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Immunogenicity of neuroblastoma tumors is controlled
by impaired activity of NF-kB and IRF1 transcription
factors.

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Alla mia famiglia

ABSTRACT

English

Low expression of major histocompatibility complex class I (MHC I) molecules on the cell surface allows tumors to evade the host T cell-based immune response. These abnormalities are often related to either genetic defects of MHC I genes or aberrant expression of antigen processing machinery (APM) components. Neuroblastoma (NB), the most common solid extracranial cancer of childhood, is not an exception. MHC I surface expression is virtually undetectable in the most NB cell lines and primary tumors, and upregulated by gamma-interferon (IFN- γ). This phenotype is compatible with defects in the regulation of antigen processing and presentation components. In this study, the molecular mechanism underlying low immunogenicity in neuroblastoma was investigated. Amplification of the MYCN oncogene characterizes the most aggressive forms of NB and is believed to downregulate expression of MHC class I molecules. Although an inverse correlation between MYCN and MHC I has been reported in human NB cell lines, a direct demonstration of the MYCN-mediated down-regulation of MHC I expression has been questioned. Herein, we demonstrate that MYCN is not responsible for low MHC I, ERAP1 and ERAP2 protein levels in human NB cell lines, since their expression is not affected by neither transfection-mediated overexpression nor siRNA suppression of MYCN. Instead, we identified NF- κ B and IRF1 as the main factors involved in the transcriptional regulation of MHC I and ERAPs proteins. By chromatin immunoprecipitation assay, we show a recruitment of p65 NF- κ B to the MHC I, ERAP1 and ERAP2 promoters that is proportional with the

expression of these genes. Moreover, low nuclear activity of both NF- κ B and IRF1 factors correlated with the MHC I, ERAP1 and ERAP2-low phenotype of the most aggressive NB cell lines. Overexpression of either the transcription factors alone rescued the MHC I, ERAP1 and ERAP2-low phenotype, but only partially and in a cell-type depending manner. Important, the co-transfection of both NF- κ B and IRF1 cooperated to strongly enhance the transactivation of MHC I, ERAP1 and ERAP2 in any cell lines. Notheworthy, NF- κ B and IRF1 acted in a synergistic manner. We found an intriguing parallel in primary NB tumors, in fact, nuclear p65 was detected in the maturing neuroblastic cells (i.e. ganglionic cells) which express higher levels of MHC I molecules in human NB specimens. These findings provide molecular insight into defective MHC I expression in NB tumors and indicate that activating NF- κ B and IRF1 in MHC I-low, aggressive NB cells could be instrumental for successful application of T cell-based immunotherapy.

Italiano

La maggior parte dei tumori riescono ad evadere il sistema immunitario inibendo l'espressione di antigeni tumorali associati alle molecole del complesso maggiore di istocompatibilità di classe I (MHC I) sulla superficie cellulare. La mancata espressione di questi complessi è spesso dovuta alla presenza di difetti strutturali dei geni codificanti le molecole MHC I, oppure all'aberrante espressione delle molecole responsabili del processamento degli antigeni legati alle molecole MHC I. Il neuroblastoma (NB), il tumore extracraniale solido più comune dell'infanzia, non è un'eccezione. Sia la maggior parte delle linee cellulari di NB, che i tumori primari esprimono bassi, se non nulli, livelli di MHC I che possono essere aumentati trattando le cellule con l'interferone-gamma (IFN- γ). Questo fenotipo è compatibile con la presenza di difetti nella regolazione trascrizionale delle molecole coinvolte nel processamento e nella presentazione dell'antigene. Il presente studio ha lo scopo di indagare il meccanismo molecolare che determina la mancata o ridotta espressione delle molecole MHC I, e delle due aminopeptidasi del reticolo endoplasmatico ERAP1 ed ERAP2. Le forme più aggressive di NB sono caratterizzate dall'amplificazione dell'oncogene MYCN. Sebbene una correlazione inversa tra l'espressione di MYCN le molecole MHC I nelle linee cellulari umane di NB sia stata riportata, un coinvolgimento diretto di MYCN nella regolazione di MHC I non è stato dimostrato. I nostri risultati dimostrano che MYCN non è responsabile dei bassi livelli di MHC I, ERAP1 ed ERAP2 nelle cellule di NB analizzate, infatti la loro espressione non è influenzata né dalla forzata espressione né dall'inibizione di MYCN. Abbiamo invece identificato due fattori di trascrizione, NF-kB e IRF1, che sono direttamente coinvolti nella regolazione delle proteine MHC I ed ERAPs. Mediante il saggio di

immunoprecipitazione della cromatina abbiamo dimostrato che il reclutamento di p65 (una subunità di NF- κ B) sui promotori di MHC I, ERAP1 ed ERAP2 è direttamente proporzionale all'espressione di questi geni. Inoltre, il fenotipo negativo per MHC I, ERAP1 ed ERAP2, caratteristico delle forme più aggressive di NB, coincide con una bassa attività nucleare di NF- κ B ed IRF1. L'overespressione dei due fattori trascrizionali da soli è in grado di recuperare solo parzialmente l'espressione di MHC I, ERAP1 ed ERAP2, inoltre il risultato dipende dalla linea cellulare trasfettata. Comunque, la trasfezione contemporanea di NF- κ B ed IRF1 produce un incremento sinergico dei geni target in tutte le linee trasfettate. Degno di nota è il fatto che l'espressione di p65 nei tumori primari di neuroblastoma è simile a quella osservata nelle linee cellulari. Infatti, solo le cellule gangliari, ovvero le cellule più differenziate presenti nel tessuto tumorale, esprimono sia l'MHC I che il p65 nucleare. Quindi, questo studio mette in luce il meccanismo molecolare responsabile della mancata espressione delle molecole MHC I, ERAP1 ed ERAP2 nei tumori di neuroblastoma più aggressivi fornendo un importante punto di partenza per lo sviluppo di protocolli immunoterapeutici più efficaci basati sull'utilizzo delle cellule T.

LIST OF PUBLICATIONS

Fruci D, Giacomini P, Nicotra MR, Forloni M, Fraioli R, Saveanu L, van Endert P, Natali PG.

Altered expression of endoplasmic reticulum aminopeptidases ERAP1 and ERAP2 in transformed non-lymphoid human tissues. *J Cell Physiol*. 2008 Sep;216(3):742-9.

Fontana L, Fiori ME, Albini S, Cifaldi L, Giovinazzi S, Forloni M, Boldrini R, Donfrancesco A, Federici V, Giacomini P, Peschle C, Fruci D.

Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One*. 2008 May 21;3(5):e2236.

Forloni M, Albini S, Limongi MZ, Cifaldi L, Boldrini R, Nicotra MR, Giannini G, Natali PG, Giacomini P, Fruci D.

NF-kappaB, and not MYCN, regulates MHC class I and endoplasmic reticulum aminopeptidases in human neuroblastoma cells.

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INTRODUCTION

I ANTIGEN PROCESSING AND PRESENTATION

Cytotoxic T lymphocytes (CD8⁺) monitor the surface of 'professional' and 'nonprofessional' antigen-presenting cells for the presence of viral infections or tumor transformation. During infection and tumor transformation, peptides from intracellular pathogens or aberrant proteins are generated and presented on the cell surface. This process is achieved through the major histocompatibility complex class I (MHC I) molecules. MHC I molecules bound to peptides (pMHC I) serve in fact as a flag for CD8⁺ T cells whose T cell antigen receptors (TCRs) recognize pMHC I and will eventually eliminate the infected or tumor cells. The formation and presentation of pMHC I complexes at the cell surface takes place in several steps. First, endogenous proteins (viral proteins in case of virus infected cells, or tumor associated antigens (TAA) in the case of tumor cells) are trimmed to peptides in the cytosol by the multicatalytic complex of the proteasome and several cytosolic peptidases. This step is followed by active translocation of peptides of 8 to 16 amino acids length across the endoplasmic reticulum (ER) membrane via proteins known as transporters associated with antigen processing (TAP1 and TAP2). While peptides of 8-10 amino acids may directly fit in the MHC I binding groove, longer peptides need to undergo further trimming to produce mature N-termini (Brouwenstijn et al., 2001; Fruci et al., 2001; Lauvau et al., 1999). In mouse this step is catalyzed by ERAAP (Serwold et al., 2002), while in human by at least two endoplasmic reticulum aminopeptidases, ERAP1 and ERAP2 (Saric et al., 2002; Saveanu et al., 2005; York et al., 2002). Finally, peptides are assembled with MHC I

molecules and the pMHC I complex translocates to the cell surface for the recognition by specific CD8⁺ T cells (Hammer et al., 2007; Lehner and Cresswell, 1996).

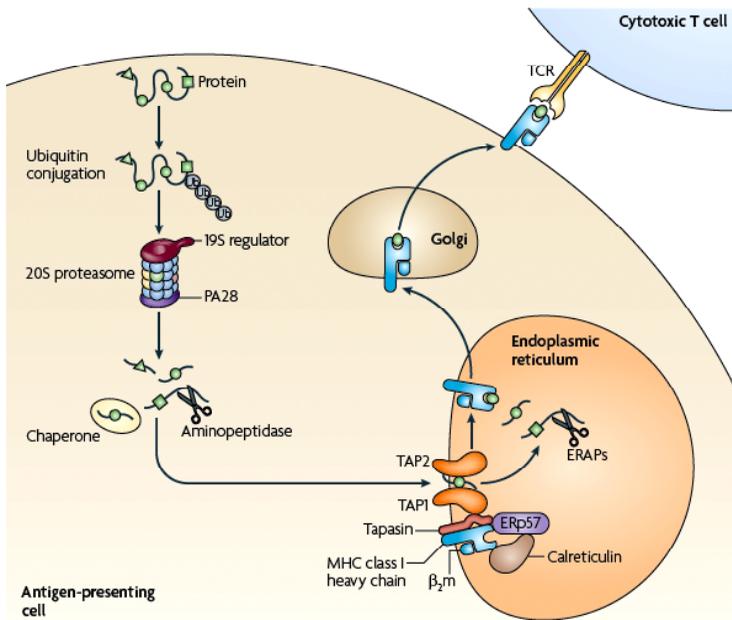


Figure 1. MHC I antigen processing and presentation. Cytosolic and nuclear proteins are degraded by the proteasome. Peptides undergo further trimming by cytosolic aminopeptidases. The transporter for antigen processing (TAP) then translocates peptide into the lumen of the ER. The MHC I molecule folds with the assistance of the chaperones calnexin and calreticulin, the adaptor molecule tapasin and the oxidoreductase ERp57. Protein disulfide isomerase (PDI) helps in formation of the disulfide bond in the α₂ domain of the MHC I molecule. ERAPs trim N-terminal extensions from antigenic precursor, thus resulting in the generation of final pMHC I complex that exit the ER and goes to the cell surface.

1.1 The ubiquitin-proteasome system

Cytosolic protein degradation is mostly carried out by a large, multicatalytic protease complex (700 kDa) called the proteasome. The proteasome is found in the nucleus and cytosol of eukaryotic cells, where is involved in non-lysosomal protein degradation of cytosolic proteins (Fehling et al., 1994; Rock et al., 1994). The ubiquitinated proteins enter into the core of the proteasome where they are broken down into short peptides of 2 to 20 amino acids. The 20S core of this complex has the shape of a barrel made of four rings. The two outer rings are identical and each is composed of seven distinct α subunits. The two inner rings are also identical. Each contains seven different β subunits, which surround a central chamber where proteolysis occurs. The proteolytic activity is exerted by three of the β subunits, namely $\beta 1$, $\beta 2$ and $\beta 5$. The 20S core proteasome can associate with two regulatory complexes. Association with the 19S cap produces the 26S proteasome particle, which is able to degrade ubiquitin-conjugated proteins, whereas association with the P28 complex is thought to increase its catalytic activity (Tanaka and Kasahara, 1998). The major role of the proteasome is the degradation of intracellular proteins. The products of this degradation represent a major source of peptides for MHC I presentation.

When cells are exposed to $\text{IFN}\gamma$ the three catalytic subunits, $\beta 1$, $\beta 2$ and $\beta 5$ are replaced by their $\text{IFN}\gamma$ -inducible homolog LMP2 ($\beta 1i$), MECL1 ($\beta 2i$) and LMP7 ($\beta 5i$), respectively. In vitro studies, performed with viral epitopes, showed that the proteasome equipped with LMP2, LMP7 and MECL1 is more efficient in processing a number of immunogenic peptides (Cerundolo et al., 1995). For this reason, it was called 'immunoproteasome' as opposed to the constitutively expressed standard proteasome. When tested in vitro on fluorogenic

substrates the catalytic activity of the immunoproteasome is characterized by a reduced cleavage after acidic amino acids and an increase in the cleavage after hydrophobic and basic residues. As the latter residues are those most frequently binding to MHC I molecules, this reinforced the notion that the immunoproteasome is more efficient than the standard proteasome for antigen presentation. It has been noted that changes in subunit composition sharpen the quantitative and qualitative ability of the proteasome to generate peptides, and thereby limit the production of self-peptides (Groettrup et al., 1996). Immunoproteasomes are constitutively expressed in immune tissues and expressed at much lower levels in other cell types where they can be induced by exposing cells to IFN γ or TNF α , cytokines released in the early stages of viral infections. The vast majority of the peptides produced during protein degradation by the cytosolic proteasome-ubiquitin system are consecutively hydrolyzed to single amino acids by multiple cytosolic peptidases. The small fraction of peptides surviving the aggressive cytosolic environment can be recruited for presentation by MHC I molecules (Kloetzel, 2004). Biochemical analysis of this mixture *in vitro* as well as in living cells has shown that proper C-termini of the final peptides are efficiently generated in the cytosol (Cascio et al., 2001). However, such peptides may frequently have to be adapted to the strict MHC I-binding requirements by one or several N-terminal-trimming steps. An accepted model proposes that peptides of 15 or more residues are shortened by cytosolic tripeptidylpeptidase II and translocate into the ER through the ATP-dependent TAP1-TAP2 heterodimer complex (Monaco et al., 1990; Neefjes et al., 1993). Both TAP1 and TAP2 are multimembrane spanning proteins with an ATP-binding cassette. The peptide transporters are selective for peptide length and the N- and C-terminal amino acids (Momburg and Hammerling, 1998; Trowsdale et al., 1990).

