Review Article

Epigenetic View on Interferon γ Signalling in Tumour Cells

(IFN-γ / epigenetic modulators / DNA methylation / histone acetylation / cancer immunotherapy)

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Abstract. IFN-γ is a pleiotropic cytokine crucial for both innate and adaptive immunity, which also plays a critical role in immunological surveillance of cancer. Genetic defects or gene silencing in the IFN-γ signal transduction pathways as well as in the expression of IFN-γ-regulated genes represent frequent mechanisms by which tumour cells can escape from immune responses. Epigenetic control of the IFN-γ signalling pathway activation associated with epigenetic changes in the corresponding regulatory gene regions, such as chromatin remodelling, histone acetylation and methylation, and DNA demethylation is frequently dysregulated in tumour cells. Epigenetic silencing of the IFN-γ regulatory pathway components, as well as of the IFN-γ-regulated genes crucial for tumour cell recognition or induction of anti-tumour immune responses, has been documented in various cancer models. Expression of both IFN-γ signalling pathway components and selected IFN-γ-regulated genes can be influenced by epigenetic modifiers, namely DNA methyltransferase and histone deacetylase inhibitors. These agents thus can mimic, restore, or boost the immunomodulatory effects of IFN-γ in tumour cells, which can contribute to their anti-tumour therapeutic efficacies and justifies their potential use in combined epigenetic therapy with immunotherapeutic approaches.

Introduction

Interferon γ (IFN-γ) is a cytokine that plays a pivotal role in both innate and adaptive immunity (Boehm et al., 1997; Schroder et al., 2004; Borden et al., 2007). Moreover, it is a crucial player in cancer immunosurveillance (Castro et al., 2018). The main organismal sources of IFN-γ are natural killer (NK) cells and natural killer T (NKT) cells, and $CD4^+$ and $CD8^+$ T lymphocytes. The IFN-γ receptor consists of two class II cytokine receptor proteins – IFN-γ receptors 1 and 2 (IFNGR1, IFNGR2). IFN-γ forms a homodimer that binds to the corresponding receptor complex on the cell surface. The signal is then transduced through the activity of JAK/STAT signalling pathway into the nucleus, where the activated pSTAT induces expression of target genes. In the nucleus, pSTAT1 binds to the γ interferon activation site (GAS) or interferon-stimulated response element (ISRE) motifs in the promoter regions of interferon-stimulated genes (ISGs). Tightly regulated IFN-γ-induced cellular responses are mediated by modulation of gene expression, including posttranscriptional regulations (Savan, 2014). The numerous IFN-γ-regulated genes responsible for different cellular processes, including genes involved in antimicrobial, antiviral and anti-proliferative pathways, can be divided into two groups, primary and secondary responsive genes (Kile and Alexander, 2001; Gysemans et al., 2008; Schneider et al., 2014). IFN-γ also increases the expression of genes of the signalling pathway itself, such as STAT1, or the negative regulators of the pathway, mainly the SOCS1 and SOCS3 proteins, which specifically down-regulate the cellular IFN-γ responses.

An important group of IFN-γ-stimulated molecules is represented by interferon regulatory factors (IRF), key players not only in the immune responses or immune

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Abbreviations: 5AC – 5-azacytidine, 5-Aza-dC – 5-aza-2'-deoxycytidine, AAc – aliphatic acids, CIITA – class II transactivator, DAP – death-associated protein, DNMT – DNA methyltransferase, DNMTi – DNA methyltransferase inhibitors, FoxP3 – forkhead box protein 3, GAS – γ interferon activation site, HAC – histone acetylase, HDAC – histone deacetylase, HDACi – histone deacetylase inhibitors, IDO1 – indoleamine 2,3-dioxygenase 1, IFNGR – IFN-γ receptor, IFN-γ – interferon γ, iNOS – inducible nitric oxide synthase, IRF – interferon regulatory factor, ISFG3 – interferon-stimulated gene factor 3, ISGs – interferon-stimulated genes, ISRE – interferon-stimulated response element, JAK – Janus kinase, NK cells – natural killer cells, NKT cells – natural killer T cells, pSTAT – phosphorylated STAT, ROS – reactive oxygen species, SB – sodium butyrate, SOCS – suppressor of cytokine signalling, STAT – signal transducer and activator of transcription, TET – ten-eleven translocation, TSA – trichostatin A.

cell development, but also in a number of other cellular processes such as cell proliferation control, metabolism, and tumour growth or suppression (Ikushima et al., 2013; Zhao et al., 2015). These transcription factors bind to IRF-E and interferon-stimulated response element (ISRE) sites in the promoter regions of other stimulated genes facilitating further response to the IFN-γ signal (Schroder et al., 2004). The main complex transmitting the IFN-γ signal is the homodimer of STAT1; however, evidence exists for the use of heterotrimer STAT1:STAT2:IRF-9 (called ISFG3). ISFG3 is traditionally used by other members of the interferon family, which can explain their partially shared effects on the cells (Platanias, 2005).

