, SK-N-BE(2), SH-SY5Y, SK-N-SH, and SK-N-SY cell lines. This latter group of NB cell lines displayed a limited variability in MHC I heavy chain and ERAP1 expression in spite of marked differences in MYCN expression. Accordingly, regression analysis of Western blotting data (Supplementary Fig. S1A) revealed the inverse correlation between MYCN and MHC I heavy chains (R^2 = 0.27; P = 0.13). A similar inverse correlation was found between MYCN and ERAP1 (R^2 = 0.23; P = 0.16). MYCN and cell surface MHC I also showed an inverse correlation (R^2 = 0.35; P = 0.07). However, none of these correlations reached the significance threshold. In contrast, highly significant correlations were detected between ERAP1

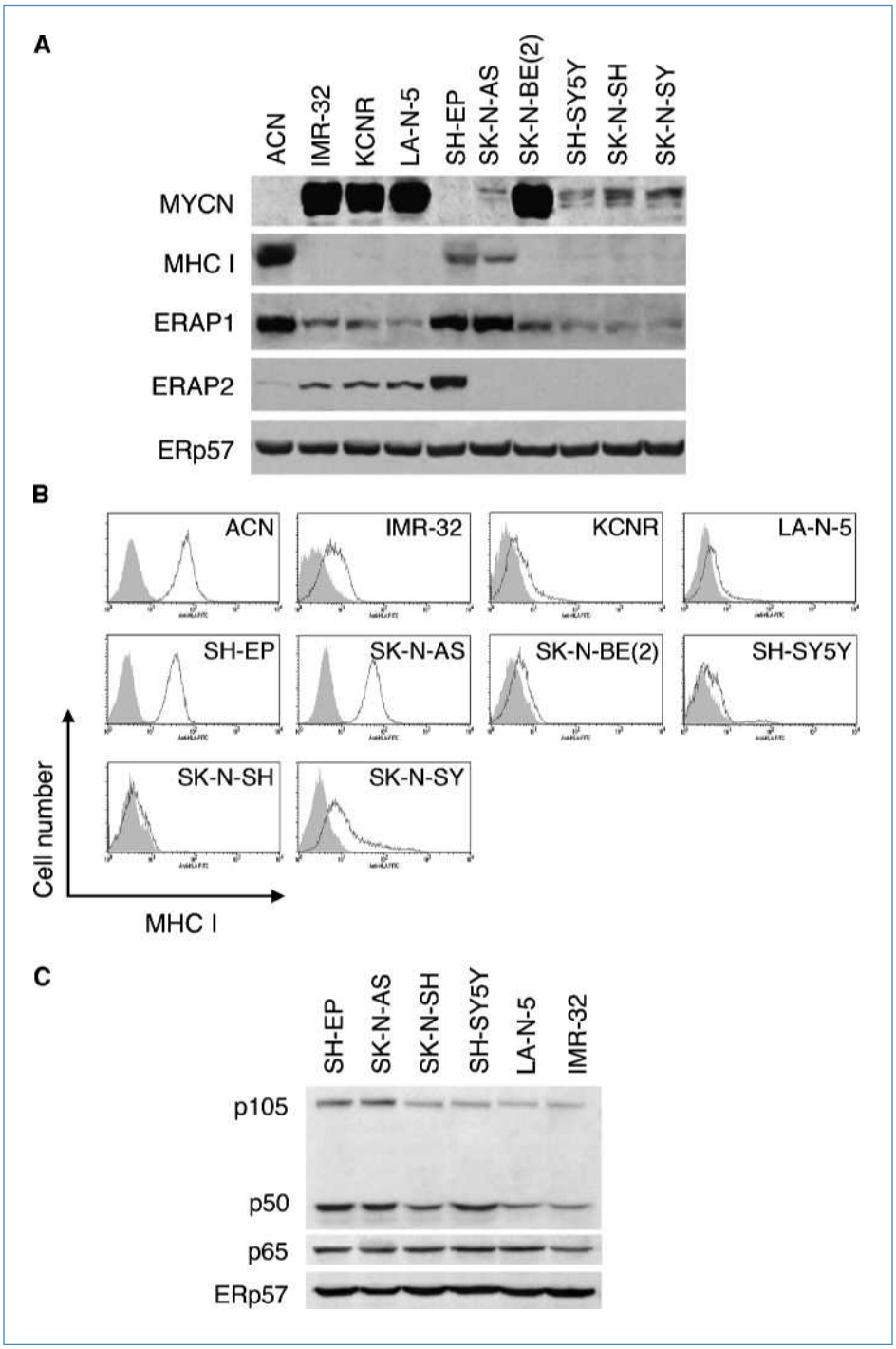

Figure 1. Expression of MYCN, MHC I, ERAP1, ERAP2, and NF-κB p105, p50, and p65 subunits in NB cell lines. A, immunoblot analysis of MYCN, MHC I, ERAP1, and ERAP2 in different NB cell lines. Equal amounts of cell lysates were resolved by SDS-PAGE and probed with the indicated antibodies. B, flow cytometry analysis of surface MHC I expression of NB cells (black lines) using the w6/32 mAb. Shaded histograms, negative controls stained with isotype-matched primary antibody. C, Western blot analysis of p105, p50, and p65 expression in whole-cell extracts of different NB cell lines. ERp57 was used for normalization in A and C.

and MHC I heavy chains (R^2 = 0.62; P = 0.0071) and also between ERAP1 and cell surface MHC I (R^2 = 0.88; P < 0.0001). This is consistent with the finding obtained for other human cell lines (13). Any of MYCN, MHC I, and ERAP1 did not significantly correlate with ERAP2.

