Published in final edited form as:

Toxicol Appl Pharmacol. 2009 May 15; 237(1): 22–28. doi:10.1016/j.taap.2009.02.021.

Down modulation of IFN- γ Signaling in Alveolar Macrophages Isolated from Smokers

Navneet K. Dhillon^{1,*}, William J. Murphy^{2,*}, Michael B. Filla², Ana J. Crespo³, Heath A. Latham², and Amy O'Brien-Ladner²

¹ Division of Pulmonary and Critical Care Medicine, Department of Molecular & Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas, USA

² Department of Medicine, University of Kansas Medical Center, Kansas City, Kansas, USA

³ The Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

Abstract

The master cytokine, IFN- γ possesses a wide spectrum of biological effects and is crucial for development of the highly activated macrophage phenotype characteristically found during inflammation. However, no data exists regarding the potential influence of cigarette smoke on the status of the expression of the cell surface receptor for IFN- γ (IFN- γ R) on alveolar macrophages (AM) of smokers. Here in, we report reduction in the expression of the IFN- γ R α -chain on AM of cigarette smokers, when compared with nonsmokers. Ensuing from the loss of receptor expression on the AM of smokers there was a decrease in IFN- γ -mediated cell signaling. This included a decrease in the phosphorylation of signal transducer and activator of transcription (STAT)-1 and induction of interferon regulatory factor (IRF)-1. Further, diminished activation/induction of transcription factors did not appear to result from induction of known members of the 'suppressors of cytokine signaling (SOCS)' family. Decreased IFN- γ signal transduction in AM from smokers may have an important implication regarding the use of therapeutic IFN- γ in the lungs of patients that develop respiratory disorders as a result of tobacco use.

Keywords

Transcription factor; monocytes/macrophage; signal transduction; inflammation; cytokine receptors

INTRODUCTION

Smoking-related morbidity encompasses many organ systems, but none more so than the lung. The acute and chronic impairment of immune function associated with smoking has been well

Corresponding Author: Amy O'Brien-Ladner, MD, Division of Pulmonary and Critical Care Medicine, University of Kansas Medical Center, 3901 Rainbow, Mail Stop 3007, Kansas City, KS 66160. Email: E-mail: ALadner@kumc.edu.

*The first two authors contributed equally to the study.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

described, yet there continues to be no cohesive model that explains precisely how smoking causes disease within the human lung.

Multiple defense mechanisms, directed against infection and malignancy, are operative throughout the human lung, with the alveolar macrophages (AM) playing a sentinel role in immune surveillance within the alveolar space. However, significant changes have been found to occur in AM isolated from smokers, when compared with those isolated from non-smokers (Harris *et al.*, 1970; Barbers *et al.*, 1987; Janoff *et al.*, 1987; Brown *et al.*, 1989). The numbers of AM have been reported to increase in smokers (Barbers *et al.*, 1987; Janoff *et al.*, 1987) and these macrophages show: a) changes in redox status (Rahman and MacNee, 1996); b) an accumulation of iron (McGowan and Henley, 1988; Thompson *et al.*, 1991; Ghio *et al.*, 1997) and ; c) an increased rate of glucose utilization (Harris *et al.*, 1970). In addition, previous studies from our lab and by others on AM from smokers suggest attenuated secretion of IL-1 (Brown *et al.*, 1989; Yamaguchi *et al.*, 1989; O'Brien-Ladner *et al.*, 1998), TNF- α (Yamaguchi *et al.*, 1993; O'Brien-Ladner *et al.*, 1998), and IL-6 (Soliman and Twigg, 1992), upon stimulation with LPS compared with AM isolated from non-smokers.

The responsiveness of macrophages to LPS, can be greatly enhanced by their prior or simultaneous exposure to interferon- γ (IFN- γ) (Gifford and Lohmann-Matthes, 1987; Scheibenbogen and Andreesen, 1991). This cytokine possesses a wide spectrum of biological effects and is crucial for development of the highly activated macrophage phenotype characteristically found during inflammation (Murray, 1988; Williams *et al.*, 1999). Activation of the IFN- γ -signaling pathway within the macrophage begins by binding of this cytokine to the cell surface IFN- γ receptor (IFN- γ R). The receptor is constitutively present on virtually every nucleated cell, but several studies have shown that the usual constitutive expression of this protein can be increased after addition of external stimuli, such as IL-1 (Krakauer and Oppenheim, 1993), TNF- α (Sanceau *et al.*, 1992; Krakauer and Oppenheim, 1993) or IL-6 (Braun *et al.*, 1998). The IFN- γ R complex consists of two subunits: a ligand-binding protein, the alpha (α)-chain; and a species-specific accessory factor, or beta (β)-chain (Bach *et al.*, 1997). All of the physiologic and pathogenic effects of IFN- γ are dependant upon its binding to α -chain of the receptor complex. The extracellular domain of IFN- γ α -chain binds the IFN- γ whereas the intracellular domain has the constitutive binding site for Janus kinase (JAK)1 and the IFN- γ -dependent tyrosine phosphorylation site for docking of transcription factor, signal transducer and activator of transcription-1 (STAT1) (Greenlund *et al.*, 1994). IFN- γ R β chain does not directly interact with ligand but is known to increase the association of IFN- γ with the α -chain and is required to confer responsiveness to IFN- γ (Skrenta *et al.*, 2000) through the binding site for JAK2 tyrosine kinase on its intracellular domain (Sakatsume *et al.*, 1995). Direct binding of IFN- γ to IFN- γ R α chain on cell surface results in dimerization of α and β chains. This binding of IFN- γ to its receptor results in transphosphorylation and activation of JAK kinases followed by phosphorylation of STAT1 (Darnell, 1998) which is then known to regulate immune/inflammatory responses, cell proliferation, apoptosis and antiviral/anti-bacterial responses (Chesler and Reiss, 2002; Bruder *et al.*, 2006; Gattoni *et al.*, 2006, b; Gattoni *et al.*, 2006a). The complete activation of Stat 1 by IFN- γ requires phosphorylation of both: a) Tyrosine 701 and, b) Serine 727 (Bach *et al.*, 1997; Stark *et al.*, 1998). While two reports from the same laboratory demonstrated that exposure of a mouse macrophage cell line to cigarette smoke constituents down regulated their responsiveness to added IFN- γ (Braun *et al.*, 1998; Edwards *et al.*, 1999) nothing is known regarding the *in-vivo* status of IFN-signaling in cigarette smokers. In the present study we evaluated the potential influence of cigarette smoke on the status of expression of IFN- γ R and the related downstream signal transduction in AM isolated from smokers.

