Pegylated-interferon–alpha treatment modulating the immune response of cytotoxic lymphocytes in cervical intraepithelial neoplasia

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Background: Interferons are inducible secretory glycoproteins with immunomodulators, antiviral, antiangiogenic and antiproliferative effects. Evaluate the mechanisms responsible by regression of patients diagnosed with Cervical Intraepithelial Neoplasia (CIN) and treated with IFN-α, systemically and locally, by Interferon-α (IFN-α) receptor 1 (IFNR1) and IFN-α receptor 2 (IFNR2) and transcription factors STAT-1 (*Signal Transducers and Activators of Transcription* 1) and IRF-7 (*Interferon Regulatory Factor 7*), as well as the endogenous produced IFN-α by total (CD3+), Helper (CD4+), cytotoxic (CD8+) T lymphocytes and monocytes (CD14+). *Methods*: A prospective study was developed in which eighteen patients diagnosed with CIN II/III in treatment protocol with Peginterferon-α. Cells were evaluated using Real-Time and flow cytometry, and the data were analyzed using Kruskal-Wallis and ANOVA tests, considering $p \leq 0.05$. *Results*: Eight patients obtained regression of the lesion, and ten did not obtain the regression. Patients who did respond positively to the treatment presented a CD8+ T lymphocyte with IFN-α increase when compared to patients who not responded positively. When analyzing CD8+ T lymphocytes during the stages of treatment in lesion regression, it is observed a significant IFNR1 ($p = 0.0391$) decrease in patients who did not achieve lesion regression. CD3 and CD14 data was not significant. *Discussion*: Immunomodulation by Interferon-alpha seems to depend on the systemic expression of IFN receptors. Our data suggest that patients who can respond to immunotherapy already have a pattern of IFN receptor expression in lymphocytes, which contributes to successful treatment.

Keywords

Immunotherapy; Interferon-α; Cervical intraepithelial neoplasia

1. Introduction

The Human Papilloma Virus (HPV) infection is the leading cause of cervical cancer and a relevant factor in the development of anogenital cancer (anus, vulva, vagina, and penis) as well as head and neck cancer. HPV 16 and HPV 18 are responsible for about 70% of all cervical cancer cases worldwide [1]. The proteins E6 and E7 from HPV are able to interfere with several mechanisms in the infected keratinocytes, including the synthesis of Interferon-alpha cytokine (IFN-α) and the inhibition of their activation pathway proteins, like STAT-1, IRF-7, proapoptotic genes, and pathogen recognition receptors [2].

The expression of STAT-1 has an essential role in viral pathogenesis, been necessary for genome amplification and maintenance of episomes, however the HPV infection, between the oncoproteins E6 and E7 suppressed this transcription factor at transcriptional level [3].

Type I interferons (IFN-α/β) induce the expression of several IFN-stimulated genes (ISGs), perform cell growth movements, anti-virus and immunomodulatory effects. An interaction of IFN-α with its receptor triggers a series of phosphorylation events, such as an activation of the receptor associated with Janus Kinase (JAK), associated with IFNR2 and Tyrosine Kinase (TYK), associated with IFNR1, which promotes a transduction of signal and activation of STAT protein transcription [4]. These pathways are signal through IRF-3 and 7, being execute by the beginning of a cascade of signals that result in the transcription of IFN type I [5].

A study realized by Li *et al.* [6] concluded that DNA binding ability and ISGF-3 (*Interferon-Stimulated Gene Factor*) transactivation are decreased in cells expressing HPV-18 E6 protein after IFN treatment, resulting in decreased phosphorylation of TYK2, STAT2, and STAT-1. This fact was explain through HPV-18 E6 proteins physical interaction with TYK-2 binding to the cytoplasmic portion of IFNAR1, thereby inhibiting the pathway to IFN activation [7].

The most common treatments of CIN are surgical, such as conization, LEEP (loop electrical excision procedure), and hysterectomy. However, the patients are of reproductive age and these surgical treatments imply difficulty in getting pregnant and maintaining the pregnancy to term, a fact before which researchers around the globe have been dedicating their efforts in order to guide and stimulate the consoli-

dation of healthy polices and secondary strategies that collaborate with prevention, diagnosis and early treatment of cervical cancer.

It is in this scenario that the potential of immunotherapy as a treatment option has been progressively acknowledge, either through its application coextending surgical procedure or in isolation. Immunotherapy currently includes the application of vaccines, recombinant viral proteins, monoclonal antibodies, cytokines, and dendritic cells [8]. In this context, the use of IFN-alpha becomes relevant by their potential to act as antiviral, immunomodulatory, antiangiogenic, and antiproliferative in several cell types. And consequently, assuming ample potential due to their antitumor effect, which has been handled in several studies on the subject. A study demonstrated that patients with cervical intraepithelial neoplasia showed a systemic increase in the number of T lymphocytes that express IFNR1 and IFNR2 [9].

The purpose of this study is elucidated the mechanisms of tumor regression involved *in vivo* immunotherapy with IFN-α in patients with CIN II and III, evaluating the interferon-alpha receptors (IFNR1 and IFNR2) and transcription factors (STAT-1 and IRF-7) intralesional and in peripheric blood. Moreover, this study seeks collaboration with the world's science in the inquiry for new methods and protocols of treating tumor lesions, especially concerning the development of more efficient treatment with IFN-α in patients with CIN.