IFN-γ is a key cytokine mediating anti-tumour immunity, although under some circumstances it can also be pro-tumorigenic (Zaidi and Merlino, 2011). Besides its immunomodulatory effects, IFN-γ displays direct effects on tumour cells that may increase their visibility to the effector immune cells (increased MHC class I expression, antigen presentation machinery genes, costimulatory molecules). IFN-γ can also inhibit proliferation of tumour cells and even induce their apoptosis via different mechanisms. Notably, IFN-γ, either alone or in combination with other cytokines, can induce cellular stress in selected tumour cells, leading to the cell death or senescence (Braumüller et al., 2013; Rakshit et al., 2014; Hubackova et al., 2016). No wonder that non-responsiveness to IFN-γ and silencing of IFN-γ-regulated genes are among the major mechanisms by which tumour cells can escape from immune surveillance (Garcia-Lorca et al., 2003). Besides "hard", irreversible changes based on the DNA changes, tumour cells can silence genes regulated by IFN-γ. This can be reversed by IFN-γ itself, by other cytokines, as well as by epigenetic modulators, suggesting that epigenetic modifications take place in these expression regulations (Setiadi et al., 2007; Vlková et al., 2014).

Despite growing understanding of the IFN-γ cellular activities, the mechanisms involved in the regulation of expression of a plethora of target genes that may frequently be dysregulated in tumour cells, and especially the role of the epigenetic processes, are not fully understood. In our review we summarize the current knowledge about the epigenetic components of IFN-γ activity in tumour cells, revealing the often overseen part of the IFN-γ signalling mechanism. We have mostly focused on DNA methylation and histone modifications that can be pharmacologically influenced by epigenetic agents. Further, epigenetic silencing of the IFN-γ-regulated genes may be responsible for aberrant antigen processing, tumour-associated antigen down-regulation, MHC down-regulation, or lack of costimulation in tumour cells, and represents an important mechanism mediating the tumour escape from immune surveillance (Tomasi et al., 2006). Epigenetic modifiers, such as DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi), may potentially revert these processes and act synergistically with IFN-γ. Indeed, a number of studies demonstrate promising results of anti-tumour therapies using combined epigenetic and immunotherapeutic agents (Dunn and Rao, 2017).

Epigenetic regulations and chromatin remodelling as participants in the IFN-γ pathway

Epigenetics is defined as the field exploring the heritable information non-written in the primary structure of DNA (Berger et al., 2009). Epigenetic mechanisms consist of modifications of DNA itself, among which the methylation of cytosine is the most studied variant, including post-translational modifications of histones and activity of many different classes of non-coding RNA molecules that are interrelated to the chromatin organization. The role of the epigenetic changes has been demonstrated in many physiological and pathological conditions, including cancer (Jones and Baylin, 2007). Global hypomethylation of CpG islands paired with local hypermethylation is characteristic of cancer cells and probably takes its role in the tumorigenesis by epigenetic silencing of tumour suppressor genes or by inhibiting antigen presentation or DNA repair and the apoptotic pathways (Hassler and Egger, 2012). Demethylation and activation of these particular genes by DNMTi thus represents an attractive chemotherapeutic approach to fighting against selected tumours. Indeed, DNA methylation-inhibiting drugs, namely 5-azacytidine (5AC; Vidaza) and 5-aza-2'-deoxycytidine (5-Aza-dC; Decitabine), have been investigated in a number of clinical trials and already approved for therapy of myelodysplastic syndrome and acute myeloid leukaemia (Ahuja et al., 2016; Jones et al., 2016).

The significance of epigenetic mechanisms in cancer therapy and immunosurveillance is illustrated by successful experience with augmenting anti-tumour immune responses using epigenetic modulators. According to West et al. (2014), the effectivity of histone deacetylase inhibitors against aggressive B-cell lymphoma and colon carcinoma relies on the functionality of the immune system, especially on the production of IFN-γ. These authors showed that HDACi were significantly less effective in immunocompromised mice compared to the immunocompetent individuals. HDACi sensitized tumour cells to the antineoplastic effects of IFN-γ, and co-administration of HDACi Vorinostat with IFN-γ inducer α-galactosylceramide prolonged survival of the tumour-bearing mice. In another experiment, murine experimental MHC class I-deficient and -positive tumours were treated with 5AC combined with immunotherapy (Šímová et al., 2011). The efficacy of the combined chemoimmunotherapy against originally MHC class I-deficient tumours became partially dependent on the $CD8^+$ cell-mediated immune responses that corresponded to the increased cell surface expression of MHC class I cell molecules on tumours explanted from the 5AC-treated animals. This increase was associated with

up-regulation of the antigen-presenting machinery-related genes, as well as of the genes encoding selected components of the IFN-γ-signalling pathway in these cells.

All of these findings suggest tight connections between epigenetic mechanisms and immunosurveillance of malignant cells, process in which IFN-γ plays an important role, and provide evidence that epigenetic modulations underlie some of the defects in the IFN-γ signalling pathway and expression/silencing of the IFN-γ-regulated genes. In this review we have mostly focused on DNA methylation and histone modifications that are involved in the IFN-γ signalling or interfere with this pathway in tumour cells. We discuss DNA methylation and histone modification separately, although these two processes are closely interrelated due to the complexity of epigenetic modifications.

