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Retinoids Hamper Inflammation in Normal and Cancer Epithelial Cell Lines

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Abstract: Inflammation is considered one of major causes of cancer and hamper inflammation is possible target of chemotherapy and chemopreventive treatment. Retinoids have potential anti-inflammatory effects. We investigated the effects of the retinoid all-trans retinoic acid (RA) and the 6-OH-11-O-hydroxyphenantrene (IIF), a specific ligand of Retinoid XReceptor (RXR), on the normal keratinocyte cell line NCTC and on the neuroepithelioma cancer cell line SKNMC. IIF and RA treatment inhibited proliferation and viability in a time and dose dependent manner. Interestingly, IIF was significantly more effective than RA and reduced of 50% SKNMC cells after 24 h of treatment at the dose 30 μM. The effects are further evident in cancer cells than normal cells where retinoids reduce slightly the viability. We stimulated inflammation with LPS and TNFα in normal and cancer cells and we observed the effects of IIF on several catechins involved in inflammation. A reduction of TNFα, IL2 expression and IL6 production after treatment with IIF in SKNMC cells was reported. Moreover, IIF reduced IL18, IL15 and IFNγ expression in inflammatory NCTC cells. Finally, inflammation induction increased while IIF treatment reduced matrix metalloproteinase-2 and -9 activity, two proteins that are involved in cell invasion and metastasis. In conclusion, we suggest that retinoids as IIF could be used to overcome inflammation in human carcinoma and it may be a powerful tool in the development of cancer therapies.

Keywords: Inflammation, Retinoids, Cancer, Nuclear Receptors, Cytokines

I. Introduction

Retinoids have many functions in the regulation of immune responses such as increase the number of immune system cells including neutrophils, NK cells, B- and T- lymphocytes. Several in vivo studies suggest that vitamin A deficiency aggravates the clinical manifestations of inflammatory reactions and can lead to a greater risk of acquiring irreversible tissue damage (Wiedermann, 1996). Actually, the lack of vitamin A in vivo is associated with an alteration in the balance between pro-inflammatory cytokines and anti- inflammatory with impaired ability to infection control (Hinds, 1997). The mechanism of action about anti-inflammatory activity of retinoids is not yet known.

Retinoids, have important effects in the regulation of inflammation through the retinoid receptors as the receptor for retinoic acid (RAR) and retinoid X receptor (RXR). These receptors are encoded by different genes that include three isotypes called RARα, β, γ and RXRα, β, γ. RARs can be activated by all-trans retinoic acid (RA), while RXRs can be activated by retinoids as 9-cis-RA (9cRA) (Evans, 2014). There are also natural and synthetic compounds with selectivity for RXRs that are called rexinoids. About rexinoids in our laboratory we have been tested a new derivate of RA called 6-OH-11-O- hydroxyphenanthrene (IIF) (Bartolini, 2006). Some RXRs ligand, as the Fenretinide [N- (4-hydroxyphenyl) retinamide], that is a synthetic analogue of vitamin A, have chemopreventive and chemotherapeutic properties in several cancers. Some studies report the efficacy of Fenretinide in inducing cell death and growth reduction of inflammation both in vitro and in vivo (Myatt, 2005). Discordant results are reported in literature about retinoids effects in inflammation control.

Retinoids are responsible in the control cytokines expression involved in inflammatory mechanisms. RA stimulates the production of immunosuppressive cytokines, such as IL- 10, while reducing TNFα synthesis and IL-12 production in macrophages (Wang, 2007). RA inhibits also the inducible form of cyclooxygenase (COX) as COX2 and the nitrous oxide synthetase (iNOS). Moreover RA can reduce production of chemokines in inflamed human chondrocytes (Hung, 2008). These argues in favour of his anti-inflammatory action or retinoids.