2. Materials and methods

2.1 Patients

A prospective study was conducted at the Maria da Glória outpatient clinic, in the Gynecology and Obstetrics Discipline of the Hospital School of the Federal University of the Triângulo Mineiro. Eighteen Patients with CIN II–III with 18 to 82 years of age, were included in the study. Were adopted as inclusion criteria for the patients in the study without any previous treatment, absence of bleeding during the examination; no sexual activity for two days preceding sample collection; no use of oral antibiotics, vaginal fungicides or creams over the previous 30 days; previous history of treatment for HPV; and no colposcopic change <1 cm. The exclusion criteria were: immunosuppressive diseases, serious cardiopathies, changes in liver or kidney function, pregnancy, a reported intolerance to IFN, or an absence of a visible lesion at colposcopy.

The clinical evaluation of the patients consisted of colposcopic examination and histological analysis. Therefore, colposcopy showed the disappearance or regression of the lesion, and it was confirmed by histological analysis from biopsy, with regression to CIN I or no CIN, the treatment was considered as a right, characterizing the responsive group. The patients were submitted to follow-up with cytology and colposcopy every 6 months. If no regression of the lesion was observed at the colposcopic examination, confirming the persistence of CIN II or III in biopsies, failure of the treatment was considered, characterizing the without response group.

All patients with CIN II and III were immediately submitted to cold knife conization (Table 1).

2.2 Application of pegylated IFN-α

Human recombinant pegylated IFN-α 2b (Pegintron®; Schering-Plough, Kenilworth, NJ, USA) was subcutaneously applied to the abdominal region at a dose of 80 mcg (flask-ampoule with lyophilic powder diluted in 0.7 mL of diluent before each application). Six injections were performed throughout the treatment, with one injection per week. Peripheral blood was collected from each patient before the first injection (Pretreatment) and on 3rd and 6th application and following ethic criterious, biopsy were collected in pretreatment and in 6th application.

2.3 Flow cytometry

Peripheral blood samples were drawn from the patients, and cells were evaluated by flow cytometry (BD FACS Calibur cytometer and cell sorter, BD Biosciences, Franklin Lakes, NJ, USA). Cytometry protocols were deployed in accordance with those suggested by the manufacturer. The peripheral blood cells were verify by: T lymphocytes (CD3+, CD4+, and CD8+) and macrophages (CD14+). The procedure was performed in prior (pretreatment), to the 3rd and 6th application.

Briefly, leukocytes were isolated from peripheral blood samples via centrifugation at 4 °C by using a standard cell lysing protocol (FACS™ Lysing Solution, BD Biosciences) in accordance with the manufacturer's instructions. Cells were resuspended in phosphate-buffered saline (PBS) for extracellular tagging with monoclonal antibodies.

After extracellular tagging, the cells were incubated at 4 °C for 30 min, rinsed twice by centrifugation with PBS, and incubated with fixation and permeabilization solution (BD Cytofix/Cytoperm™) for 20 min at 4 °C. The cells were rinsed twice with Perm/Wash buffer (BD Biosciences) before the second tagging.

For intracellular verification, the cells were incubated with the following antibodies (according to the fluorochrome extracellular antibodies) for 30 min at 4 °C: IFNR1, IFNR2, IFN-α, STAT-1 or IRF-7. In all experiments and for all patients, we used as negative control isotope antibodies intracellular and extracellular conjugated with fluorochromes according to the reference antibody. After intracellular tagging, cells were incubated at 4 °C for 30 min and resuspended in 500 μL of PBS for cytometric analysis with a BD FACSCalibur™ cytometer. For the specific determination of the cells corresponding to lymphocytes and macrophages, we identified the region to be analyzed by constructing gates according to controls for relative size (forward scatter; FSC) and granularity and complexity (side scatter; SSC) in each experiment and for each patient.

2.4 Quantitative real-time PCR

RNA was extracted from cervical cells of biopsies using Trizol reagent (Invitrogen) obtained from all patients. cDNA synthesis was performed with Superscript III rt (Invitrogen).

Table 1. Clinical charateristics, histological diagnosis by biopsy, and conduct in each case, after immunotherapy with pegylated IFN-α.

Patient	Age	Smoker	Initial diagnosis	Final diagnosis	Treatment outcome
1	36	No	CIN III	Normal Epithelium	Regression
2	82	No	CIN III	CIN III	Without Response
3	54	No	CIN III	Normal Epithelium	Regression
4	28	No	CIN II	Normal Epithelium	Regression
5	32	No	CIN III	CIN III	Without Response
6	35	No	CIN III	CIN III	Without Response
7	18	No	CIN II	CIN II	Without Response
8	34	No	CIN II	CIN II	Without Response
9	38	No	CIN III	CIN II	Regression
10	37	No	CIN III	CIN III	Without Response
11	34	No	CIN III	CIN II	Without Response
12	47	No	CIN III	Normal Epithelium	Regression
13	26	No	CIN III	Normal Epithelium	Regression
14	24	Yes	CIN II	CIN II	Without Response
15	28	Yes	CIN II	Normal Epithelium	Regression
16	60	No	CIN III	CIN III	Without Response
17	42	No	CIN III	CIN III	Without Response
18	33	No	CIN III	CIN III	Without Response

CIN, Cervical Intraepithelial Neoplasia.

Table 2. Nucleotide sequence primer and anneling temperature.