We show here that chronic exposure of the alveolar space to cigarette smoke may lead to a loss of IFN- γ R α -chain expression on AM and a consequent down regulation of IFN- γ -mediated

signaling. These findings indicate that the augmentative role played by IFN- γ during immune surveillance may be significantly curtailed in the lungs of smokers. This observation may have widespread significance with respect to infection, the development of cancers, and the treatment of such, in the lungs of smokers.

MATERIALS & METHODS

Volunteer subjects

Eight healthy, smoking volunteers and four nonsmoking volunteers underwent bronchoalveolar lavage for the isolation of AM. Mean ages were 39.5 ± 3.0 years for smokers and 40 ± 4.7 for non-smokers. Smokers reported an average consumption of 39 ± 13.8 pack-years [pack-years = (number of pack/day) $\times$ (number of years smoked)]. None of the subjects used for this study were taking medication, had a history of pulmonary disease, or had a recent upper respiratory tract infection. Subjects were given physical exams, each of which proved normal, and no subject showed evidence of lung disease, as judged by pulmonary function tests. All gave informed consent according to a protocol that had received prior approval by the institutional human subject's committee.

Alveolar macrophage isolation and treatment

AM were recovered from BAL as described previously (O'Brien-Ladner *et al.*, 1998). Lavage fluid was filtered through four layers of sterile gauze and cells were pelleted by centrifugation ($400 \times g$ for 10 min). The cell pellet was washed three times with RPMI-1640 followed by resuspension in RPMI-1640 medium supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin and 10% heat inactivated fetal bovine serum. Cell viability was determined by trypan blue exclusion, and cells were counted in a hemocytometer. A differential cell count and purity of AM was determined using a cytopsin slide preparation stained with Diff-Quik. Approximately 2.5×10^6 AM were re-suspended into 10 ml of RPMI medium and then seeded onto 100 mm tissue culture dishes and allowed to adhere for 1h. AM isolated from either smokers or from non-smokers, had no difference in their ability to adhere to the surface. Dishes with 80–90% confluent adherent cells were then subsequently incubated for various time intervals either in the presence of medium alone or medium that contained 200 U/ml human IFN- γ (Upstate Biotechnology, Lake Placid, NY).

Preparation of protein extracts

After 1 hour adherence to assure macrophage purification, AM isolated from both smokers and non-smokers, were used for soluble extract (SE) preparation. Briefly, AM were washed twice with ice cold phosphate buffered saline (PBS) followed by resuspension of cells in SE buffer, which consisted of PBS containing 1% Triton X-100, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 2 mM EDTA. The lysate was incubated on ice for 30 min and then centrifuged at $100,000 \times g$ for 30 min at 4°C . The SE was then aliquoted and stored at -70°C until used for analysis.

Whole cell extracts (WCE) were prepared from cultured AM that were incubated in the presence or absence of IFN- γ . Extracts were prepared as described by Gao *et al.* (Gao *et al.*, 1998) and then aliquoted and stored at -70°C until used.

Protein extracts of mouse brain were used as a positive control for the binding of antibodies raised against 'suppressor of cytokine signaling-1' (SOCS-1) and SOCS-3, (Santa Cruz Biotech, Santa Cruz, CA).

Western blot analysis

Equal amount of protein (25µg) samples from SE or WCE, were run on a 12% SDS-polyacrylamide gel in reducing conditions followed by transfer onto a PVDF membrane as described by Gao *et al* (Gao *et al.*, 1998). The blots were blocked with 5% non-fat dry milk in phosphate buffered saline. Western blots were probed with anti-human-IFN-γR α- and β-chain antibodies (Santa Cruz Biotech), anti-p84/p91 antibodies (anti-total 'signal transducer and activator of transcription 1' (Stat1) protein), anti-tyrosine-phosphorylated Stat1 (Zymed Laboratories, Inc., South San Francisco, CA), anti-serine-phosphorylated Stat1 antibodies (Zymed Laboratories, Inc.), anti-'interferon regulatory factor-1' (IRF-1), and anti-SOCS-1 and anti-SOCS-3 proteins (each from Santa Cruz Biotech). The secondary antibodies used were horseradish peroxidase-conjugated anti mouse or anti rabbit (1:5000, Pierce Chemical Co., Rockford, IL) and detection was performed using the enhanced chemiluminescence system (Pierce Chemical Co.). Where appropriate, membranes were stripped, reblocked, and sequentially reprobed with subsequent antibodies. Autoradiographic images were densitometrically scanned using a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI and analyzed with the ImageQuaNT software package (Molecular Dynamics).