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Recently, we demonstrated that nuclear receptors agonists reduced the inflammation dependent survival of breast cancer stem cells (Papi, 2012). In this manuscript, we tested the effects of RA and IIF on viability and inflammation pathway in two cell lines: the NCTC,

that is a keratinocyte cell line obtained from normal human skin and the SKNMC, that is a neuroepithelioma cell line derived from metastatic site in supra-orbital area.

II. MATERIAL AND METHODS

2.1. Cell culture and Reagents

NCTC and SKNMC cell lines were purchased from the American Type Culture Collection (Rockville, USA). All cell lines were maintained in RPMI (Sigma,USA) supplemented with 10% FBS (Euroclone, USA), 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml) and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. IIF (provided by Dr. Khodor Ammar, Italy, pat. WIPO W0 00/17143) and RA (Sigma, USA), were dissolved in propylene glycol and ethanol respectively. Lipopolysaccharide (LPS) and Tumor necrosis factorα (TNFα) were purchase from ImmunoTools (Germany) and used at 10 ng/mL at the final concentration. Theconcentration of the solvent in the highest dose of drugs used and LPS or TNFα did not affect cell viability (data not shown).

2.2. Cell Viability

Cell viability was measured by Sulphorodamine B (SRB) assay and MTT assay as previously described (Papi, 2010). Cells were grown in 96-well plates (10^4/well) and treated with EGCG and/or IIF or RA for 24-72 h at different concentrations. After treatment, the cells were treated with MTT or fixed and marked with SRB according to the protocol instructions (Sigma, USA). Finally, the absorbance was measured in a microplate reader (Bio-Rad, USA) at 570 nm.

2.3. Elisa Test

Determination of IL6 level in cell lines supernatant were evaluated by ELISA (S.I.C., Rome, Italy). Briefly, cells were seeded in a 6-well plate at the density of 3×10^5 cells per well and treated in serum-free medium for 24 h. The harvested medium was centrifuged at 500 *g* for 5 min (4°C) to remove floating cells and the supernatants were collected and assayed following the customer's instructions.

2.4. Western Blot (WB) Analysis

The cells were treated and lysate in lysis buffer as previously described (Papi, 2009). Cell lysates were size fractionated in 10-12% SDS-polyacrylamide before transferring to Hybond TM-C Extra membranes (GE Healthcare, UK). Membranes were blocked and incubated overnight at 4°C with the antibodies. The following antibodies were used: anti- Jun (Assay Design, USA), anti-TNFα, anti-IL2, anti-IL15, anti-IFNγ, anti-IL1β and anti-IL18 (ImmunoTools, Germany). The primary antibodies were diluted as indicated by manufacturer's instructions and the anti-rabbit/mouse peroxidase conjugated antibodies were diluted 1:1000 (GE Healthcare, UK). Bands were quantified by using a densitometric images analysis software (Quantity One, Bio-Rad, USA). Molecular mass were determined using a wide range protein marker 8-200 KDa (Sigma, USA). Protein loading was controlled by anti-actin (1:1000) (Sigma) detection. Experiments were performed in triplicate, normalized against actin control and statistically evaluated. Stripping solution (Pierce, USA) was used for re-probe the same membranes. Densitometric data were reported.

2.5. Zymography

Cells were seeded and after 18 h were placed in serum-free medium (RPMI) with treatments for 24 h. MMP2 and MMP9 activity was determined by gelatin zymography as previously described (Papi, 2010). Only the active form of gelatinases were detected: 81 KDa for MMP9 and 63 KDa for MMP2. The MMPs activities, indicated by clear bands of gelatin digestion on a blue background, were quantified by using densitometric image analysis software (Quantity One).

2.6. Statistical Analysis

Statistical significance was assessed by ANOVA multiple comparison test with standard deviation (SD), as appropriate, using PRISM 5.1 (GraphPad, USA). The level for accepted statistical significance was $p< 0.05$.