Primer	Nucleotide sequence primer	Temperature	Primer	Amostra
β-actina Forward	5′GTGGGGCGCCCCAGGCACCA3′	60 ºC	2 μL	1 μL
β-actina Reverse	5′CTCCTTAATGTCACGCACGATTTC3′	60 ºC	2 μL	1 μL
IFNR1 Forward	5′-CTTTCAAGTTCAGTGGCTCCACGC-3′	60 °C	1.5 μL	3 μL
IFNR1 Reverse	5′-TCACAGGCGTGTTTCCAGACTG-3′	60 °C	1.5 μL	3 μL
IFNR2 Forward	5′-GAAGGTGGTTAAGAACTGTGC-3′	60 °C	1 μL	2 μL
IFNR2 Reverse	5′-CCCGCTGAATCCTTCTAGGACGG-3′	60 °C	1 μL	2 μL
IFN-α Forward	5′-ACTTTGGATTTCCCCAGGA-3′	60 °C	1 μL	2 μL
IFN-α Reverse	5′-CAGGCACAAGGGCTGTATT-3′	60 °C	1 μL	2 μL

Interferon-α (IFN-α) receptor 1 (IFNR1) and IFN-α receptor 2 (IFNR2).

Quantitative PCR was performed with GoTaq qPCR Master Mix (Promega) using the 7900 HT Fast Real-Time PCR System (Applied Biosystems). Nucleotide sequence of *prime* with annealing temperature is in Table 2.

2.5 Statistical analysis

An electronic database was developed for the statistical analysis. Variables were analysed with the GraphPad Prism 4.0 program. Values were submitted to Mann-Withney or Kruskal Wallis test. Differences with $p \leq 0.05$ were considered to be statistically significant.

3. Results

The Tables 3 and 4 demonstrate the means of the general values of flow cytometry, representing the mean of values of the number of lymphocytes and monocytes peripheric and the intensity of fluorescence of transcriptions factors involved of activation of IFN-α pathway in these cells, separated in patients with regression of lesion and patients without regression of lesion.

When comparing treatment phases with lesion regression in systemic helper T lymphocytes (CD3+, CD4+), patients who did not achieve lesion regression showed a significant increase on both IFNR1 ($p = 0.0336$) and IFNR2 ($p = 0.0165$) during the 3rd application and a decreasing during the 6th application. Likewise, the STAT-1, IRF-7, and IFN-α factors increased during the 3rd application and decreased during the 6th application in patients who did not achieve lesion regression (Fig. 1). There was a difference between the systemic expression of IFNR1 and IFNR2 as well as the expression of STAT-1 and IRF-7, when the moments of treatment were compared.

By analyzing systemic cytotoxic T lymphocytes (CD3+, CD8+), when comparing the treatment phases with the lesion regression, a significant reduction in IFNR1 ($p = 0.0391$) was observed in patients who did not obtain lesion regression when comparing the 3rd and 6th applications (Fig. 2). Unlike helper T lymphocytes, cytotoxic T lymphocytes showed no discrepancy in IFNR1 and IFNR2 expression.

Table 3. Mean of values of Fluorescence Intensity (MIF) and % gate of the patients with regression of lesion.

Celular type and transcriptions factors		Groups				
		% Gate (mean of fluorescence intensity)				
		Pre-therapy		3rd Application		6th Application	
	IRF-7	7.22	(29.47)	16.08	(29.77)	7.25	(31.16)
	STAT-1	12.28	(49.02)	15.83	(29.92)	6.747	(31.84)
CD4	IFN-alpha	8.07	(28.56)	15.91	(30.69)	6.42	(30.29)
	IFNR1	7.72	(28.77)	13.96	(30.39)	8.42	(38.13)
	IFNR2	8.45	(30.49)	14.21	(30.08)	8.63	(33.42)
	IRF-7	17.27	(38.53)	18.86	(39.18)	12.08	(29.49)
	STAT-1	17.33	(41.47)	18.95	(39.25)	13.06	(30.86)
CD8	IFN-alpha	16.71	(37.29)	18.74	(37.77)	12.07	(29.17)
	IFNR1	17.61	(41.75)	19.77	(42.04)	13.52	(30.03)
	IFNR2	17.97	(39.45)	19.91	(41.30)	13.54	(30.89)
	IRF-7	0.60	(46.98)	2.19	(54.60)	5.49	(55.58)
	STAT-1	0.82	(47.95)	2.40	(55.71)	2.21	(56.79)
CD14	IFN-alpha	0.63	(49.15)	1.84	(55.65)	1.36	(58.65)
	IFNR1	4.18	(133.2)	2.34	(55.88)	3.94	(52.34)
	IFNR2	1.28	(44.49)	2.27	(54.94)	2.77	(53.55)

Distribution of values mean expression of IFN-alpha, IFNR1 and IFNR2, IRF-7, STAT-1 in T lymphocytes helper (CD4+), cytotoxic (CD8+) and monocytes (CD14+) obtained from periferic blood of patients with regression of lesion.

Fig. 3 shows the local expression of IFNR1, IFNR2 and IFN-α. The analysis of IFNR1 expression shows that patients who did not obtain lesion regression displayed a higher tendency to this receptor. However, despite the increase mentioned above, this does not seem to be statistically significant. In this scenario, when analyzed the local response to the treatment, results may indicate that IFNR1 expressed homogeneously among all patients. From the IFNR2 analysis, it is possible to detach that patients who achieved lesion regression presented a higher tendency to this factor expression. However, despite the increase, the same is not statistically significant. The analysis of IFN-α expression indicates that all patients presented a balance in the expression of this cytokine, indicating that treatment with Peginterferon-alpha can enable pathways to produce endogenous IFN-α.