DNA methylation

DNA methylation, an epigenetic mark located directly on DNA, was first described in the 1940s, but its epigenetic role was found many years later – in the 1980s, when several experiments clearly demonstrated its role in the regulation of gene expression. Since that time DNA methylation has been recognized as one of the major epigenetic mechanisms, and therefore it is the object of many epigenetic studies aiming to understand the exact mechanisms of its action and its meaning in different genomic contexts (Moore et al., 2013). In the human genome, DNA methylation is usually found in the context of CpG dinucleotides concentrated to the regions called CpG islands. These CpG islands are located in the promoter sequences of genes and in the gene bodies with a different pattern of effects in both sites. DNA methylation in a promoter is typically associated with inhibition of gene expression due to the changes in electric charges in the vicinity of the DNA chain resulting in more tightly packed chromatin unapproachable by transcription machinery (Schübeler, 2015). On the other hand, DNA methylation in the gene bodies is linked with actively transcribed genes, challenging the traditional view on the role of the oldest known epigenetic mechanism (Jones, 2012). Together with the discovery of other modifications of cytosine, such as 5-hydroxymethylation or formation of 5-carboxycytosine and 5-formylcytosine, whose existence is tightly connected with the activity of ten-eleven translocation (TET) enzymes and their suspected ability to stimulate active demethylation pathways in cells, the old paradigm of stable, unchanging, and only found DNA modification in humans is transformed to the model of a more complex, flexible mechanism (Wu and Zhang, 2010).

The possible relationships between DNA methylation and the interferon signal transduction pathway was first partially documented in experiments using DNMTi. In 1999, a team at the Huntsman Cancer Institute investigated the effect of 5-Aza-dC treatment on gene expression with the finding that the treatment preferentially induced IFN-responsive genes in HT29 colon adenocarcinoma cells. Moreover, the observed changes correlated with the induction of transcription factors STAT 1, 2 and 3 (Karpf et al., 1999). These results were confirmed by another comparative analysis of the gene expression profile of immortalized cells treated with 5-Aza-dC, which revealed 85 genes with methylation-dependent silencing, among them 39 involved in the interferon pathway, such as IRF7, components of HLA class I, AIM2 or STAT1. Interestingly, treatment with DNMTi induced a senescence-like state, suggesting a role for gene silencing in early carcinogenesis (Kulaeva et al., 2003). Examples of genes associated with the IFN-γ signalling pathway that are activated by DNMTi are shown in Table 1.

Reversal of the IFN-γ unresponsiveness using epigenetic modifiers was documented in human prostate adenocarcinoma cell line LNCaP, which was described unresponsive to IFN-γ due to the silenced expression of JAK1. This expression was induced upon treatment of cells with a combination of inhibitors of DNA methyltransferases and histone deacetylases (Dunn et al., 2005). Anti-tumour immune responses are frequently abrogated by reversible or irreversible alterations in classical or non-classical MHC class I and class II as well as antigen presentation genes expression. Methylation of the corresponding genes that underlies these defects is well documented and has been studied in detail (Serrano et al., 2011). Our previous work has also shown that the abilities of the epigenetic agents or IFN-γ to restore expression of selected silenced antigen-presenting machinery genes was associated with changes in the DNA methylation of the corresponding gene regulatory sequences in several class I-deficient murine tumour cell lines (Manning et al., 2008). Further, by comparative transcriptome analysis in distinct MHC class I-deficient murine tumour cell lines upon their treatment with either IFN-γ or epigenetic agents we documented that although the expression patterns corresponded to the treatments, a number of genes were regulated in the same manner (Vlková et al., 2014). Epigenetic down-regulation of the *TAP1* gene, a principal component of the antigen-presenting machinery, by hypermethylation has been recently documented in breast cancer stem cells (Sultan et al., 2018).

Another epigenetic mechanism by which tumour cells can abrogate MHC class I expression and antigen presentation was described in melanoma cells. Selective silencing and IFN-γ responsiveness of the *HLA-A3* gene associated with promoter CpG methylation close to the site-α and TATA box was observed, besides other HLA class I antigen-processing defects, in the COPA-159 cell line (Chang et al, 2015). This could be reversed by DNA methyltransferase 1 depletion. A previous study also demonstrated hypermethylation in the *HLA-A* and *B* genes that resulted in transcriptional defects in MSR3-mel melanoma cells (Serrano et al., 2001). Again, treatment with 5-Aza-dC restored cell surface expression of HLA class I antigens and subsequent tumour cell recognition by MAGE-specific cytotoxic T lymphocytes. A tight connection between HLA class I and NKG2D ligand down-regulation and RAS and DNA methyltrans-

Table 1. IFN-γ-responsive genes with enhanced expression after treatments with DNMTi