In some experiments, SE were first treated with peptide *N*-glycosidase F (PNGase F) prior to western blot analysis to remove N-linked oligosaccharides from the peptide backbone (Fischer *et al.*, 1990). Briefly, cellular lysate was denatured by boiling for 10 min in extraction buffer supplemented with 0.5% SDS and 1% β-mercaptoethanol, cooled and mixed with 50 mM sodium phosphate buffer (pH 7.5) and 1% NP-40. Samples were then digested with PNGase F (New England Biolabs, Beverly, MA) at 4×10^5 U/ml for 1h at 37°C followed by western blot analysis.

Statistical Analysis

Statistical analysis was performed using one-way or two-way analysis of variance with a post hoc Student t test. Results were judged statistically significant if $p < 0.05$ by analysis of variance.

RESULTS

Reduced expression of IFN-γR α-chain on AM isolated from smokers

In order to examine the effects of cigarette smoking on expression of the IFN-γR α-chain on the surfaces of AM, western blot analysis was performed using soluble extract prepared from cells isolated from 4 non-smokers and 8 smokers (Figure 1). The receptor protein displayed a diffuse banding pattern due to the heterogeneous addition of carbohydrate moieties to the polypeptide backbone. Nonetheless, the diffuse staining pattern of around 90kDa, evident in extracts prepared from AM of non-smokers, was considerably reduced or absent in extracts prepared from smokers' AM (Figure 1A). Densitometric analysis of band intensities revealed a significant decrease (p value=0.005) in IFN-γR α-chain/ β-actin ratio obtained from smokers compared with non-smokers as shown in figure 1A, lower panel.

Further, to confirm the cell surface nature of the broad bands detected in Figure 1A, and to sharpen the resultant banding pattern, we treated protein extracts with PNGase F prior to western blot analysis. PNGase F is an endoglycosidase that removed the majority of *N*-linked polysaccharide from the IFN-γR α protein backbone. Figure 1B shows a representative blot of SE from AM of 3 non-smokers and 3 smokers treated with or without PNGase F. It can be readily seen in the figure that the broad banding pattern of IFN-γR α-chain observed without treatment were converted to sharper, major band of around 70kDa as reported earlier (Fischer *et al.*, 1990). A comparison of PNGase F-treated and untreated extracts derived from smokers' AM revealed very little difference in banding patterns or band intensities. Densitometric

scanning analysis of PNGase F-treated samples revealed significant decrease (p value=0.05) in expression of N-deglycosylated IFN- γ R α -chain in smokers compared to non-smokers (Figure 1B, lower panel), consistent with the changes observed in the analysis of native IFN- γ R.

In order to determine the alterations in the β -chain of IFN- γ R of AM isolated from smokers, western blots were probed with antibodies against IFN- γ R β -chain. This protein is also very heterogeneous in size and was difficult to detect in western blots of extracts, not digested with PNGase F. However, as shown in Figure 1C, a protein band of appropriate size (38kDa), was observed in PNGase F-treated samples that was not found in untreated samples. β -chain protein was found to be present in both non-smokers and smokers. Interestingly, the blots revealed upregulation of β -chain in smokers compared with non-smokers but this increase was not statistically significant when analyzed by two-tail, t -test (p value=0.06) (Figure 1C, lower panel).

Diminished IFN- γ -mediated signaling in AM of smokers

The primary pathway by which IFN- γ acts as an immunomodulator is through regulation of gene expression via the JAK (Janus kinase)/STAT tyrosine kinase-dependent cascade (Darnell, 1998). To elucidate whether the loss of IFN- γ R α -chain expression observed in smokers' AM resulted in a concomitant loss of IFN- γ -induced responsiveness, we investigated the activation of transcription factor Stat1. Human AM isolated from non-smokers or smokers were stimulated with IFN- γ for 0.5 and 4h followed by cell lysis and analysis of transcription factors. As shown in one of the representative western blot analysis (Figure 2A), treatment of AM with IFN- γ resulted in dramatic phosphorylation of Stat1 at Tyr701 (Stat1-PY) at both time intervals tested in non-smokers, with more phosphorylation at 0.5h compared to 4h stimulation. However, very little Tyr-phosphorylation STAT-1 was observed in AM from smokers, on stimulation with IFN- γ . To determine whether the decreased phosphorylation of Stat1 was the result of diminished accumulation of Stat1 proteins, we stripped and reprobed the blot with anti-Stat1 (p84/p91) antibodies which can detect both isoforms of Stat1; Stat1 α (91kDa) and the splice variant Stat1 β (84kDa). Both Stat1 α and Stat1 β were detectable in untreated extracts prepared from the nonsmoker (Figure 2A). This level of Stat1 α / β expression was elevated after treatment of AM with IFN- γ for either 0.5h or 4h consistent with a previous report from our laboratory (Gao *et al.*, 1998). However, the overall levels of detectable steady-state levels of Stat1 α / β proteins were observed to be significantly decreased in the smokers. As shown in Figure 2B, the level of total Stat-1 protein was lower in AM from smokers than in AM from non-smokers, when normalized with β -actin (p value <0.05 non-smokers vs. smokers at 0h). While treatment of smokers' AM with IFN- γ increased the accumulation of Stat1 α and Stat1 β proteins, this level of expression was less than that observed in non-smokers (p <0.01 at 0.5h and p <0.05 at 4.0h, smokers vs. non-smokers). Although, the levels of Stat-1 in response to IFN- γ stimulation were significantly decreased in AM from smokers compared to AM from non-smokers, the fold increase in Stat1 α / β on IFN- γ stimulation relative to before stimulation (at 0h) within the group was almost same. We found 2.6 & 2.1 fold increase at 0.5h and 4.0h, respectively in AM from non-smokers and 2.55 & 1.93 fold increase in AM from smokers relative to un-stimulated cells.