1.2 Endoplasmic reticulum aminopeptidases

Peptides, in the ER, need to be shortened to the right length to fit the peptide binding groove of MHC I molecules (Brouwenstijn et al., 2001; Fruci et al., 2001). The enzyme responsible for this trimming has been identified as ERAAP in mice (Serwold et al., 2002) and ERAP1, in the human (Saric et al., 2002; York et al., 2002). ERAAP/ERAP1 appear to be specialized to produce peptides presented on MHC I molecules and, indeed, it has been found that purified ERAP1 trimmed peptides that are ten residues or longer, but spared eight-residue peptides (Chang et al., 2005; Hammer et al., 2006). By destroying longer peptides in normal human cells, ERAP1 reduces the overall supply of antigenic peptides. Like other components of the antigen processing and presentation pathway, ERAP1 is also induced by IFN γ . It has been demonstrated that after IFN γ treatment, which causes proteasome to produce more N-terminal extended antigenic precursors, ERAP1 increases the supply of peptides for MHC I antigen presentation (Saric et al., 2002; York et al., 2002). Further evidence for ERAP1 as a key player in shaping the peptide-MHC I repertoire come from analysis of ERAP1-deficient mice generated by three independent group (Firat et al., 2007; Hammer et al., 2006; Yan et al., 2006). These works demonstrated that ERAP1 may have positive, neutral, or negative contribution in the generation of individual class I epitopes. More recently, peptide trimming in the endoplasmic reticulum has been found to be essential for protective immunity to the parasite *Toxoplasma gondii* (Blanchard et al., 2008).

A second putative human trimming peptidase has been identified based on its homology with ERAP1 (Tanioka et al., 2003). Like ERAP1, this leukocyte-derived arginine aminopeptidase (L-RAP, also called ERAP2) is localized in the ER and is induced by IFN γ

(Tanioka et al., 2005). *In vitro* trimming of several synthetic precursor peptides by recombinant L-RAP suggested that the enzyme might also be involved in precursor peptide trimming in the ER (Tanioka et al., 2003). This hypothesis has been confirmed by Saveanu and colleague's work, that demonstrated that ERAP1 and ERAP2 formed complexes and combined their restricted specificities to remove N-terminal extension by ensuring highly efficient trimming of diverse precursor peptides (Saveanu et al., 2005).

1.3 The MHC class I

The MHC I genes encode polymorphic membrane glycoproteins that present peptides of foreign or self-origin to CD8⁺ T cells. These molecules play critical roles in immune responses to viruses, malignant transformation and tissue rejection. MHC I proteins are encoded by three highly polymorphic genes, HLA-A, HLA-B and HLA-C, that are expressed in all eukaryotic cells (Little and Parham, 1999; Uhr et al., 1979), and three less polymorphic genes, HLA-E, HLA-F and HLA-G that are expressed in a tissue-restricted fashion (Wake, 1986).

In human, MHC I consist of two polypeptide chains, an heavy or α chain encoded in the MHC locus and a smaller non polymorphic light chain, β_2 -microglobulin, which is encoded elsewhere (Cunningham, 1977).

The MHC-encoded polypeptide folds into three separate domains called α_1 , α_2 and α_3 . β_2 -microglobulin is non-covalently associated with the α_3 domain (Ohnishi, 1983). The α_3 domain and β_2 -microglobulin have a folded structure that closely resembles that of an immunoglobulin domain. The most remarkable feature of MHC I molecules is the structure of the folded α_1 and α_2 domains in which are site of polymorphism. These two domains fold to make up a region bounded by a β -

pleated sheet on the bottom and two α helices on the sides. This groove, capable of binding via non-covalent interactions a small peptide of about 8-10 amino acids, is the peptide-binding site. The small peptide presented defines the antigenic epitope that can be recognize by CD8⁺ T-cell.

1.4 The peptide-loading complex

The peptide loading complex (PLC) is a multisubunit structure in the ER containing TAP at its centre assembled with MHC I molecules, β_2 -microglobulin, tapasin (tpn), calreticulin, ERp57, and possibly protein disulfide isomerase (PDI) (Cresswell et al., 2005; Elliott and Williams, 2005). Tpn, ERp57 and PDI are required for the stabilization of TAP and regulation of the redox state of a disulfide bound in the peptide-binding groove of the MHC I heavy chain (Cabrera, 2007; Chambers et al., 2008). Upon peptide loading, the PLC dissociates and the complex consisting of the MHC I and β_2 -microglobulin is released and transported via the trans-Golgi to the cell surface to be exposed to CD8⁺ cytotoxic T lymphocytes. Deficiency in components of the PLC can have dramatic consequences on the expression and quality of the pMHC I repertoire on the cell surface (Garbi et al., 2000).

II TRANSCRIPTIONAL REGULATION OF MHC I AND ERAPs

2.1 Transcriptional regulation of MHC I

Expression of MHC I genes is tightly regulated and vary considerably amongst the different tissue types. In particular, certain reproductive and developmental tissues lack expression of MHC I molecules, similar to cells of the nervous system and eyes. The highest levels of MHC I gene expression occur in tissues and cells of the immune system. (Garrido et al., 1993; Singer and Maguire, 1990). Activation of MHC I gene promoter is mediated by three major regulatory elements: enhancer A, IFN stimulated response element (ISRE), and the SXY module (van den Elsen et al., 1998a; van den Elsen et al., 1998b). These *cis*-acting sequences mediate different routes of tissue-specific and cytokine-induced transcription of MHC I genes and are localized in a region extending approximately from nucleotides -220 to -95 upstream of the transcription initiation site. Nucleotide sequence variation of enhancer A and ISRE in different MHC I promoters affects the binding of specific factors to these elements. As a result, the promoter activation differs among the various MHC I loci (Girdlestone, 2000; Gobin et al., 1998; Gobin et al., 1999). Notably, the level of expression of the different MHC I loci also differs between cell types, revealing cell-type-specific basal and inducible expression levels (Johnson, 2003).

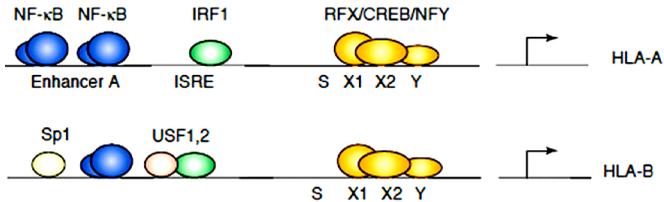


Figure 2. cis-acting elements on the MHC I promoter. Shown are the upstream regulatory modules of the HLA-A and HLA-B promoters, comprising the enhancer A, ISRE and their interacting factors. The SXY (S-X1-X2-Y) is cooperatively bound by a multiprotein complex which acts as an enhanceosome driving transactivation of the MHC I genes.

2.1.1 Enhancer A and NF-κB binding

The Enhancer A is the target-binding site for transcription factors of the NF-κB/Rel family and is thought to be essential for constitutive and cytokine-induced gene expression. The κB motif (GGGGATTCCCC) of enhancer A is highly conserved in MHC I gene promoters, particularly in HLA-A and HLA-B loci (Le Bouteiller, 1994). It is a symmetrical variant of the more divergent κB motif of the promoter of Ig *k*-light chain gene (GGGACTTCC (Sen and Baltimore, 1986)). Although the κB motif is the principal target sequence for proteins of the NF-κB/Rel family, it is also bound by several other DNA-binding proteins, such as the high mobility group protein (HMG) I(Y) and proteins that belong to the leucine zipper family of transcription factors (Miyamoto and Verma, 1995).

The NF-κB/Rel family of transcription factors comprises at least five members: p50, p65 (also termed RelA), p52, c-Rel, and RelB, that bind DNA either as homo- or heterodimers (Baeuerle and

Henkel, 1994; Miyamoto and Verma, 1995). These dimers possess different binding affinities for *kB* sites and their half-sites, as well as different transcriptional properties (Urban and Baeuerle, 1990; Urban et al., 1991). The p50-p65 heterodimer, termed NF-*kB*, is present in virtually all differentiated cells and is the most abundant of the NF-*kB*/Rel dimers. NF-*kB* is normally retained in the cytoplasm complexed to the inhibitory protein I*kB* (Finco and Baldwin, 1995; Thanos and Maniatis, 1995). Following activation by stimuli such as TNF α or phorbol esters, I*kB* is phosphorylated and degraded, after which NF-*kB* is released and subsequently translocated to the nucleus (Finco and Baldwin, 1995; Thanos and Maniatis, 1995). Interaction between NF-*kB* and the *kB* site results in transactivation of MHC I genes and a variety of other molecules, including cytokines. The level of gene transcription of the various MHC I loci is determined by *a*) the specific expression level of the NF-*kB*/Rel family proteins at the specific tissue, *b*) the binding affinity of the NF-*kB*/Rel family proteins for a particular *kB* site, and *c*) the transactivation capacities of different NF-*kB*/Rel dimers (Baeuerle and Henkel, 1994; Miyamoto and Verma, 1995).

The NF-*kB* subunits p65 and c-Rel both have a transactivation domain, although p65 is the more potent of the two. The NF-*kB* subunit p50, lacking such transactivation domain, is thought to have no transactivating capacity as a homodimer but rather to fulfill an ancillary function for the p65 and c-Rel subunits. However, since in cell-free assays the p50 homodimer can transactivate MHC I (Fujita et al., 1992), its exact role in transactivation is still unclear.

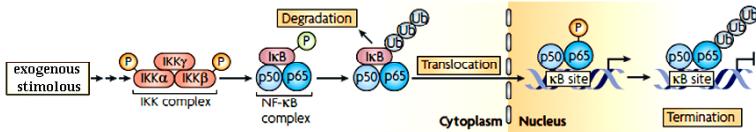


Figure 3. Relevant regulatory nodes in NF- κ B signalling pathway. An external stimulus activates the IKK (inhibitor of NF- κ B (I κ B) kinase) complex. This results in the phosphorylation of I κ B proteins and consequently their degradation by proteasome. Released NF- κ B dimers translocate to the nucleus and bind κ B site in the promoters of target genes. Note that the green phosphate signifies phosphorylation that results in negative regulation of the phosphorylated protein, whereas the yellow phosphate indicates activation. Ub, ubiquitin.

2.1.2 ISRE and IRF family members

The IFN stimulated response element ($^A/GNGAAANNGAAACT$) is a binding site for transcription factors of the interferon regulatory factor (IRF) family (Darnell et al., 1994). The IRF family members belong to a large family of transcription regulators, comprising nine members: IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8/ICSBP, IRF9/ISGF3g (Mamane et al., 1999; Taniguchi et al., 2001). IRFs were first characterized as transcriptional regulators of type I interferon and IFN-inducible genes, but recent studies have revealed that this family plays a pivotal role in the regulation of the host defence beyond its function in the IFN system. IRFs exert several functions in regulating important biological processes, such as innate immune responses, development and differentiation of both immune and non-immune cells, cell growth, apoptosis and oncogenesis (Honda and Taniguchi, 2006; Ozato et al., 2007; Tamura et al.,

2008). ISRE sequence mediates the induction of MHC I transcription by the IFNs, of which IFN γ is the most potent (Girdlestone et al., 1993; Johnson and Pober, 1994; Le Bouteiller, 1994). IFN γ exerts its biological effects through the signal transduction pathway, which involves binding to its receptor, activation of Janus kinases (Jak) 1 and 2, and phosphorylation of latent Stat1 (Bach et al., 1997; Schindler and Darnell, 1995). A homodimer of activated Stat1 can bind the IFN γ activation site (GAS), or the ISRE in combination with p48 (also called ISGF3), thereby transactivating genes bearing either of these sequences in their promoter (Darnell et al., 1994). IRF1, IRF2, and IFN consensus sequence binding protein (ICSBP) are induced by this route. These molecules form a group of secondary transcription factors that regulate gene transcription in a positive or negative manner, or act as helpers of protein/DNA complex formation. This cascade of events results in the transactivation of a number of genes important in the immune system, including MHC I heavy chain, β_2 -microglobulin, and other genes, the products of which are important for class I antigen processing and presentation (Boehm et al., 1997).