Name	Full name	Agent	Reference
STAT1	Signal transducer and activator of transcription 1	5-Aza-dC	Karpf et al., 1999, Kulaeva et al., 2003
ISG15	Interferon-stimulated gene 15 (IFN-inducible 17-kDa protein)	5-Aza-dC	Karpf et al., 1999, Kulaeva et al., 2003
P2Y5	Purinergic receptor	5-Aza-dC	Karpf et al., 1999
MIC-1	Macrophage inhibitory cytokine 1	5-Aza-dC	Karpf et al., 1999
IFI27	IFN-α-inducible protein 27	5-Aza-dC	Karpf et al., 1999
MHC class I	Major histocompatibility complex class I	5-Aza-dC	Karpf et al., 1999, Chang et al., 2015, Serrano et al., 2001
OAS3	2'-5'-Oligoadenylate synthetase 3	5-Aza-dC	Karpf et al., 1999
CXCL10 (IP-10)	IFN-inducible protein 10	5-Aza-dC	Karpf et al., 1999
CFH	Complement factor H	5-Aza-dC	Kulaeva et al., 2003
RSAD2	Radical S-adenosyl methionine domain containing 2	5-Aza-dC	Kulaeva et al., 2003
TRAIL	TNF-related apoptosis-inducing ligand	5-Aza-dC	Kulaeva et al., 2003
IL-8	Interleukin 8	5-Aza-dC	Kulaeva et al., 2003
HLA-C	Major histocompatibility complex class I antigen heavy chain HLA-C	5-Aza-dC	Kulaeva et al., 2003
HLA-F	Major histocompatibility complex class I F	5-Aza-dC	Kulaeva et al., 2003
SLURP1	Ly-6-related protein gene	5-Aza-dC	Kulaeva et al., 2003
OAS-2	2'-5'-Oligoadenylate synthetase 69/71 kDA isoform	5-Aza-dC	Kulaeva et al., 2003
IGFBP4	Insulin-like growth factor binding protein-4	5-Aza-dC	Kulaeva et al., 2003
TAP-1	Transporter 1, ATP binding cassette subfamily B member	5AC	Manning et al., 2008
TAP-2	Transporter 2, ATP binding cassette subfamily B member	5AC	Manning et al., 2008
LMP-2 (PSMB9)	Proteasome subunit β 9	5AC	Manning et al., 2008
LMP-7 (PSMB8)	Proteasome subunit β 8	5AC	Manning et al., 2008
HLA-B	Major histocompatibility complex class I antigen HLA-A heavy chain	5-Aza-dC	Nie et al., 2001
MX1	Interferon-inducible protein p78	5-Aza-dC	Kulaeva et al., 2003

ferase activities was demonstrated in colorectal cancer cells (Sers et al., 2009). DNMT deficiency and MEK inhibition of HCT116 cells resulted in demethylation of the regulatory sequences of both genes encoding HLA-A1/A2 and *ULBP2* gene encoding a ligand for activating NK receptor NKG2D, and their subsequent expression.

The class II transactivator (CIITA), which plays a pivotal role in the expression of MHC class II genes and their accessories (invariant chain [Ii] and HLA-DM), is also regulated by IFN-γ (Muhlethaler-Mottet et al., 1997). The association between impaired antigen presentation and *CIITA* silencing and gene methylation was demonstrated in developmental tumour cell lines (van der Stoep et al., 2002). It was not clear whether this status rather reflected the mode of *CIITA* expression during early development than the processes of oncogenic transformation. Further experiments were performed using a variant of human promyelocytic cell line THP-1. This cell line normally expresses high levels of HLA class II upon IFN-γ treatment. In a selected variant that did not express HLA class II after the IFN-γ treatment, this unresponsiveness was associated with methylation of the *CIITA* promoter. DNA demethylation using 5AC restored IFN-γ-dependent HLA class II expression (De Lerma Barbaro et al., 2008). Epigenetic *CIITA* silencing has also been demonstrated in rhabdomyosarcomas, highly malignant paediatric sarcomas. However, no hypermethylation but rather block of hyperacetylation was observed in cell line RD (embryonal rhabdomyosarcoma), and only modest hypermethylation was seen in SJRH30 (alveolar rhabdomyosarcoma) cells (Londhe et al., 2012). In agreement with these findings, TSA alone restored the *CIITA* expression in RD cells, while its combination with DNMTi was required for its up-regulation in SJRH30 cells. Hypermethylation of the *CIITA*-promoter IV locus leading to reduction of the CIITA expression and subsequent MHC class II antigen presentation was also indicated in human squamous cell carcinoma of the head and neck cell line PCI-52 (Meissner et al., 2008).

Research has also been focused on the role of DNA methylation in the regulation of the prolonged-hand of IFN-γ, acting downstream of the JAK/STAT signalling, the IRFs. Transcription factors IRFs are stimulated by interferons. They regulate transcription of selected interferon-inducible genes, thus promoting interferon effects on cells and bridging innate and adaptive immunity. IRFs are also active players in the control of oncogenesis; especially IRF1 and IRF8 are considered tumour

suppressor genes. No wonder that IRFs can carry both genetic and epigenetic modifications, and their interrelations with the IFN unresponsiveness and association with tumour progression have been described in a number of studies (Fragale et al., 2013). Analysis of a number of tumour cell lines revealed that particular IRFs were differentially expressed in various gastric cancer cells, and frequent suppression of IRF4, IRF5 and IRF8 was documented (Yamashita et al., 2010). These differences in expression correlated with their DNA methylation status.