NF-κB has been known to be involved in MHC I expression (11, 12) and the p50 NF-κB subunit has been claimed to directly correlate with MHC I expression in the rat B104 NB cell line (8–10). To see whether NF-κB is a transcription factor also of ERAP1 and ERAP2, NF-κB p65 and p50 subunits, as well as the p105 precursor, were assessed for the whole-cell lysates in representative NB cell lines by Western blotting (Fig. 1C). Densitometric data were analyzed for the correlation with those of MHC I heavy chain, ERAP1, and ERAP2 (Fig. 1A) and cell surface MHC I (Fig. 1B). Representative plots are displayed in Supplementary Fig. S1B. This analysis

revealed that MHC I heavy chain correlated significantly with p50 (R^2 = 0.84; P = 0.01) and with p105 (R^2 = 0.64; P = 0.05) and that ERAP1 also correlates with p50 (R^2 = 0.86; P = 0.008) and p105 (R^2 = 0.88; P = 0.006). Similar correlations were found for cell surface MHC I with p50 (R^2 = 0.92; P = 0.03) and with p105 (R^2 = 0.94; P = 0.001). On the other hand, no significant correlations were found for p65 with any of MHC I heavy chain, ERAP1, and ERAP2 and cell surface MHC I. MYCN showed inverse and nonsignificant correlation with p50 (R^2 = 0.45; P = 0.14), p65 (R^2 = 0.30; P = 0.26), and p105 (R^2 = 0.57; P = 0.08).

Thus, MHC I, ERAP1, and the NF-κB p105 and p50 subunits are linked each other by statistically significant patterns of coordinated expression, whereas their correlations with MYCN were inverse and nonsignificant, and ERAP2 and p65 behaved as completely independent variables.

MYCN does not regulate the expression of MHC I, ERAP1, ERAP2, and NF-κB subunits in NB cell lines. To directly determine whether MYCN downregulates MHC I and NF-κB expression and affects ERAP1 and ERAP2 expression in human NB cell lines, we induced rapid changes in the functional expression of MYCN in two MYCN-transfected NB cell line: SH-EP MYCN-ER (16) and Tet-21/N (16). SH-EP MYCN-ER carries 4-OHT–responsive MYCN transgene fused to a mutagen estrogen-responsive domain (ER), whereas Tet-21/N carries a tetracycline-repressible MYCN transgene. The expression of MYCN is activated with 4-OHT treatment in SH-EP MYCN-ER and suppressed with doxycycline treatment in Tet-21/N. These two cell lines, untreated or treated with 4-OHT or doxycycline, were tested by Western blotting for the expression of MHC I, ERAP1, ERAP2, as well as NF-κB p105, p50, and p65. MYCN expression was drastically activated with 4-OHT treatment in SH-EP MYCN-ER, as indicated by the strong upregulation of the known MYCN target gene *HMGA1* (*high mobility group A1*; ref. 26) determined by quantitative reverse transcription-PCR (qRT-PCR; Supplementary Fig. S2), and drastically suppressed by doxycycline treatment in Tet-21/N, as determined by Western blotting (Fig. 2B). In spite of this clear change of MYCN expression, no noticeable change was seen in any of MHC I heavy chain, ERAP1, ERAP2, and NF-κB p105, p50, and p65 in both cell lines tested (Fig. 2A and B). Furthermore, MYCN silencing by RNA interference in SH-SY5Y cells did not affect the levels of any of MHC I, ERAP1, ERAP2, and NF-κB p105, p50, and p65 as compared with the controls (Fig. 2C).

Thus, MYCN is not directly involved in the regulation of MHC I, ERAP1, ERAP2, and NF-κB expression in NB cell lines.

Effect of TNFα on the expression of MHC I, ERAP1, ERAP2, and NF-κB. The coordinated expression of MHC I, ERAP1, and NF-κB indicates the possibility that ERAP1 is regulated by the same transcription factor as MHC I (i.e., NF-κB). To address the regulatory role of NF-κB in the MHC I and ERAP1 expression, NB cell lines that differ in MHC I, ERAP1, and ERAP2 protein level were treated with TNFα, a major inducer of NF-κB nuclear translocation, and the NF-κB p50 and p65 subunits were assayed for the nuclear extracts.

As shown in Fig. 3A, TNFα mediated detectable nuclear translocation of both p50 and p65 subunits in SH-EP and SK-N-AS that were high in constitutive MHC I and ERAP1 expression. This was associated by steady-state accumulation of MHC I, ERAP1, and ERAP2 in SH-EP and MHC I and ERAP1 in SK-N-AS (Fig. 3A) and also by surface enhancement of MHC I molecules (Fig. 3B). These results were confirmed at the mRNA level by qRT-PCR (Supplementary Fig. S3). The observed enhancing effects of TNFα were not seen in other NB cell lines that were low in MHC I, ERAP1, and ERAP2 expression (Fig. 3A and B).