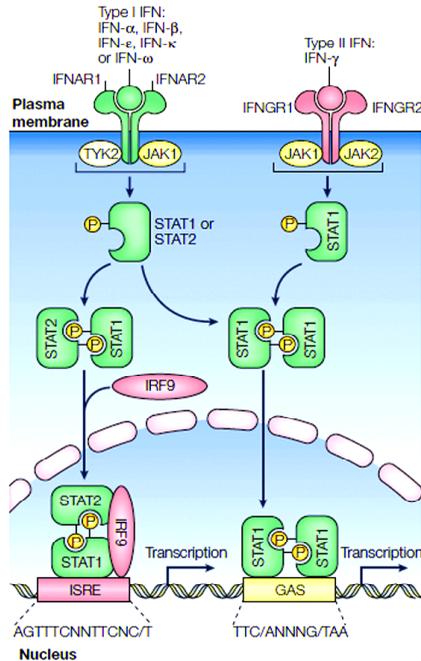


Figure 4. Interferon receptors and activation of classical JAK-STAT pathway by type I and II interferons. Type I and Type II interferons bind different receptors. Type I IFN receptor is associated with the janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1. The only type II IFN receptor is associated with JAK1 and JAK2. Activation of the JAKs that are associated with the type I IFN receptor results in tyrosine phosphorylation of STAT2 (signal transducer and activator of transcription 2) and STAT1; this leads to the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) complexes, which are known as ISGF3 (IFN-stimulated gene (ISG) factor 3) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind GAS (IFN- γ -activated site) elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. The consensus GAS element and ISRE sequences are shown. N, any nucleotide.

2.2 Transcriptional regulation of the ER aminopeptidases

ERAP1, like many other antigen processing and presenting components, can be enhanced by IFN γ (Beninga et al., 1998; Saric et al., 2002; York et al., 2002). Several potential transcription factor binding motifs, such as MIZ-1, IRF1/2, C/EBPa/b, Sp1, and NF-kB were identified by bioinformatics analysis in the promoter region of the ERAP1 gene. In a recent work Doody *et al.* demonstrated that both IRF1 and IRF2 bind to ERAP1 promoter at the constitutive level (Doody et al., 2007). Niizeki *et al.* observed that the expression of ERAP1 increased during *in vitro* differentiation of mouse embryonic stem (ES) cells into endothelial cells (EC) (Niizeki et al., 2004). They showed that ERAP1 expression in mouse endothelial cells was regulated, at least in part, by the transcription factor PEBP2 (Niizeki et al., 2004).

Like for ERAP1, ERAP2 is also upregulated by IFN γ (Tanioka et al., 2003). Bioinformatics analysis of the ERAP2 promoter detected several transcription factor binding sites, such as NF-kB, IRF1/2, ets-1 and Oct-1. Deletion and site-directed mutagenic analyses of the 5'-flanking region of the ERAP2 gene, and electrophoretic mobility shift assay indicated that while IRF2 is important in the basal condition, IRF1 is the primary regulator of IFN γ -mediated enhancement of ERAP2 expression (Tanioka et al., 2005). In addition, PU.1, a member of the E26 transformation-specific family of transcription factors, also play a role in the regulation of ERAP2 transcription. The maximum expression of ERAP2 gene was achieved by coexpression of IRF1 and PU.1 in HEK293 cells, while IRF2 suppressed the IRF1-mediated enhancement of gene expression (Tanioka et al., 2005).

III CANCER IMMUNE EVASION

The concept of immune surveillance of tumors implies that the immune system has evolved for the elimination of neoplastic cells, thereby providing protection of an organism from abnormal cell growth and subsequent tumor development. Thus, tumor development and progression must result from a failure of the immune system either to recognize or to mediate destruction of tumor cells. In fact, tumors have evolved a number of well-defined mechanisms by which cancerous cells are able to evade recognition by immune system; i.e. elimination of the immune cells, suppression of their function by releasing inhibitory factors that directly or indirectly suppress immune function, such as TGF- β , IL-10 or prostaglandins (Ahmad et al., 2004; Bin et al., 2002; Tsuruma et al., 1998), or downregulation of cell surface peptide-MHC I complexes that are essential for the presentation of TAAs to the cells of the immune system (Garrido et al., 1993). Moreover, appropriate TAA processing and presentation is a prerequisite for the successful outcome of T-cell-based immunotherapy of malignant diseases (Storkus et al., 2007; van Endert, 1999). Optimization of the design of the T-cell epitope greatly benefits from detailed knowledge of the pathophysiology of the MHC I and II (MHC II) antigen-processing machinery and of the molecular defects used by tumor cells to escape from T-cell recognition.

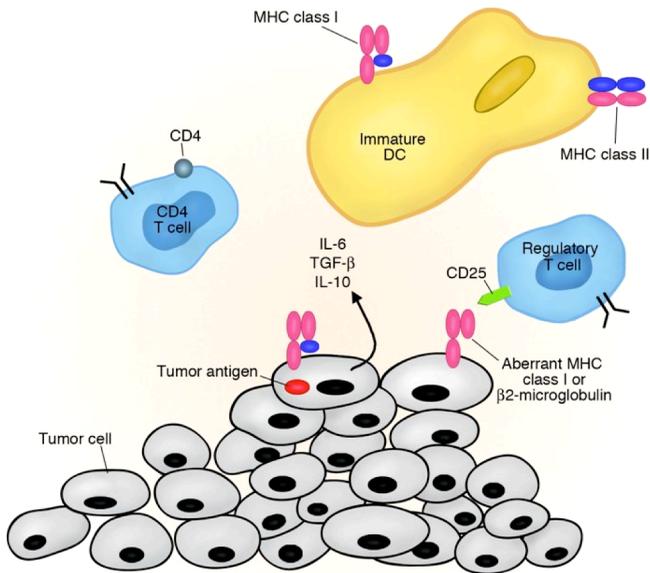


Figure 5. Tumor escape from the host immune system. Tumor may escape immune detection through several mechanisms. For example, secreting a number of suppressive cytokines, such as IL-6, IL-10, and TGF- β . Again, tumor cells may express aberrant MHC I molecules, resulting in inadequate antigen recognition of cancer by effector T cells.

3.1 MHC class I abnormalities

The recognition of tumor cells by effector T cells is based upon a complex pathway of processing and presentation of endogenous TAAs in the context of MHC I molecules. Given the complexity of this process, it can be predicted that tumor cells acquired defects in some components of this pathway and thus escape detection by the immune system. In fact, this has been shown to be the case. It has been reported that 40% of tumors have

alterations in expression of MHC I molecules (Garrido et al., 1993). A direct correlation between the degree of tumor differentiation and expression of MHC I, in which poor differentiated tumor cells have reduced expression of MHC I, has been reported for a variety of tumor types, including breast, lung, basal cell carcinomas, and larynx (Esteban et al., 1989; Garrido et al., 1993; Lopez-Nevot et al., 1989). Furthermore, an association between metastasis, tumor progression, and poorer prognosis for tumors having reduced expression of MHC I has been described (Aptsiauri et al., 2007; Ogino et al., 2006; Ruiz-Cabello et al., 1989). These data provide compelling evidence for loss of MHC I determinants representing a major factor in tumor progression. Interestingly, hepatocellular carcinoma and leukaemia are two exceptions to these general findings (Shen et al., 2009; Wetzler et al., 2001).

Loss of MHC I expression can be based upon mutations in any of the proteins involved in processing antigenic peptides or in MHC I molecules themselves. Given the relatively high number of changes in MHC I expression in tumors, it does not seem likely that alterations in the coding regions of structural genes for MHC I heavy chains are primarily responsible for this loss of expression, as this would require multiple mutations to affect expression. Therefore, mutations of antigen processing protein-coding genes, or alterations in transcription of MHC I as well as in other antigen processing molecules are more likely candidates for affecting this mechanism of immune evasion.

3.2 Antigen Processing Machinery (APM) defects

There are substantial evidences for the reduced expression of the antigen processing machinery components in cancer; for instance, downregulation of expression of LMP2, LMP7, TAP1, and TAP2 in a variety of tumor types, including small-cell lung carcinomas (Lou et al., 2005), esophageal squamous cell carcinomas (Liu et al., 2009), melanomas (Tao et al., 2008), and renal cell carcinomas (Seliger et al., 1996), has already been demonstrated. In a recent work, Fruci *et al.* demonstrated that also the expression of the ER aminopeptidases ERAP1 and ERAP2 is imbalanced in several tumor cell lines, including leukaemia/lymphoma, melanoma and carcinoma cell lines, compare to EBV-B cells from healthy donors (Fruci et al., 2006). Moreover, they found that in these cell lines ERAP1 expression significantly correlated with MHC I level (Fruci et al., 2006). Defective expression of the antigen-processing components may be due to several reasons, such as mutation or deletion in either the promoter or the coding region of these genes, or a defective regulatory mechanism. Several works have supported the latter hypothesis (Johnsen et al., 1998; Singal et al., 1996). A comparative study by Johnsen *et al* investigated the expression of multiple components of the MHC I antigen processing pathway in different tumor cell lines, representing a variety of histological types (Johnsen et al., 1998). Combined deficiencies in the expression of antigen-processing genes, such as TAP1, TAP2, LMP2 and LMP7 were demonstrated in several cell lines. So, the existence of deficiencies in the expression of genes at dispersed loci suggested that it is due to a defective regulatory mechanism, as opposed to mutation or deletion of these genes.

Thus, in most tumors, abnormalities in the APM reflect dysfunctional rather than structural mechanisms. These deficiencies may cause defects in peptide generation,

translocation and/or loading onto β_2 -microglobulin-MHC I heavy chain complexes. As a consequence, these complexes are retained in the ER and, being unstable, are rapidly degraded. $\text{INF}\gamma$ treatment usually restores peptide supply by up-regulating APM component expression (Johnsen et al., 1998; Seliger et al., 2000) and reverses MHC I antigen downregulation.

IV NEUROBLASTOMA

4.1 Biology of neuroblastoma

Neuroblastoma is a tumor derived from primitive cells of the sympathetic nervous system, the so-called neural crest progenitor cells. It is the most common solid extracranial neoplasm of infancy and childhood, accounting for approximately 7-10% of all childhood malignancies, and responsible for approximately 1-5% of all childhood cancer deaths. The clinical hallmark of neuroblastoma is heterogeneity, associated with a wide variety of likelihood of tumor progression. The most significant clinical predictors of outcome are age and stage, although an assessment of the patient's prognosis solely on the basis of clinical parameters is limited due to diverse biological tumor behaviour and subsequent survival rates, even at distinct clinical stages (Maris and Matthay, 1999). Therefore, the heterogeneity of this tumor entity requires cellular and molecular markers in order to distinguish the different biological characteristics. Established molecular markers such as *MYCN* copy number and loss of heterozygosity for chromosome 1p36 may help predicting poor outcome. Based on the age-related prevalence and distinguishing genetic features of biologically favourable and unfavourable tumors, Brodeur *et al.* proposed that NB are at least two or three diseases with distinct clinical and biological characteristics (Brodeur *et al.*, 2001). However, there is evidence that traditional prognostic parameters, such as analysis of *MYCN* amplification, which is considered an essential component of disease evaluation and treatment stratification, do not ensure completely accurate prognostic grouping (Kawa *et al.*, 1999). Identifying new prognostic markers can increase the accuracy of risk assessment

and can also identify biologically relevant targets for developing new therapies.

4.2 Chromosomal abnormalities

Unbalanced gain of distal 17q material is the most common genetic abnormality detected in primary neuroblastoma tissue specimens. This event is associated with adverse prognostic features and is present in the vast majority of neuroblastomas with *MYCN* amplification (Bown et al., 1999). Unbalanced 1;17 translocations occur frequently in primary neuroblastomas and often result in loss of distal 1p with concomitant gain of distal 17q material (Savelyeva et al., 1994; Van Roy et al., 1994). Thus, 17q21-qter is likely to harbour a gene (or genes) that contributes to neuroblastoma tumorigenesis when present in increased copy number and/or overexpressed. Other genetic abnormalities include chromosomal deletions. Deletions of the short arm of chromosome 1 (1p) are found in approximately 35% of primary tumors and are highly correlated with *MYCN* amplification (Fong et al., 1989). Hemizygous deletions of the long arm of chromosome 11 occur in approximately 40% of human neuroblastomas (Srivatsan et al., 1993). In addition, constitutional rearrangements of chromosome 11q, including interstitial deletions, have been observed in neuroblastoma patients. Thus, 11q likely harbours a neuroblastoma-suppressor gene, but in contrast to 1p deletions, there is a striking inverse relationship of 11q loss of heterozygosity (LOH) with *MYCN* amplification, despite the fact that this abnormality is correlated with advanced stage disease (Guo et al., 1999). Likewise, deletion of the short arm of chromosome 3 and distal long arm of chromosome 14 (Ejeskar et al., 1998; Fong et al., 1992) are also a common abnormality in neuroblastomas, present in about 20% to 25% of cases. LOH for 14q is highly correlated with 11q LOH and

inversely related to 1p36 LOH and *MYCN* amplification. Taken together, it appears that inactivation of genes located at 3p, 11q, and/or 14q may be required during the malignant evolution of high-risk neuroblastomas that do not contain *MYCN* amplification. There are other regions of the genome that are frequently deleted in neuroblastoma, suggesting the existence of additional tumor-suppressor genes. There are reports of chromosomal deletions at 4p (Caron et al., 1996), 9p (Marshall et al., 1997) and 18q (Reale et al., 1996), but these appear less common than loss at the other loci noted above.

