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Twenty-five years of type I interferon-based treatment: A critical analysis of its therapeutic use

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ABSTRACT

The clinical exploitation of type I interferon (IFN) as an antiviral and antineoplastic agent is based on the properties originally attributed to this cytokine family, with schedules reflecting only their antiviral and antiproliferative activities. Nevertheless, type I IFN has emerged as a central activator of the innate immunity. As current schedules of treatment for chronic hepatitis C and for hematological and solid tumors, based on the continuous administration of recombinant type I IFN or pegylated formulations, disregard viral resistance, host genetic variants predicting treatment outcome and mechanisms of refractoriness, new administration schedules, the combination of type I IFN with new drugs and the increased monitoring of patients' susceptibility to type I IFN are expected to provide a new life to this valuable cytokine.

1. Introduction

In his book on the history of interferon (IFN), Toine Pieters affirms that IFN can be considered one of modern medicine's most famous and infamous drugs, the history of which has represented an example of therapeutic survival in the face of several cycles of promise and disappointment as a 'miracle drug'. The therapeutic use of IFN has enhanced our understanding of how drug manufacturing and marketing has played a role in pushing the boundaries of research, from the post penicillin era to the genetics revolution in medicine [1]. It is worth noting that, despite the enormous efforts to produce natural and recombinant type I IFN, and the huge amount of research performed on its biology, the mechanisms of IFN action remain in part elusive and its exploitation in the clinic is still based on the knowledge of its biology from an earlier era. The questions also arise as to whether there is still room for a more appropriate use of type I IFN in infectious diseases and in cancer, and whether the volumes of new information about mechanism of action have been properly incorporated into clinical applications. Before specifically addressing the above issues, it is useful to briefly retrace the history of type I IFN as an anticancer and antiviral drug.

Hyped as a potential antiviral drug, the study of IFN from the very beginning attracted wide attention [2]. Because the efforts to molecularly define and purify IFN proteins remained fruitless for about 20 years, many scientists were openly skeptical about properties ascribed to IFN, including its very existence. Nevertheless, a method for production of IFN had been developed by culturing human leukocytes in Gresser's laboratory [3]. In addition, natural IFN was produced and partially purified at the Finland Red Cross by Cantell [4], for use in the first clinical trial in osteosarcoma patients [5], following the observations on type I IFN antitumor effects obtained in experimental models by Gresser et al. [6]. Many attempts were made to demonstrate the activity of natural IFN on other cancer types – those potentially associated with a viral origin – such as juvenile laryngeal papillomatosis, human condylomata acuminata, Hodgkin's disease, acute leukemia in children, multiple myeloma and others. Early results were considered encouraging but the treatment schedules required optimization and combination schemes required formulation for optimal effect [7].

With regard to true viral infections, the recognition that hepatitis B virus (HBV) may cause a chronic infection leading to cirrhosis and hepatocellular carcinoma suggested that virus-infected patients could be efficaciously treated with IFN. All the "IFN enthusiasts" remember the famous sentence "It is a pleasant

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surprise to learn that interferon may have an effect on an established chronic infection caused by a poorly understood but extremely important DNA virus" written in 1976 in New England Journal of Medicine by Ho after the discovery made by Greenberg and his colleagues on the ability of IFN to depress Dane-particle-associated DNA, DNA polymerase and core antigen in chronic active hepatitis [8,9]. Based on these very preliminary "positive" results, IFN-based antiviral and antitumor therapies, quickly became more than a hope among the potential medical armamentarium against tumor and viral infections (Figs. 1 and 2). However, it was only after the successful cloning of Type 1 IFN cDNA (the first cytokine ever cloned) and the identification of the IFN gene family that IFN research joined the mainstream of the scientific enterprise [10–12]. Specifically, Shigekazu Nagata and Sidney Pestka independently identified and expressed IFN alpha 2 in *Escherichia coli*; the protein was then rapidly purified with monoclonal antibodies and used for research and clinical trials [11,13]. Thanks to the expression of the recombinant protein in yeast [14], pharmaceutical companies then produced these two types of recombinant IFN in substantial amounts for clinical use in oncology and infectious diseases. IFN alpha 2a and IFN alpha 2b were subsequently approved for the therapeutic treatment of hairy cell leukemia in 1986 (Fig. 1). IFN beta was also cloned, and produced in sufficient quantities for clinical use [15,16] against tumors and viral diseases. IFN alpha 2a and 2b were registered in 1990 for the treatment of hepatitis C virus (HCV) infection; subsequently, IFN therapy for chronic HBV infection documented two complementary activities of IFN; in HBeAg-positive disease IFN may act as immunomodulatory agent while in HBeAg-negative disease IFN may function as a direct antiviral agent [17].

IFNs have been the standard of care for certain diseases for more than a decade. It is opinion of the authors that during this period, there has not been an adequate effort made to address the actual mechanism of IFN action or to characterize the factors that could influence IFN treatment outcome. Hence our aim is to discuss some of these issues in the belief that they should be carefully addressed to fully appreciate the efficacy of IFNs as therapeutic agents.

2. Past and current use of IFN

The Type I IFN family in humans consists of 13 IFN alpha subtypes, plus IFN beta, IFN epsilon, IFN kappa, and IFN omega [18]. Most of the available IFN preparations used in clinical practice belong to the type I IFN family (Table 1). All type I IFN subtypes elicit antiviral, antiproliferative and immunomodulatory responses by binding to shared cell surface receptors. Interestingly, IFN alpha subtypes have different gene induction profiles and variable antiproliferative, antiviral and immunological properties [19–21]. However, only a few subtypes of type I IFN, namely IFN alpha 2a and 2b, have been used in therapeutic practice, and the remaining IFN subtypes represent an untapped reservoir of opportunity. The following section summarizes the most common applications of IFN alpha in oncology and virology.