Densitometry analysis of Stat1-PY showed significant decrease in Stat1-PY when normalized with total STAT1 at both 0.5h (p value=0.03) and 4h (p value=0.05) post-stimulation in smokers' AM compared to an increase in density of Stat1-PY band with IFN- γ treatment within non-smokers' AM (Figure 2C). So apart from a decrease in the amount of available total Stat1 α / β there was reduced IFN signaling that resulted in diminished ability of IFN- γ to induce Stat1 phosphorylation at Tyr 701, in smokers' AM as compared to non-smokers.

Similarly, the phosphorylation of Stat1 α at Ser 727 (Stat1-PS) when normalized to total STAT1 was also increased following treatment with IFN- γ within AM acquired from non-smokers. IFN- γ also induced Stat1 α phosphorylation at Ser 727 in smokers' AM, but the extent to which Stat1-PS/total STAT1 ratio increased was greatly reduced in smokers when compared to non-smokers at corresponding post-stimulation times (Figure 2A). Densitometric analysis of scanned images revealed a significant decrease in Stat1-PS/Stat1 ratio in IFN- γ stimulated AM isolated from smokers at both the time points tested (p value<0.001) when compared with non-smokers (Figure 2D). The Stat1 β protein, a truncated form of Stat1 α , lacks the necessary serine residue (Schindler *et al.*, even in the un-stimulated AM from non-smokers whereas Stat1-PS was observed only by IFN- γ -stimulation in AM from smokers.

Activation of Jak/Stat pathway leads to increase in expression of many IFN-responsive target genes including transcription factors like interferon regulatory factor (IRF). In order to examine the ability of IFN- γ to induce new gene expression, we assayed for the induction of IRF-1 in AM isolated from non-smokers and smokers. Consistent with previous reports (Hayes and Zoon, 1993) we observed constitutive expression of IRF-1 in untreated AM isolated from non-smokers (Figure 2A). Because the appearance of newly induced IRF-1 requires both *de novo* gene transcription and protein synthesis, there is little additional IRF-1 protein detected after 0.5h of IFN- γ treatment, but considerable new protein after 4h of treatment. Nevertheless, the pattern of IRF-1 expression in AM from smokers differed markedly from that observed in AM from non-smokers. In smokers, IRF-1 was virtually undetectable in both untreated AM and in AM that were treated with IFN- γ for 30 minutes. Densitometric scans of untreated AM or treated with IFN- γ for 0.5h (Figure 2E) confirmed the absence of IRF-1 in smokers. However, after AM had been treated with IFN- γ for 4 hours, differences in IRF-1 levels of smokers and non-smokers were not statistically significant (p = 0.22), presumably because of the high variability in expression of IRF-1 in smokers.

Reduction in IFN- γ -responsive signaling in smokers does not correlate with increase in cytokine signaling inhibitors

A wide array of cytokines and growth factors induce the synthesis of multiple suppressors of cytokine signaling (SOCS) proteins, which in turn inhibit the JAK /STAT signaling pathway (Starr *et al.*, 1997). To determine whether SOCS proteins might be involved in the suppression of IFN- γ -signaling, we assayed, by western blot analysis, for the presence or absence of the two most commonly induced SOCS family members, SOCS-1 and SOCS-3. When blots of the protein extracts from AM of smokers and non-smokers were probed with anti-SOCS-1 antibody, no protein was detectable in either the non-smokers or smokers group (data not shown), although the SOCS-1 band did appear in the tissue extract from mouse brain used as a positive control. However, when blots were probed with anti-SOCS-3, it was found that AM from both non-smokers (n =4) and smokers (n =8) expressed equivalent amounts of SOCS-3 protein (Figure 3A). Analysis of densitometric scans showed no statistical significant difference between the ratio of SOCS-3/ β -actin band intensities in AM extracts from smokers and non-smokers (P = 0.23) (Figure 3B). We conclude from this study that there is no difference in SOCS-3 expression between the AM of smokers and non-smokers and, consequently, that reduced IFN- γ -mediated signaling in the smokers' AM is not due to the result of over-expression of the SOCS family of suppressors.

DISCUSSION

In this study we have shown a significant down-regulation in expression of the α -chain of the IFN- γ R, on surfaces of AM acquired from smokers, when compared with non-smokers whereas no significant change was demonstrated in the expression of IFN- γ R β -chain within the groups. Furthermore, signal transduction and gene induction, in response to added IFN- γ , is reduced

in the AM of smokers, in comparison with non-smokers. These results are important in that they demonstrate a potential defect in the IFN- γ -directed immune defense of the smoker's lung.

The differential regulation of the α - and β -chain of IFN- γ R, in response to tobacco smoke, is likely due to the distinct organization of their promoters. The α -chain promoter contains several candidate *cis*-acting DNA elements that might be responsive to external stimuli. These include Sp1, AP-1, AP-2, CREB (Merlin *et al.*, 1997). The β -chain gene, on the other hand, appears to have a more limited potential for regulation because its promoter primarily contains candidate Sp1 and AP-2 sequences, and one candidate Pu-box (Rhee *et al.*, 1996). Thus, differences in the promoters of the receptor subunits' genes may account, at least in some part, for the discrepancy in expression of α -chain of the IFN- γ R protein as compared to the IFN- γ R β -chain protein. Furthermore in CD4+T cells (Sakatsume and Finbloom, 1996), down regulation of β -chain on differentiation into the Th1 type, has been reported to be dependent on IFN- γ binding whereas down regulation of α -chain on T cell activation, has been reported to be IFN- γ independent (Skrenta *et al.*, 2000). Similar differential regulation of α - and β -chain may exist in macrophages and the increase in β -chain of the receptor, although not significant, in AM smokers, therefore, may be the result of a feedback response of the cells to the lack of IFN- γ -signaling. However, previous studies by Celada *et al.* (Celada and Schreiber, 1987) demonstrated no change in the expression of IFN- γ receptor on macrophages on binding with IFN- γ . They suggested that constant presence of intracellular receptors and constitutive recycling of receptors to the cell surface, maintains same level of cell-surface receptors on macrophages in the absence or presence of IFN- γ . Furthermore, no report exist on the alteration or regulation of IFN- γ receptor in response to cigarette smoking, however, an alternative mechanism similar to that found by recent study by HuangFu *et al.* (HuangFu *et al.*, 2008) on mouse fibroblasts, reporting down-regulation of IFNAR1 chain of IFN α / β receptor on exposure to cigarette smoke through phosphorylation-dependent ubiquitination, may be possible. Due to the concern regarding risk to human subjects with bronchoscopy we limited this study after finding statistically significant changes in the protein expression of the IFN- γ receptor. Certainly, future experiments are needed to examine the molecular mechanisms involved in the down-modulation of IFN- γ R- α -chain in response to cigarette smoke exposure.

