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Inflammatory signaling compromises cell responses to interferon alpha

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Abstract

Interferon alpha (IFNα) is widely used for treatment of melanoma and certain other malignancies. This cytokine as well as the related IFNβ exerts potent anti-tumorigenic effects; however, their efficacy in patients is often suboptimal. Here we report that inflammatory signaling impedes the effects of IFNα/β. Melanoma cells can secrete pro-inflammatory cytokines that inhibit cellular responses to IFNα/β via activating the ligand-independent pathway for the phosphorylation and subsequent ubiquitination and accelerated degradation of the IFNAR1 chain of Type I IFN receptor. Catalytic activity of the p38 protein kinase was required for IFNAR1 downregulation and inhibition of IFNα/β signaling induced by proinflammatory cytokines such as Interleukin 1 (IL-1). Activation of p38 kinase inversely correlated with protein levels of IFNAR1 in clinical melanoma specimens. Inhibition of p38 kinase augmented the inhibitory effects of IFNα/β on cell viability and growth *in vitro* and *in vivo*. The role of inflammation and p38 protein kinase in regulating cellular responses to IFNα/β in normal and tumor cells are discussed.

Keywords

inflammation; cancer; interferon; receptor; ubiquitin; melanoma

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INTRODUCTION

Malignant melanoma affects millions of people worldwide and, considering its rapidly rising incidence and high mortality, represents a major health problem. Although surgery is effective in early melanoma stages, high resistance of melanoma cells to chemo- and radiation therapy compromises post-surgical efforts that prevent recurrence in metastatic disease (Fecher and Flaherty 2009, Rigual et al 2008, Soengas and Lowe 2003). Adjuvant therapy with a recombinant version of endogenous cytokine IFNα is one of the few FDA-approved treatments (Ascierto and Kirkwood 2008, Kaehler et al 2010, Yao et al 2009). Although IFNα exhibits potent anti-proliferative and anti-survival effects towards both tumor cells and endothelial cells of tumor vasculature in vitro (Bracarda et al 2010), its therapeutic efficacy is limited (reviewed in (Mocellin et al 2010)). Overcoming these limitations requires a better understanding of the mechanisms that temper the effects of pharmaceutical IFNα in patients.

Some of the clues in regard to these mechanisms could be gleaned from the ability of melanoma to grow and invade despite the presence of endogenous IFNα or related IFNβ in the tumor microenvironment. Recent years have witnessed a renewed interest in the important role of tissue inflammation in human cancers in general. This interest is supported by a wealth of mechanistic, epidemiologic and clinical data (reviewed in (Balkwill and Mantovani 2010, Grivennikov et al 2010, Mantovani et al 2008)). Roles of inflammatory signaling in promoting tumor growth, angiogenesis and metastatic progression of malignant melanoma have been well documented (Melnikova and Bar-Eli 2009).

Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukins (IL-1, IL-6, etc) and others play important roles in mechanistically linking the pathogenetic cascades of tumorigenesis and inflammatory responses. These cytokines can be produced by various types of cells including malignant melanoma (Bennicelli et al 1989, Kock et al 1989, Moretti et al 1999, Okamoto et al 2010, Sander and Boeryd 1996). Pro-inflammatory cytokines stimulate diverse receptors, however, these signaling pathways often converge on activating several key protein kinases including stress activated protein kinases (JNK and p38) and IκB kinase (IKK, reviewed in (Germano et al 2008, Lin and Karin 2007)). The importance of these pathways in regulating the responsiveness of cells to IFNα/β is largely unknown.

All effects of IFNα/β on cells are elicited through interaction with a cognate cell surface Type I IFN receptor that consists of IFNAR1 and IFNAR2c chains. This interaction leads to catalytic activation of Janus kinases (JAK1 and TYK2), tyrosine phosphorylation of Signal Transducers and Activators of Transcription (STAT1/2) proteins and activation of transcription of IFN-stimulated genes whose products restrict cell proliferation and viability (reviewed in (Aaronson and Horvath 2002, Platanias 2005, Uze et al 2007)). Upon completion of the transcriptional program, the expression of negative regulators of JAK and STAT activities limits the magnitude and duration of this signaling. However, much earlier the ability of cells to further react to additional IFNα/β molecules is rapidly eliminated by the ligand-induced downregulation of Type I IFN receptors from the cell surface (Coccia et al 2006).