To rule out that MYCN expression per se might cause the difference in the responsiveness to TNFα treatment of NB

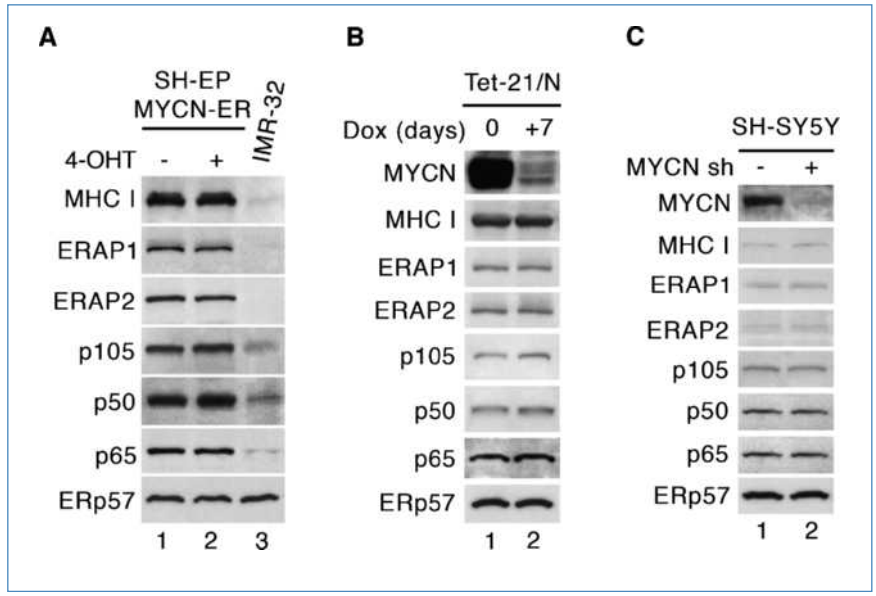

Figure 2. MYCN expression does not affect expression of the p50 NF-κB subunit, MHC I, ERAP1, and ERAP2 in NB cell lines. Immunoblot analysis of MYCN, MHC I, ERAP1, ERAP2, and NF-κB p105, p50, and p65 subunits in (A) SH-EP MYCN-ER grown in the presence and absence of 4-OHT for 66 h, (B) Tet-21/N cells either left untreated (*0*) or treated with doxycycline (*Dox*; *+7*) for 7 d, and (C) SH-SY5Y transfected with either MYCN siRNAs or control scrambled. The MYCN-amplified IMR-32 NB cell line was included as a control in A. ERp57 was used for normalization.

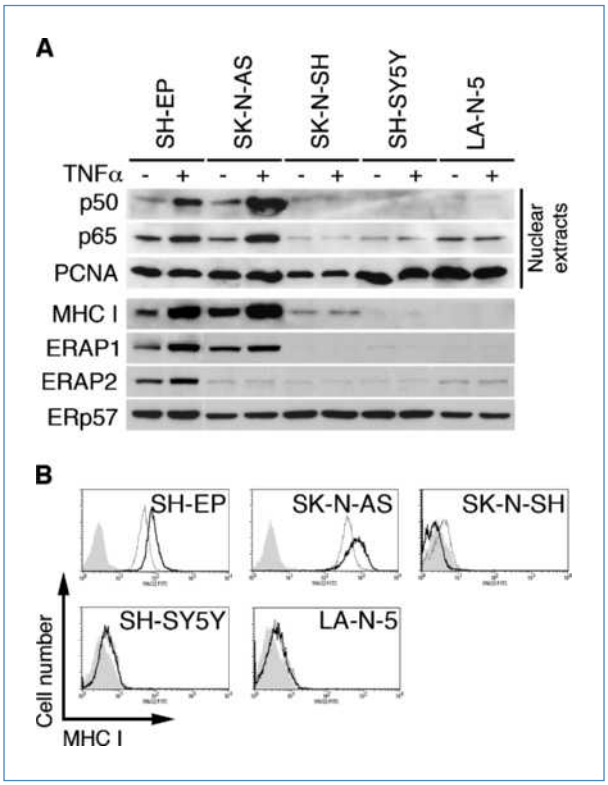

Figure 3. TNFα enhances the expression of MHC I, ERAP1, and ERAP2 in a set of NB cell lines. A, immunoblot analysis of NB cell lines grown in the presence and absence of TNFα for either 1 h (top three panels, nuclear extracts) or 48 h (bottom four panels, whole-cell extracts). PCNA and ERp57 were used for normalization. B, flow cytometry analysis of surface MHC I expression of NB cells either TNFα treated for 48 h (black lines) or untreated (gray lines) using the W6/32 mAb. Shaded histograms, isotype-matched negative controls.

cell lines, the Tet-21/N cell line was treated with TNFα in the presence and absence of doxycycline and tested for p50 and p65 in the nuclear extracts. In spite of drastic MYCN repression, no changes were detected in either constitutive nuclear expression or TNFα-mediated translocation of p50 and p65 (Supplementary Fig. S4).

Altogether, it seems that the availability of nuclear NF-κB subunits correlates with MHC I, ERAP1, and ERAP2 levels in all the tested NB cells regardless of MYCN expression and that some factors other than MYCN repress nuclear translocation of NF-κB in NB cell lines.

NF-κB regulates MHC I, ERAP1, and ERAP2 in NB cell lines. To explore further the relevancy of NF-κB to ERAP1 and ERAP2 expression, we used three different approaches: (*a*) to inhibit NF-κB nuclear translocation with specific inhibitors, (*b*) to inhibit NF-κB expression by silencing p65 subunit expression with a p65-specific siRNA, and (*c*) to enhance NF-κB p65 subunit by transfection of a p65 expression vector.