III. DATA ANALYSIS

We investigated the viability effect of RA and IIF on normal cell line NCTC and cancer cell line SKNMC. We tested cell lines for 24 to 72 h at different concentration of RA and IIF (10-30 μM) with SRB assay (**Figure 1**). Proliferation data were confirmed by MTT assay (data not shown). Interestingly, the anti-proliferation effects of retinoids, and in particular of IIF, were higher on SKNMC cancer cell (Figure 1C-D) than NCTC normal cells (**Figure 1A-B**).

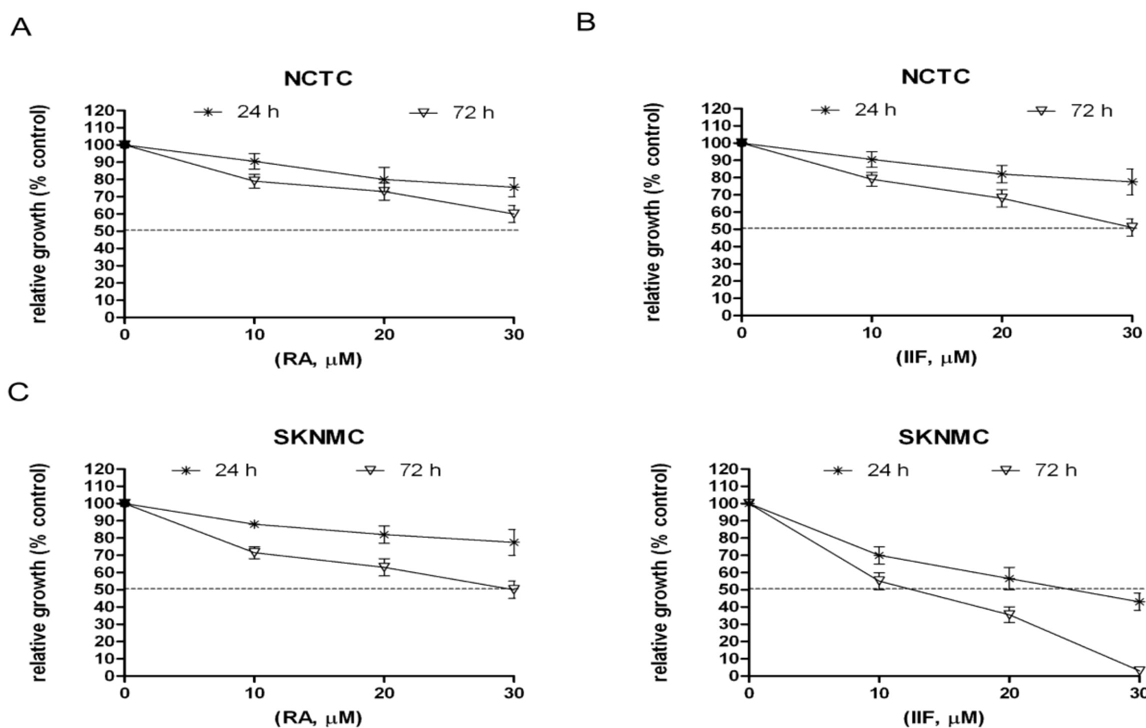

Figure 1

Fig1. *Effect of RA and IIF on cell viability*

SRB assay after 24 and 72 h treatment with RA (10- 30 μM) or IIF (10-30 μM) in NCTC (a-b) and SKNMC (c-d) cell lines. Each bar represents the mean (± SD) of six replicate from three independent experiments.

Already after 24 h, IIF reduced approximately 70% viability arriving at a total inhibition after 72 h of treatment. The effect of the RA, was not only delayed, but it was also lower than the IIF, with reduction of viability around 18% after 24 h and about 40% after 72 h. We then tested the effect of retinoids on cells stimulated with LPS 10 ng/mL and TNFα 1 ng/mL for 24 h. We observed that LPS and TNFα did not have effects on cell viability and on retinoids effects (data not shown).