Moreover, treatment with DNA methyltransferase inhibitor 5-Aza-dC restored their expression and enhanced suppression of malignant cells by interferons α, β and γ. Methylation of the promoter of *IRF4* gene in chronic myeloid leukaemia cells is associated with its repression, and 5-Aza-dC increased its expression at both, mRNA and protein levels. On the other hand, *in vitro* methylation of the *IRF4* construct decreased the promoter activity, illustrating that DNA methylation could be the critical mechanism of regulation (Ortmann et al., 2005). Frequent promoter hypermethylation of *IRF5* resulting in gene silencing was reported in hepatocellular carcinoma tissue samples, as well as in Epstein-Barr virus-associated gastric carcinomas (Shin et al., 2010; Dong et al., 2015). However, the clinical implications have to be further studied. In 2008, it was found that DNA methylation levels of the promoter of *IRF8* gene were inversely correlated with its expression in human colorectal carcinoma cells *in vivo*. This silencing was mediated through the cooperation of suppressor complex MBD1 and pSTAT1 inhibitor PIAS. Inhibition of DNA methylation restored the activation of *IRF8* expression with IFN-γ (McGough et al., 2008). Another study focused on multiple myeloma and published that same year ended up with similar conclusions (Tshuikina et al., 2008). In three out of 13 multiple myeloma cell lines and one out of nine primary cell lines, the lower expression levels were associated with methylation of the *IRF* gene promoter sequence. The role of DNA methylation in the suppression was further supported by *in vitro* methylation and treatment with 5-Aza-dC, which led to decreased or increased expression, respectively. However, in the other cell lines with decreased expression of *IRF8* compared to healthy cells this suppression was not accompanied by hypermethylation, suggesting another potential regulating mechanism. In a murine study, B16-F10 melanoma cells were transplanted into *IRF8*-deficient mice. Rapidly growing tumours displayed inhibited *IRF8* expression. Upon 5-Aza-dC treatment, *IRF8* expression was restored, which was accompanied by increased leukocyte infiltration and tumour growth arrest (Mattei et al., 2012).

IRF1 is a crucial factor mediating not only immune responses, but also other cellular processes including tumour suppression (Tanaka et al., 1994). In melanoma cell line ESTDAB-159 with lost IFN-γ inducibility of HLA class I expression, not methylation of the *IRF1* gene itself but epigenetic blocking of the downstream IRF1 transactivation was suggested to be responsible for this defect, since IFN-γ induced IRF1 expression, but only the combination with 5-Aza-dC treatments induced HLA class I expression (Rodríguez et al., 2007).

Recently, the methylation status of selected *IRF*s has been investigated in lung cancer tissues and correlated to the *CD274* (*PD-L1*) expression (Lai et al., 2018). Highly methylated *IRF1* and *IRF7* genes were associated with low *CD274* expression. Hypomethylation of *IRF1/7* upon the 5-Aza-dC treatments restored the PD-L1 levels. This is in agreement with an older study on non-small cell lung cancer cell lines. These cells frequently displayed hypermethylation and low expression of *IRF7* (Wrangle et al., 2013). Importantly, the treatment with 5AC also up-regulated PD-L1. Taken together, both these studies support the idea to epigenetically sensitize tumour patients to immune responses and combine this treatment with a PD-1/L1 checkpoint blockade.

Indoleamine 2,3-dioxygenase-1 (IDO1) is an IFN-γ-inducible enzyme that plays an important immunoregulatory/immunosuppressive role through up-regulation of tryptophan catabolism (Munn et al., 1999). Its epigenetic activation upon combined treatment with zebularine and IFN-γ has been demonstrated (Xue et al., 2012). Hypothetically, this activation should induce immunosuppression promoting tumour growth, but on the other hand, alleviating autoimmunity and chronic inflammation. Another study focused on triple-negative breast cancer showed that the expression and IFN-γ-mediated inducibility of IDO were down-regulated by gene methylation in oestrogen receptor-positive cell lines, which was reversed by the 5-Aza-dC treatment in MCF7 cells (Noonepalle et al., 2017). This is thus an example showing that epigenetic silencing is a double-edged sword in terms of immune activation and that demethylation can also induce immune suppression. This has been well documented in the regulation of the forkhead box protein 3 (*FoxP3*) gene, expression of which is crucial for immune regulatory functions of T-regulatory cells (Tregs). The expression is controlled by DNA methylation of the Treg-specific demethylated region and is inducible by 5AC (Polansky et al., 2008). In this case, the 5AC effect may be opposite to that of the IFN signalling pathway, since IRF1 has been documented as an important negative regulator of the *FoxP3* expression (Fragale et al., 2008).

It is noteworthy that not only aberrant DNA methylation of the IFN-γ-signalling components and IFN-γ controlled genes in tumour cells, but also epigenetic silencing of IFN-γ production by lymphocytes in tumour-bearing individuals can inhibit the IFN-γ-mediated immunity in the course of cancer development. Epigenetic regulation, and especially DNA methylation of the *IFNg* gene, has been well documented as a crucial mechanism in the T-cell development and differentiation (Penix et al., 1996; Chang et al., 2007; Schoenborn et al., 2007; Spilianakis et al., 2007; Aune et al, 2013; Scharer et al., 2013). Promoter methylation is responsible for the regulation of the *IFNg* gene during *in vitro* differentiation

of human peripheral blood T cells into a Th2 population (Yano et al., 2003). The capacity of 5AC to restore IFN-γ production by murine cell line BFS upon IL-2 treatment was described more than 30 years ago (Farrar et al., 1985). Down-regulation of the *IFNg* gene expression associated with the gene hypermethylation was observed in $CD4^+$ T lymphocytes in lung cancer (Wang F. et al., 2013), as well as in T lymphocytes in cervical and oral cancer tissues (Ma et al., 2014; Tian et al., 2017).