2.1. Past and current use of IFN in oncology

After the first attempts to use IFN in osteosarcoma, many other tumors were tested for their sensitivity to IFN treatment, and among them, a number of sensitive tumors were identified (see Table 1). The first responsive malignancy was Hairy cell leukemia (HCL), in which IFN induced the reduction of cytopenia and the elimination of hairy cells from the blood, as well as the reduction of bone marrow fibrosis [22]. Although IFN is no longer considered a first-line therapy in HCL, it is still recommended for cytopenic patients to increase granulocyte levels, in order to mount a stronger anti-infective response to antibiotic or anti-fungal therapy.

Since then, IFN has been used primarily in hematologic malignancies such as chronic myelogenous leukemia, multiple myeloma, non-Hodgkin lymphomas, Kaposi's sarcoma in AIDS patients and mycosis fungoides. In all these malignancies, despite a clear *in vitro* effect on malignant cell proliferation and despite numerous clinical trials, its exact role in the management of disease remained uncertain. Continuous administration (about 3–6 million IU every other day), alone or in combination with other

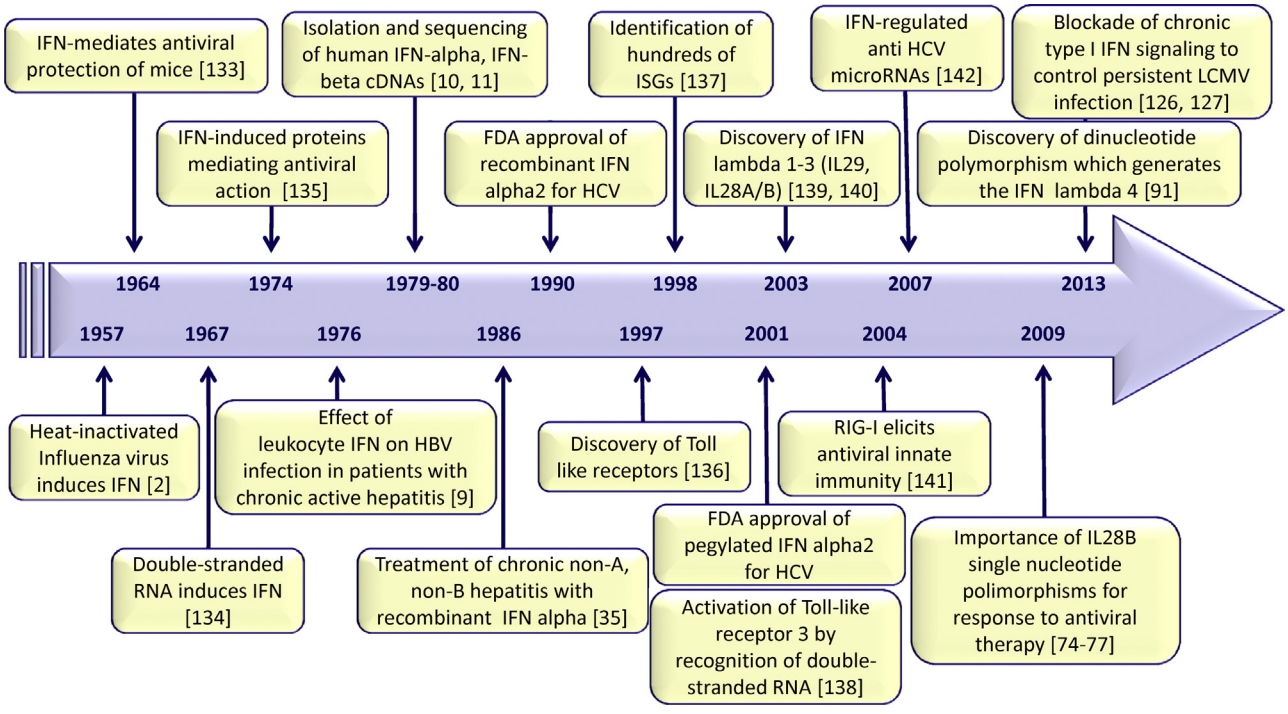

Fig. 1. Timetable of the most relevant findings in the clinical exploitation of type I IFN in infectious diseases.