The graphs of Fig. 4 illustrate de comparation of % Gate mean in systemic T helper lymphocytes and the expression of IFNR1, IFNR2, IFN-α in local tissue. The analysis of IFNR1 expression shows that patients who did not obtain lesion regression displayed a higher tendency to this factor expression. However, despite the aforementioned increase, this does not seem to be statistically significant. In this scenario, when analyzed the local response to the treatment, results may indicate that IFNR1 expressed homogeneously among all patients. From the IFNR2 analysis it is possible to detach that patients who achieved lesion regression presented a higher tendency to this factor expression. However, despite the increase, the same is not statistically significant. The analysis of IFN-α expression indicates that all patients presented a balance in the expression of this cytokine, indicating that treatment with Peginterferon-α can enable pathways to produce endogenous IFN-α (Fig. 5).

4. Discussion

Interferons (IFNs) are pleiotropic cytokines (they have multiple effects on different cells) and have been widely studied for the treatment of tumors. They were discovered in the 1950s and initially classified as proteins produced by cells of the immune system in response to viral infections [10].

IFNs are known for their ability to induce an activated state of infected cells, having an important characteristic of inducing antiviral factors, interfering in various stages of the viral replication cycle [11]. In addition, they have functions that influence the innate and adaptive immune response, not only in viruses, but also in bacterial pathologies, as well as having a potent antiproliferative activity, essential for the blocking of the growth and immune survival of tumor cells [12].

Peginterferon-α immunotherapy provides the development of critical biological functions, such as activating transcription factors and producing specific cytokines for the immune system activation to eliminate neoplastic cells and inhibit viral replication. Dunn *et al.* [13] have shown in their study that IFN-α/β is essential for the rejection of highly immunogenic sarcomas in mice and reduces the proliferation of tumor-induced by primary carcinogens. It is especially in cervical intraepithelial neoplasia is very important to develop a clinical treatment to avoid surgical complications, although the lesions are prevalent in patients in reproductive age that could be complications in a future pregnancy, like spontaneous aborts, premature birth.

Table 4. Mean of values of Fluorescence Intensity (MIF) and % gate of the patients without regression of lesion.

Celular type and transcriptions factors		Groups				
		% Gate (mean of fluorescence intensity)				
		Pre-therapy		3rd Application		6th Application
	IRF-7	4.50	(23.41)	2.53	(30.55)	6.57	(28.86)
	STAT-1	9.14	(20.30)	4.47	(25.59)	9.43	(27.26)
CD4	IFN-alpha	8.16	(24.05)	2.50	(22.21)	5.70	(23.11)
	IFNR1	6.15	(24.07)	2.61	(29.01)	6.67	(31.19)
	IFNR2	6.26	(24.32)	2.90	(30.80)	7.11	(28.46)
	IRF-7	11.73	(36.33)	12.93	(35.89)	16.95	(31.17)
	STAT-1	10.32	(33.32)	14.34	(40.53)	15.18	(30.99)
CD8	IFN-alpha	10.40	(36.17)	13.95	(41.07)	14.85	(29.68)
	IFNR1	13.32	(38.49)	15.24	(40.67)	16.96	(31.50)
	IFNR2	13.40	(37.55)	13.44	(39.38)	13.62	(28.99)
	IRF-7	1.82	(100.9)	1.85	(54.65)	4.26	(53.33)
	STAT-1	2.40	(68.28)	1.88	(53.39)	3.13	(52.65)
CD14	IFN-alpha	1.61	(54.89)	2.14	(61.52)	2.77	(51.89)
	IFNR1	1.63	(86.02)	2.92	(56.86)	2.77	(55.83)
	IFNR2	2.64	(74.00)	2.12	(54.40)	4.94	(54.19)

Distribution of values mean expression of IFN-alpha, IFNR1 and IFNR2, IRF-7, STAT-1 in T lymphocytes helper (CD4+), cytotoxic (CD8+) and monocytes (CD14+) obtained from periferic blood of patients without regression of lesion.

Several data of our group indicate that there is essential differences between the in lesion regression when the patients were submitted to immunotherapy. A case report has shown a successful pregnancy after the patient was treated with intralesional IFN-α 2b for vaginal tumors. It demonstrated that conservative treatment is favourable and efficient for young women of childbearing age [14]. Conservative treatment with IFN-α for patients with CIN II/III is advantageous by preserving reproductive capacity. Ramos *et al.* [15] demonstrated that patients with a satisfactory response (60%) to IFN-alpha-2b treatment expressed more cytokines of Th1 profile (IFN-γ, TNF-α, IL-2) and a significant reduction in high-risk HPV viral load. Patients with failed therapy were smokers and had higher expression of Th2 (IL-4) or Treg (TGF-beta2 and TGF-beta3) cytokines.

Experimental and clinical data have demonstrated that locally and systemically cytokine treatment can induce tumor regression. Moltó *et al.* [16] concluded that patients with bladder tumors had no recurrence of the disease and had a significant increase in proliferative response in peripheral mononuclear cells after underwent treatment with IFN-α. Patients with chronic genital condyloma acuminatum caused by HPV, and giant condyloma acuminatum of Buschke-Löwenstein, treated with recombinant IFN-α2a, and IFN-α2b demonstrated reduction of lesions and increased rates of remission of the complete disease [17–21]. The significant increase in expression of IFNAR1 e IFNR2 genes has been pointed out because of several chronicle viral infections, such as hepatitis B and C [22, 23] and tumors, like adenocarcinoma, for instance [24]. Moreover, IFNAR1-blocking antibodies led to a significant decrease proliferation of Treg

associated with multiple myeloma [25].