NCTC cells are used in the study because they provide a good model for detectable basal cytokines expression and this expression can be increase after stimulation with LPS or UV rays; we used as model LPS stimulation. Moreover, we tested the effect of retinoids on SKNMC cancer cells, in order to investigate if the antitumoural action of retinoids performed also through modulation of pro-inflammatory cytokines.

TNFα have a dual action in cancer physiology: the first is as defence against tumour cells, in fact is a inducer of apoptosis, the second is as inductor of tumour inflammation, in fact is a pro-inflammatory cytokine. The basal production of TNFα in normal and cancer cells increased after treatment with LPS and TNFα expression was reduced in significant manner after 24 h of treatment with IIF only in SKNMC cancer cell line (**Figure 2A**). We also evaluated the IL-2 expression: LPS stimulated the production of IL-2 that was increased by 20% in NCTC and 40% in SKNMC cells compared to baseline. The treatment with IIF, used either alone or in the presence of LPS, reduced IL2 expression approximately 20% in NCTC and 40% in SKNMC cell line (**Figure 2B**).

IL6 is the principal inflammatory cytokine that induce cancer stem cells growth (Papi, 2012). We considered the basal production of IL-6 in NCTC cells and SKNMC (1000 pg/ml and 1500 pg/mL) through enzyme immunoassay ELISA. The basal production of IL6 was doubled in cells stimulated with LPS and was reduced 5-fold after treatment with IIF. The treatment with IIF in cells stimulated with LPS, reduced to very low levels the production of IL-6, from about 2000 pg/ml to about 400 pg/ml (**Figure 2C**).