IFNα/β-induced downregulation of Type I IFN receptor is governed by ubiquitination of its IFNAR1 chain mediated by the $SCF^{\beta Trcp2/HOS}$ E3 ubiquitin ligase (Kumar et al 2003, Kumar et al 2007a). This ligase is recruited to IFNAR1 upon IFNα/β-induced and JAK kinase activity-dependent phosphorylation of the Ser residues (e.g., Ser535) within the degron of IFNAR1 (Kumar et al 2004, Liu et al 2008, Marijanovic et al 2006) mediated by protein kinase D2 (Zheng et al 2011). Intriguingly, degron phosphorylation of IFNAR1 can also occur via a ligand/JAK-independent pathway (Liu et al 2008); this pathway can be activated in some melanoma cells (Huangfu et al 2010).

Intrinsic or acquired refractoriness to extracellular signals that inhibit cell growth and proliferation (such as Type I IFN) represents a major hallmark of cancer (Hanahan and Weinberg 2000). Among the potential mechanisms, downregulation of IFNAR1 that is expected to render cells refractory to IFNα/β became a focus of our investigation. Low levels of IFNAR1 indeed correlate with poor prognosis for patients with metastatic melanoma (Messina et al 2008). Furthermore, several melanoma cell lines can secrete soluble factors that promote degradation of IFNAR1 and ensuing inhibition of JAK-STAT signaling specifically induced by Type I IFN (Huangfu et al 2010). Here we report the identification of pro-inflammatory cytokines (such as interleukin 1, IL1) as factors that can be secreted by melanoma to stimulate the phosphorylation and degradation of IFNAR1. We also report that induction of this inflammatory pathway depends on activity of p38 kinase and leads to inhibition of cell responses to IFNα/β.

RESULTS

Melanoma-secreted factors accelerate the ligand-independent degradation of IFNAR1

Several melanoma cell lines can secrete soluble factors that inhibit IFNα signaling and promote phosphorylation of IFNAR1 degron (S535), and ensuing ubiquitination and degradation of IFNAR1 (Huangfu et al 2010). We sought to characterize these factors and determine how they induce the phosphorylation of IFNAR1 degron on Ser535. This phosphorylation can be mediated via either ligand-inducible or ligand independent pathway. The latter occurs in the absence of IFNα/β (Liu et al 2008) but requires activity of casein kinase 1α (CK1α (Liu et al 2009)), and is stimulated by a priming phosphorylation of IFNAR1 on Ser532 (Bhattacharya et al 2010). Addition of the neutralizing antibodies against IFNα/β to the melanoma-conditioned media did not affect the media's ability to induce Ser535 phosphorylation on endogenous IFNAR1 in HeLa cells (Figure 1A) or in 293 cells (Figure S1). Intriguingly, the effect of secreted factors was sensitive to the presence of CK1 inhibitor D4476 (Figure 1B). Furthermore, treatment of cells with the melanoma-conditioned media induced the priming phosphorylation of IFNAR1 on Ser532 (Figure 1A), and mutation of this site prevented induction of the degron phosphorylation on Ser535 by melanoma-conditioned media but not by IFNα (Figure 1C). In addition, efficient induction of IFNAR1 ubiquitination (Figure 1D) and acceleration of IFNAR1 degradation (Figure 1E) in cells treated with secreted factors seemed to depend on the integrity of the priming site (Ser532). Consistent with our recent report (Huangfu et al 2010), melanoma-conditioned media robustly inhibited IFNα-induced STAT1 phosphorylation (Figure 1F). This inhibition could be partially alleviated by expression of exogenous IFNAR1 proteins. Although the

magnitude of this rescue of IFNα signaling might be underestimated due to the limitations in transfection efficacy, IFNAR1^{S532A} mutant was noticeably more active than the wild type receptor under these conditions (Figure 1F). These results suggest that melanoma-secreted factors accelerate IFNAR1 degradation and downregulation in a manner dependent on the priming phosphorylation of IFNAR1 on Ser532; this mechanism might contribute to the inhibition of IFNα signaling.