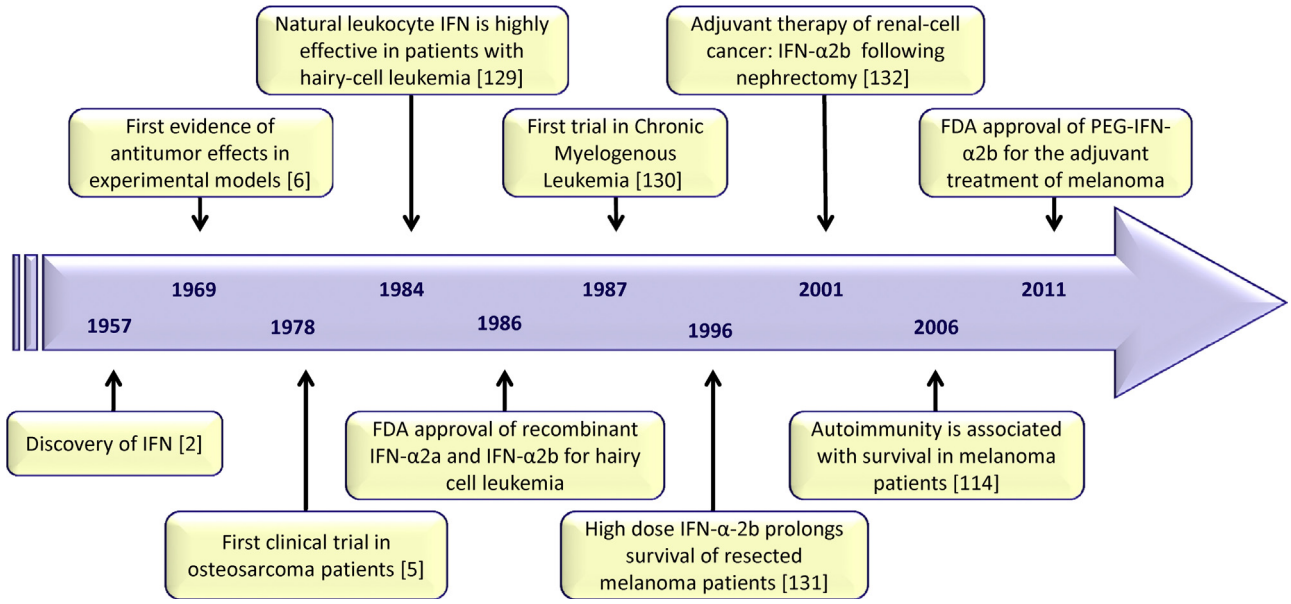

Fig. 2. Timetable of the most relevant findings in the clinical exploitation of type I IFN in oncology.

Table 1
Interferon (IFN) alpha formulations in clinical practice.

Type of IFN	Brand name	Chemical modification	Structure	Company	Source	Recommendation
Lymphoblastoid IFN alpha N1	Wellferon®	Not done	Mixture of natural human IFNs alpha subtypes	Glaxo Wellcome	Lymphoblastoid (Namalva) cells	Hairy cell leukemia, juvenile laryngeal papillomatosis, condylomata acuminata, chronic hepatitis B or C
Natural human leukocyte IFN alpha	Alfaferone®	Not done	Mixture of natural human IFNs alpha subtypes	Alfa Wassermann	Human leukocyte	Hairy cell leukemia, multiple myeloma, non-Hodgkin lymphoma, follicular lymphoma, chronic myelogenous leukemia, malignant melanoma, AIDS-related Kaposi's sarcoma, chronic hepatitis B or C
HuIFN alpha-Le	Multiferon®	Not done	Human leukocyte IFN alpha (IFN alfa-1, 2b, 8, 10, 14, 21)	Swedish Orphan International	Human leukocyte	Malignant melanoma; treatment of patients who initially respond to recombinant IFN-alpha, but for whom treatment subsequently fails, most likely as the result of neutralizing antibodies
IFN alpha 2b	Intron A®	Not done	165 amino acids (19 kDa) Arginine at position 23, deletion at position 44	Schering-Plough	Trasformed *E. coli*	Chronic hepatitis B or C, hairy cell leukemia, follicular lymphoma, condylomata acuminata, AIDS-related Kaposi's sarcoma and malignant melanoma
IFN alpha 2a	Roferon-A®	Not done	165 amino acids (19 kDa) Lysine at position 23, deletion at position 44	Hoffmann – La Roche Inc.	Trasformed *E. coli*	Chronic hepatitis B or C, hairy cell leukemia, chronic myelogenous leukemia
Consensus IFN (IFN alfacon-1)	Infergen®	≈89% homology with IFN alpha and 30% homology with IFN beta	166 amino acids (19.4 kDa)	Kadmon Corporation	Trasformed *E. coli*	Chronic hepatitis C, hairy cell leukemia
Pegylated IFN alpha 2b	PegIntron®	12 kDa linear pegylated molecule	165 amino acids (19 kDa)	Schering-Plough	Trasformed *E. coli*	Chronic hepatitis C
Pegylated IFN alpha 2a	Pegasys®	40 kDa branched pegylated molecule	165 amino acids (19 kDa)	Hoffmann – La Roche Inc.	Trasformed *E. coli*	Chronic hepatitis B, chronic hepatitis C
Pegylated IFN alpha 2a	Reiferon Retard® – Egypt	20 kDa linear pegylated molecule	165 amino acids (19 kDa)	Rhein-Minapharm	Trasformed *Hansenula polymorpha*	Chronic hepatitis C
Pegylated IFN alpha 2b	Sylatron™	31 kDa pegylated molecule	165 amino acids (19 kDa)	Schering Corporation	Trasformed *E. coli*	Melanoma after surgery

chemotherapeutic drugs, was considered the best treatment available in the induction as well as in the maintenance phase of treatment. In multiple myeloma, for example, among the patients who had an objective response to induction chemotherapy, those treated with IFN had a significantly longer duration of survival [23]. In contrast, with indolent non-Hodgkin lymphoma and follicular lymphoma, IFN treatment was not associated with an overall survival benefit, although it might prolong progression-free survival. Currently, the use of IFN competes with "new drugs": thalidomide, lenalidomide and bortezomib are now successfully used in myeloma treatment; imatinib and tyrosine kinase inhibitors are the mainstay of treatment in chronic myelogenous leukemia, and IFN is rarely used in these malignancies. Despite evidence that addition of IFN to current protocols (CEOP-Bleo, CHOP, CVP, CHVP, MOPP) as maintenance therapy for follicular lymphoma improved progression-free survival, a net benefit for overall survival was less evident, and IFN was associated with significant toxicities that may have a major impact on the patient's quality of life [24]. In mantle cell lymphoma, rituximab in combination with CHOP or bendamustine have been carefully evaluated in clinical trials, while bortezomib (NFkB inhibitor) or lenalidomide are the approved agents [25]. Similarly, Kaposi's sarcoma and mycosis fungoides patients now have different treatment options in addition to IFN, but the relatively low number of cases actually reduces the quality of the analyses necessary to properly define a superior treatment regimen relative to other treatments [26,27].