IFN- γ activates the Jak/Stat pathway through, α - and β -subunits of IFN- γ receptor (Darnell, 1998; Lackmann *et al.*, 1998). In almost all cells IFN- γ binds to the α - and β -subunits of its receptor leading to activation of Jak1 and Jak2 kinases followed by phosphorylation of Stat1 (Muller *et al.*, 1993; Shuai *et al.*, 1993; Darnell, 1998). The IFN- γ -induced accumulation of Stat1 α / β in AM from either smokers or non-smokers, and the decreased availability of these proteins in AM isolated from smokers in this study, is consistent with our previous observations in mouse macrophages (Gao *et al.*, 1998). In mouse macrophages we demonstrated induction of Stat1 α / β protein synthesis by IFN- γ as well as by IFN- β and LPS. Furthermore, peritoneal macrophages prepared from mice in which the IFN- γ gene had been targeted for deletion showed considerably lower steady-state levels of Stat1 α and β (Gao *et al.*, 1998) similar to the decreased availability of Stat1 α / β in AM isolated from smokers in the present study. Thus, we hypothesize that fully functional IFN- γ related receptor/ligand interactions may be necessary for the maintenance of homeostatic levels of Stat1 α / β . In addition, the same levels of IFN- γ -induced increase in the expression of Stat1 α / β relative to the un-stimulated levels within smokers or non-smokers may indicate that both groups were equally responsive to IFN- γ but differ in the number of functional receptors as confirmed by the decrease in α -chain of the IFN- γ R in smokers. However, following treatment of smokers' AM with IFN- γ , phosphorylation of Stat1 at Tyr 701 and at Ser 727 when normalized with total Stat1 α / β was significantly lower in smokers than in non-smokers and this may be due to changes in IFN-induced activity. Absence of IRF-1 expression in un-stimulated AM from smokers and decreased induction of IRF-1 in response to IFN- γ further confirmed the defect in IFN- γ -signaling in smokers

Although this study was limited by the availability of human AM acquired by bronchoscopy, the results reported may provide insight into the variable effect of IFN- γ on both lung cancer cell lines and on AM isolated from lung cancer patients, as well as into the general failure of IFN- γ -based immunotherapy modalities for the treatment of lung cancer. IFN- γ had a variable effect on proliferation of several small cell lung cancer (SCLC) cell lines and induced the synthesis of MHC class I molecules by more than twofold in only 4 of eight of these lines (Ball *et al.*, 1986). IFN- γ also failed to inhibit colony formation among 3-of-4 non-small cell lung cancer (NSCLC) cell lines tested by Hong *et al.* (Hong *et al.*, 1987). Clonogenic assays similarly showed a variable antiproliferative effect of IFN- γ on SCLC and NSCLC cell lines (Jabbar and Twentyman, 1990), with SCLC lines apparently being more susceptible to the effects of IFN- γ than were the NSCLC lines (Jabbar and Twentyman, 1990; Prior *et al.*, 1998). Furthermore, AM isolated from 3-of-7 lung cancer patients, in the study of Halme *et al.* (Halme *et al.*, 1995), failed to show an IFN- γ -induced increase in oxidative burst, over pre-IFN- treatment samples.

Clinical trials, utilizing an intravenous (Schiller *et al.*, 1989) or subcutaneous (Jett *et al.*, 1994; van Zandwijk *et al.*, 1997) route of IFN- γ administration, have failed to demonstrate improved survival or time-to-progression among either SCLC (Jett *et al.*, 1994; van Zandwijk *et al.*, 1997) or NSCLC (Schiller *et al.*, 1989) patients. In addition, IFN- γ failed to improve upon the efficacy of either cisplatin or etoposide in combinatory trials (Schiller *et al.*, 1989). Interestingly, intrapleural instillation of IFN- γ showed potential as a therapeutic agent in that NSCLC cells disappeared from the pleural effusion after treatment (Schiller *et al.*, 1989; Yanagawa *et al.*, 1997). In none of the studies cited above was the question of expression of the receptor for IFN- γ , either on AM, lung cancer cells, or lung cancer cell lines, addressed. Because the vast majority of lung cancers arise as the result of cigarette smoking, determining the presence or absence of the IFN- γ R is of paramount importance.

Although the IFN- γ R is constitutively present on virtually every nucleated cell type, it is clear that expression of the receptor may be modulated negatively. Thus, the potential exists for the restoration of IFN- γ R levels in the lungs of smokers and this could also lead to the development of more efficacious treatment therapies, based on the instillation of the IFN- γ into the lung. In any case, full understanding of the molecular mechanisms involved in the regulation of IFN- γ R expression is essential.