Posttranslational modification of histones and regulation of the IFN-γ-controlled genes

Histones, basic proteins shaping the chromatin structure, are involved in another important and widely studied mechanism regulating the transcriptional activity of genes (Cosgrove et al., 2004). Compared to the DNA methylation, the world of the histone language is from the beginning much more complex. The spectrum of possible histone modifications is wide; beginning from methylation, to acetylation and phosphorylation, to ubiquitination or sumoylation in many different sites. The combination of different modifications in the same locus together makes up the patterns characteristic of many types of genes with different levels, time, and spatial organization of activity (Berger, 2007; Su and Denu, 2016). Changes in a number of immune-related genes, including those regulated by IFN-γ, have been well documented (reviewed by Hull et al., 2016), and extensive research is focused on the role of histone modifications in the regulation of the IFN-γ pathway components, uncovering the IFN-γ effects on the expression of a varied palette of epigenetic modifiers, the epigenetic activity of IRFs, or cooperation of histone-modifying enzymes with other factors driving expression or suppression of interferon-stimulated genes. Further, a number of genes encoding the components of the IFN-γ signalling pathway or IFN-γ-regulated genes can be activated in tumour cells by HDACi, as can be seen in Table 2.

Early research in this area was focused on the effect of inhibition of histone deacetylase activity by TSA on binding of RNA polymerase II after the treatment with IFN-β. In contrast to the expectations arising from the known association of increased histone acetylation with transcriptionally active regions, the TSA treatment suppressed expression of selected IFN-β-stimulated immediate early genes that are activated by STAT1/STAT2. It turned out that the binding of RNA polymerase II to interferon-stimulated response element (ISRE) was dependent on the HDAC activity. However, the binding on the *IRF1* promoter remained unaffected. The TSA treatment had no influence on the phosphorylation or binding of STAT1 or STAT2, and the inhibitory effect of TSA was fully reversed by ectopic expression of the first 326 amino acids of IRF9 (Sakamoto et al., 2004). Despite the fact that the research was focused on IFN-β signalling, it is known that interferons share not only the effect, but also some factors and signal transmitters, including different IRFs or STAT. Hence the evidence for connection between STATs, IRFs and activity of HDAC seems thought-provoking from the point of view of IFN-γ research.

Another study by Jin et al. (2017) was also interested in IRF9, but from the opposite standpoint. In this study, the interactions between H3K4 demethylase, lysine-specific demethylase (LSD1) and H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) were investigated. These proteins form an important part of repressor complexes called CoREST or PRC2, respectively. The study revealed that knockdown of both these proteins up-regulated expression of the proteins involved in the type I IFN pathway. Changes were also discovered in the *IRF9* locus, where their interaction stabilized the binding of LSD1 to the promoter and modified the histone methylation in this locus. These results suggest that coordinated action of different histone-modifying enzymes may serve as a double lock system for precise regulation of the expression of interferon-stimulated genes. Again, even though the work looked at the proteins in type I IFN pathway, IRF9 has a well-described role in IFN-γ signalling (Schroder et al., 2004) and its ability of cooperation with histone-modifying enzymes should therefore be taken into account.

Another methylation-modifying enzyme involved in the regulation of IFN-γ is histone demethylase PHF8 responsible for H4K20me1 demethylation. Affinity purification and mass spectrometry analysis identified coop-

Table 2. IFN-γ-responsive genes with enhanced expression after treatments with HDACi

Name	Full name	Agent	Reference
TAP-1	Transporter 1, ATP binding cassette subfamily B member	TSA, AAc	Setiadi et al., 2008 Khan et al., 2008
TAP-2	Transporter 2, ATP binding cassette subfamily B member	TSA, AAc	Setiadi et al., 2008, Khan et al., 2008
LMP-2 (PSMB9)	Proteasome subunit β 9	TSA, AAc	Setiadi et al., 2008, Khan et al., 2008
TAPBP	TAP binding protein (Tapasin)	TSA	Setiadi et al., 2008
LMP-7 (PSMB8)	Proteasome subunit β 8	AAc	Khan et al., 2008
CD86	CTLA-4 counter-receptor B7	SB, AAc	Maeda et al., 2000 Khan et al., 2008
MHC class I	Major histocompatibility complex class I	AAc	Khan et al., 2008
HLA-DR	Major histocompatibility complex, class II, DR α	TSA	Magner et al., 2000

eration of PHF8 with SIN3A and HDAC1 corepressors. PHF8 bound to IFN-γ-responsive promoters before stimulation; after the treatment, PHF8 was phosphorylated and released from the promoter, which was associated with increased H4K20me1 mark and reduced H4K20me3 mark, increased H3K4me3 levels, and transcriptional activation. In PHF8 knock-down cells, a significant increase in the H4K20me1 levels was observed, confirming its role in sustaining the low levels of this mark at some promoters (*IRF1*, *SP100*, *IF16*, *UBE2L6*) before activation (Asensio-Juan et al., 2017). All of these findings illustrate the possible mechanism of prevention of non-specific activation of the IFN signalling pathway while still allowing its fast re-activation when needed.