Renal cell carcinoma (RCC) and melanoma are two solid tumors that demonstrate some success with IFN therapy, although the armamentarium for the systemic therapy of these two tumors has undergone dramatic changes in recent years. In particular, cytokine-based therapy including IFN for RCC has been replaced by vascular-endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) inhibitors. For low and intermediate risk disease, pazopanib, sunitinib or the combination of bevacizumab plus IFN are considered, whereas combinations of targeted agents (*e.g.* sunitinib combined with IFN) have generally been plagued by high grade toxicity. Therapy for malignant melanoma has been object of intense research to assess the efficacy of immunotherapeutic strategies; while no treatments have yet shown superior efficacy to IFN in the adjuvant phase, the efficacy of IFN treatment in this setting at low or high doses is still under debate. A number of trials are ongoing to assess whether IFN, associated with different vaccination strategies, reduces the risk of recurrence in resected metastatic melanoma patients [28,29].

In conclusion, despite the multiple effects of IFN in a variety of malignancies that range from antiangiogenic [30] to potent immunomodulatory [31], differentiation-inducing [32], antiproliferative and proapoptotic [33], IFN is still administered following the schedule used in the first clinical trials, and its use has been superseded by new, more effective and less toxic drugs.

2.2. Past and current use of IFN as antiviral agent

There are currently several approved IFN formulations used to treat chronic viral infections (see Table 1 for the main indications). In addition to hepatitis B virus (HBV) and HCV infections, other chronic viral infections (such as herpes zoster, herpes simplex, cytomegalovirus, HIV, papillomavirus infections) have been effectively treated with both IFN alpha and IFN beta [34]. In parallel, prophylaxis and treatment of acute respiratory viral infections (such rhinovirus, influenza virus or coronavirus) have been evaluated; the introduction of effective antiviral compounds such as acyclovir and its analogs for herpesviruses or azidothymidine and protease inhibitors for HIV with greater therapeutic effect (and reduced side effects), rapidly reduced the commercial interest for IFNs as antivirals.

Evaluation of IFN treatment against all viral infections is an impossible task, thus our discussion is focused only on type I IFN in the treatment of HCV infection. Indeed, the use of IFNs as an anti-HCV agent is an excellent example of "a mix of partial successes and pending challenges", and reflects the difficulties encountered in the therapeutic use of such a powerful biological weapon. The initial choice of IFN alpha as a potential treatment for chronic hepatitis C (CHC) in 1986 by Hoofnagle was rather empirical [35]. Since then, natural IFN or recombinant IFN alpha formulations were the only drugs available for the treatment of patients suffering from CHC but were associated to limited rates of sustained virological response (SVR), in the range of 12–16%. The addition of ribavirin (RBV) to recombinant IFN alpha increased the SVR rate to about 50%. In 2001, PEG-IFN alpha formulations, specifically PEG-IFN alpha 2a and PEG-IFN alpha 2b, in which inert polyethylene glycol was attached to conventional IFN molecules, were introduced into clinical practice, on the basis of a longer half-life (Fig. 2). The improvement in pharmacokinetic properties of standard IFN alpha preparations, led to a 40–50 SVR in patients infected with HCV genotype (GT) 1. It was therefore not surprising that, after the entry into the market of pegylated forms of IFN alpha (PEG-IFN), a number of companies developed modified IFN or novel IFN delivery systems in the hope of achieving improved pharmacokinetic and pharmacodynamic properties, more potent immunomodulatory effects, and better tolerability. The list of new IFN alpha preparations currently available is provided in Table 2.

More recently, the availability of several directly acting antiviral agents (DAAs) for HCV, mainly inhibitors of NS3, NS5A and NS5B viral proteins, has offered the possibility of IFN-free, anti-HCV treatment. Although moving to DAAs therapies will greatly increase SVR rate and offer new treatments for ineligible or non-responding HCV positive patients, the actual price of DAAs is exceedingly high and these new drugs are associated with development of drug resistance, thus calling into question the choice to abandon the "old and consolidated" therapeutic road. At the same time, new antiviral agents that boost host innate immunity or target specific cellular pathways (host-targeting antivirals (HTAs)), are also emerging as alternative anti-viral therapies [36]. Among them are Toll-like receptor (TLR)7, TLR9 (ANA773, GS-9620 and IMO-2125) and retinoic acid-inducible gene I (RIG-I) (*e.g.* 5′pppRNA) agonists and antimicroRNA122 (Miravirsen). All these strategies are linked with the IFN system, again emphasizing the critical role of IFN in orchestrating the innate immune response and the necessity to unravel the pleiotropic biological actions of IFN [36–38].

Type I IFN preparations remain a valid therapeutic option, due to their broad-spectrum of antiviral properties and their ready availability for the treatment of emerging zoonotic viral diseases, including those in which the time to vaccine availability precludes vaccination at the onset of an outbreak. Since the 2002–2003 severe acute respiratory syndrome coronavirus (SARS-COV) outbreak, several other examples illustrate the ability of IFN alpha/beta to inhibit the replication of new emerging viruses [*e.g.* Middle East Respiratory Syndrome coronavirus (MERS-COV), avian and pandemic H1N1influenza viruses [19,39–44]].

3. Factors affecting the response to type I IFN

The intense clinical research on the therapeutic use of type I IFN has not been paralleled by similar efforts in defining the determinants that confer cell sensitivity or refractoriness to it. More specifically, efforts to characterize the factors affecting IFN therapy outcome have not been comparable in oncology and virology, in large part because viral therapy is more suited to the

Table 2
New interferon alpha formulations in clinical practice.