In the first approach (*a*), sulfasalazine, a chemical inhibitor of nuclear NF-κB translocation, was shown to prevent nuclear translocation of p65 in SH-EP (Supplementary Fig. S5) and SK-N-AS (data not shown) cells in a dose-dependent fashion. At the optimal dosage, sulfasalazine treatment strongly reduced the constitutive and/or TNFα-induced expression of MHC I, ERAP1, and ERAP2 in these cells (Fig. 4A). Furthermore, a stable SH-EP transfectant carrying an IκBα mutant (M-IκBα) that is refractory to TNFα (18) was shown to be drastically impaired in nuclear translocation of p65, as assessed by immunofluorescence in a time course experiment (Supplementary Fig. S6), and in MHC I, ERAP1, and ERAP2 mRNA accumulation (Fig. 4B). The effect of the mutant IκBα was specific because IFNγ enhanced the expression of MHC I, ERAP1, and ERAP2 mRNA equally well in SH-EP cells transfected with both M-IκBα and the empty vector, whereas TNFα enhanced expression of the tested genes less efficiently in SH-EP cells transfected with IκBα than cell with the empty vector (Fig. 4B).

In the second approach (*b*), p65-specific siRNA was shown to knock down p65 and reduced MHC I, ERAP1, and ERAP2 expression in SH-EP cells, as assessed by Western blotting (Fig. 4C).

In the last approach (*c*), five representative NB cell lines that differ in MYCN, MHC I, ERAP1, and ERAP2 protein level (Fig. 1A) were transfected with a vector bearing p65 cDNA (p65) or the empty vector (pcDNA3) and looked for expression of p65, MHC I heavy chain, ERAP1, and ERAP2 by Western blotting and for cell surface MHC I by flow cytometry (Fig. 4D). As shown in Fig. 4D, p65 transfection enhanced expression of p65 in all the tested NB cell lines. MHC I heavy chain was enhanced in all the tested cell lines, particularly well in SH-SY5Y, IMR-32, and SK-N-BE(2)c, except LAN-5, whereas ERAP1 was clearly enhanced in SH-EP, SH-SY5Y, and SK-N-BE(2)c. Flow cytometry of the four cell lines in which p65 effectively upregulates MHC I heavy chain showed that MHC I surface expression was fairly proportional to the level of MHC I heavy chain in SH-EP and SH-SY5Y but not in IMR-32 and SK-N-BE(2)c in which only a marginal increase was noticeable (Fig. 4D). This poor surface MHC I expression may result from impaired expression of some components in MHC I–presenting machinery, such as β_2m and TAP, other than MHC I heavy chain.

These results indicate that NF-κB upregulates MHC I, ERAP1, and ERAP2 in NB cell lines, and the low expression of these genes is due to a low efficiency of NF-κB in activating target gene transcription, presumably consequence of a poor constitutive NF-κB nuclear expression, and/or the nuclear translocation as suggested by the TNFα unresponsiveness in several tested NB cell lines (Fig. 3A).

Recruitment of NF-κB to ERAP1 and ERAP2 promoter regions. Because NF-κB directly binds enhancer A elements in the promoters of *MHC I* genes (27), we looked for similar sequences in the promoter regions of ERAP1 (28) and ERAP2 (29). Enhancer A elements were detected in both promoters at positions –79 and –14 and displayed 90% and 70% analogies with the canonical MHC I enhancer A sequence, respectively. Of interest, enhancer A elements were close to ISRE sequences, as in MHC I genes (Fig. 5A).

To evaluate the recruitment of NF-κB to these promoter regions, PCR primers were designed for the different gene promoters and ChIP assays were performed using an anti-p65

antibody to precipitate chromatin fragments from SH-EP cells, either untreated or treated (1 hour) with TNFα. The interleukin-8 (IL-8) gene promoter, a known target of NF-κB (30), was used as a control. As shown in Fig. 5B, p65 specifically binds all the tested promoters (*lane 3*), and the signal is strongly increased following TNFα stimulation (*lane 5*). Remarkably, the increased recruitment of p65 to these promoter regions results in the expected enhancement in surface MHC I expression as shown by flow cytometry with HLA-A–specific mAb TU155 (Fig. 5C). These data show that ERAP1 and ERAP2 are regulated through the NF-κB/enhancer A pathway and provide a molecular basis for the coordinated expression of these genes with MHC I in NB cell lines.

Expression of MHC I and NF-κB in primary NB samples. To determine whether the correlation between MHC I expression and the expression/nuclear localization of NF-κB might also be observed *in vivo*, 32 primary NB lesions were tested by immunohistochemistry. Like in previous studies (31–34), no MHC I expression could be detected in the neuroblastic cell components. However, careful inspection revealed scattered W6/32-positive cells with distribution and morphology reminiscent of gangliar cells (e.g., most differentiated neuroblastic cells) in eight tumors (Fig. 6A). Remarkably, only these cells displayed nuclear p65 staining (Fig. 6B). These results suggest *in vitro-in vivo* analogies in MHC I regulation by NF-κB.