The interaction of IFN-α with its receptors (IFNR1 and IFNR2) triggers a series of phosphorylation events, such as activation of the receptor associated with proteins Janus kinase (JAK) and Tyrosine kinase (TYK), which promote signal transduction and activation of transcription of STAT proteins [26]. A study made by Vitale *et al.* [27] showed that the expression of type I IFN receptors, mostly localized on the membrane, was significantly increased in pancreatic tumor of BxPC-3 type (cell line most sensitive to IFN). However, in models utilizing resistant cell line to IFN (Panc-1), 60 to 70% of cells were negative for IFNR2 expression, and most of it was present on cellular cytoplasm. Another study pointed out that human cell lines of pancreatic cancer respond in variable ways to IFN-α/β, and the expression level of type I IFN has a predictive value for antitumor effects [28].

There is also the presence of soluble cytokine receptors in body fluids that can modulate immunologic activity during homeostasis and disease. Soluble receptors of IFNR2 are found in serum, urine, salve, peritoneal fluid, and they may inactivate the action of IFN-α by avoiding the coupling with IFNR1 on the cellular membrane [29].

Besides host cells to produce IFNARs during viral infections, certain viruses have evolved to produce a soluble form of IFNR as a mean of evading the immune system. The poxvirus, for example, encodes a homologous soluble receptor of IFN that neutralizes type I IFN, which becomes essential for its virulence and is an escape mechanism of the immune system [30].
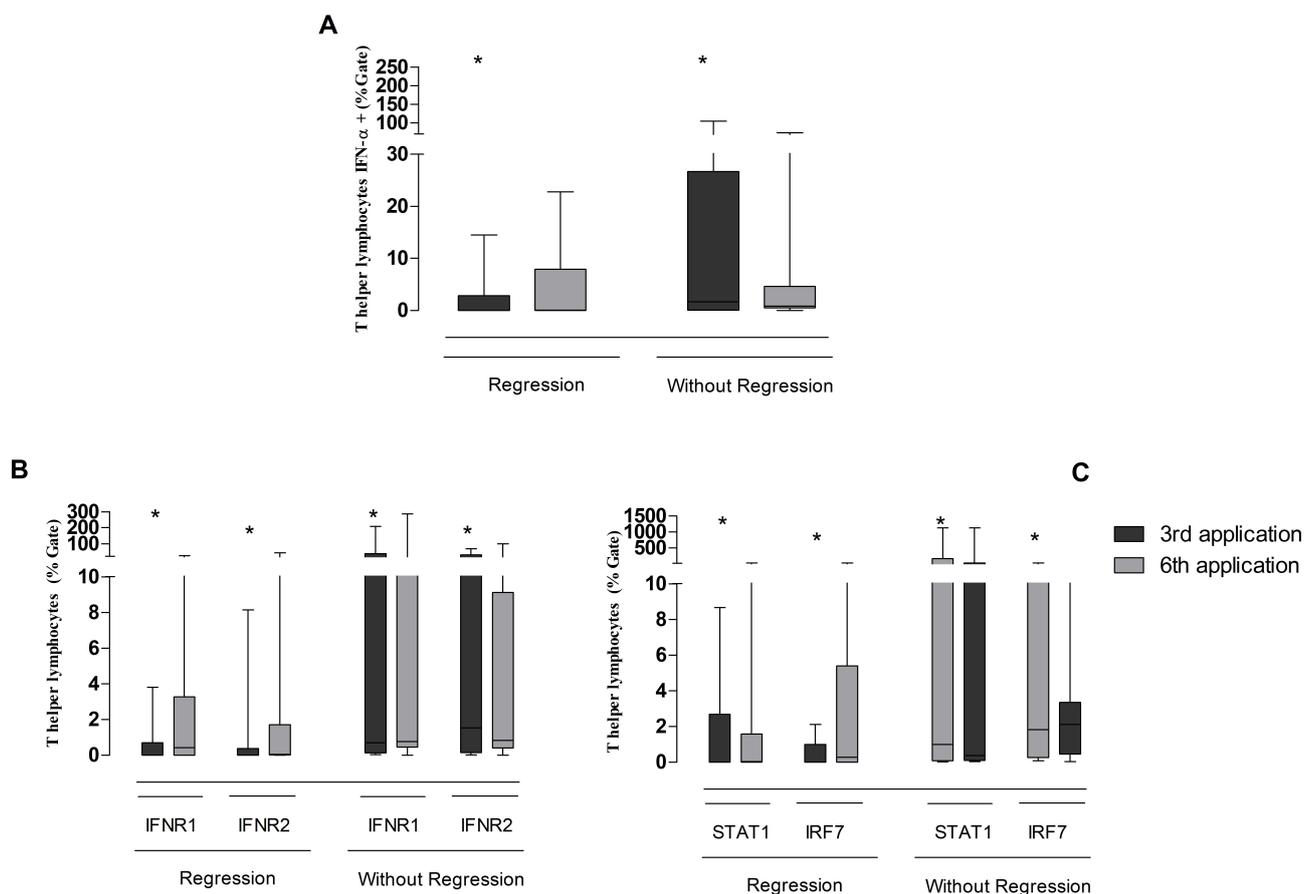
Fig. 1. Number (%) of helper T lymphocytes positive for IFN-α, receptors (IFNR1 e IFNR2) and transcription factors (STAT-1 e IRF-7) obtained from patients with CIN and submitted to immunotherapy. Comparation patients with good or worst response to IFN-α Immjunotherapy in systemic helper T lymphocytes verifying the IFN-α, receptors and transcription factors in during the 3rd application/Pretherapy and 6th application/pretherapy in patients submitted to. (A) IFN-α. (B) IFNR1, IFNR2. (C) STAT-1, IRF-7. The values represent a comparative analysis between the treatment stages: 3rd application and pre-therapy; 6th application and pre-therapy. The analysis were grouped according to the time of treatment: dark gray representation the 3rd application and light gray, the 6th * Values of $p < 0.05$.