Type of IFN	Brand name	Chemical modification	Structure	Company	Source	Recommendation
Albinterferon alpha 2b (Albuferon)	JOULFERON® Zalbin™	r-Human albumin modified IFN alpha 2b	165 amino acids (19 kDa)	Human Genome Sciences Inc./ Novartis International AG	Transformed *Kluyveromyces*	Chronic hepatitis C
IFN alpha 2b	Locteron®	PolyActive® technology[a]-based controlled-release recombinant formulation	165 amino acids (19 kDa)	Biolex Therapeutics	Trasformed *E. coli*	Chronic hepatitis C
IFN alpha 2b	IFN alfa-2b XL	Medusa® technology[b]-based recombinant formulation	165 amino acids (19.4 kDa)	Flamel Technologies	Trasformed *E. coli*	Chronic hepatitis C
IFN alpha	Belerofon®	Single aa mutation that lowers sensitivity to protease-mediated degradation	N.A.[c]	Nautilus Biotech	N.A.[c]	Chronic hepatitis C
IFN alpha	Novaferon	Artificial design technology combining DNA-shuffling and High throughput screening	82% sequence identity to IFN alpha 2b (19 kDa)	N.A.	N.A.	N.A

[a] Biodegradable polymeric drug delivery system.
[b] Nanoparticles delivery system.
[c] Not available.

complex relationships between host, virus and IFN response. Likewise, knowledge acquired in the virological field has not been fully exploited to explain the failure of IFN treatment in cancer patients.

3.1. General mechanisms of refractoriness to IFN

IFN activity is mediated by the binding to its receptor and the activation of the JAK–STAT signaling pathway, resulting in the induction of the expression of hundreds of IFN-stimulated genes (ISGs), as reviewed in [45]. All type I IFNs signal through a common heterodimeric receptor composed of a low- (IFNAR1) and a high-affinity (IFNAR2) subunit and, through an as yet non-completely elucidated mechanism, drive different biological signals [46]. When administered at high doses, IFNs are not devoid of toxic effects, indicating that their action must be fine-tuned through opposing enhancing and suppressive signals. Upon ligand engagement, the signaling complex is rapidly internalized by endocytosis and, subsequently ubiquitinated and degraded. Marijanovic et al. showed that while the surface half-life of IFNAR1 is 4 h in unstimulated cells, its half life is reduced to 1 h in cells stimulated with IFN alpha [47]. The non-responsive state of IFN-treated cells was shown to last up to 3 days [48], whereas after IFN removal, expression of IFNAR1 at the cell surface returned to nearly 100% control levels within 3 h [47]. The consequence of these observations is that continuous administration of high dose IFN or the pegylated formulations can determine an unwanted decrease of receptor expression at the cell surface, and represents one of the earliest activated mechanisms of refractoriness to IFN. Given that all effects of type I IFN on infected, malignant and immune cells are mediated by its receptor, modulation of IFNAR levels may be expected to play a dominant role in shaping the extent and the duration of the type I IFN response. Altogether, these observations argue that rational design of IFN-based therapies should carefully consider these aspects of IFN receptor biology, indicating that new strategies to stabilize IFNAR1 and inhibit its degradation are needed. Similarly, evaluation of the basal expression of the IFNAR complex and of its down-regulation following treatment should be a component of standard care in IFN-treated patients.

Mice repeatedly injected with murine IFN alpha were shown to become refractory to further stimulation within a few hours after the first injection [49]. Suppressor of cytokine signaling (SOCS) proteins, in particular SOCS1 and SOCS3, was shown to be important for the negative regulation of type I and type II IFN signaling, through the inhibition of STAT1 binding to IFNAR and suppression of JAK activity [50] respectively. Similarly, ubiquitin carboxy-terminal hydrolase 18 (USP18)/Ubiquitin protease 43 (UBP43) was shown to be required for induction of a long-lasting desensitized state [49]. Binding of UBP43 to IFNAR2 *in vivo* displaced JAK1 from IFNAR2 and led to the inhibition of the downstream phosphorylation cascade and other signaling events [51]. Of note, administration of PEG-IFN alpha 2b in patients with CHC was shown to activate the JAK/STAT pathway only during the first day following injection, and then rapidly induced SOCS1, SOCS3 and UBP43 expression, despite the fact that the serum concentrations of PEG-IFN alpha 2 remained high for the entire week. These observations argued that the superior antiviral efficacy of PEG-IFN alpha was not related to constitutive activation of IFN signaling pathways, but rather the induction of immune response-associated genes by PEG-IFN was the mechanism underlying increased efficacy [52].

Since the deficiency of either SOCS1 or UBP43 can amplify IFN antiviral and proinflammatory actions, both activities may represent promising therapeutic targets to improve the benefits of IFN treatment in cancer as well as in viral diseases. In this regard, UBP43 was identified as a poor prognostic marker of IFN alpha therapy in patients with chronic hepatitis C [53], and its *in vitro* silencing in human cells potentiated the antiviral activity of IFN [54]. Similarly, UBP43 levels were increased in several human tumors, including non-small cell lung cancer, and its silencing in mouse lung cancer cells rendered the cells less tumorigenic and more sensible to the growth-inhibitory and pro-apoptotic effects of IFN *in vitro* [55].