Acknowledgements

The authors dedicate this paper to the fond memory of Joseph C. Cates. This work was supported, in part, by an NIH IDeA grant (P20 RR11825) (AOL), Wilkinson Endowment for Cancer Research, American Lung Association - Kansas (AOL. and WJM.), Joseph C. Cates Family Foundation (AOL), Parker B. Francis Research Fellowship (ND) and by a scholarship (BD/9586/96) from Fundação para Ciência e Tecnologia, Portugal (AC.).

Abbreviations

AM	alveolar macrophage
LPS	bacterial lipopolysaccharide
IFN-γ	interferon- γ
IFN-γR	IFN- γ receptor

IRF-1	interferon regulatory factor-1
NSCLC	non-small cell lung cancer
PNGase F	peptide <i>N</i> -glycosidase F
SCLC	small cell lung cancer
SOCS	suppressor of cytokine signaling
SE	soluble extract
STAT (or Stat)	signal transducer and activator of transcription
WCE	whole cell extract

References

- Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annual review of immunology* 1997;15:563–591.
- Ball ED, Sorenson GD, Pettengill OS. Expression of myeloid and major histocompatibility antigens on small cell carcinoma of the lung cell lines analyzed by cytofluorography: modulation by gamma-interferon. *Cancer research* 1986;46:2335–2339. [PubMed: 3008987]
- Barbers RG, Gong H Jr, Tashkin DP, Oishi J, Wallace JM. Differential examination of bronchoalveolar lavage cells in tobacco cigarette and marijuana smokers. *The American review of respiratory disease* 1987;135:1271–1275. [PubMed: 3592403]
- Braun KM, Cornish T, Valm A, Cundiff J, Pauly JL, Fan S. Immunotoxicology of cigarette smoke condensates: suppression of macrophage responsiveness to interferon gamma. *Toxicology and applied pharmacology* 1998;149:136–143. [PubMed: 9571981]
- Brown GP, Iwamoto GK, Monick MM, Hunninghake GW. Cigarette smoking decreases interleukin 1 release by human alveolar macrophages. *The American journal of physiology* 1989;256:C260–264. [PubMed: 2784033]
- Bruder D, Srikiatkachorn A, Enelow RI. Cellular immunity and lung injury in respiratory virus infection. *Viral Immunol* 2006;19:147–155. [PubMed: 16817757]
- Celada A, Schreiber RD. Internalization and degradation of receptor-bound interferon-gamma by murine macrophages. Demonstration of receptor recycling. *J Immunol* 1987;139:147–153. [PubMed: 2953810]
- Chesler DA, Reiss CS. The role of IFN-gamma in immune responses to viral infections of the central nervous system. *Cytokine Growth Factor Rev* 2002;13:441–454. [PubMed: 12401479]
- Darnell JE Jr. Studies of IFN-induced transcriptional activation uncover the Jak-Stat pathway. *J Interferon Cytokine Res* 1998;18:549–554. [PubMed: 9726434]
- Edwards K, Braun KM, Evans G, Sureka AO, Fan S. Mainstream and sidestream cigarette smoke condensates suppress macrophage responsiveness to interferon gamma. *Hum Exp Toxicol* 1999;18:233–240. [PubMed: 10333308]
- Fischer T, Thoma B, Scheurich P, Pfizenmaier K. Glycosylation of the human interferon-gamma receptor. N-linked carbohydrates contribute to structural heterogeneity and are required for ligand binding. *The Journal of biological chemistry* 1990;265:1710–1717. [PubMed: 2136857]