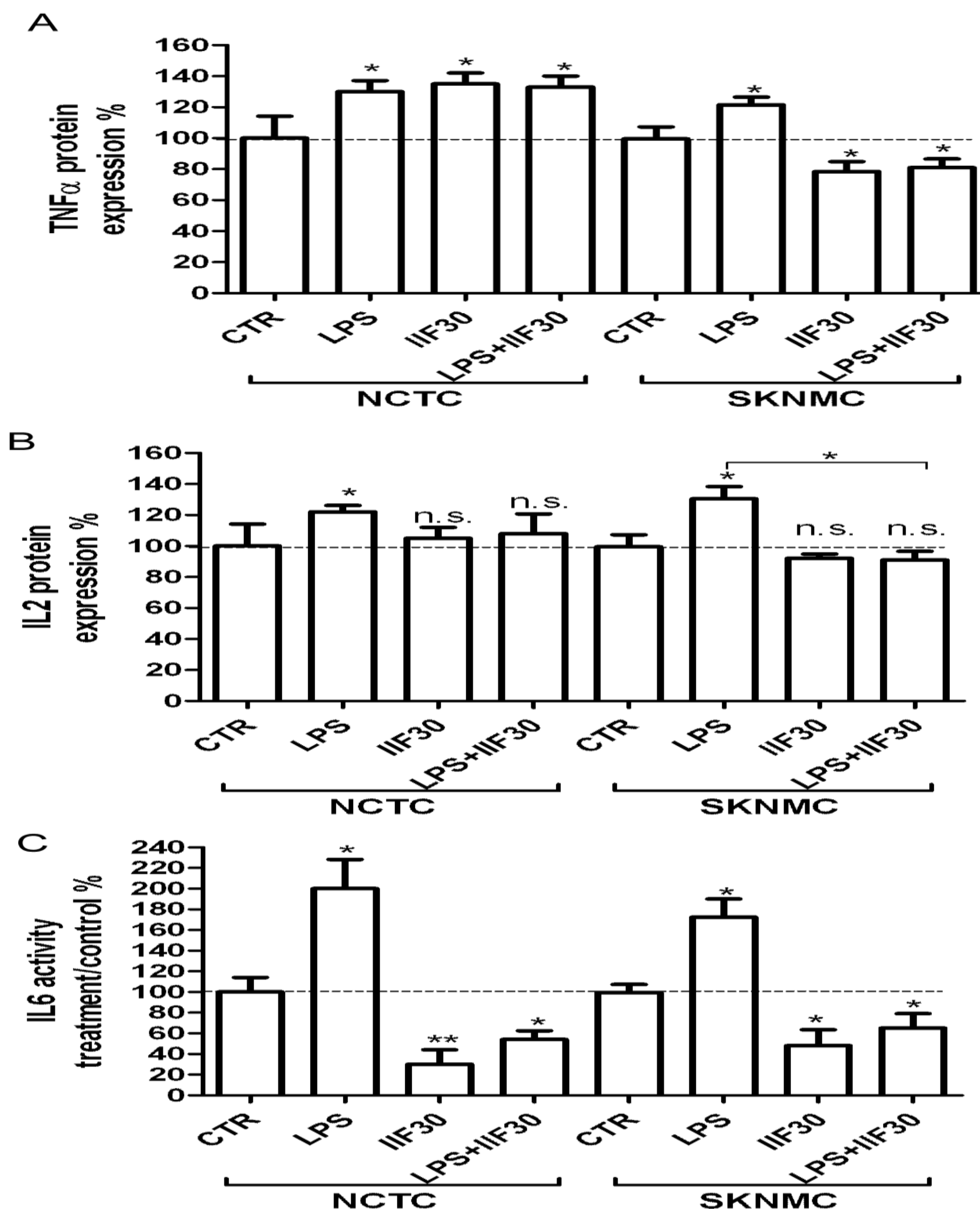

Fig2. *Effect of IIF on cytokine expression in normal and cancer cells.*

(a) TNFα and (b) IL2 protein level (WB) and (c) IL6 activity after 24 h of treatment with LPS (10 ng/mL) and/or IIF 10 μM in NCTC and SKNMC cell lines. Untreated cells: CTR. Each bar represents the mean (± SD) of three independent experiments. * $p<0.05$. ANOVA, n=3.

Other cytokine involved in the retinoid inflammation regulation were investigated. The expression of IL1β, IFNγ and IL18 were increased by LPS treatment in NCTC cells. IIF reduced this increase after 24 h of treatment (**Figure 3A-C**). In our experimental conditions, it was not possible to detect the presence of IL1β, IFNγ and IL18 in SKNMC cells. In parallel we observed IL15 expression only in SKNMC cells; LPS increase IL15 expression that was hamper by IIF treatment (**Figure 3D**).