Several recent papers suggest a major role for different microRNAs (miR) in regulating the IFN response. In this regard, miR-155 broadly suppresses the expression of IFNAR–JAK–STAT pathway components in CD8+ T cells [56], while miR-221 suppresses SOCS1/SOCS3 functions, thus acting as enhancer of the JAK–STAT signaling [57]. Importantly, patients with hepatocellular carcinoma with reduced miR-26 expression in tumors displayed a significant improvement in overall survival after receiving adjuvant therapy with IFN alpha, indicating that miR-26 expression status can indeed help to stratify patients with respect to response to IFN therapy [58].

3.2. Resistance and variability to IFN alpha treatment in CHC patients

Since the beginning of IFN in therapy for CHC, it was evident that HCV infected subjects responded differently to IFN administration. Such a variability in responsiveness was totally unexpected on the basis of results obtained by *in vitro* experimentations, and this variability has stimulated intense

efforts aimed at understanding the underlying mechanisms of resistance to IFN treatment, as well as characterizing predictive biomarkers that could be useful in identifying patients who were likely to respond to IFN treatment. It is important to emphasize that although many determinants of the IFN response have been identified, much work is still required to fully appreciate the potential of type I IFN action. Two variables that modulate the IFN response – viral and host determinants – will be discussed separately below.

3.2.1. Viral determinants

The highly variable response rate observed in HCV patients treated with IFN alpha depends on well-established viral factors such as viral load; patients with high pretreatment viral loads have poor long-term outcomes compared to patients with low viral loads, and an increased risk of treatment failure is observed against the most predominant HCV GT, GT1 (subtypes 1a and 1b). The HCV decay kinetics is also an important factor in estimating the effectiveness of antiviral therapy [59].

To fully appreciate the variability of the IFN response in HCV patients, it is also important to consider the polymorphism of the HCV genome. For instance, in 1995 Enomoto et al., found that a HCV GT1b virus with four or more amino acid substitutions in the NS5A 2209–2248 region, termed IFN-sensitivity-determining region (ISDR), prior to therapy was strongly correlated with the SVR to IFN alpha therapy in Japanese patients, and the number of amino acid substitutions was thought to be an independent predictor of IFN treatment success [60,61]. These initial findings were further confirmed by other studies in Japanese patients [62,63]. However, several reports from Europe and United States failed to show a correlation between ISDR mutations and IFN responsiveness [64]. Later it was established that the ISDR was necessary but not sufficient for the interaction between NS5A and the IFN induced PKR enzyme; an additional 26 amino acids, called PKR binding domain (PKRBD), distal to the ISDR was also required; the latter region was shown to hinder PKR dimerization and resulted in the repression of PKR function on the eIF-2alpha translation factor [64]. Indeed, mutations within the PKRBD of HCV GT1 have been associated with a long-term sustained response to IFN alpha and PEG-IFN alpha/RBV therapy [65–67].

Other regions of HCV, especially E2 (PKR/eIF-2alpha phosphorylation homology domain (PePHD)) or the NS5A region (V3 in the C-terminal portion) have been shown to contain specific sequences in different HCV genotypes that differentially affect the IFN response [64,68–70]. Interestingly most of these HCV proteins can subvert the type I IFN response *in vitro* [18] but whether these HCV evasion strategies are active *in vivo* and influence the IFN treatment response are unclear. All the above considerations, together with the indication of a close association between HCV genetic variability and antiviral treatment outcome lead to an important general question – "Is the infection of specific HCV GTs and/or mutations in specific regions of the genome able to affect the endogenous production/action of IFNs and expression of ISGs, or does such a high variability simply reflect an authentic adaptation of HCV to the immune response including type I IFN expression and function"? Some attempts to answer these questions have been made but the issue remains largely unresolved [71–73].

3.2.2. Host determinants

Since the initial use of IFN for CHC therapy, it has been understood that different pre-treatment host factors (such as age, sex, body weight, insulin resistance, and liver fibrosis) significantly affect the virological response to IFN or PEG-IFN alpha-ribavirin therapy. The variability of IFN response however could not entirely be explained by the above-mentioned factors; recently, two single nucleotide polymorphisms (SNPs) rs12979860 (T/C) and rs8099917 (T/G), in the region of interleukin (IL) 28B/IFN lambda 3 on chromosome 19, have been definitively associated with spontaneous and IFN treatment-induced viral clearance in HCV infection [74–77].

Together with the discovery of the above SNPs, it became clear that the concerted action of multiple ISGs must also be considered to fully understand the limits of IFN alpha treatment. Administration of IFN induces an up-regulation of ISGs in PBMC and the liver of HCV-infected individuals; the type, and especially the magnitude, of the IFN induced response differed between responder and non-responder HCV positive individuals [53,78–80]. HCV infected patients who were less likely to respond to the IFN alpha therapy exhibited paradoxically a higher constitutive ISG expression compared to those that achieved a good response [53,78,80–83]. Notably, the presence of high basal levels of ISGs was associated with a lack of increased expression after IFN administration [83,84]. The molecular mechanism linking baseline ISG induction to IFN non-response remains currently unknown.