Our study, during the 3rd application, demonstrated patients who did not obtain regression of their lesions showed a significant increase in IFNR1 and IFNR2 in CD4+ T lymphocyte when compared to patients who obtained regression. However, this increase was not observed during the 6th application. In CD8+ T lymphocytes, the number of IFNR1-labeled cells in patients who did not have lesion regression was significantly reduced from the 3rd to the 6th applications. These data demonstrated that patients who do not respond to the treatment have higher levels of sistemic T helper cells with IFNR1 and IFNR2 than those who respond to the treatment.

Zhang *et al.* [31] concluded in their study that cells from bladder tumors presented low expression of IFNR1 and IFNR2 when compared to cells from healthy tissue, which demonstrates resistance to immune therapeutic treatment with IFN-α. In our study, the local expression of interferon receptors was different. Nonresponsive patients showed higher expression of IFNR1 and lower expression of IFNR2, despite not being statistically significant.

Already the interaction between IFNRs and cytoplasmic transcriptional factors is significant for the activation of immune response activator genes. Researches have shown that cells that express E6 protein from HPV-18 demonstrated to have a decreased capacity of binding on DNA and transactivation of ISGF-3 transcription factors. E6 proteins impair phosphorylation of STAT-1 and STAT2 by physically interacting with Tyk-2 [32].