As has been mentioned above, epigenetic silencing of the MHC class I expression and expression of antigen-presenting machinery genes can be restored by HDACi, which increases histone acetylation in the corresponding regulatory gene regions (Khan and Tomasi, 2008; Manning et al., 2008). In the experiments using murine MHC class I-deficient TC-1/A9 or B16F10 tumour cell lines, the treatments increased susceptibility of these cells to their lysis by specific cytotoxic T lymphocytes. *In vivo* experiments demonstrated that the anti-tumour effects of HDACi Trichostatin A against the TC-1/A9 tumours were dependent on a functional immune system, since no therapeutic effects were observed in immunodeficient (*Rag -/-*) mice CIT (Setiadi et al., 2008). In these experiments, the HDACi partially mimic the effects of IFN-γ on the genes encoding the antigen-presenting machinery. In another experiment, it was documented that HDACi enhanced inhibited IFN-γ-inducible genes, including *IRF1* or *CIITA*, in murine trophoblast cell lines. These results suggest that histone acetylation could modulate the IFN-γ responses and block the processes that could lead to the immune destruction of placenta (Choi et al., 2009).

The complex language of epigenetics – interaction between DNA and histone modifications in the IFN-γ responses

It is known that epigenetic marks are not solo players and that the definitive state of the gene activity is determined by their combination. Several interesting facts about the complex epigenetic regulation of IFN-γ response are already known, including some more detailed description of the kinetics of the entire epigenetic machinery. Using chromatin immunoprecipitation, Morris et al. (2002) mapped the time course of transcription factor binding and epigenetic modifications in the promoter region of *CIITA*, the transcriptional coactivator and major regulator of the HLA class II transcription. They found that in the first five minutes after IFN-γ treatment, STAT1 was bound to the Promoter IV (PIV) sequence. The STAT1 binding was accompanied by acetylation of histones H3 and H4, completed in less than 30 min. However, for initiation of the transcription, yet another factor is needed – IRF1, whose synthesis and binding is completed after no less than 120 min. Another interesting fact emerging from these measurements is that non-responsiveness of trophoblast cells to the IFN-γ induction of *CIITA* expression is associated with hypermethylation of its promoter, which probably prevents the binding of both factors.

A remarkable contribution to the debate on the cooperation of different epigenetic marks in the IFN-γ pathway was brought by the study focused on the regulation of GPR109A, receptor for short-chain fatty acids, which according to this study is silenced in human colon carcinomas and activated by IFN-γ (Bardhan et al., 2015). After binding to its receptor, IFN-γ induces phosphorylation of STAT1, which activates transcription of p300. p300 then binds to the promoter of *GPR109A* and induces H3K18 acetylation without affecting the high level of promoter DNA methylation, showing that the level of DNA methylation may not be the crucial mechanism for the cellular effects of pSTAT1, which surprisingly binds directly to the still methylated but hyperacetylated *GPR109* promoter and restores its transcription.

Complex epigenetic dysregulation may also take place in the IFN-γ-regulated pathways controlling tumour cell death and resistance to chemotherapy. Death-associated protein kinase (DAP kinase) is a positive regulator of programmed cell death that is induced by IFN-γ. Analysis of gastric cell lines, as well as of primary colorectal and gastric tumours, demonstrated that DAP silencing was associated with 5' CpG island methylation (Satoh et al., 2002). Methylation inversely correlated with acetylation of histones H3 and H4 in the 5' region of the gene, and DAC treatment restored the *DAP* gene expression. Downregulation of caspase 8, a key player in TRAIL-induced apoptosis, is frequently downregulated in a number of tumours and may contribute to resistance to cytotoxic therapy. Combination treatment with 5-Aza-dC or HDACi and IFN-γ restored caspase 8 expression and sensitized resistant neuroblastoma and medulloblastoma cells to TRAIL-induced apoptosis (Fulda and Debatin, 2006; Häcker et al., 2009).

The epigenetic modifications are in close connection with the chromatin organization. It is known that a higher DNA methylation level is associated with higher condensation of chromatin or that a higher level of histone acetylation usually corresponds to more open chromatin. However, a study published in 2007 provides an interesting view on the IFN-γ pathway from the point of higher-order chromatin remodelling and its relation to induction of expression of the MHC complex (Christova et al., 2007). According to this observation, JAK/STAT signalling resulting in phosphorylation of STAT1 also moderated looping of the MHC class locus out of the chromosome 6 territory, which seems to be essential for chromatin decondensation, RNA polymerase binding and hyperacetylation. All of that can be prevented by point mutations interfering with the phosphorylation of STAT1 or lack of BRG1, which is a catalytic subunit of the chromatin-remodelling SWI/SNF complex.