4.3 MYCN amplification

MYCN belongs to the Myc family of proto-oncogenes, which encode for transcription factors of the basic/helix-loop-helix/zipper (b/HLH/Z) class. *MYCN* is fundamental for the development of the peripheral and central nervous systems (PNS and CNS). The *MYCN* protein has a very restricted expression pattern: it is mainly expressed during embryonic development, and detected in adults only in B-cell precursors. *MYCN* amplification is a strong prognostic marker for neuroblastoma. Brodeur and colleagues originally demonstrated that *MYCN* amplification, that occurs in approximately 25% of primary neuroblastomas, associates with advanced stages of disease, rapid tumor progression, and a poor prognosis (Brodeur et al., 1984; Seeger et al., 1985). Amplification is found in 30% to 40% of patients with advanced disease and in only 5% to 10% of patients with low stage of disease and stage 4S (Brodeur, 1995; Brodeur and Fong, 1989). *MYCN* amplification is almost always present at the time of diagnosis (Brodeur et al., 1987), so it appears to be an intrinsic biologic property of a subset of very aggressive tumors. Interestingly, overexpression of a *MYCN* transgene in murine peripheral neural crest cells causes neuroblastic tumors with high

penetrance and remarkably similar phenotype as in humans (Weiss et al., 1997). In general, there is a significant correlation between *MYCN* copy number and its expression at the mRNA and protein levels (Slavc et al., 1990). Thus, *MYCN* overexpression in the context of amplification consistently identifies a subset of neuroblastomas with highly malignant behaviour. However, it is controversial whether or not overexpression of *MYCN* has prognostic significance in non-amplified tumors (Seeger et al., 1988; Slavc et al., 1990). Some neuroblastoma cell lines express high levels of *MYCN* mRNA or protein without gene amplification (Seeger et al., 1988; Slavc et al., 1990; Wada et al., 1993). This may be a result of alterations in normal protein degradative pathways, rather than loss of *MYCN* transcriptional regulation (Cohn et al., 1990; Otto et al., 2009). In addition, some studies have suggested that *MYCN* expression correlates inversely with survival probability (Chan et al., 1997), whereas others did not find such correlation (Bordow et al., 1998). Further studies in a larger cohort of consistently treated patients will be necessary to determine if quantitative assessment of *MYCN* expression in tumors lacking *MYCN* amplification provides additional prognostic information.

4.4 Spontaneous regression

The most enigmatic clinical behaviour of NB is its spontaneous regression (Carlsen, 1990). This is particularly prevalent in Evans Stage IV-S patients. There is evidence attributing this behaviour to an immune response. Beckwith and Perrin demonstrated that nest of neuroblastoma cells in the adrenal glands of stillborn infants occur at a much higher frequency than tumors in the general population (Beckwith and Perrin, 1963). Additional evidence of an immune mechanism for spontaneous regression is the report that Stage IV-S NBs express a higher level of MHC I

antigen than NBs at Stages I-IV (Squire et al., 1990). Alternate mechanisms that might explain tumor regression include the ‘two-hit’ theory of oncogenesis described by Knudson and Meadows (Knudson and Meadows, 1980), a relationship of regression to *MYCN* oncogene amplification (Nakagawara et al., 1990), a coexpression of the mRNA for both the TRK gene and the low-affinity nerve growth factor (NGF) receptor (Kogner et al., 1993), and an alteration of apoptosis (Pritchard and Hickman, 1994).

V IMMUNE EVASION OF NEUROBLASTOMA

Several data show that neuroblastoma may escape the control mediated by cytotoxic T lymphocyte and natural killer cells through multiple mechanisms; i.e. expression of suppressive factors like TGF- β or IL-10 which may prevent activation and expansion of tumor-infiltrating lymphocytes (Rivoltini et al., 1992), downregulation of costimulatory molecules such as CD40, CD80 or CD86 (Airoldi et al., 2003), and aberrant expression of MHC I antigens. Among these, the downregulation of MHC I molecules play a key role and occurs by different mechanisms.

5.1 MHC I and APM expression in neuroblastoma

Over the past decades several works have demonstrated the paucity of MHC I expression on neuroblastoma cell lines and tumors (Lampson and George, 1986; Wolfi et al., 2005). Metastatic neuroblastoma in bone marrow has been shown to express HLA-A, -B, -C molecules at very low level (Lampson et al., 1983; Lampson and Whelan, 1983). Moreover, defects in β_2 -microglobulin, β_2 -microglobulin free heavy chain and TAP1 molecules, which can be corrected by IFN γ , have been described in neuroblastoma cell lines (Corrias et al., 2001; Lampson and George, 1986). Recently, Raffaghello and colleagues studied the expression of MHC I molecules and the antigen processing machinery components in primary neuroblastoma tissues. They showed that, as compared to normal adrenal medulla, primary neuroblastoma tissues expressed low levels of tapasin, TAP1 or TAP2, MHC I heavy chain, β_2 -microglobulin, LMP2 and LMP7, thereby demonstrating the existence of a link between downregulation of the APM components and the paucity of MHC I molecules on neuroblastoma tumors (Raffaghello et al., 2005). Interestingly, they found that incubation of neuroblastoma cell

lines with IFN γ caused upregulation of both MHC I molecules and APM components. Thus, defects in APM components explain reduced peptide loading on MHC I molecules and the instability and failure of MHC I to be expressed on the cell surface.

The molecular mechanisms underlying defects in the expression of the antigen processing and presenting molecules in neuroblastoma remain to be investigated. The elucidation of these mechanisms may allow a more accurate selection of patients as candidates to receive cellular immunotherapy and eventually improve their outcome.

5.2 MYCN as a putative regulator of MHC I expression

The role of the oncogene MYCN in regulating MHC I expression in neuroblastoma is yet unclear. During the past years, several contrasting works have been published demonstrating either the involvement or not of MYCN in the transcriptional regulation of MHC I molecules. Bernardis *et al.* demonstrated that transfection of the MYCN gene in a rat neuroblastoma cell line drastically reduced the cell surface expression of MHC I antigens (Bernardis *et al.*, 1986). The MYCN-dependent MHC I repression seemed to be associated with reduced binding of a transcription factor to the enhancer A region of the MHC I promoter. Afterwards, this transcription factor has been identified being the p50 subunit of NF-kB (Lenardo *et al.*, 1989; van 't Veer *et al.*, 1993); indeed, MYCN downregulated MHC I expression by repressing the p50 mRNA transcription. Versteeg *et al.* supported these data by demonstrating that by switching off MYCN expression, after somatic cell fusion of a human and a murine neuroblastoma cell line, it was possible to obtain the restoration of the MHC I antigens in a MHC I negative neuroblastoma cell line (Versteeg *et al.*, 1990). However, as compelling as these findings appear, an analysis of a large number of neuroblastoma and

neuroepithelioma cell lines showed no consistent relationship between MHC I expression and *MYCN* copy number (Reynolds et al., 1988). Observation that neuroblasts in a 24-wk-old fetal adrenal gland do not express β_2 -microglobulin, whereas the adult adrenal medulla does, provides evidence that MHC I antigen expression is regulated developmentally as neuroblasts differentiate to form the adrenal medulla (Feltner et al., 1989). Moreover, transfection of the *MYCN* gene into different human neuroblastoma cell lines didn't revert the MHC I positive phenotype (Feltner et al., 1989; Gross et al., 1994). So, these data suggest that variation in MHC I expression among human neuroblastoma cells may reflect the developmental stage at which neuroblasts were arrested during tumorigenesis and/or that other transcription factors, instead of *MYCN*, are responsible for the different MHC I expression observed between human neuroblastoma cell lines.

5.3 IRFs and NF-kB

In the past, few works focused on the identification of the molecular mechanisms underlying the MHC I paucity on neuroblastoma tumors, even if most of the transcription factors involved in the transcriptional regulation of these molecules are well known. Murphy *et al.* by carrying out a detailed molecular analysis of the extended MHC I promoter revealed the presence of a series of negative regulatory elements. Moreover, they showed that these silencer elements mediated the repression of the MHC I gene in a neuroblastoma cell line, concluding that the MHC I promoter is active in that neuroblastoma cell line but that the failure in expression is the result of active repression (Murphy et al., 1996).

Since, MHC I heavy chain, β_2 -microglobulin and many of the antigen processing components contain in their promoter region

ISRE-like sequences capable of binding the transcription factor IRF1, some works analyzed the effect of IFN γ on MHC I and antigen processing molecules expression in neuroblastoma cell lines. Corrias *et al.* demonstrated that the defective expression of MHC I, β_2 -microglobulin, TAP1 and TAP2 can be restored by IFN γ treatment and that upregulation of all these genes appeared to correlate with induction of IRF1 expression (Corrias *et al.*, 2001). Drew *et al.* found that the low expression of MHC I and β_2 -microglobulin in neuroblastoma cell lines resulted from low factor binding activity to the *cis*-elements on the promoter of these genes. Treatment of neuroblastoma cells with IFN γ and TNF α induced factor binding to the *cis*-elements and led to increased MHC I and β_2 -microglobulin gene transcription (Drew *et al.*, 1993). Moreover, treatment with both cytokines resulted in a synergistic enhancement of the MHC I and β_2 -microglobulin cell surface expression. TNF α induced binding of the p65 and p50 NF-kB subunits to the NF-kB-like element of the MHC I promoter, and IFN γ induced binding of IRF1 to the adjacent interferon consensus sequence (ICS) (Drew *et al.*, 1995; Drew *et al.*, 1993). It was demonstrated that NF-kB and IRF1 physically interact with each other and cooperatively induce MHC I promoter transactivation when cotransfected into a neuroblastoma cell lines (Drew *et al.*, 1995). All these findings support a model by which TNF α and IFN γ synergistically induce the expression of a variety of genes involved in immune responses, including MHC I, and suggest the hypothesis that lack of binding factor, like NF-kB and IRF1, in neuroblastoma cell lines could account for low constitutive MHC I expression.

AIM

Here, I will show my PhD project that focused on the identification of the molecular defects underlying classical MHC class I antigen and ER aminopeptidases downregulation or loss by neuroblastoma cells, hoping that this information may contribute to the understanding of the molecular basis of immune escape mechanisms utilized by neuroblastoma cells and may suggest strategies to correct these defects.

RESULTS AND DISCUSSION

MYCN, MHC I, ERAP1, ERAP2 and NF- κ B expression in NB cell lines

Since MYCN is inversely correlated with MHC I (Bernards et al., 1986; Lenardo et al., 1989; van 't Veer et al., 1993), and MHC I is positively correlated with ERAP1 expression in human cell lines of various lineages (Fruci et al., 2006; Fruci et al., 2008), 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. 1D) 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 Fig. 1B.

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 (Fig. 1B) 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 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 (Fruci et al., 2006). Any of MYCN, MHC I and ERAP1 did not significantly correlate with ERAP2.

NF- κ B has been known to be involved in MHC I expression (Girdlestone et al., 1993; Mansky et al., 1994) and the p50 NF- κ B subunit has been claimed to directly correlate with MHC I expression in the rat B104 NB cell line (Bernards et al., 1986; Lenardo et al., 1989; van 't Veer et al., 1993). To determine whether NF- κ B is a transcription factor also of ERAP1 and ERAP2, NF- κ B 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 analysed for the correlation with those of MHC I heavy chain, ERAP1 and ERAP2 (Fig. 1A) and cell surface MHC I (Fig. 1D). Representative plots are displayed in Fig. 1B. 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 correlated 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 non-significant 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- κ B p105 and p50 subunit are linked each other by statistically significant patterns of coordinated expression, whereas their correlations with MYCN were inverse and non-significant, and ERAP2 and p65 behaved as completely independent variables.

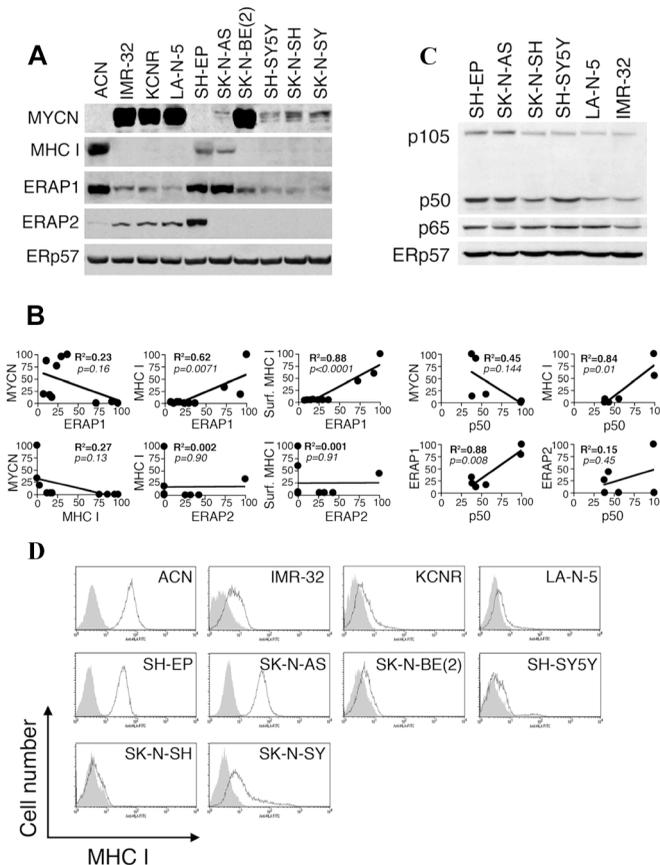


Figure 1. Expression of MYCN, MHC I, ERAP1, ERAP2 and NF- κ B p105, p50 and p65 subunits in NB cell lines. (A) Immunoblot analysis of MYCN, MHC I, ERAP1 and ERAP2 in different NB cell lines. Equal amounts of cell lysates were resolved by SDS-PAGE and probed with the indicated antibodies. (B) Representative regression analysis of western blotting data in (A) and (C) and surface MHC I in (B). (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). (D) Surface MHC I expression of different NB cell lines was analysed with the W6/32 mAb by flow cytometry (black lines). Negative controls stained with isotype-matched primary Ab are indicated by shaded histograms.