It has been proposed that the refractoriness to exogenous IFN could reflect the presence of high pretreatment levels of the UBP43 negative regulator and/or reflect that IFN transduction pathways are saturated [49,80,85]. The up-regulation of ISGs in liver-derived biopsies of chronically HCV infected patients is largely sustained by hepatocytes [86], although Kupffer cells can be a local source of IFN that promoted basal expression of ISG in hepatocytes of non-responders [87]. In addition, there is no significant correlation between serum or intrahepatic viral loads with ISG expression levels, suggesting that this response is ineffective in term of HCV replication control [80]. It is also not clear whether type I or III IFN is the driver of basal ISGs induction. In this regard, type I and III IFN differ in their kinetics of production and their level of ISG induction with a clearly detectable hierarchy [88,89]; these pathways are severely impaired in liver-derived tissues of chronically HCV infected patients [86]. The lack of a specific signature of type I and III IFN in the liver of HCV positive subjects, characterized by the presence of high endogenous levels of ISGs, could be indicative of a severe impairment of early host defense pathways. In addition we speculate that pretreatment ISG response may reflect type I and III IFN produced in response of HCV replication, as well as an IFN-independent mechanism triggered by HCV; alternatively other pathways, for instance mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/ERK) and the phosphoinositide-3 kinase (PI-3K), could be involved in the process. Another possibility is that the basal ISGs signature is induced by IFN beta rather than by IFN alpha subtypes, or reflects a deficient IFN gamma signal as observed in acute *vs.* chronic HCV infection [85]. In this regard, a unique IFN beta signaling axis mediated *via* the receptor IFNAR1 has been recently identified [90].

Several studies have demonstrated that the IL28B/IFN lambda 3 poor-response minor alleles (both the above rs120979860 and rs8099917) are associated with high basal levels of ISGs, providing compelling evidence for the involvement of a genetic predisposition [80]. It should also be mentioned that the very recent discovery of a dinucleotide polymorphism ss469415590 TT/ΔG upstream of IL28B/IFN lambda 3, which generates the novel IFN lambda 4 (variant allele, ΔG) protein, may reveal an alternative scenario to understand the functional architecture of type III IFN genomic regions and its influence on the outcome of HCV infection, and also to identify the relationship between endogenous ISG up-regulation and poor response to IFN alpha treatment [91]. As the IFN lambda 4 creating allele ΔG is correlated with the unfavorable rs12979860 allele T, ss469415590 TT was a better predictor of HCV clearance than rs12979860, and a role of ss469415590 TT in predicting response to anti-HCV therapy with or without IFN has been reported (for a review see [92]). Interestingly, carriers of the

IFN lambda 4 creating ΔG allele were found to have significantly higher amounts of ISG mRNA than patients homozygous for the disruptive TT allele [93]. Moreover, by relating actual IFN lambda 4 transcription in carriers of the ΔG allele to ISG induction, HCV infected patients with measurable quantities of IFN lambda 4 mRNAs presented significantly stronger ISG induction than those without [93]. This observation may provide a possible explanation as to why HCV positive patients show ISG stimulation in their livers in the apparent absence of an induction of other IFN subtypes [93].

As HCV therapy is evolving rapidly from IFN-based to DAA-only regimens, the relevance of the endogenous IFN system for IFN-free therapy treatment outcome could be questioned. However, recently it has been demonstrated that HCV clearance achieved during IFN-free treatment with a DDAs regimen is accompanied by hepatic down-regulation of type II and III IFN, their receptors, and ISGs [94]. Furthermore, the ability to restore intrahepatic type I IFN signaling is associated with prolonged HCV suppression [94]. Altogether, the above findings together with the observation that RBV has been shown *in vitro* to up-regulate a narrow spectrum of ISGs [95,96], highlight the importance of evaluating whether variability in the expression of components of the IFN system may affect the clinical outcome of IFN-free regimen in HCV patients.

3.3. Immunomodulatory properties of type I IFN

Although the immunoregulatory properties of type I IFN have been appreciated since the 1970s [97,98], IFNs are still administered with schedules and modalities that reflect their antiviral and antiproliferative activities. Early studies in mouse tumor models demonstrated that the generation of a long-lasting antitumor response to type I IFN depended on host immune mechanisms [99,100], in part reflecting the identification of type I IFN as a regulator of class I histocompatibility antigen expression [101]. IFNs are indeed important regulators of several components of the host immunity, with activities ranging from the stimulation of lymphocyte- and monocyte-mediated cytotoxicity [102], to the activation of macrophages, natural killer (NK) [103], CD8+ memory T cells [104] and dendritic cells (DC) [105,106]. IFN alpha/beta, in fact, induces DC maturation, up-regulates their co-stimulatory activity and enhances their capacity to kill tumor cells and present or cross-present antigens [107]. IFN alpha also plays a key role in the polarization of T-helper cells toward a Th-1 subtype [108], in enhancing the primary antibody response to soluble antigens [109] and in deactivating the suppressive function of mice [110] and human regulatory T cells (Treg) [111], thus enhancing T helper cell functions and NK cell tumor cytotoxicity.

All the immunomodulatory activities of type I IFN and, in particular, the effects on the innate immune response and on immunological memory can account for its efficacy as a vaccine adjuvant, as demonstrated in an influenza infection model in mice [112], as well as in melanoma patients [28]. Recently, IFN was shown to have a pivotal role in rendering immunogenic the cell death induced by chemotherapies, thus supporting the participation of the immune system in their cytocidal activity [113]. The observed correlation between a favorable response to systemic administration of IFN alpha and the appearance of autoimmunity in metastatic melanoma patients, strongly corroborate the hypothesis that the induction of a host immunostimulatory mechanism is a critical factor predicting the antitumor efficacy of IFN [114]. Nevertheless, in the large majority of clinical studies, the effect of IFN administration on immune cells is not monitored and therefore an important part of its mechanism of action is disregarded, not allowing a mechanistic-based improvement of the therapeutic efficacy.