Clinical relevance

Epigenetic dysregulations of the IFN-γ pathway and IFN-γ-controlled gene expression in cancer and other diseases are potential therapeutic targets. The therapeutic effects of the epigenetic agents, some of which have been approved for therapy, can be partially based on their capacity to augment or restore anti-tumor immunity (Héninger et al., 2015). It has been suggested and experimentally proved that treatments with epigenetic modifiers possess the potential to reverse some pathological epigenetic modifications, limiting the effectivity of immune response (like the silencing genes of the antigen-presenting machinery, costimulatory genes, or tumour-associated antigens, some of them controlled by IFN-γ in tumour cells (Maeda et al., 2000; Magner et al., 2000; Nie et al., 2001)), and thus sensitize tumour cells to immune responses and immunotherapy, and therefore enhance the therapeutic results (Wang L. X. et al., 2013; Wastowski et al., 2013; Li et al., 2014; Terracina et al., 2016, Barrero, 2017). It has been demonstrated recently, using a murine model for epithelial ovarian cancer, that the epigenetic treatment with 5AC induced type I interferon responses and viral defence genes, which was associated with increased survival (Stone et al., 2017). It is therefore plausible that epigenetic therapy may also either partially mimic or boost the IFN-γ effects as well, and thus increase the immunogenicity of tumour cells and/or their recognition by the immune system, or that this therapy may augment the efficacy of immunotherapy in a synergistic manner. This strategy may be effective especially against tumours with epigenetically down-regulated HLA antigen expression (Campoli and Ferrone, 2008; Reiniš, 2010).

As in the research focused on the general mechanism of epigenetic action, a number of clinically based studies were focused on *IRF* gene expression. The *IRF8* gene has been found epigenetically silenced in nasopharyngeal, oesophageal and other carcinomas (Lee et al., 2008). In renal carcinoma, *IRF8*, suggested to function as a tumour suppressor, is frequently down-regulated by methylation both in the cell lines and primary tumours, which is associated with a poor prognosis (Zhang et al., 2014). IFN-γ alone was able to induce expression in unmethylated or weakly methylated *IRF8* promoters; however, strong methylation successfully abolished IFN-γ-mediated induction of *IRF8* expression. Pharmacological and genetic demethylation could restore *IRF8* expression, indicating a direct epigenetic mechanism. Further experiments using ectopic expression of *IRF8* in tumour cells lacking its expression suggested *IRF8* as a functional tumour suppressor. *IRF8* down-regulation was also demonstrated in breast cancer cell lines and primary tumours, mainly due to promoter methylation (Luo et al., 2017). Again, restoration of *IRF8* expression blocked proliferation of tumour cells, induced apoptosis and cell cycle arrest, and inhibited canonical β-catenin signalling. These findings support the hypothesis of *IRF8* as a tumour suppressor. Another study revealed the role of IRF1 in hyperacetylation of histone H4 through interaction with many different histone acetylases in primary monocytes of patients with systemic lupus erythematosus, perhaps explaining the gene dysregulation found in this autoimmune disease (Leung et al., 2015). It is also known that epigenetics plays a role in IFN-γ and cell-specific induction of extracellular superoxide dismutase in pulmonary arteries, which plays a role in development of pulmonary hypertension and other pulmonary diseases, so that understanding of the underlying mechanism is important for finding novel therapies (Zelko et al., 2011).

It is noteworthy that epigenetic therapy can also induce immune suppression, either directly by activation of immunosuppressive genes or cell lineages, or indirectly as a feedback control of the immune cell activation. This theoretically opens the field to combinations of the epigenetic treatments with checkpoint inhibitors. This approach is supported by the results of a study in which 5-Aza-dC or 5AC treatments could enhance responsiveness of the lung cancer cells to IFN-γ, but at the same time also increased PD-L1 expression, suggesting that combined treatment with 5-Aza-dC and anti-PD-L1 antibody could be effective for the treatment of lung cancer patients with anti-PD-1/L1 therapy resistance (Lai et al., 2018). Similarly, class I HDACi up-regulated PD-L1 in various tumour cell lines including melanomas, providing a rationale that they can be effectively combined with anti-PD-1/L1 inhibition (Wrangle et al., 2013; Woods et al., 2015; Briere et al., 2018).

Conclusions

Activation of the IFN-γ signalling pathway results in changes in the expression of hundreds of genes, which is naturally associated with modifications of the cellular epigenetic landscape. Better understanding of the epigenetic mechanisms can contribute to the explanation of the different outcomes of IFN-γ pathway activation in distinct cell lineages under various circumstances, the role of the IFN-γ pathway and its abrogation in the development of tumour cells and, finally, some of the therapeutic effects of the epigenetic modulators used as anti-tumour agents. Better knowledge of the interference of the epigenetic agents with the IFN-γ pathway in tumour cells can optimize the therapeutic protocols and possible combined therapy with immunotherapy, including checkpoint inhibitors. Further, IFN-γ non-responsiveness represents a frequent mechanism by which tumour cells can escape from the immune responses and limits the efficacy of immunotherapeutic strategies. In some cases, and not only in oncology, this could be changed after taking the epigenetic components into play.

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