The present study demonstrated that there was a significant increase in STAT-1-labeled CD4+ T lymphocytes in patients who did not obtain regression of lesion during the 3rd application, however, this increase was not maintained until the 6th application. The production of cytokines in the tumor microenvironment influences the expression of transcriptional factors. Nguyen *et al.* [33] has presented that the absence of STAT-1 leads to inhibition of the IFN-α/β pathway, and the cytokines produced induce expression of IFN-γ. These results indicate that the activation pathways of type I IFNs occur through STAT-1-dependent mechanisms and that efficient induction of IFN-γ expression by IFN-α/β re-
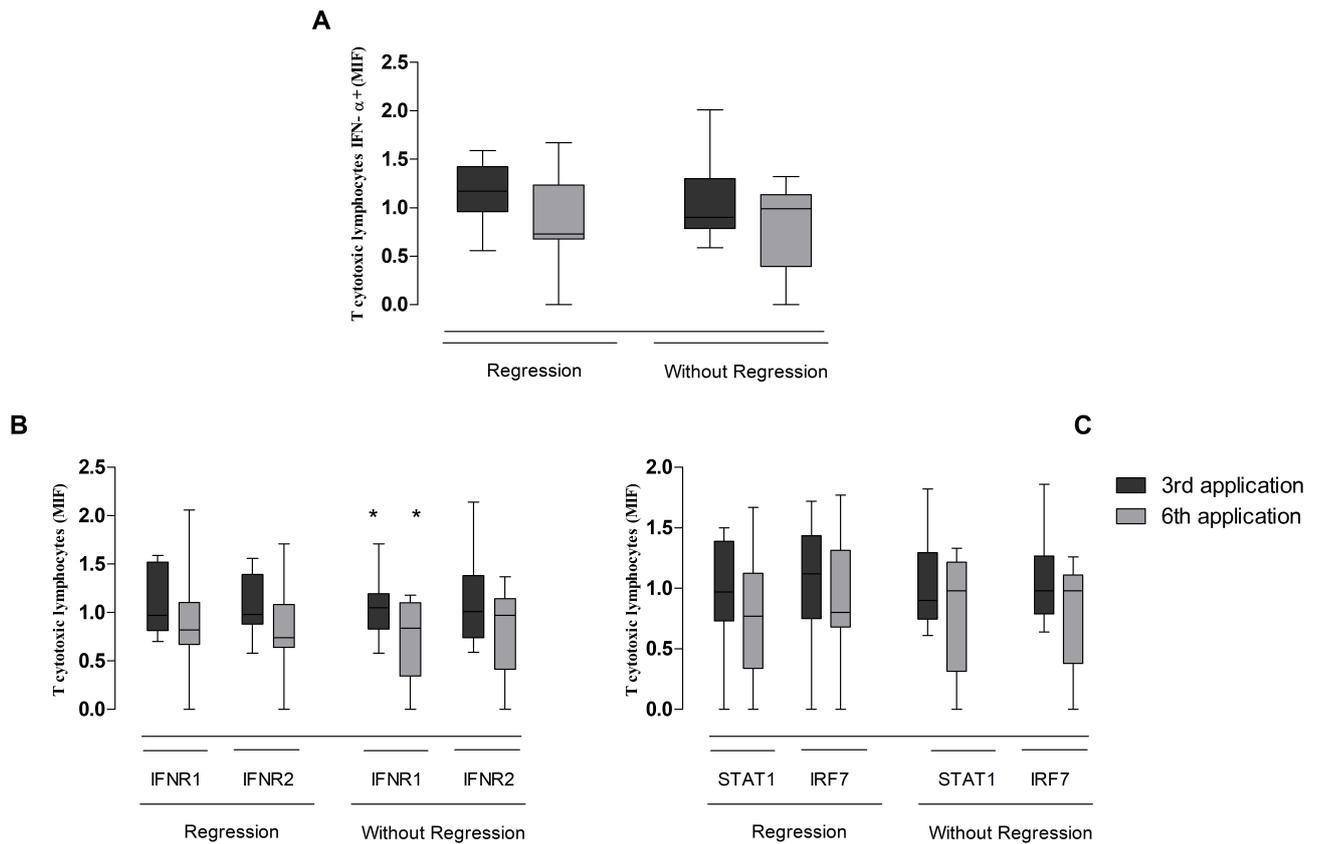
Fig. 2. Expression (MFI) of IFN-α, receptors (IFNR1 e IFNR2) and transcription factors (STAT-1 e IRF-7) in cytotoxic T lymphocytes obtained from patients with CIN and submitted to immunotherapy. Comparison of the mean values of fluorescence intensity (MIF) in periperic helper lymphocytes obtained from patients with regression or without beside the immunotherapy with IFN-α in the 3rd pplication/Pretherapy and 6th application/pretherapy of the cytotoxic T lymphocytes positive for (A) IFN-α, (B) IFNR1, IFNR2, (C) STAT-1, IRF-7. The values represent the comparative analysis between the treatment stages: 3rd application and pre-therapy; 6th application and pre-therapy. The analysis were grouped according to the time of treatment: dark gray representation the 3rd application and light gray, the 6th * Values of $p < 0.05$.
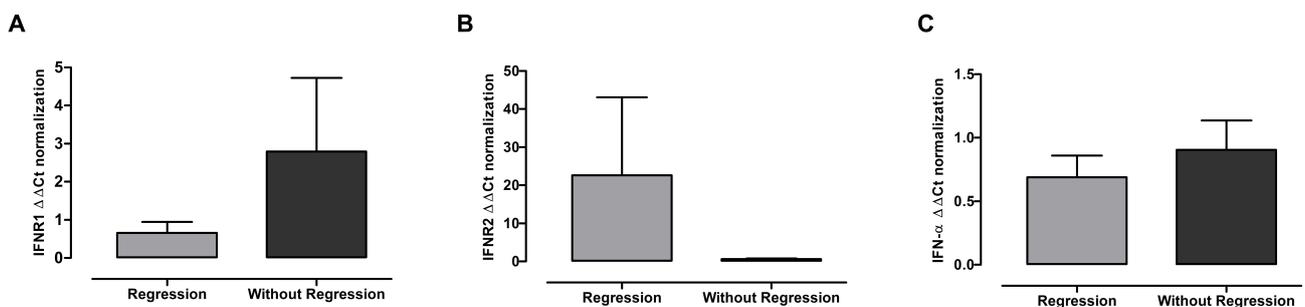
Fig. 3. Expression of IFN-α and their receptors (IFNR1 and IFNR2) in lesions obtained from patients with regression and without regression. Mathematical normalization values of $\Delta\Delta Ct$ which represents the number of times the IFNR1 (A), IFNR2 (B) and IFN-α (C) genes were expressed in local tissue.