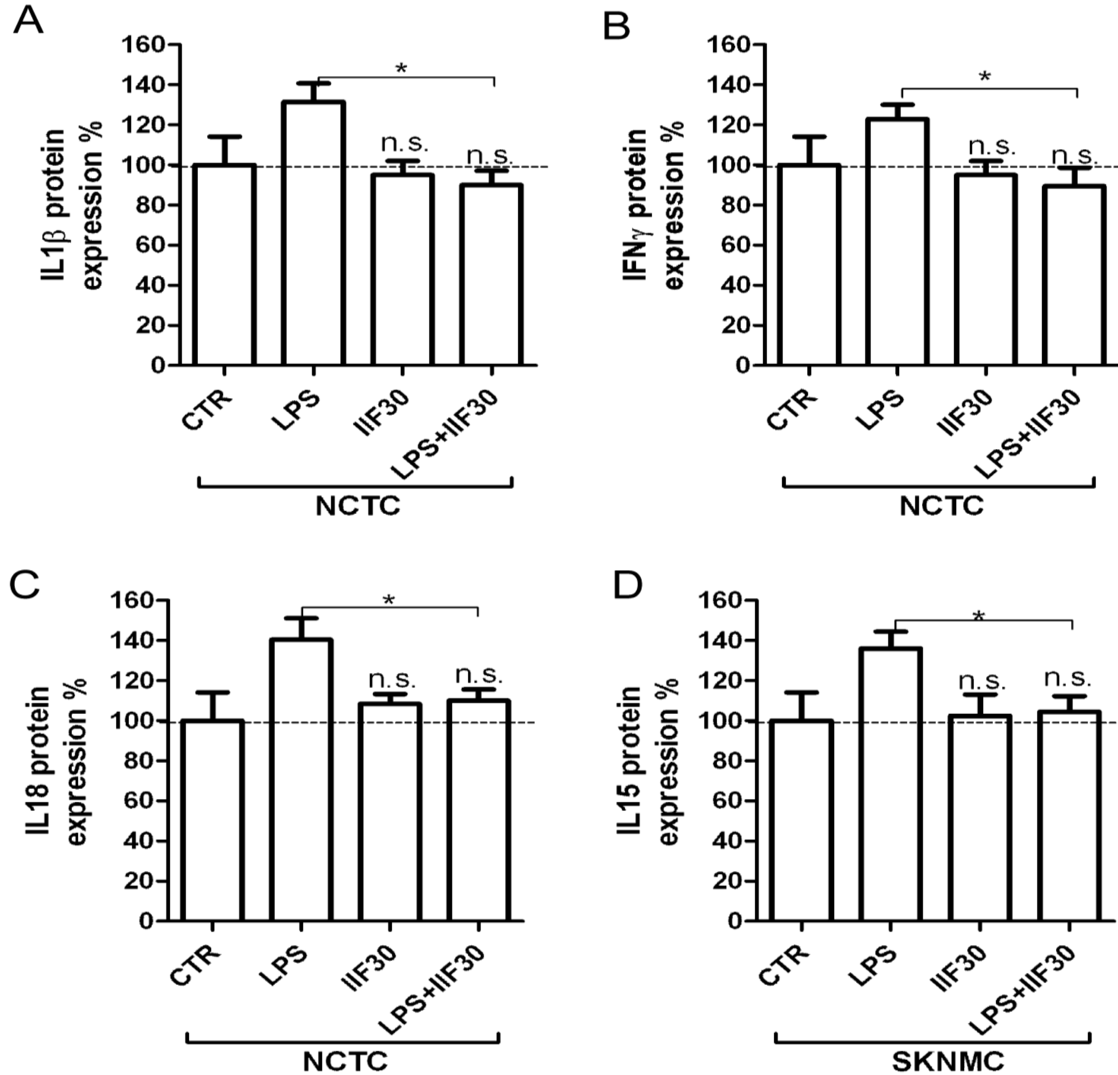

Fig3. *Effect of IIF on cytokine expression*

(a) IL1β, (b) IFNγ, (c) IL18 protein level (WB) in NCTC cell line and (d) IL15 protein level (WB) in SKNMC cell line after 24 h of treatment with LPS (10 ng/mL) and/or IIF10 μM. Untreated cells: CTR. Each bar represents the mean (± SD) of three independent experiments. * $p<0.05$. ANOVA, n=3.

Tumour invasiveness is regulated by specific metalloproteinases (MMPs) called gelatinases that include MMP2 and MMP9 (Han, 2002). Since, the transcription factor AP- 1 can regulate MMPs expression and activity (Ho, 2005) we verified that inflammation can up-regulated the constitutive protein of AP-1 called c-Jun in NCTC and SKNMC cell lines and IIF can block this effect (**Figure 4A**). We verified a significant reduction of MMP9 and MMP2 activity after treatment with IIF in SKNMC cell line. In parallel LPS and TNFα inflammatory treatment increased MMPs activity that was hamper by IIF treatment (**Figure 4B**)