Discussion

Overexpression of MYCN has been shown to interfere with the expression of MHC I by blocking the transcription of p105 NF-κB precursor in a rat NB cell line (8–10). NF-κB is a well-known transcription factor that binds a consensus regulatory region upstream of *MHC I* genes, known as enhancer A (11). Although an inverse correlation between MYCN and MHC I has been reported in human NB cell lines, a direct

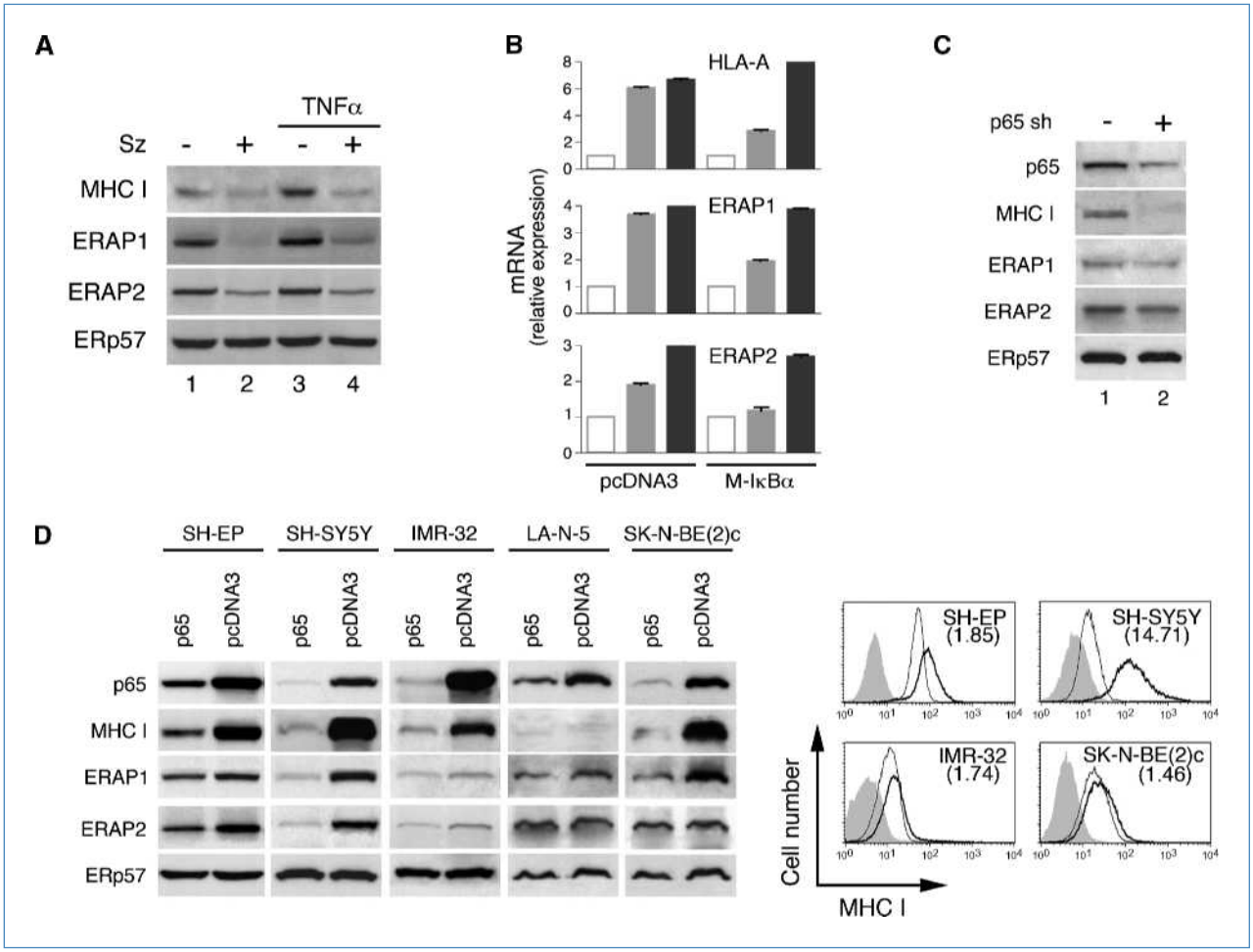

Figure 4. NF-κB regulates the expression of MHC I, ERAP1, and ERAP2 in NB cell lines. A, immunoblot of SH-EP cells preincubated for 30 min with 2 mmol/L sulfasalazine (*Sz*) and then grown for 8 h in the presence and absence of TNFα. B, qRT-PCR analysis of mRNAs from SH-EP-pcDNA3 and SH-EP M-IκBα transfectants either left untreated or grown for 48 h in the presence of TNFα or IFNγ. C, Western blot of SH-EP cells transfected with p65 siRNA (*p65 sh*) or control scrambled. D, Western blotting (left) and flow cytometry analysis with W6/32 mAb (right) of the indicated NB cell lines transfected with either an empty vector (*pcDNA3* in the left panel and gray line in the right panel) or a vector expressing p65 (*p65* in the left panel and black line in the right panel). Right, fold increases for each NB cell line are indicated in parenthesis. Shaded histograms, isotype-matched negative controls.