- Gao JJ, Filla MB, Fultz MJ, Vogel SN, Russell SW, Murphy WJ. Autocrine/paracrine IFN- α mediates the lipopolysaccharide-induced activation of transcription factor Stat1 α in mouse macrophages: pivotal role of Stat1 α in induction of the inducible nitric oxide synthase gene. *J Immunol* 1998;161:4803–4810. [PubMed: 9794412]
- Gattoni A, Parlato A, Vangieri B, Bresciani M, Derna R. Interferon-gamma: biologic functions and HCV therapy (type I/II) (2 of 2 parts). *Clin Ter* 2006a;157:457–468. [PubMed: 17147054]
- Gattoni A, Parlato A, Vangieri B, Bresciani M, Derna R. Interferon-gamma: biologic functions and HCV therapy (type I/II) (1 of 2 parts). *Clin Ter* 2006b;157:377–386. [PubMed: 17051976]
- Ghio AJ, Pritchard RJ, Dittrich KL, Samet JM. Non-heme (Fe³⁺) in the lung increases with age in both humans and rats. *The Journal of laboratory and clinical medicine* 1997;129:53–61. [PubMed: 9011591]
- Gifford GE, Lohmann-Matthes ML. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. *Journal of the National Cancer Institute* 1987;78:121–124. [PubMed: 3099049]
- Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *Embo J* 1994;13:1591–1600. [PubMed: 8156998]
- Halme M, Maasilta P, Repo H, Ristola M, Taskinen E, Mattson K, Cantell K. Inhaled recombinant interferon gamma in patients with lung cancer: pharmacokinetics and effects on chemiluminescence responses of alveolar macrophages and peripheral blood neutrophils and monocytes. *International journal of radiation oncology, biology, physics* 1995;31:93–101.
- Harris JO, Swenson EW, Johnson JE 3rd. Human alveolar macrophages: comparison of phagocytic ability, glucose utilization, and ultrastructure in smokers and nonsmokers. *The Journal of clinical investigation* 1970;49:2086–2096. [PubMed: 4319967]
- Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide responses: expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. *Infection and immunity* 1993;61:3222–3227. [PubMed: 8335353]
- Hong WS, Jett JR, Sasaki Y, Takahashi H, Nakano H, Nakagawa K, Hoshi A, Saijo N. Colony inhibitory effect of recombinant human tumor necrosis factor (rH-TNF) and/or recombinant human interferon (rH-IFN)-alpha, -beta and -gamma on human lung cancer cell lines. *Japanese journal of clinical oncology* 1987;17:49–55. [PubMed: 2435930]
- HuangFu WC, Liu J, Harty RN, Fuchs SY. Cigarette smoking products suppress antiviral effects of Type I interferon via phosphorylation-dependent downregulation of its receptor. *FEBS Lett* 2008;582:3206–3210. [PubMed: 18722370]
- Jabbar SA, Twentyman PR. The use of clonogenic assays in assessing the response of human lung cancer cell lines to alpha and gamma interferons alone or in combination with adriamycin. *International journal of cancer* 1990;46:546–551.
- Janoff A, Pryor WA, Bengali ZH. NHLBI workshop summary. Effects of tobacco smoke components on cellular and biochemical processes in the lung. *The American review of respiratory disease* 1987;136:1058–1064. [PubMed: 3310769]
- Jett JR, Maksymiuk AW, Su JQ, Mailliard JA, Krook JE, Tschetter LK, Kardinal CG, Twito DI, Levitt R, Gerstner JB. Phase III trial of recombinant interferon gamma in complete responders with small-cell lung cancer. *J Clin Oncol* 1994;12:2321–2326. [PubMed: 7964947]
- Krakauer T, Oppenheim JJ. IL-1 and tumor necrosis factor-alpha each up-regulate both the expression of IFN-gamma receptors and enhance IFN-gamma-induced HLA-DR expression on human monocytes and a human monocytic cell line (THP-1). *J Immunol* 1993;150:1205–1211. [PubMed: 8432976]
- Lackmann M, Harpur AG, Oates AC, Mann RJ, Gabriel A, Meutermans W, Alewood PF, Kerr IM, Stark GR, Wilks AF. Biomolecular interaction analysis of IFN gamma-induced signaling events in whole-cell lysates: prevalence of latent STAT1 in high-molecular weight complexes. *Growth factors (Chur, Switzerland)* 1998;16:39–51.
- McGowan SE, Henley SA. Iron and ferritin contents and distribution in human alveolar macrophages. *The Journal of laboratory and clinical medicine* 1988;111:611–617. [PubMed: 3373107]

- Merlin G, van der Leede BJ, McKune K, Knezevic N, Bannwarth W, Romquin N, Viegas-Pequignot E, Kiefer H, Aguet M, Dembic Z. The gene for the ligand binding chain of the human interferon gamma receptor. *Immunogenetics* 1997;45:413–421. [PubMed: 9089099]
- Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, Silvennoinen O, Harpur AG, Barbieri G, Witthuhn BA, Schindler C, et al. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature* 1993;366:129–135. [PubMed: 8232552]
- Murray HW. Interferon-gamma, the activated macrophage, and host defense against microbial challenge. *Annals of internal medicine* 1988;108:595–608. [PubMed: 3126690]
- O'Brien-Ladner AR, Blumer BM, Wesselius LJ. Differential regulation of human alveolar macrophage-derived interleukin-1beta and tumor necrosis factor-alpha by iron. *The Journal of laboratory and clinical medicine* 1998;132:497–506. [PubMed: 9851740]
- Prior C, Thaler J, Huber H. Comments on Role of recombinant interferon-gamma maintenance in responding patients with small cell lung cancer. A randomised phase III study of the EORTC Lung Cancer Cooperative Group, van Zandwijk et al., *Eur J Cancer* 1997, 33, 1759–1766. *Eur J Cancer* 1998;34:1638. [PubMed: 9893643]
- Rahman I, MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free radical biology & medicine* 1996;21:669–681. [PubMed: 8891669]
- Rhee S, Ebensperger C, Dembic Z, Pestka S. The structure of the gene for the second chain of the human interferon-gamma receptor. *The Journal of biological chemistry* 1996;271:28947–28952. [PubMed: 8910544]
- Sakatsume M, Finbloom DS. Modulation of the expression of the IFN-gamma receptor beta-chain controls responsiveness to IFN-gamma in human peripheral blood T cells. *J Immunol* 1996;156:4160–4166. [PubMed: 8666783]
- Sakatsume M, Igarashi K, Winestock KD, Garotta G, Larner AC, Finbloom DS. The Jak kinases differentially associate with the alpha and beta (accessory factor) chains of the interferon gamma receptor to form a functional receptor unit capable of activating STAT transcription factors. *The Journal of biological chemistry* 1995;270:17528–17534. [PubMed: 7615558]
- Sanceau J, Merlin G, Wietzerbin J. Tumor necrosis factor-alpha and IL-6 up-regulate IFN-gamma receptor gene expression in human monocytic THP-1 cells by transcriptional and post-transcriptional mechanisms. *J Immunol* 1992;149:1671–1675. [PubMed: 1387149]
- Scheibenbogen C, Andreesen R. Developmental regulation of the cytokine repertoire in human macrophages: IL-1, IL-6, TNF-alpha, and M-CSF. *Journal of leukocyte biology* 1991;50:35–42. [PubMed: 2056245]
- Schiller JH, Storer B, Dreicer R, Rosenquist D, Frontiera M, Carbone PP. Randomized phase II–III trial of combination beta and gamma interferons and etoposide and cisplatin in inoperable non-small cell cancer of the lung. *Journal of the National Cancer Institute* 1989;81:1739–1743. [PubMed: 2553993]
- Schindler C, Fu XY, Improta T, Aebersold R, Darnell JE Jr. Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha. *Proceedings of the National Academy of Sciences of the United States of America* 1992;89:7836–7839. [PubMed: 1502203]
- Shuai K, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ, Darnell JE. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. *Nature* 1993;366:580–583. [PubMed: 7504784]
- Skrenta H, Yang Y, Pestka S, Fathman CG. Ligand-independent down-regulation of IFN-gamma receptor 1 following TCR engagement. *J Immunol* 2000;164:3506–3511. [PubMed: 10725704]
- Soliman DM, Twigg HL 3rd. Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *The American journal of physiology* 1992;263:L471–478. [PubMed: 1415725]
- Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annual review of biochemistry* 1998;67:227–264.
- Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ. A family of cytokine-inducible inhibitors of signalling. *Nature* 1997;387:917–921. [PubMed: 9202125]