MYCN does not regulate the expression of MHC I, ERAP1, ERAP2, and NF-kB subunits in NB cell lines

To directly determine whether MYCN downregulates MHC I and NF-kB 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-stably transfected NB cell line, SH-EP MYCN-ER (Schulte et al., 2008) and Tet-21/N (Schulte et al., 2008). SHEP MYCN-ER carries a 4-hydroxy-tamoxifen (4-OHT)-responsive MYCN transgene fused to a mutagen oestrogen-responsive domain (ER), while Tet-21/N carries a tetracycline-repressible MYCN transgene. The expression of MYCN is activated with 4-OHT treatment in SHEP 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-kB 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 *HMGAI* (high mobility group A1) (Giannini et al., 2005) determined by qRT-PCR (Fig. 2A, lower panel), 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-kB p105, p50, and p65 in both cell line tested (Figs. 2A and B). Furthermore, MYCN silencing by RNAi in SH-SY5Y cells, a MYCN overexpressing cell line (Fig. 1A), did not affect the levels of any of MHC I, ERAP1, ERAP2 and NF-kB p105, p50 and p65 as compared to the controls (Fig. 2C).

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

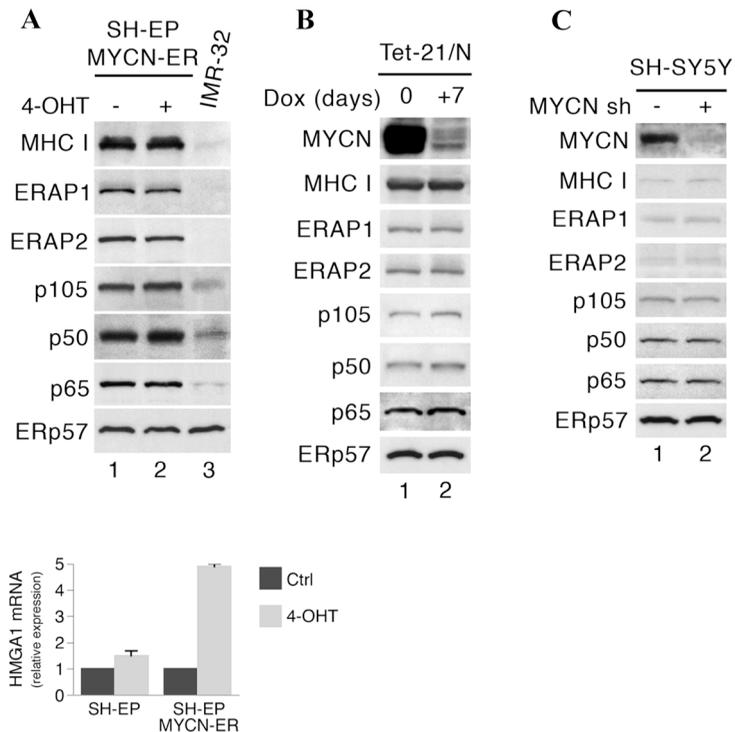


Figure 2. MYCN expression does not affect expression of the p50 NF- κ B subunit, MHC I, ERAP1 and ERAP2 in NB cell lines. Immunoblot analysis of MYCN, MHC I, ERAP1, ERAP2 and NF- κ B p105, p50 and p65 subunits in SH-EP MYCN-ER grown in the presence or absence of 4-OHT for 66 hours (A, upper panel), Tet-21/N cells either left untreated (0) or treated with doxycycline (+7) for 7 days (B), SH-SY5Y transfected with either MYCN siRNAs or control scrambled (C). HMGA1 mRNA expression was tested by qRT-PCR as control for 4-OHT-induced MYCN activation (A, lower panel). The MYCN-amplified IMR-32 NB cell line was included as a control in (A). ERp57 was used for normalization.

Effect of TNF α on the expression of MHC I, ERAP1, ERAP2 and NF-kB

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

As shown in Fig. 3A, TNF α 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). The observed enhancing effects of TNF α were not seen in other NB cell lines that were low in MHC I, ERAP1 and ERAP2 expression (Figs. 3A and 3B).

To rule out that MYCN expression *per se* might cause the difference in the responsiveness to TNF α treatment of NB cell lines, the Tet-21/N cell line was treated with TNF α in the presence or 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 TNF α -mediated translocation of p50 and p65 (Fig. 3C).

Altogether, it appears that the availability of nuclear NF-kB 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-kB in NB cell lines.

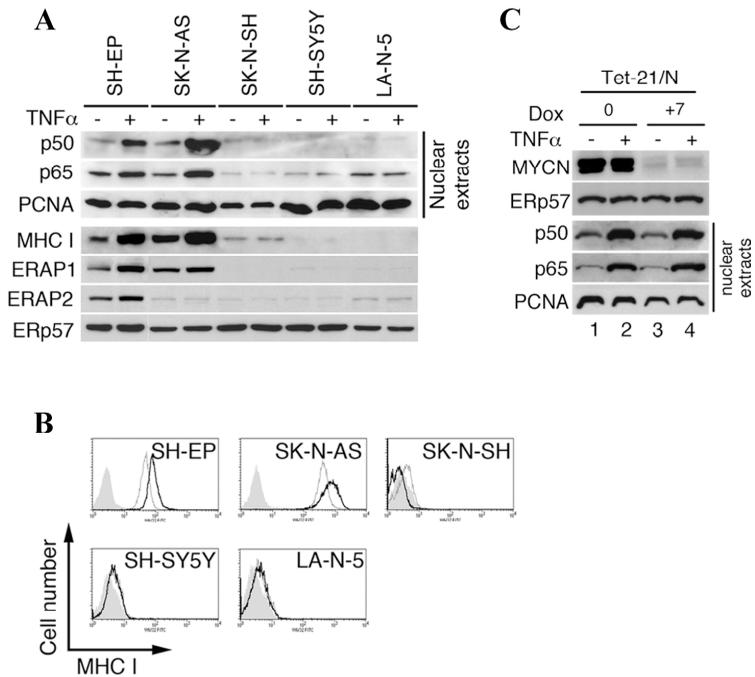


Figure 3. TNF α enhances the expression of MHC I, ERAP1 and ERAP2 in a set of NB cell lines. (A) Immunoblot analysis of NB cell lines grown in the presence or absence of TNF α for either 1 hour (top three panels, nuclear extracts), or 48 hours (bottom four panels, whole-cell extracts). (B) Flow cytometry analysis of surface MHC I expression of NB cells either TNF α -treated for 48 h as in A (black lines) or untreated (grey lines), using the W6/32 mAb. Isotype-matched negative controls are displayed as shaded histograms. (C) Immunoblot analysis of TET21/N treated with TNF α for 1 hour or left untreated in either presence (+7) or absence (0) of doxycycline. PCNA and ERp57 were used for normalization.

NF- κ B regulates MHC I, ERAP1 and ERAP2 in NB cell lines

To explore further the relevancy of NF- κ B to ERAP1 and ERAP2 expression, we used three different approaches: (a) to inhibit NF- κ B nuclear translocation with specific inhibitors; (b) to inhibit NF- κ B expression by silencing p65 subunit expression with a p65-specific siRNA; and (c) to enhance NF- κ B p65 subunit by transfection of a p65 expression vector.

In the first approach (a), sulfasalazine (Sz), a chemical inhibitor of NF- κ B nuclear translocation, was shown to prevent nuclear translocation of p65 in SH-EP (Fig. 4A, upper panel) and SK-N-AS (not shown) cells in a dose-dependent fashion. At the optimal dosage, Sz treatment strongly reduced the constitutive and/or TNF α -induced expression of MHC I, ERAP1 and ERAP2 in these cells (Fig. 4A, lower panel). Furthermore, a stable SH-EP transfectant carrying an I κ B α mutant (M-I κ B α) that is refractory to TNF α (Davies et al., 2005) and defective in p65 nuclear translocation, as assessed by immunofluorescence in a time course experiment (not shown), was shown to be drastically impaired in MHC I, ERAP1 and ERAP2 mRNA accumulation (Fig. 4B). The effect of the mutant I κ B α was specific, since IFN γ enhanced the expression of MHC I, ERAP1 and ERAP2 mRNA equally well in SH-EP cells transfected with either the M-I κ B α or the empty vector, whereas TNF α enhanced expression of the tested genes less efficiently in SH-EP cells transfected with M-I κ B α than in cells transfected 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 tested for the expression of p65, MHC I heavy chain, ERAP1 and ERAP2 by Western blotting (Fig 4D, left panel), and for cell surface MHC I by flow cytometry (Fig. 4D, right panel). As shown in Fig. 4D (left panel), 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, except LAN-5, particularly well in SH-EP, SH-SY5Y and SK-N-BE(2)c, and to a lesser extent in IMR32. ERAP1 expression paralleled that of MHC I in p65-transfected cell lines, while ERAP2 was upregulated just in two cell lines out of five, i.e. SHEP and SH-SY5Y. Flow cytometry of the 4 cell lines in which p65 upregulated 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, right panel). This poor cell surface MHC I expression may result from impaired expression of some components in MHC I-presenting machinery, such as β_2m , TAP and so on, other than MHC I heavy chain.

These results indicate that NF-kB up-regulates MHC I, ERAP1 and ERAP2 in NB cell lines, and the low expression of ERAP1 and MHC I is due to a low efficiency of NF-kB in activating target gene transcription, presumably consequence of a poor constitutive NF-kB nuclear expression and/or the nuclear translocation as suggested by the TNF α unresponsiveness in several tested NB cell lines (Fig. 3A). Unlike ERAP1 and MHC I, ERAP2 transcription seems to be regulated by different factors, other than NF-kB, in most NB cell lines.

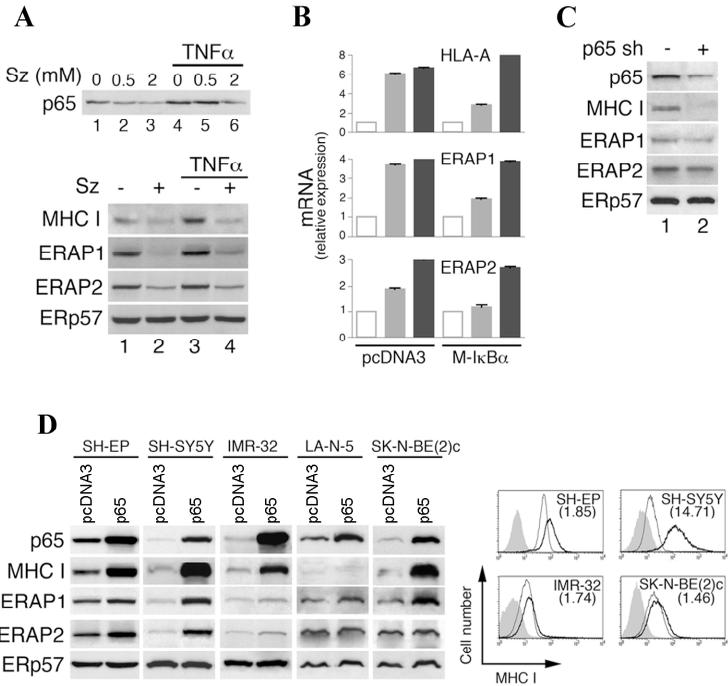


Figure 4. NF- κ B regulates the expression of MHC I, ERAP1 and ERAP2 in NB cell lines. (A) SH-EP cells pre-incubated for 30 min with different concentration of Sz (upper panel) or 2 mM of Sz (lower panel) in either presence or absence of TNF α (8h). (B) qRT-PCR analysis of mRNAs from SH-EP-pcDNA3 and SH-EP M-lkBa transfectants, either left untreated or grown for 48 h in the presence of TNF α (grey columns) or IFN γ (black columns). (C) Western blot of SH-EP cells transfected with p65 siRNA (p65 sh) or control scrambled. (D) Western blotting (left panel) and flow cytometry analysis with W6/32 mAb (right panel) of the indicated NB cell lines transfected with either an empty vector (pcDNA3 in left panel and grey line in right panel), or a vector expressing p65 (p65 in left panel and black line in right panel). In right panels, fold-increases for each NB cell line are indicated in parenthesis and isotype-matched negative controls are displayed as shaded histograms.