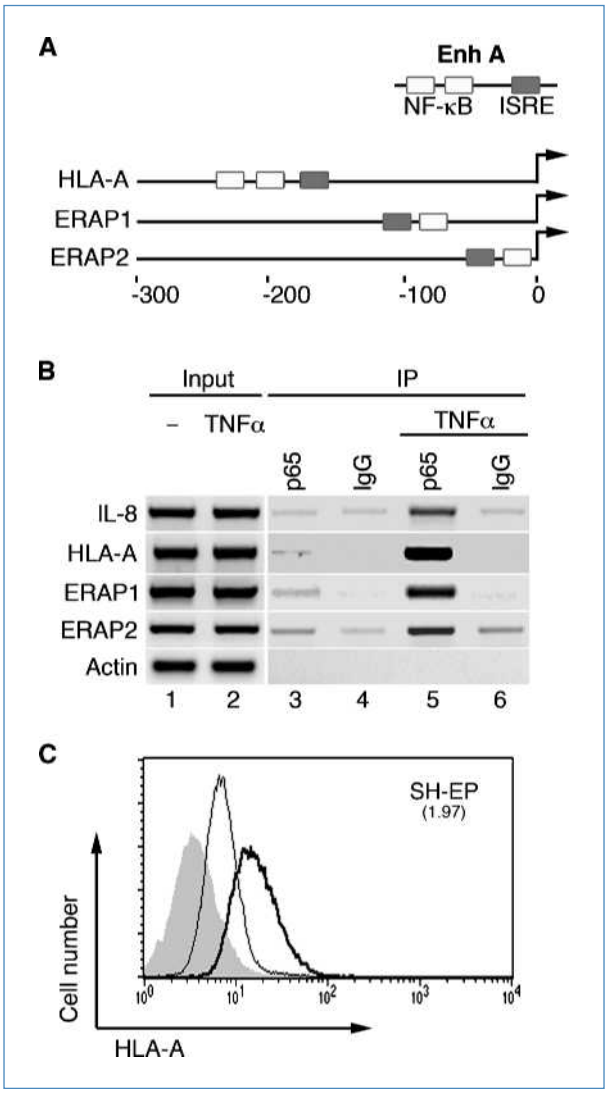

Figure 5. NF-κB interacts *in vivo* with the promoters of *MHC I, ERAP1*, and *ERAP2*. A, schematic representation of the promoter regions of the *HLA-A, ERAP1*, and *ERAP2* genes. Enhancer A (*Enh A*) and ISRE elements are indicated. B, chromatin immunoprecipitation from SH-EP cells grown for 30 min in the presence and absence of TNFα using an anti-p65 antibody and a control anti-IgG antibody. IL-8 and β-actin (*Actin*) promoters were included as positive and negative controls, respectively. C, flow cytometry analysis of surface HLA-A expression of SH-EP cells either TNFα treated for 48 h (black lines) or untreated (gray lines) using mAb TU155. Shaded histogram, isotype-matched negative control. Fold increase in the presence of TNFα is indicated in parenthesis.

demonstration of the MYCN-mediated downregulation of MHC I expression in human NB cells has been questioned by the observation that MYCN overexpression does not repress MHC I expression in the human SK-N-AS NB cell line (7). In the present report, we confirm that MYCN is inversely related to MHC I expression in human NB cell lines (Fig. 1) but also show that it does not have a direct effect on either the levels of MHC I molecules or the expression and nuclear translocation of the tested NF-κB subunits because none of them is detectably affected by MYCN overexpression (on transfection) or suppression (by siRNAs) in human NB cell lines (Fig. 2). In the context of the available literature (7–10, 34), the present data clearly show that MYCN does not regulate MHC I and NF-κB expression in human NB cell lines. It will be of interest to assess the effect of MYCN on MHC I expression in rat NB cell lines other than B104.

In addition, we confirm that NF-κB is a master transactivator of MHC I in NB because overexpression of the p65 NF-κB subunit reverses the MHC I–low phenotype of NB cell lines, and conversely, inhibition of NF-κB accumulation and/or nuclear translocation by either chemical inhibitors or overexpression of a dominant-negative IκBα gene causes a reduction in MHC I expression both at steady-state level and following TNFα treatment (Figs. 3 and 4). Moreover, we provide evidence that NF-κB is the immediate upstream regulator of MHC I expression in NB because it binds the MHC I promoter directly and proportionally to constitutive and TNFα-mediated activation (Fig. 5). Thus, whereas a direct involvement of MYCN in human MHC I expression may be excluded, the involvement of NF-κB and the underlying mechanism are clearly shown by the present data.

Herein, we distinguished two different NB cell phenotypes depending on the constitutive activation of NF-κB: phenotype (a), NB cells that express high levels of nuclear p65 NF-κB and consequently high levels of NF-κB target genes, such as MHC I molecules; phenotype (b), NB cells that express neither nuclear p65 NF-κB nor high levels of MHC I molecules. These two cell phenotypes differ also in terms of responsiveness to TNFα, suggesting that the two conditions, constitutively activated versus constitutively inactivated (and not TNFα-activable) NF-κB, are essential for their survival.