4. Conclusions and recommendations

IFNs and their receptors represent early elements in innate and adaptive immunity evolution and their physiological activity consists of stimulating immune defenses against the invasion of foreign pathological elements by a potent, but transient, action which is quickly extinguished by self-regulatory mechanisms. After the discovery of IFN, early studies focused only on two of its multiple properties, the antiviral and anti-proliferative activities. The identification of additional members of the IFN family, characterization of the complex properties of distinct IFN subtypes, and increasing knowledge of the regulation of the IFN response have all progressed rapidly in the realm of pre-clinical research, but have yet to be incorporated into the use of IFN in treatments for infectious disease or cancer. Clinically, IFN has been used at high doses, with the intent to maintain high concentrations in the body, through frequent injections or sustained release. The overall therapeutic results of such a strategy have been beneficial in some infectious or neoplastic diseases, but in other diseases, especially when used as a single agent, the value of IFN therapy has been limited, particularly in light of systemic toxicity. For example, high doses of IFN are effective in drastically reducing the viremia in HCV-infected patients after the first treatment cycle, but subsequently, virus loads rise and ribavirin is required for therapeutic efficacy. Furthermore, the beneficial effects of continuous exogenous IFN (*i.e.* after PEG-IFN) are hampered by the induction of different mechanisms of refractoriness – receptor internalization/degradation, rapid induction of UBP43 and SOCS negative regulators – thus limiting PEG-IFN activity, despite its persistence in serum [51]. Nevertheless, PEG-IFN alpha, with its favorable pharmacokinetics and pharmacodynamics, improves the outcome in CHC patients, although it is not clear that its activity is related to the acute induction of ISGs. Indeed, since its approval in the early 1990s for CHC, IFN alpha and PEG-IFN alpha has been used in HCV patients regardless of whether a pre-existing type I IFN signature could make the patient intrinsically resistant to IFN therapy, and may reflect an activated IFN transduction pathway, as in the case of chronic viral infection or continuous IFN administration [80,115,116]. The relationships between type I IFN production, ISG expression, host genetic determinants, virus variability and clearance of HCV are not understood for IFN alpha-based therapy; likewise the above properties of the IFN response may also be important in the new era of DAAs against HCV, since some evidence indicates that the introduction of DAAs into the IFN regimen may affect innate immune activation [94,117]. It is thus questionable whether IFN alpha and/or DAAs can be used effectively without first assessing endogenous type I IFN activation. Indeed, IFN has known anti-fibrotic action and there is no proof that patients with advanced stage liver disease who clear viral infection with DAAs completely arrest liver disease progression [118].

In addition to the anti-angiogenic effects of high dose IFN, which lead to the reduction of tumor vasculature, most of the antitumor therapeutic effects of IFN are associated with immune modulation. A brief but intense production of type I IFN by plasmacytoid dendritic cells stimulates innate immunity [119] and, similarly induces the proliferation of memory CD4+ and CD8+ T cells [104] and activates NK cells [120]. In the physiology of the immune response, type I IFN plays a crucial but brief role, whereas continuous IFN production leads to pathological consequences as demonstrated in systemic lupus erythematosus [121]. These considerations should lead to a reconsideration of the scheduling of IFN treatment in favor of administration given at progressively delayed time intervals, to avoid the refractory mechanisms induced by IFN. Furthermore, IFN therapy should be directed to its target (vascular endothelium, monocytes/dendritic cells, T lymphocytes, tumor cells *etc.*), through targeted delivery strategies, which require lower doses and therefore produce less

systemic side effects [122]. Also the pro-apoptotic and immuno-adjuvant activities of IFN should be combined with chemo- or radio-therapeutic treatment to increase the immunogenicity of dying tumor cells [113] and their up-take by dendritic cells [107]. Monitoring of immune cell activation should be considered in IFN treated patients for a mechanistic-based improvement of the therapeutic efficacy.

Based on the observations that genetic variability (*i.e.* SNPs in the IL28B/IFN lambda 3 and IFN lambda 4 on chromosome 19) rendered CHC patients prone to virus clearance after IFN treatment, IFN treatment should be personalized to consider genetic variations that may impact treatment of viral infections or cancer. Similarly, monitoring the known mechanisms of "resistance or escape" used by viruses and tumors, or developing drugs that reduce resistance, could improve IFN treatment effectiveness.

The observations that type I IFN response can be deleterious for the host in secondary bacterial or fungal infections, several autoimmune diseases, and certain chronic viral infections, should prompt us to reflect whether IFN alpha has been optimally used in clinical practice [123]. For instance the detrimental effects of type I IFN action are clearly demonstrable during lymphocytic choriomeningitis virus (LCMV), pathogenic simian immunodeficiency virus (SIV) and HIV infections where a direct causal link between type I IFN/ISGs expression and chronic immune activation and dysfunction has been reported [124–127]. It is not surprising, then, that the possibility of therapeutically targeting either type I IFN or its production is now emerging as a new therapeutic strategy against viral diseases. However, recently it was demonstrated that blockade of the type I IFN receptor using type I IFN receptor antagonist after SIV infection of rhesus macaques diminished antiviral gene expression, increased SIV reservoir size and accelerated CD4+ T-cell depletion with progression to AIDS, despite decreased T-cell activation [128]. Thus, an elevated type I IFN signature can be deleterious in some chronic viral infections, further emphasizing the difficulties associated with in the *in vivo* manipulation of this complex biological system. All these observations demonstrate that the consequences of manipulation of IFN signaling are difficult to predict *in vivo*, and therapeutic intervention in patients should be conducted with caution, with careful consideration of the physiology of the IFN action.

Conflict of interest statement

None.

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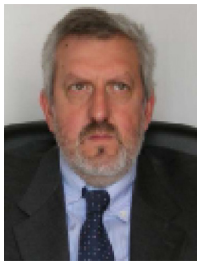

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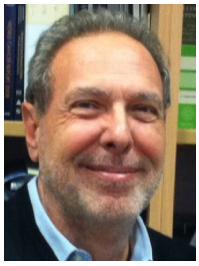

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