- Thompson AB, Bohling T, Heires A, Linder J, Rennard SI. Lower respiratory tract iron burden is increased in association with cigarette smoking. *The Journal of laboratory and clinical medicine* 1991;117:493–499. [PubMed: 2045717]
- van Zandwijk N, Groen HJ, Postmus PE, Burghouts JT, ten Velde GP, Ardizzoni A, Smith IE, Baas P, Sahmoud T, Kirkpatrick A, Dalesio O, Giaccone G. Role of recombinant interferon-gamma maintenance in responding patients with small cell lung cancer. A randomised phase III study of the EORTC Lung Cancer Cooperative Group. *Eur J Cancer* 1997;33:1759–1766. [PubMed: 9470829]
- Williams MA, Newland AC, Kelsey SM. The potential for monocyte-mediated immunotherapy during infection and malignancy. Part I: apoptosis induction and cytotoxic mechanisms. *Leukemia & lymphoma* 1999;34:1–23. [PubMed: 10350328]
- Yamaguchi E, Itoh A, Furuya K, Miyamoto H, Abe S, Kawakami Y. Release of tumor necrosis factor-alpha from human alveolar macrophages is decreased in smokers. *Chest* 1993;103:479–483. [PubMed: 8432140]
- Yamaguchi E, Okazaki N, Itoh A, Abe S, Kawakami Y, Okuyama H. Interleukin 1 production by alveolar macrophages is decreased in smokers. *The American review of respiratory disease* 1989;140:397–402. [PubMed: 2788378]
- Yanagawa H, Haku T, Hiramatsu K, Nokihara H, Takeuchi E, Yano S, Hanibuchi M, Sone S. Intrapleural instillation of interferon gamma in patients with malignant pleurisy due to lung cancer. *Cancer Immunol Immunother* 1997;45:93–99. [PubMed: 9390200]

Figure 1. Comparison of expression of α - and β -chains of the IFN- γ R on AM isolated from smokers and non-smokers

(A) Western blot analysis of soluble extract (SE) protein from AM of 4 non-smokers and 8 smokers using anti-IFN- γ R α -chain antibodies. The blot was reprobed with antibodies raised against human β -actin. The histogram shows the densitometric analysis of the ratio of broad band of IFN- γ R α -chain of around 90-kDa (*bracketed* in the figure) normalized to corresponding β -actin band. (B) Protein from SE shown in (A) was treated with PNGase F, followed by western blot analysis for IFN- γ R α -chain. Densitometric analysis, of the area denoted by the *small bracket* corresponding to PNGase F-treated samples (+) was performed and normalized with β -actin band intensities, as shown by the histogram. (C) Western blot analysis of soluble extract (SE) protein from AM of non-smokers and smokers using anti-IFN- γ R β -chain antibodies. The *arrowhead* demarcates the 35kDa, deglycosylated β -chain (*bottom panel*). The same relative molecular mass is indicated by the *arrowhead* in the untreated samples (*top panel*). Histogram represents the average densitometric ratio of 35kDa, deglycosylated β -chain to β -actin in non-smokers and smokers groups. Statistical significance was calculated using a two-tail, independent, t-test. (** $p \leq 0.01$, * $p \leq 0.05$).

Figure 2. Comparison of IFN- γ R-related signal transduction events in AM isolated from smokers and non-smokers

AM, isolated from non-smokers and smokers, were plated and incubated with or without IFN- γ (200 U/ml) for the times indicated. Following treatment, whole cell extracts (WCE) were prepared and then analyzed by western blot. Representative western blot on WCE of AM from two non-smokers and two smokers stimulated with IFN- γ for 0.5h or 4h is shown (A). Blots were sequentially probed, stripped, and reprobed with: anti-phosphotyrosine Stat1 antibodies; anti-phosphoserine Stat1 antibodies; anti-IRF-1 antibodies; anti-Stat1 (p84/p91); and human anti- β actin antibodies, as shown by the representative blot in the figure. Histograms represent the average densitometric ratio of total STAT-1/ β -actin (B), phosphorylated STAT1 at Tyr701 (STAT1-PY)/STAT-1 (C), phosphorylated STAT1 at Ser 727 (STAT1-PS)/STAT-1 (D), and IRF-1/ β -actin (E) obtained from AM of smokers (n=8) and non-smokers (n=4) on stimulation with IFN- γ (mean + SEM, *p<0.05, **p<0.01, ***p<0.001 vs. non-smokers' AM stimulated for corresponding times).

Figure 3. Constitutive expression of SOCS-3 protein in SE prepared from AM isolated from smokers and non-smokers

A) Aliquots of SE of AM from non-smokers and smokers, represented in Figure 1A were electrophoresed, electroblotted and probed with anti-SOCS-3 antibodies followed by re-probing with anti-β-actin antibodies. As a positive control of SOCS-3 antibody binding, a cellular extract was prepared from mouse brain (see the Materials and Methods) and electrophoresed alongside the AM samples (*lane C*). B) Densitometric analysis of SOCS-3 revealed no significant change between the AM of smokers and non-smokers.