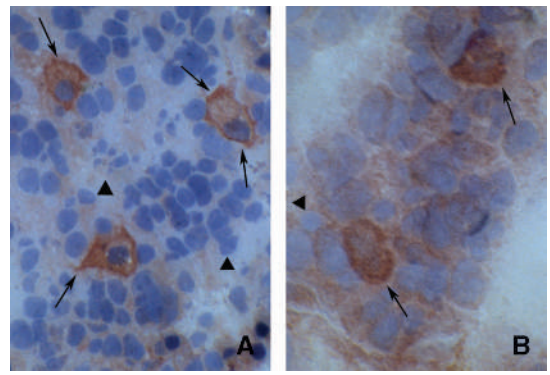

Figure 6. Expression of MHC I and NF-κB in primary NB lesions. Staining of cryostatic sections of NB lesion for MHC I and p65 NF-κB. A, MHC I expression is undetectable in neuroblasts (arrowheads) and strongly expressed in gangliar cells (arrows). Original magnification, ×250. B, weak cytoplasmic p65 expression in neuroblasts (arrowhead) and more intensive both cytoplasmic and nuclear p65 staining in gangliar cells (arrows). Original magnification, ×400. Scale bars, 15 μm.

NF-κB is a transcription factor known to promote cell survival and induce apoptosis depending on cell type and context (35). Two recent studies have focused on the importance of NF-κB in the survival of NB cells (36, 37). They show that NF-κB activity and function differ according to different NB cell phenotype. In agreement with our data, they detect a constitutively active NF-κB in SH-EP and SK-N-AS cell lines (37) and a constitutively inactive NF-κB in SH-SY5Y and IMR-32 cell lines (36) and show that these conditions are required for the cell survival because the NF-κB inhibition in the former, as well as the NF-κB activation in the latter, induces apoptosis (36, 37).

To data, no evidence has been advanced about the identification of these two NB cell phenotypes *in vivo*. Herein, we provide evidence that ganglion cells (e.g., the most maturating neuroblastic cells) selectively express high levels of MHC I molecules and high levels of nuclear p65, whereas immature neuroblasts express neither nuclear p65 nor MHC I molecules (Fig. 6).

During embryonic life, the development of the central and peripheral nervous system is characterized by massive cellular death of still-immature neuroblasts before complete differentiation. It is possible that the lack of nuclear p65 in immature neuroblast component of NB may represent the fundamental requisite for survival of this cell type.

Finally, we show that changes in the expression and nuclear translocation of NF-κB similarly affect the peptide-trimming ERAP1 and ERAP2 enzymes. A molecular basis for this coordinated downregulation is the presence in the promoters of these genes of functional NF-κB–binding enhancer A sequences resembling those of MHC I genes. In the context of the available evidence, this is to our knowledge the first demonstration of a molecular mechanism underlying the coordinated downregulation of antigen-processing functions in tumors at a single promoter site. Although under the control of NF-κB, ERAP2 is not tightly coordinated with MHC I and ERAP1, supporting our previous suggestion (13) that there are additional ERAP2-specific regulatory factors.

It will be of interest to identify the NB gene repressor(s) upstream of NF-κB responsible for the drastic reduction of antigen-processing functions in neuroblastic cell types. This putative repressor may eventually provide the missing link between tumorigenesis and immune escape in NB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. B. Berwanger and M. Schwab for providing SH-EP MYCN-ER and Tet-21/N cells; Drs. M. Levrero and M. Cippitelli for providing pcDNA3 neo p65 NF-κB and PUSE amp IκBα (S32A/S36A; M-IκBα), respectively; Prof. P. van Ender, Dr. N. Tanigaki, and Dr. M. Cippitelli for helpful suggestions and critical reading of the manuscript; and E. Giorda and S. Petrini for their skillful assistance in fluorescence-activated cell sorting analysis and confocal microscopy, respectively.

Grant Support

Associazione Italiana per la Ricerca sul Cancro (Milan, Italy) IG grant (D. Fruci) and fellowship (M.Z. Limongi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 7/13/09; revised 11/11/09; accepted 11/27/09; published OnlineFirst 1/26/10.

References

1. Schwab M, Varmus HE, Bishop JM, et al. Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. Nature 1984;308:288–91.
2. Attiyeh EF, London WB, Mosse YP, et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. N Engl J Med 2005;353:2243–53.
3. Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111–6.
4. Seliger B, Ritz U, Ferrone S. Molecular mechanisms of HLA class I antigen abnormalities following viral infection and transformation. Int J Cancer 2006;118:129–38.
5. Versteeg R, Kruse-Wolters KM, Plomp AC, et al. Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. J Exp Med 1989;170:621–35.
6. Versteeg R, Noordermeer IA, Kruse-Wolters M, Ruiter DJ, Schrier PI. c-myc down-regulates class I HLA expression in human melanomas. EMBO J 1988;7:1023–9.
7. Feltner DE, Cooper M, Weber J, Israel MA, Thiele CJ. Expression of class I histocompatibility antigens in neuroectodermal tumors is independent of the expression of a transfected neuroblastoma myc gene. J Immunol 1989;143:4292–9.
8. Bernards R, Dessain SK, Weinberg RA. N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. Cell 1986;47:667–74.
9. Lenardo M, Rustgi AK, Schievella AR, Bernards R. Suppression of MHC class I gene expression by N-myc through enhancer inactivation. EMBO J 1989;8:3351–5.
10. van 't Veer LJ, Beijersbergen RL, Bernards R. N-myc suppresses major histocompatibility complex class I gene expression through down-regulation of the p50 subunit of NF-κB. EMBO J 1993;12:195–200.
11. Girdlestone J, Isamat M, Gewert D, Milstein C. Transcriptional regulation of HLA-A and -B: differential binding of members of the Rel and IRF families of transcription factors. Proc Natl Acad Sci U S A 1993;90:11568–72.
12. Mansky P, Brown WM, Park JH, Choi JW, Yang SY. The second κB element, κB2, of the HLA-A class I regulatory complex is an essential part of the promoter. J Immunol 1994;153:5082–90.
13. Fruci D, Ferracuti S, Limongi MZ, et al. Expression of endoplasmic reticulum aminopeptidases in EBV-B cell lines from healthy donors and in leukemia/lymphoma, carcinoma, and melanoma cell lines. J Immunol 2006;176:4869–79.
14. Fruci D, Giacomini P, Nicotra MR, et al. Altered expression of endoplasmic reticulum aminopeptidases ERAP1 and ERAP2 in transformed non-lymphoid human tissues. J Cell Physiol 2008;216:742–9.
15. Saveanu L, Carroll O, Lindo V, et al. Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. Nat Immunol 2005;6:689–97.
16. Schulte JH, Horn S, Otto T, et al. MYCN regulates oncogenic MicroRNAs in neuroblastoma. Int J Cancer 2008;122:699–704.
17. Lutz W, Stohr M, Schurmann J, Wenzel A, Lohr A, Schwab M.