Inflammatory cytokines stimulate IFNAR1 degradation and attenuate IFNα signaling

To identify the factor(s) responsible for IFNAR1 phosphorylation and degradation, we profiled the levels of 38 cytokines, growth factors and soluble receptors secreted by melanocytes or melanoma cells using the Luminex protein array system. Several of these factors, including IFNα, IFNγ, IL-6, IL-7 and IL-1α, were detectable in media conditioned by the 1205Lu melanoma cells but not by melanocytes (Supplementary Table). Treatment of cells with recombinant IL-1α but not with IFNγ, IL-6 or IL-7 robustly increased phosphorylation of endogenous IFNAR1 on priming Ser532 (Figure 2A). IL-1α induces diverse intracellular signaling pathways upon activation of IL-1 receptor. IL-1β, a related cytokine that was not dramatically upregulated in melanoma cells tested (Supplemented Table), yet known to utilize the same receptor as IL-1α, also stimulated priming phosphorylation of IFNAR1 on Ser532 (Figure 2A) and its degron phosphorylation on Ser535 (data not shown and Figure 4D–F). Intriguingly, another pro-inflammatory cytokine, tumor necrosis factor alpha (TNFα) also stimulated phosphorylation of Ser535 on either endogenous (Figure 2B) or exogenous IFNAR1. Phosphorylation of IFNAR1 protein was dependent on the integrity of Ser532 (Figure S2) and phosphorylation of this residue in response to TNFα was observed as well (Figure S3). These data suggest that some pro-inflammatory cytokines such as IL-1α/β and TNFα can stimulate the signaling pathways that lead to phosphorylation of the IFNAR1.

Pre-treatment of cells with an antagonist of IL-1 receptor, Anakinra, attenuated priming phosphorylation and ubiquitination of IFNAR1 induced by melanoma-conditioned media (Figure 2C). For further analyses of effects that occur downstream of IL-1 receptor, we chose readily available recombinant IL-1β. This cytokine stimulated ubiquitination of wild type Flag-IFNAR1 but not of its mutant lacking Ser532 (Figure 2D). These results indicate that activation of the IL-1 receptor is partially responsible for the effects of melanoma media on IFNAR1 ubiquitination and that the latter process can be stimulated by pro-inflammatory cytokines.

An accelerated degradation of IFNAR1 was observed in HeLa cells treated with IL-1β (Figure 3A) or TNFα (data not shown). Conversely, treatment with Anakinra slowed down the IFNAR1 turnover induced by melanoma-conditioned media (Figure 3B) and increased total levels of IFNAR1 in 1205Lu melanoma cells (Figure 3C). Intriguingly, pre-treatment of HeLa cells with recombinant IL-1 noticeably decreased the extent of STAT1 phosphorylation in response to IFNα (Figure 3D). Furthermore, adding Anakinra to the melanoma cell-conditioned media partially compromised its ability to inhibit IFNα-induced STAT1 phosphorylation in HeLa cells (Figure 3E). Finally, treatment with Anakinra augmented IFNα signaling in 1205Lu cells (Figure 3F). These results indicate that pro-

inflammatory cytokines signal to promote downregulation and degradation of IFNAR1 and this mechanism contributes to suppression of IFNα signaling.

Role of p38 kinase in modulation of cellular responses to IFNα by pro-inflammatory cytokines

We next sought to delineate the mechanisms underlying induction of priming phosphorylation of IFNAR1 in response to pro-inflammatory cytokines. Proximal IL-1-induced signaling was shown to involve activities of TRAF6 E3 ubiquitin ligase, Ubc13 E2 ubiquitin conjugating enzyme and TAK1 protein kinase. Distal IL-1-stimulated signaling events are common with those induced by TNFα and include activation of IκB kinases (IKK), Jun N-terminal kinase (JNK), p38 stress activated protein kinase and Erk (reviewed in (Lin and Karin 2007, Weber et al 2010)). Expression of dominant negative mutant of Ubc13 inhibited priming phosphorylation of IFNAR1 on Ser532 induced by IL-1β in HeLa cells (Figure 4A). Furthermore, knockdown of either TRAF6 or TAK1 (monitored by a decreased efficacy of JNK phosphorylation) also attenuated priming phosphorylation (Figure 4B). Treatment of cells with p38 kinase inhibitors SB203580 or VX-702 (but not with IKK inhibitor NBD or JNK inhibitor SP600125 or PI3K inhibitor LY294002) dramatically inhibited phosphorylation of IFNAR1 in response to either IL-1β (Figure 4C–D) or TNFα (Figure S3 and data not shown). These results implicate p38 kinase functions downstream of TRAF6, Ubc13 and TAK1 in mediating the priming phosphorylation of IFNAR1 in response to pro-inflammatory cytokines.