Recruitment of NF- κ B to ERAP1 and ERAP2 promoter regions

Since NF- κ B directly binds enhancer A elements in the promoters of MHC I genes (Gobin et al., 1998), we looked for similar sequences in the promoter regions of ERAP1 (Hattori et al., 2001) and ERAP2 (Tanioka et al., 2005). 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- κ B 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 h) with TNF α . The IL-8 gene promoter, a known target of NF- κ B (Jamaluddin et al., 2007), was used as a positive control. As shown in Fig. 5B, p65 specifically binds all the tested promoters (lane 3), and the signal is strongly increased following TNF α 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 demonstrate that ERAP1 and ERAP2 are regulated through the NF- κ B/enhancer A pathway and provide a molecular basis for the coordinated expression of these genes with MHC I in NB cell lines.

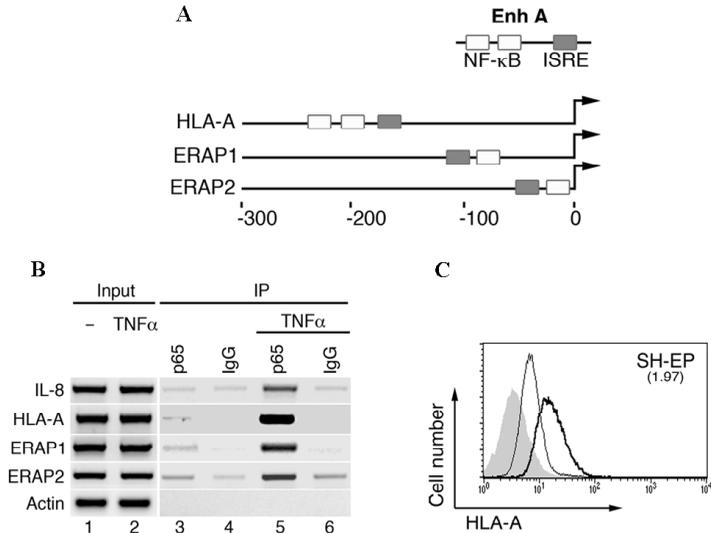


Figure 5. *NF- κ B* interacts in vivo with the promoters of MHC I, ERAP1 and ERAP2. (A) Schematic representation of the promoter regions of the HLA-A, ERAP1 and ERAP2 genes. Enhancer A and ISRE elements are indicated. (B) Chromatin immunoprecipitation from SH-EP cells grown for 30 min in the presence and absence of TNF α , using an anti-p65 antibody and a control anti-IgG antibody. Interleukin-8 (IL-8) and b-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 TNF α -treated for 48 h (black lines) or untreated (grey lines) using mAb TU155. Isotype-matched negative controls are displayed as shaded histograms. Fold-increase in the presence of TNF α is indicated in parenthesis.

Expression of MHC I and NF- κ B in primary neuroblastoma samples

To determine whether the correlation between MHC I expression and the expression/nuclear localization of NF- κ B might also be observed *in vivo*, 32 primary neuroblastoma lesions were tested by immunohistochemistry. Like in previous studies (Cooper et al., 1990; Raffaghello et al., 2005; Whelan et al., 1985; Wolfl et al., 2005), 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 8 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- κ B.

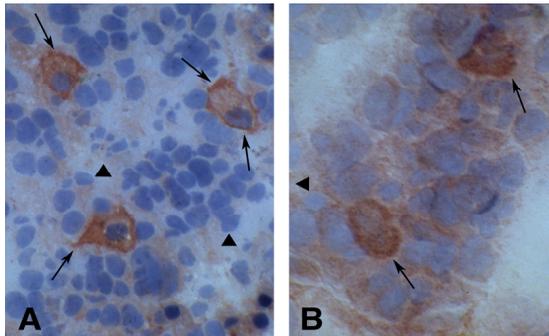


Figure 6. Expression of MHC I and NF- κ B in primary NB lesions.

Staining of cryostatic sections of NB lesion for MHC I and p65 NF- κ B. (A) MHC I expression is undetectable in neuroblasts (arrowhead) and strongly expressed in gangliar cells (arrows) (original magnification 250X). (B) Weak cytoplasmic p65 expression in neuroblasts (arrowhead) and more intensive both cytoplasmic and nuclear p65 staining in gangliar cells (arrows) (original magnification 400X). Bars=15 μ m.

IRF1 and IRF2 expression in neuroblastoma cell lines

Although p65 transfection strongly enhanced MHC I heavy chain expression in most NB cell lines tested, except the LAN-5 cell line, IMR-32 and SK-N-BE(2)c showed only a faint increase in the cell surface MHC I expression (Fig. 4D, right panel). We reasoned that defective expression of components involved in the MHC I antigen processing could have affected the MHC I surface expression. Indeed, we observed that ERAP1 and ERAP2 were upregulated in 3 and 1 out of 5 cell lines, respectively (Fig. 4D). Thus, it is conceivable that in these NB cell lines other factors might cooperate with NF- κ B in the transcriptional regulation of the antigen processing and presentation components. Previous reports demonstrated that IRF1 and IRF2 directly bind the ISRE element in the promoter of MHC I (Gobin et al., 1999), and ERAP1 (Doody et al., 2007) genes. Moreover, while IRF1 mediates IFN γ -dependent upregulation of ERAP2, IRF2 is involved in its constitutive expression (Tanioka et al., 2005). Thus, we asked whether IRF1 and IRF2 would regulate MHC I, ERAP1 and ERAP2 genes in NB cell lines, and if lack of these factors in less immunogenic NB cells would account, at least in part, for the absence of MHC I, ERAP1 and ERAP2 expression. Firstly, we assayed the expression of IRF1 and IRF2 in a panel of NB cell lines that differ in MHC I, ERAP1 and ERAP2 protein level. As shown in Fig. 7A, IRF1 and IRF2 were expressed at high levels in the SH-EP and SK-N-AS cell lines, that were high in constitutive MHC I, ERAP1 and ERAP2 expression, and at low levels in the NB cell lines with low or null expression of MHC I, ERAP1 and ERAP2 molecules. In this latter group, the exceptions were the SH-SY5Y and the SK-N-BE(2)c cells, that showed high level of IRF2 protein. Moreover, nuclear IRF1 and IRF2 displayed the same expression pattern as in the whole-cell extracts (Fig. 7B). These findings, indicate that the low

expression level of IRF1 and IRF2 accounts for the absence of these transcription factors in the nucleus, and suggest the existence of a functional correlation between the nuclear availability of IRF1 and IRF2, and the expression of ERAP1, ERAP2 and MHC I genes.

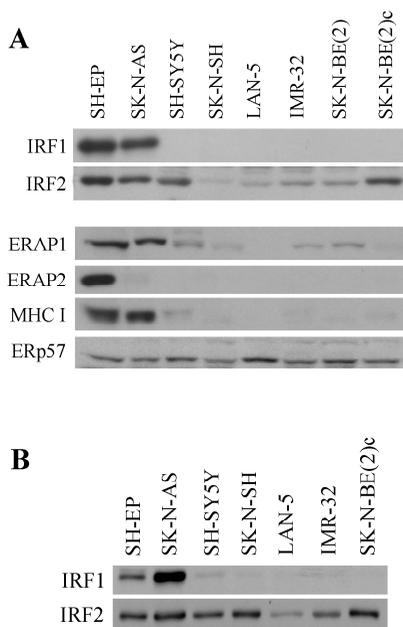


Figure 7. Expression of MHC I, ERAP1, ERAP2, IRF1 and IRF2 in NB cell lines. Immunoblot analysis of MHC I, ERAP1, ERAP2, IRF1 and IRF2 in whole-cell extracts (A) and of IRF1 and IRF2 in nuclear extracts (B) of different NB cell lines. Equal amounts of cell lysates were resolved by SDS-PAGE and probed with the indicated antibodies. ERp57 was used for normalization in (A), while red Ponceau was evaluated for a correct gel loading in (B).

Effect of IFN γ on the expression of MHC I, ERAP1 and ERAP2 expression in neuroblastoma cell lines

IFN γ is known to induce MHC I antigen processing and presentation components (Zhou, 2009). Moreover, IRF1 has been demonstrated to play a key role in the IFN γ -dependent signalling (Taniguchi et al., 2001). Thus, we asked whether ERAP1, ERAP2 and MHC I molecules, as well as IRF1 and IRF2 would increase their expression upon IFN γ treatment in NB cells. Accordingly with previous findings (Corrias et al., 2001), we observed a strong upregulation of IRF1 expression in all IFN γ -treated NB cell lines (Fig. 8A). Otherwise, IRF2 protein underwent to a slight increase only in NB cell lines with low constitutive ERAP1, ERAP2 and MHC I expression. Interestingly, IFN γ -treatment strongly enhanced both MHC I and ERAP1 expression. Moreover, IFN γ treatment upregulated ERAP2 in 4 out of 7 NB cell lines. Indeed, the SK-N-AS, SK-N-BE(2) and SK-N-BE(2)c cells did not express ERAP2, neither upon IFN γ stimulation (Fig 8A). As shown in Fig 8B, the IFN γ treatment resulted also in a strong increase of the cell surface MHC I expression. These data, confirm that in NB cells both ERAP1 and MHC I genes are functional, and that the low level of these molecules, in less immunogenic NB cell lines, is mostly due to regulatory mechanisms. Otherwise, depending on the cell type, the expression of ERAP2 may rely on either regulatory mechanisms or structural defects. Furthermore, it appears that the availability of the IRF1 subunit correlates with MHC I, ERAP1 and ERAP2 levels, indicating IRF1 as positive transactivator of these genes in NB cells.

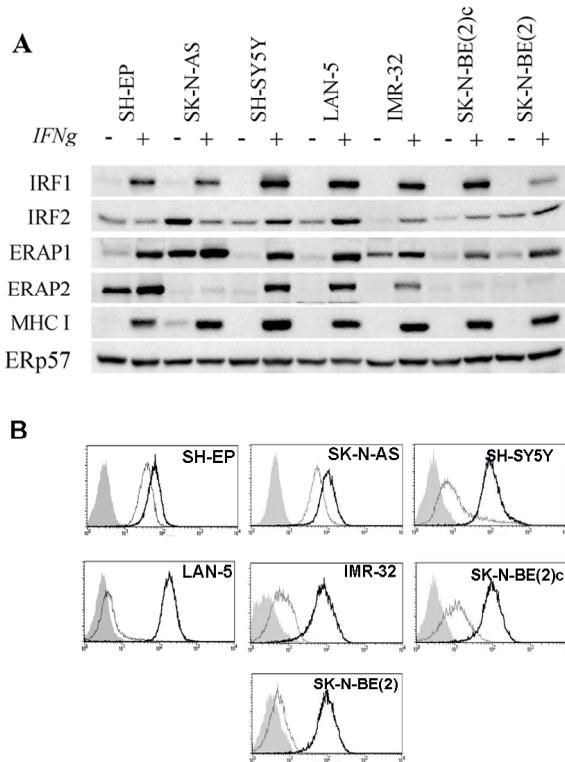


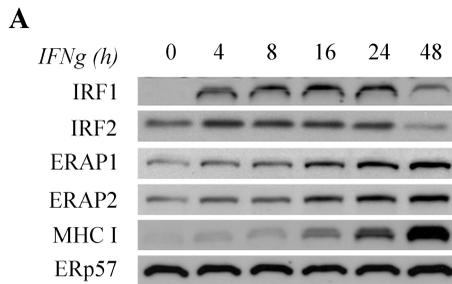
Figure 8. IFN γ -induced MHC I, ERAP1, ERAP2 and IRF1, IRF2 overexpression in NB cell lines. NB cell lines were either left untreated or treated for 48 hours with IFN γ for 48 hours. (A) Immunoblot analysis of MHC I, ERAP1, ERAP2, IRF1 and IRF2. ERp57 was used as loading control. (B) Flow cytometry analysis of surface MHC I expression of NB cells either IFN γ -treated as in (A) (black line) or untreated (grey line) using the W6/32 mAb. Negative controls stained with isotype-matched primary Ab are indicated by shaded histograms.

IFN γ enhances ERAP1, ERAP2 and MHC I expression through IRF1

The above results led us to further investigate the role of IRF1 in IFN γ -induced upregulation of ERAP1, ERAP2 and MHC I in NB cells. IFN γ -mediated IRF1 activation is based on *de novo* protein synthesis of IRF1 itself (Taniguchi et al., 2001). Thus, SH-EP cells were treated with IFN γ and assayed for IRF1, IRF2 and ERAP1, ERAP2 and MHC I expression at different times. As shown in Fig 9A, IRF1 protein, and to a lesser extent IRF2, peaked as early as at 4 h of IFN γ treatment and remained unchanged for up to 24 h. At 48 h, both IRF1 and IRF2 expression dropped down to the initial level. Otherwise, ERAP1, ERAP2 and MHC I followed a slower kinetics than IRF1 and IRF2, and the protein level elevated gradually only after 16 h, reaching the highest expression at 48 h of IFN γ treatment. Noteworthy, the IFN γ -dependent upregulation of ERAP1, ERAP2 and MHC I followed the same kinetics, suggesting that they may be regulated by the same transcription factors.