Conditional expression of N-myc in human neuroblastoma cells increases expression of α-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 1996;13:803–12.
18. Davies CC, Bem D, Young LS, Eliopoulos AG. NF-κB overrides the apoptotic program of TNF receptor 1 but not CD40 in carcinoma cells. Cell Signal 2005;17:729–38.
19. Fontana L, Fiori ME, Albini S, et al. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. PLoS One 2008;3:e2236.
20. Hattori A, Kitatani K, Matsumoto H, et al. Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells. J Biochem 2000;128:755–62.
21. Barnstable CJ, Bodmer WF, Brown G, et al. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. Cell 1978; 14:9–20.
22. Nakamuro K, Tanigaki N, Pressman D. Common antigenic structures of HL-A antigens. VI. Common antigenic determinants located on the 33,000 Dalton alloantigenic fragment portion of papain-solubilized HL-A molecules. Immunology 1975;29:1119–32.
23. Hutter H, Hammer A, Blaschitz A, et al. Expression of HLA class I molecules in human first trimester and term placenta trophoblast. Cell Tissue Res 1996;286:439–47.
24. Moschonas A, Kouraki M, Knox PG, Thymiakou E, Kardassis D, Eliopoulos AG. CD40 induces antigen transporter and immunoproteasome gene expression in carcinomas via the coordinated action of NF-κB and of NF-κB-mediated *de novo* synthesis of IRF-1. Mol Cell Biol 2008;28:6208–22.
25. Saccani S, Pantano S, Natoli G. p38-dependent marking of inflammatory genes for increased NF-κB recruitment. Nat Immunol 2002;3: 69–75.
26. Giannini G, Cerignoli F, Mellone M, et al. High mobility group A1 is a molecular target for MYCN in human neuroblastoma. Cancer Res 2005;65:8308–16.
27. Gobin SJ, Keijsers V, van Zutphen M, van den Elsen PJ. The role of enhancer A in the locus-specific transactivation of classical and nonclassical HLA class I genes by nuclear factor κB. J Immunol 1998; 161:2276–83.
28. Hattori A, Matsumoto K, Mizutani S, Tsujimoto M. Genomic organization of the human adipocyte-derived leucine aminopeptidase gene and its relationship to the placental leucine aminopeptidase/oxytocinase gene. J Biochem 2001;130:235–41.
29. Tanioka T, Hattori A, Mizutani S, Tsujimoto M. Regulation of the human leukocyte-derived arginine aminopeptidase/endoplasmic reticulum-aminopeptidase 2 gene by interferon-γ. FEBS J 2005;272:916–28.
30. Jamaluddin M, Wang S, Boldogh I, Tian B, Brasier AR. TNF-α-induced NF-κB/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. Cell Signal 2007;19:1419–33.
31. Wolfl M, Jungbluth AA, Garrido F, et al. Expression of MHC class I, MHC class II, and cancer germline antigens in neuroblastoma. Cancer Immunol Immunother 2005;54:400–6.
32. Raffaghello L, Prigione I, Bocca P, et al. Multiple defects of the antigen-processing machinery components in human neuroblastoma: immunotherapeutic implications. Oncogene 2005;24:4634–44.
33. Whelan JP, Chatten J, Lampson LA. HLA class I and β 2-microglobulin expression in frozen and formaldehyde-fixed paraffin sections of neuroblastoma tumors. Cancer Res 1985;45:5976–83.
34. Cooper MJ, Hutchins GM, Mennie RJ, Israel MA. β2-Microglobulin expression in human embryonal neuroblastoma reflects its developmental regulation. Cancer Res 1990;50:3694–700.
35. Graham B, Gibson SB. The two faces of NFκB in cell survival responses. Cell Cycle 2005;4:1342–5.
36. Bian X, McAllister-Lucas LM, Shao F, et al. NF-κB activation mediates doxorubicin-induced cell death in N-type neuroblastoma cells. J Biol Chem 2001;276:48921–9.
37. Bian X, Opipari AW, Jr., Ratanaproeksa AB, Boitano AE, Lucas PC, Castle VP. Constitutively active NFκB is required for the survival of S-type neuroblastoma. J Biol Chem 2002;277:42144–50.