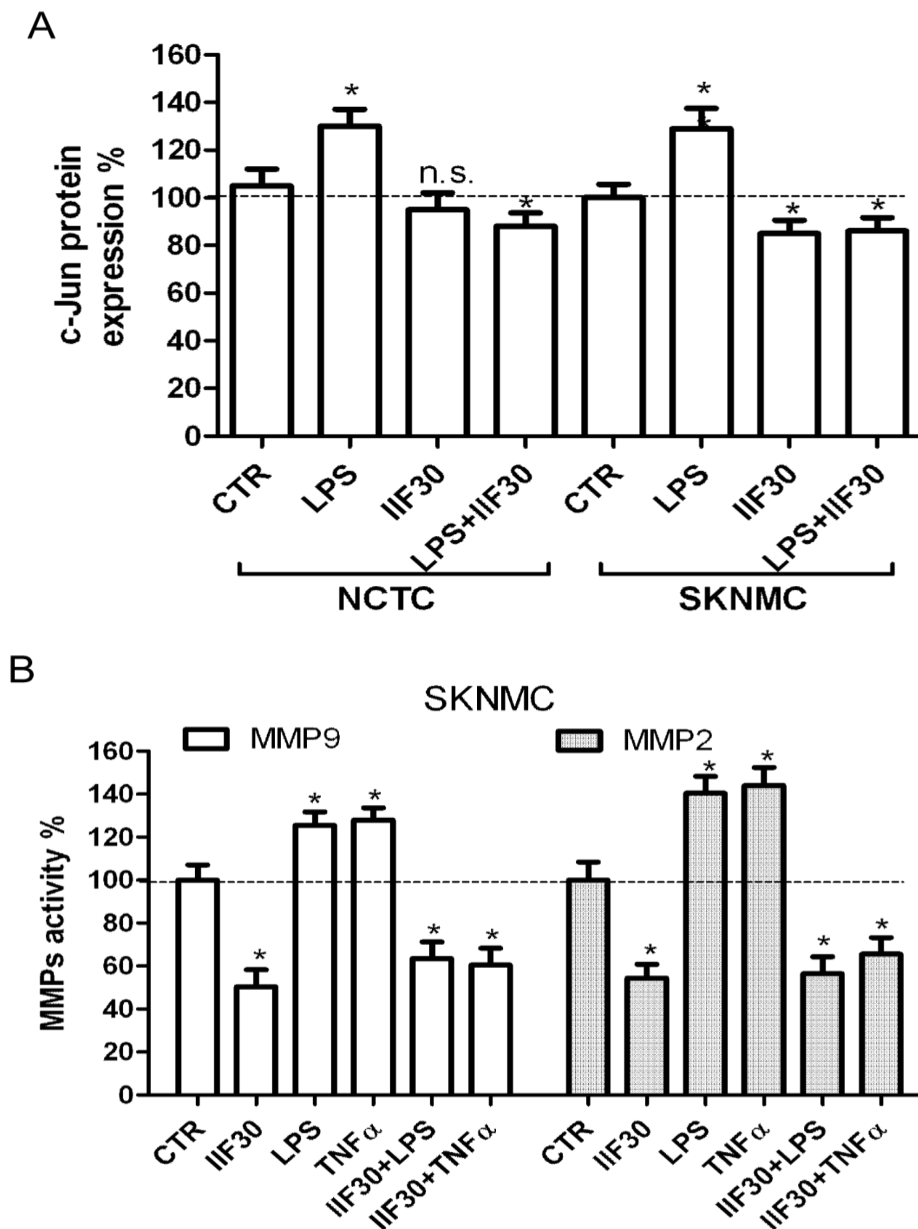

Fig4. *Effects of IIF on MMPs*

(a) c-Jun and (b) MMP2/MMP9 after 24 h of treatment with LPS (10 ng/mL) and/or IIF10 μM in NCTC and SKNMC cell lines. Untreated cells: CTR. Each bar represents the mean (± SD) of three independent experiments. * $p<0.05$. ANOVA, n=3.

IV. Discussion

We have been demonstrated the strong antitumoural activity of the retinoid IIF on many cancer cell lines, and its activity was consistently stronger than that exerted by RA (Bartolini, 2006; Papi, 2007-2012). Our data demonstrated that IIF had a strong antiproliferative effect, always higher than that exerted by RA in SKNMC cancer cell line. Instead in normal NCTC cell line both retinoids reduced only slightly the viability. This result is particularly interesting in view of the potential use of IIF in the treatment of tumours of epithelial origin, because it

confirms what has already been observed in our laboratory on lymphocytes and in vivo, IIF is only weakly toxic against non-cancer cells while it is very toxic in tumour cells (Papi, 2009).