Moreover, to obtain a definitive evidence of the involvement of IRF1 in mediating the IFN γ -dependent upregulation of the ERAP1, ERAP2 and MHC I expression, we performed an experiment by using Cycloheximide (CHX), a drug that inhibits protein synthesis. As shown in Fig. 9B (upper panel), *de novo* protein synthesis of IRF1 in IFN γ -treated SH-EP and SH-SY5Y cells was inhibited by CHX. Then, we looked for ERAP1, ERAP2 and HLA-C mRNA expression by treating both SH-SY5Y and SH-EP cells as described above, then collecting the cells at different times. IFN γ -treatment induced a gradual increase of the ERAP1, ERAP2 and HLA-C mRNA expression in both cell lines. The higher fold increase observed in SH-SY5Y cells compare to that observed in SH-EP cells is likely due to the lower basal ERAP1, ERAP2 and HLA-C mRNA level presented by SH-

SY5Y cells compare to SH-EP cells. Interestingly, the presence of CHX almost completely inhibited IFN γ -induced ERAP1, ERAP2 and HLA-C mRNA upregulation in both cell lines. Altogether the data, confirm the role of IRF1 as the main mediator of the IFN γ -induced ERAP1, ERAP2 and MHC I upregulation in NB cells.



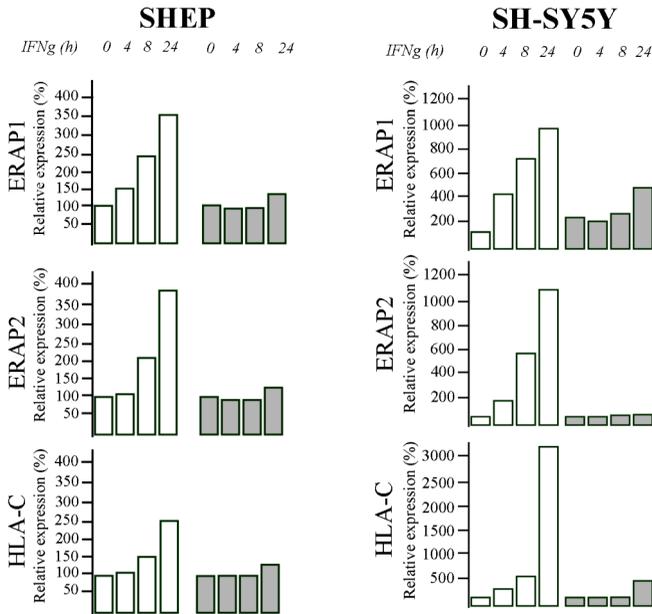
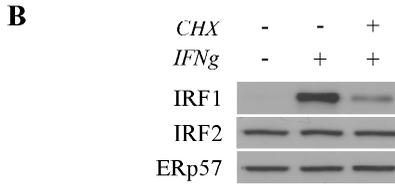


Figure 9. IRF1 mediates the *IFN* γ -induced MHC I, ERAP1 and ERAP2 upregulation in NB cell lines. (A) SH-EP cells were treated with *IFN* γ for the indicated time points, then followed by immunoblotting to detect MHC I, ERAP1, ERAP2, IRF1 and IRF2. (B, upper panel) Western Blot of IRF1 and IRF2 of SH-EP cells treated with or without *IFN* γ , in presence or absence of cycloheximide (CHX, 10ug/ml); (B, lower panel) SH-EP and SH-SY5Y cells, treated as described above, were collected at the indicated time points and then subjected to qRT-PCR to analyse ERAP1, ERAP2 and HLA-C mRNA expression

IRF2 regulates the constitutive expression of MHC I, ERAP1 and ERAP2 in NB cell lines

Subsequently, we sought to determine the IRF1 and IRF2 involvement in the regulation of the constitutive ERAP1, ERAP2 and MHC I expression. To this end, SH-EP cells, which express both ERAPs and MHC I proteins, were transfected with IRF1- and IRF2-specific siRNAs. Silencing of IRF1 or IRF2, or both proteins caused a specific downregulation of the gene targets (Fig. 10A). Downregulation of IRF1 caused just a faint decrease in all of the target genes, whereas silencing of IRF2 provoked a marked downmodulation of the genes studied, with the stronger effect on the ERAP1 protein. A further slight decrease of the ERAP2 and MHC I expression was observed when both IRF1 and IRF2 were silenced (Fig. 10A). These data demonstrate that IRF2, and to a lesser extent IRF1, mediates the constitutive expression of ERAP1, ERAP2 and MHC I in NB cells.

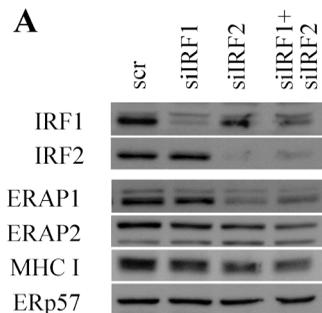


Figure 10. IRF2 regulates constitutive activation of ERAP1, ERAP2 and MHC I genes. (A) SH-EP cells were transfected with siRNA against IRF1 (siIRF1), IRF2 (siIRF2), IRF1 and IRF2 (siIRF1+IRF2), or with negative control (scr). The cells were collected 48h later and tested for expression of MHC I, ERAP1, ERAP2, IRF1 and IRF2 by Western blotting.

IRF1 and p65 synergistically cooperate to induce ERAP1 and MHC I expression

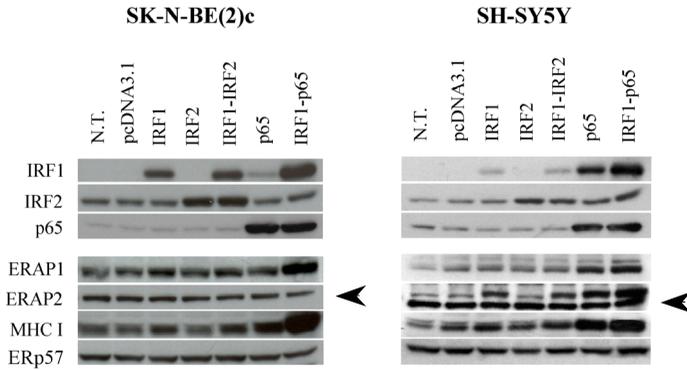
Based on the above results, we reasoned that in MHC I-negative NB cell lines the low ERAP1, ERAP2 and MHC I expression may rely on defective activation of NF- κ B and the lack of other positive transactivators, i.e. IRF1 and IRF2. In order to verify this, three representative NB cell lines were transfected with vectors bearing IRF1 or IRF2 cDNAs (IRF1, IRF2) or the empty vector (pcDNA3.1), and tested for the expression of MHC I heavy chain, ERAP1 and ERAP2 by Western blotting (Fig. 10A), and for cell surface MHC I expression by flow cytometry (Fig. 10B). As shown in Fig. 10A, IRF1 *per se* enhanced the expression of ERAP1 in 1 out of 3 cell lines, and of ERAP2 only in the SH-SY5Y cell lines. The MHC I heavy chain was weakly induced by IRF1 in any NB cell line tested, and similarly behaved the cell surface MHC I (Fig. 10B). Unlike IRF1, IRF2 overexpression didn't influence neither the ERAPs nor the MHC I protein level in any cell line tested. No synergic or cooperative effect was detected following overexpression of both IRF1 and IRF2.

As shown in Fig. 4D, the overexpression of the p65 NF- κ B subunit in MHC I negative NB cell lines positively affects both ERAP1 and MHC I expression. We confirmed these finding and, accordingly with a previous report (Ohmori et al., 1997), we observed an enhancement of IRF1 in p65 transfected cells (Fig. 10A). Interestingly, the magnitude of this induction was cell type-specific. Several reports have demonstrated that p65 and IRF1 synergistically cooperate to induce the expression of the targeted genes (Sanceau et al., 1995; Saura et al., 1999). To examine whether this cooperation would also lead to a synergistic induction of ERAP1, ERAP2 and MHC I in NB cells, IRF1 and p65 were cotransfected in NB cell lines. As shown in fig 10A, cotransfection of these transcription factors strongly enhanced the

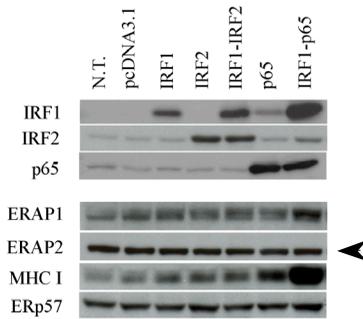
expression of MHC I and ERAP1 in any cell line tested, and of ERAP2 in the SH-SY5Y cells (Fig 10A). Interestingly, ERAP2 failed to be induced in SK-NB-E(2) and SK-BN-E(2)c by both cotransfection of IRF1 and p65 (Fig. 10A) and IFN γ treatment (Fig 8A), suggesting that a structural defect in this gene may exist in these cell lines.

Likewise, cotransfection of IRF1 and p65 promoted a stronger upregulation of MHC I surface expression as compare to transfection of each transcription factor (Fig. 10B). Noteworthy, the two transcription factors cooperate in a synergistic way to both induce the targeted proteins (Fig. 10A) and enhance the cell surface presentation of the MHC I molecules (Fig. 10B). Collectively, these data demonstrate that the low expression of the MHC I antigen processing and presenting components, observed in the less immunogenic NB cell lines, is due to a defective expression of IRF1 and p65. Most relevant, their synergistic cooperation suggest that both transactivators are essential for a strong and effective presentation of peptide-MHC I complexes on the cell surface.

A



SK-N-BE(2)



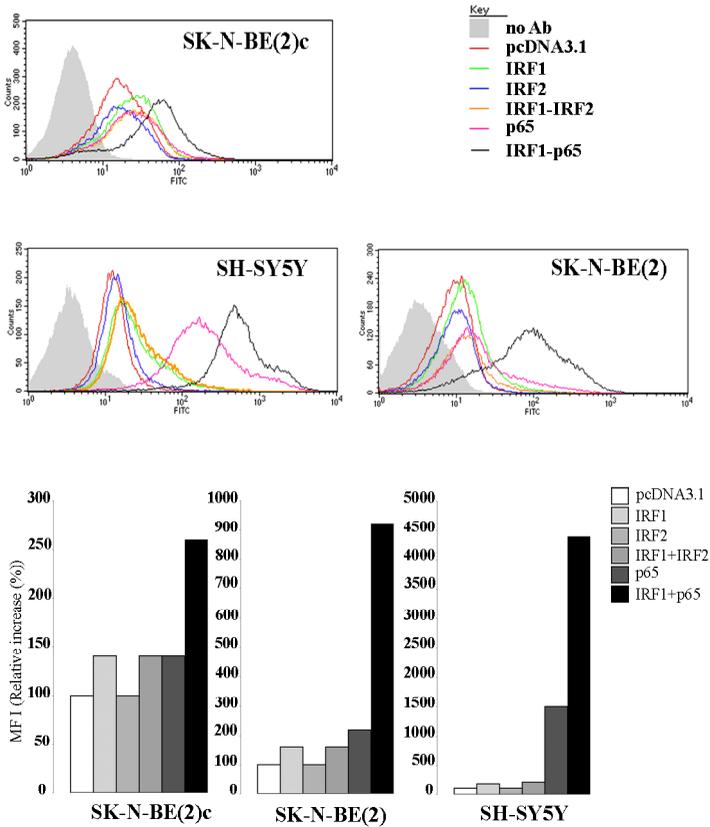
B

Figure 11. IRF1 and p65 synergistically enhance ERAP1 and MHC I expression. Western blotting (A) and flow cytometry analysis (B) with W6/32 mAb (colored lines) of the indicated NB cell lines transfected with either an empty vector or vectors expressing IRF1 (IRF1) or IRF2 (IRF2) and/or the p65 NF- κ B subunit (p65). In (A), arrowheads indicate unspecific bands. In (B) isotype-matched negative controls are displayed as shaded histograms. Histograms (lower panel) represent the fold increase of the mean fluorescence intensity (MFI) for each NB cell line.

CONCLUSION

Antigen processing and presentation have a crucial role in immune surveillance. ER aminopeptidases ERAP1 and ERAP2 play a key role by producing peptides with the correct length and composition to be assembled on MHC I molecules and then presented on the cell surface to the immune cells. Most tumors, including NB, display defective expression of the MHC I antigen processing and presentation components, thereby evading the immune surveillance (Lampson and George, 1986; Lou et al., 2005; Raffaghello et al., 2005; Wolfl et al., 2005) (Tao et al., 2008). Genetic defects and aberrant expression of specific transcription factors have been identified as principal determinants of the low immunogenic activity of tumor cells (Johnsen et al., 1998; Singal et al., 1996). However, in NB tumors the transcriptional mechanism leading to inefficient expression of the MHC I antigen processing and presentation pathway needs to be elucidated.

In this study, we dissected the molecular mechanism underlying the MHC I-, ERAP1- and ERAP2-low phenotype of NB cell lines. Indeed, we demonstrated that: *a*) both IRF1 and NF- κ B synergistically cooperate to induce the coordinated expression of MHC I as well as ERAP1 and ERAP2, and that *b*) the low level of both IRF1 and NF- κ B mostly accounts for the lack of cell surface MHC I expression in NB cells. Furthermore, we provided supporting evidence for a parallel in primary NB tumors.