quires regulation of STAT-1. This pathway is essential for the activation of both innate and adaptive immune responses against viral infections.

STAT-1 transcription factor plays an essential role in responses mediated by IFN-α through controlling the activation pathway of type I IFNs [34]. In the absence of STAT-1, the endogenous production of type I IFN has antiprolifera-

tive activity and increases the survival of CD4+ and CD8+ T lymphocytes [35].

The family of the Regulator Factor of IFN-α counts various genes involved in antiviral responses such as IRF-3 and IRF-7, which are the most important modulators of IFN-α through the activation of TBK1/IKK [36, 37] Knockout mice for IRF-7 have marked a reduction in serum levels of IFN-α.
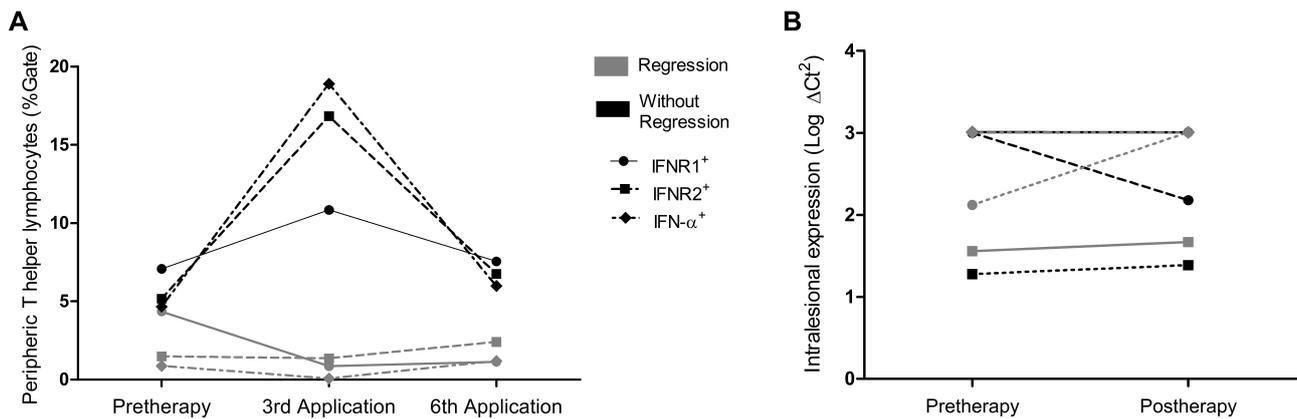
Fig. 4. Comparation of IFN-α and their receptors (IFNR1 and IFNR2) in peripheric blood and intralesional from patients with regression and without regression. (A) Percentage of T helper lymphocytes positive for IFNR1, IFNR2 and IFN-α throughout the treatment. The values represent a mean of % Gate of systemic CD4+ cells. (B) Expression pretherapy and posttherapy of intralesional of IFNR1, IFNR2 and IFN-α by real-time PCR. The analysis were grouped according to the response to treatment: dark gray representation regression of the lesion and black, without regression.
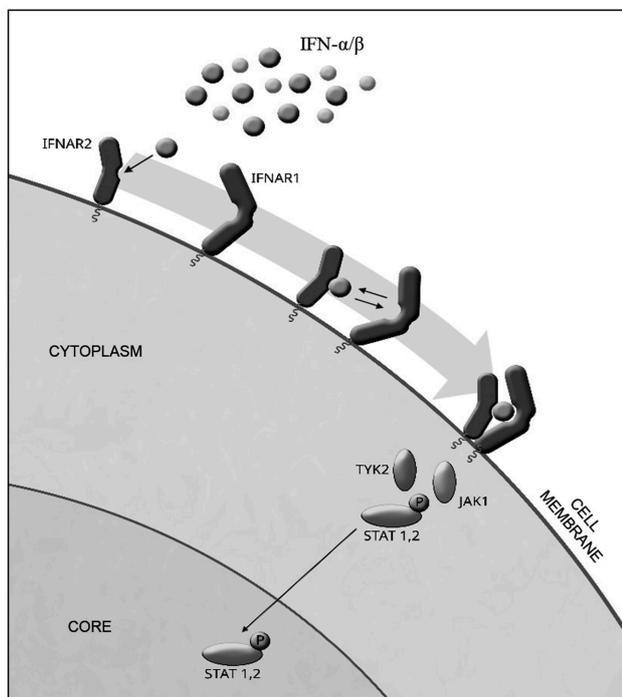
Fig. 5. The balance in the cellular expression of IFNR1, IFNR2 results in a good response to treatment with IFN-alpha, activating the intracellular pathways.

They are more vulnerable to viral infection, which indicates that the activation pathway through IRF-7 may be essential in systemic antiviral responses activated by IFN-α [38].

By analyzing the systemic immune response through the evaluation of the presence of IRF-7 in helper and cytotoxic T lymphocytes, it was observed that patients who did not have regression of lesion demonstrated increasing of positive cells during the 3rd application but not after it. Additionally, despite the attempt of IRF-7 activation during the 3rd application, this result points to the impossibility of maintaining this

pathway in non-responsive patients.

When assessing the integrity of the IFN-α activation pathway in auxiliary and cytotoxic T lymphocytes, systemically, through the evaluation of the presence of the transcriptional factors STAT-1 and IRF-7, we can observe that in patients who did not obtain the regression of the lesion, despite showing increased positive cells during the 3rd application, this was not maintained. This result points to the impossibility of maintaining this route in unresponsive patients, despite the attempt to activate it during the 3rd application.

This result demonstrates the attempt to respond to treatment with Peginterferon-α, which was not maintain and thus did not eliminate the lesion. Several studies have shown that immunotherapy with Peginterferon-Alfa may have increased efficacy and decreased toxicity when administered subcutaneously in the same way it may reduce metastases, increase the ability of antigen presentation, and cytotoxic activity by the immune system [39–41].

Studying the inflammatory infiltrate in the tumor microenvironment, Silva *et al.* [42] concluded that there is a predominance of CD3+ and CD20+ lymphocytes in patients with CIN III compared to samples from patients with invasive cancer and that cell migration seems to be proportional to progression of the injury. Another study showed that there is a positive expression of CD3+ T lymphocytes in patients with recurrence, after conization by CIN III [43].

A study by Fernandes and collaborators [44] investigated the number and function of circulating neutrophils in patients with cervical neoplasia and observed an increase in the number of cells in patients with microinvasion. Soluble mediators released by tumor cells, such as nitric oxide, could interfere with the ability of neutrophils to migrate, thus impairing the host's immune response.

One of the first signs of viral infection is pain. An established study that type I IFNs (IFN-α and IFN-β) can act directly on nociceptors of the dorsal root ganglion (DRG) to

cause pain sensitization through stimulate JAK/STAT signalling [45]. Therefore, this pathway may be associated with immune responsiveness to Type 1 Interferons also in neurons.

All of these studies may point out that interferon immunotherapy is influenced not only by the expression of receptors in systemic or tissue immune cells, but also by the cells of the cervix itself, developing the ability to eliminate the HPV virus and set local tissue homeostasis. Thus, further studies are need, which include the investigation of the inflammatory infiltrate and cervical tissue of these patients.

We concluded with immunomodulation by interferon-alpha seems to depend on the systemic expression of IFN receptors. Our data suggest that patients who can respond to immunotherapy already have a pattern of IFN receptors expression in lymphocytes, which contributes to successful treatment. The local and systemic distribution of IFN receptors is different, demonstrating that there may be a different expression of these receptors in the local stroma, which needs to be evaluated.

Author contributions

MAM and EFCM conceived, designed the experiments; LMS performed the experiments, analyzed the data and wrote the paper; RSN and MAT selected the patients, accompanied and performed all clinical procedures. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all subjects involved in the study. All procedures performed followed the criteria developed by the Ethics Committee (approval number: CEP/UFTM Nos. 759 and 1525).

Conflict of interest

The authors declare no conflict of interest.

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