The inflammatory cytokine pathways and the activation of pro-inflammatory regulator as IL6 are essential to sustain cancer proliferation. For this reason it is important to target cancer by using selective inhibitors of inflammation (Korkaya, 2011). Retinoids have long been investigated in preclinical models and clinical data for cancer are reported in literature but the effects on inflammation remain contradictory (Hung, 2008). In literature was reported that retinoids inhibit TNFα expression on peripheral blood mononuclear cells (Nozaki, 2006) and induce IL2 expression in T-lymphocytes (Engedal, 2004). We demonstrated that the rexinoid IIF can reduce both TNFα and IL2 expression in inflammatory cancer cells. Recently, we observed that retinoids dampen IL6 expression in breast cancer stem cells (Papi, 2012); in this manuscript, we demonstrated that IIF reduce IL6 production in inflammatory NCTC and SKNMC cell lines.

In literature was reported that there is a binding site for RAR in IL1β gene and RA treatment stimulates the production of IL1β in keratinocytes, monocytes and in smooth muscle cells of the aorta (Blanton, 1989; Wagsater, 2006). It is known that some retinoids hamper IFNγ expression or blocking its pro-inflammatory activity (Nozaki, 2006) and that IFNγ is in turn triggered by the IL2 transcription signal (Hung, 2008). IL18 is another pro-inflammatory cytokine produced by many cell types, including leukocytes, in the form of inactive precursor, which is activated by a caspase. This caspase is not present in cells under normal conditions, but only after activation by, for example, phorbol 12-myristate 13- acetate (PMA) or LPS. The major producers of IL-18 in humans are keratinocytes. The high expression of IL18 induced in keratinocytes from UV ray plays a critical role through its effect on cells of the immune system and in the skin transformation into malignant cells (Park, 2001). Our data about confirming the literature data that retinoids can reduce IL-1β, IFNγ and IL18 pro-inflammatory activity in NCTC keratinocytes (Kang, 2008).

This inflammatory effect are induced by the transcription factor NF-κB and AP1 (activating protein-1) stimulation. The transcription factor AP-1 is a collective term referring to dimeric transcription factors composed of Jun and Fos subunits that bind to a common DNA site, the AP-1-binding site (Patil, 2015). We observed a reduction expression of the factor c-Jun in inflammatory cancer cells after treatment with IIF.

The cancer cells begin to invade the tissues thanks to the secretion of several proteolytic enzymes, especially matrix metalloproteinases (MMPs), which digest the extracellular matrix, thus promoting the diffusion and expansion of the tumour. MMPs produced by the tumour cells are in particular the MMP9 and MMP2 that are induced by AP-1 (Patil, 2015). In previous research we have been showed that RA and IIF, are capable of limiting the invasiveness of the tumour, reducing the synthesis and activity of these two MMPs (Papi, 2007). We confirmed that inflammation with LPS and TNFα stimulation induces the synthesis of MMPs in tumour cells as previously demonstrated (Han, 2002). Moreover, we verified for the first time that IIF reduce MMP9 and MMP2 activity in SKNMC cancer cells in basal and inflammatory condition.

V. Conclusion

The retinoid RA and the rexinoid IIF, are potent antiproliferative agents in the tumour- derived neuroectodermal SKNMC cells. Moreover, IIF reduces only slightly the proliferation in the NCTC keratinocytes cell line, confirming its low toxicity in non-tumour cells (Papi, 2012). Moreover, IIF modulate the production of numerous pro-inflammatory cytokines as IL6 and the transcription factor AP-1. Consequently, IIF have an anti-invasive effect reducing the MMP2 and MMP9 activity in SKNMC cells.

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Conflict of interest: The authors declare that they do not have any conflict of interest.

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