MYCN has been previously shown to down regulate MHC I expression, in a rat NB cell line, by directly inhibiting the transcription of the p50 NF- κ B subunit (Lenardo et al., 1989; van 't Veer et al., 1993). The finding that MYCN inversely correlated

with MHC I, ERAP1 and ERAP2 in human NB cells (Fig. 1) prompted us to investigate the role of this oncogene in the regulation of the aforementioned genes. Here, our data demonstrate that the high level of the MYCN oncogene did not affect neither the p50 NF-kB nor the expression of MHC I, ERAP1 and ERAP2 in human NB cell lines (Fig. 2). Comparison of our results with previous findings (Feltner et al., 1989; van 't Veer et al., 1993) indicates that MYCN may regulate MHC I expression in rat, but not in human NB cell lines. Otherwise, we would speculate that the aberrant expression of MHC I, ERAP1 and ERAP2 in NB cell lines might rather reflect the defective expression of common transcription factors involved in their regulation.

Consistent with this view, all the components of the antigen processing and presentation machinery can be coordinately enhanced by both IFN γ and TNF α (Corrias et al., 2001; Drew et al., 1993; Goldberg et al., 2002; Marques et al., 2004). IRF1 and NF-kB have been demonstrated to be the main mediators of the IFN γ - and TNF α -induced signalling, respectively.

Thus, we treated several NB cell lines with these cytokines and observed that any NB cell line responded to the IFN γ stimulus by up regulating MHC I (Corrias et al., 2001), as well as ERAP1 and ERAP2 expression (Fig. 8), while only MHC I-high NB cell lines responded to TNF α treatment (Fig. 3A), by inducing NF-kB activation and upregulation of MHC I as well as of ERAP1 and ERAP2. This NF-kB-mediated upregulation relied on the presence of NF-kB binding elements proximal to both the MHC I and ERAP1, ERAP2 gene transcription start site that bind to p65 *in vivo* (Fig. 5). In agreement with our results, NF-kB has been previously demonstrated to mediate the CD40-induced upregulation of several antigen processing molecules, such as

TAP1, TAP2 and several subunits of the immunoproteasome in carcinoma cells (Moschonas et al., 2008).

In MHC I-low NB cells, the MHC I and the β_2 -microglobulin (β_2 -m) promoters lack binding of transcription factor specific for both the NF- κ B-like site (κ B site) and the IFN-responsive elements (IRE) (Drew et al., 1993). Consistently, constitutive expression of NF- κ B, IRF1 and IRF2 is faint in MHC I- and ERAPs-low NB cell lines (Fig. 7). Evidence for a functional *in vitro-in vivo* link derived from the results obtained in primary NB lesions, which resembled those found in NB cell lines. Indeed, ganglionic cells, the most maturing neuroblastic cells, selectively expressed high levels of MHC I (Squire et al., 1990) and nuclear p65, whereas the immature neuroblasts displayed neither nuclear p65 nor MHC I molecules (Fig 6).

Constitutively active NF- κ B is required for the survival of the MHC I-high NB cell lines (Bian et al., 2002). Moreover, doxorubicin-dependent NF- κ B activation induces cell death in MHC I-low NB cell lines (Bian et al., 2001). Thus, our results are consistent with previous findings and suggest that the lack of both constitutively active and TNF α -activable NF- κ B may be essential for the survival of less immunogenic NB tumors.

In light of the above observations, we sought to determine whether overexpression of IRF1 and/or NF- κ B in MHC I-, ERAP1- and ERAP2-low NB cells would induce the rescue of the MHC I, ERAP1 and ERAP2 expression. The effect of either the IRF1 or NF- κ B overexpression on the transactivation of MHC I, ERAP1 and ERAP2 differed depending on the NB cell line tested. When transfected alone IRF1 and NF- κ B behaved in some cases as weak transactivators, or completely failed to induce the target genes (Fig. 11). However, a strong and synergistic enhancement of MHC I, ERAP1 and ERAP2 protein and an effective MHC I cell surface expression was observed after co-transfection of both

transcription factors in any NB cell lines. Thus, the cooperative functional interaction between NF- κ B and IRF1 ensure transactivation of components of the antigen processing and presentation machinery, i.e. MHC I and ERAPs protein, the synchronous synthesis of which is most likely required for the engagement of an effective antitumor immune response and for immune function. Remain to be determined whether other antigen processing components, besides the ER aminopeptidases, are coordinately enhanced by both IRF1 and NF- κ B in NB cell lines. Moreover, it will be of interest to investigate the functional relevance of the augmented cell surface expression of MHC I in term of immune recognition of the tumor cells.

In summary, we show the existence of a tightly coordinated regulation of MHC I, ERAP1 and ERAP2 in NB cells. We identify IRF1 and the p65 NF- κ B subunit as being the transcriptional factors that play major role in modulating MHC I cell surface expression in NB cells. Most important, we demonstrate that the coordinated downregulation of MHC I, ERAP1 and ERAP2 in less immunogenic NB cells is attributable to a low transcriptional availability of both NF- κ B and IRF1 transcription factors. Thus, we believe that the present work provide an explanation for the failure of NB cells to express MHC I molecules and represent a rationale background for the design of CTL- or NK cell-based immunotherapeutic strategies.

MATERIAL AND METHODS

Tumor cell lines and reagents

The following human neuroblastoma cell lines were used: SH-EP, SK-N-AS, SK-N-SH, ACN, SK-N-SY, SH-SY5Y, LAN-5, IMR-32, KCNR, SK-N-BE(2) and SK-N-BE(2)c. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas). SH-SY5Y and SK-N-SH cells were grown in DMEM, and MEM, respectively. The other cell lines were maintained in RPMI 1640. The mediums were supplemented with 10% FCS (HyClone), glutamine, 100 µg/ml penicillin and 50 µg/ml streptomycin. The SH-EP MYCN-ER cell line, which conditionally expresses MYCN under the control of 4-hydroxy-tamoxifen (4-OHT), was kindly provided by B. Berwanger (Marburg, Germany). The Tet-21/N cell line expressing MYCN under the control of a doxycycline-repressible promoter was kindly provided by M. Schwab (Heidelberg, Germany). 4-OHT and doxycycline, both from Sigma-Aldrich, were used at 200 nM and 10 ng/ml, respectively. Cycloheximide (Sigma-Aldrich) was used at 10 µg/ml. Recombinant human TNF α (PeproTech) and IFN γ (R & D Systems) were used at 50 ng/ml and 500 U/ml, respectively. Sulfasalazine (Sz) was from Sigma-Aldrich and used at 2 mM. The p53 siRNAs were purchased from Cell Signalling Technology, while the siRNAs against MYCN, IRF1 and IRF2 were from Sigma-Aldrich.

DNA constructs and transfections

The mutant Ser³²→Ala/Ser³⁶→Ala IKB α (M-IkBa) in pUSE, the NF-kB p53 subunit in pcDNA3 (kindly provided by M. Cipitelli,

and M. Levrero, respectively), the expression vectors pRc/CMV-IRF1 and pRc/CMV-IRF2 (kindly provided by A. Battistini) and the corresponding empty vectors were transfected using LipofectAMINE 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Cells were seeded in tissue culture plates to achieve 80% confluence. 24 h later, the DNA-lipofectAMINE complexes were added in OptiMEM. 15 h following transfection the complexes were replaced with fresh medium, and the cells were treated as indicated. Stable SH-EP and SK-N-AS transfectants were selected in 450 mg/ml G418 (Invitrogen Life Technologies) for 2-3 weeks.

siRNA experiments

All small interfering RNA (siRNA) duplex oligonucleotides were synthesized and subsequently annealed for use. For p65 silencing, cells were transiently transfected with 5 nM p65 siRNA or the corresponding scrambled siRNA, using 1.5 ml Hyperfect (Qiagen). For MYCN, IRF1 or IRF2 silencing, cells were transfected with 80 nM siRNAs, using LipofectAMINE 2000. Cells were seeded at a confluence of 50%. After 24h, cells were transfected with siRNA at the indicated concentration. The complexes were removed after 15 h of transfection. Cells were harvested 48 h later, or were cultured for an additional 10 h, and then the medium was exchanged with either the medium alone or medium containing IFN γ , depending on the experimental setting.

Semiquantitative and quantitative (RT)-PCR

Total RNA was extracted with Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Aliquots of 1 μ g of total RNA were treated with DNase I (Ambion), and the single-stranded cDNAs were obtained by retro-transcription with Superscript II (Invitrogen) according to the manufacturer's

instructions. Real-time qRT-PCR was performed with Taqman probes (Applied Biosystems) in the Applied Biosystems 7700 Sequence Detection system (Applied Biosystems). The expression of each mRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) after normalization with reference to expression of 18S RNA.

Antibodies and immunoblotting

The rabbit polyclonal Ab A-LAP and the murine mAb 3F5 were raised against recombinant human ERAP1 and ERAP2, respectively (Hattori et al., 2000; Saveanu et al., 2005). The rabbit polyclonal Ab R5996-4 and the mouse mAb W6/32 to denatured and β_2 microglobulin-associated MHC class I heavy chains (Barnstable et al., 1978; Nakamuro et al., 1975) were used to detect MHC class I antigens by Western blotting and flow cytometry, respectively. The following antibodies were all from Santa Cruz Biotechnology: MYCN (sc-791), NF- κ B p50 (sc-8414), NF- κ B p65 (sc-109), IRF1 (sc-497), IRF2 (sc-498) and PCNA (sc-56). For total extracts cells were lysed in 50 mM Tris pH 7.5 and 250 mM NaCl containing 1% Nonidet P-40 (NP-40) in the presence of a mixture of protease inhibitors (10 mM Leupeptin, 10 mM Pepstatin A, 1 mM PMSF, and 10 mM Aprotinin). Nuclear extracts were obtained by suspending cells in hypotonic buffer (300 mM Sucrose, 10 mM HEPES-KOH pH 7.9, 0.1 mM EDTA, 1.5 mM $MgCl_2$ and proteinase inhibitors) for 10' on ice. Following addition of 0.2% NP-40, the solution was subjected to shearing with a 22-gauge needle. After mild centrifugation, nuclear pellets were lysed in extraction buffer (10 mM HEPES-KOH pH 7.9, 400 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol and protease inhibitors) for 30' on ice. The extracts were assayed for protein concentration by the BCA assay

kit (Pierce). Equal amount of protein extracts were resolved on 8-10% SDS-PAGE and electroblotted on a nitrocellulose membrane (Whatman). Filters were blocked with 5% nonfat milk, and probed with primary Ab, followed by incubation with peroxidase-coupled secondary Ab. Immunoreactivity was detected using the ECL detection system (Amersham Biosciences). Equal loading of whole-cell and nuclear extracts was checked by Abs to ERp57 and PCNA, respectively.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as described previously (Saccani et al., 2002), with some modifications. Cells were treated with 1% formaldehyde for 10 min, and the reaction was stopped with 0.125 M glycine. Cells were lysed and chromatin was sheared by sonication to 300-1000 bp fragments, and then incubated with 5 mg of mAb sc-109 to p65. The resulting immunocomplexes were absorbed on protein G beads and washed once with dilution buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% NP-40), twice with low salt buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.25% NP-40, 0.05% SDS, 2 mM EDTA), twice with high salt buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.5% NP40, 0.05% SDS, 2 mM EDTA), and once in TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). All buffers were supplemented with protease inhibitors. Immune complexes were extracted in TE containing 1% SDS and 100 mM NaHCO₃. DNA crosslink was reverted by heating at 65°C. After proteinase K digestion, DNA was extracted and subjected to PCR. The PCR primers were designed to amplify about 400 bp DNA fragments encompassing NF-κB binding sites upstream to the HLA-A, ERAP1, ERAP2, IL-8 and β-actin genes. Primer sequences were: *HLA-A*: forward 5'-TCCAGGGACAGAGATTACGG-3' and reverse 5'-

CTTCTCTGGAAACCCGACAC-3'; *ERAP1*: forward 5'-CGCAACGCTAAACAGTGAAA-3' and reverse 5'-GGACCGAAAGTGAAAGTGGA-3'; *ERAP2*: forward 5'-TCTTTCATGGTTTGGCTTC-3' and reverse 5'-CAGATTTGACTGAAGGGGAAT-3'; *IL-8*: forward 5'-GTTGTAGTATGCCCTAAGAG-3' and reverse 5'-CTCAGGGCAAACCTGAGTCATC-3'; *β -actin*: forward 5'-CCCTCCAAGAGCTCCTTCTG-3' and reverse 5'-TGTGCTCGCGGGGCGGACGC-3'.

Flow cytometry and immunohistochemistry

Surface expression of MHC class I molecules was determined by flow cytometry with mAb W6/32. 5×10^5 cells, for experimental point, were incubated for 1h at 4°C with the W6/32 antibody. Cells were washed with PBS containing 0,5% FCS. The cells were then incubated for 1h at 4°C with FITC-labeled goat anti mouse Ig Fc (1:100). The cells were washed with PBS containing 0,5% FCS and analyzed with a FACScan flow cytometer (Becton Dickinson). Primary NB lesions were obtained upon informed parental consent and snap frozen in liquid nitrogen. Four- μ m thick sections were fixed in either cold absolute acetone and stained by W6/32 to MHC I, or in acetone: methanol (1:1 vol/vol) and stained for p65. Staining was revealed by a super-sensitive immunohistochemistry kit (biogenex). After the samples were counterstained with haematoxylin, the localization of MHC I and p65 was examined by light microscopy.

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 statistical software. A level of $P < 0.05$ was considered statistically significant.

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