Indeed, transient knockdown of p38α kinase in HeLa cells inhibited Ser532-phosphorylation induced by either IL-1β or melanoma-conditioned media (Figure 4E). Under these conditions (but not upon treatment of cells with IFNα), we also observed a decrease in phosphorylation of IFNAR1 degron on Ser535. Furthermore, murine IL-1β stimulated degron phosphorylation on Ser526 (analogue of human Ser535) of IFNAR1 in wild type mouse embryo fibroblasts (MEF). The ratio between phosphorylated and total IFNAR1 signals was noticeably lesser in MEF from p38α knockout mice treated with IL-1β (Figure 4F). Given that Ser532 does not conform to a proline-directed phospho-acceptor site, which p38 kinase was reported to prefer (reviewed in (Roux and Blenis 2004)), we tested the potential contribution of several protein kinases known to function downstream of p38 kinase. However, pre-treatment of cells with either validated RNAi reagents against MSK1/2 or MK2/3/5 or available Mnk1/Msk inhibitors did not affect IL-1β-induced Ser532 phosphorylation of endogenous IFNAR1 in HeLa cells (data not shown).

A direct phosphorylation of bacterially produced GST-IFNAR1 fusion protein on Ser532 was detected in an in vitro kinase reaction using the Flag antibody-immunoprecipitated material from HeLa cells that expressed wild type Flag-tagged p38α kinase (Figure 4G). This activity (robustly induced by pre-treatment of cells with IL-1β) was not observed when the lysates were prepared from cells that express catalytically inactive Flag-p38α^{AGF} mutant or when GST-IFNAR1 was mutated on Ser532 (Figure 4G).

We next tested the possibility that priming phosphorylation is mediated by a kinase that associates with p38α. GST-tagged p38α was expressed in HeLa cells (treated with IL-1β) and purified. These preparations were used as a source of kinase in an in vitro

phosphorylation of Ser532 on GST-IFNAR1. Under these conditions, addition of p38 kinase inhibitor SB203580 (but neither Msk1 inhibitor HB806 nor Mnk1 inhibitor CGP57380) directly to the reaction led to a dose-dependent decrease in Ser532 phosphorylation (Figure 4H). Together these data strongly suggest that p38α kinase mediates priming phosphorylation of IFNAR1 in response to IL-1, although the contribution of other p38 forms or downstream kinases cannot be ruled out (see Discussion).

We next tested the role of p38 kinase in downregulation of IFNAR1 using genetic approaches and analyses of two independent materials of human melanoma specimens. Knockdown of p38α in HeLa cells noticeably up-regulated IFNAR1 in cells treated with either recombinant IL-1β or melanoma-conditioned media (Figure 5A). Furthermore, a decrease in cell surface levels of IFNAR1 in response to murine IL-1β (but not to murine IFNβ) was noticeably tempered in MEFs from p38α knockout mice (Figure 5B).

In agreement with low levels of IFNAR1 reported in some patients with metastatic melanoma (Messina et al 2008), our analysis of benign skin and metastatic melanoma tissues obtained from the same patient revealed that total levels of IFNAR1 were lower in melanoma cells than in normal melanocytes (Figure S4). Similarly, a noticeably stronger immunoreactivity for IFNAR1 was observed in normal skin cells compared to melanoma cells from the same patients in seven out of eight case-matched tissue samples (Figure 5C, patients #1–4 and 6–8). Based on these data together with the in vitro evidence of downregulation of IFNAR1 levels by activated p38 kinase we predicted that levels of IFNAR1 in human melanoma specimens would be inversely correlated to activation of p38 kinase (assessed by measuring the levels of phospho-p38 signal). We first used AQUA fluorescence-based immunoprofiling of histologic sections of a pilot material of 24 human melanomas and observed a negative correlation between levels of phosphop-38 and IFNAR1 (Figure 5D–E, rho = −0.42; p = 0.043). We then repeated the analysis on a larger material of primary and metastatic melanomas from Melanoma Spore Tissue Microarray (described in (Nazarian et al 2010)) and found a similar negative correlation (Figure S5, rho = −0.213; p = 0.013; N = 134). This material included both thin and thick melanomas, and the negative correlation was particularly evident among thin melanomas (Figure S6, rho = −0.355; p = 0.011; N=50). These results collectively indicate that activation of p38 kinase plays an important role in downregulation of IFNAR1 in cells that respond to proinflammatory cytokines and in malignant melanoma tissues.

We next investigated the role of p38 kinase in regulation of IFNAR1 stability and of cellular responses to IFNα/β. Treatment of melanoma cells with p38 inhibitor SB203580 noticeably delayed IFNAR1 turnover upon addition of protein synthesis blocker cycloheximide (Figure 6A). Similar results were obtained in HeLa cells where acceleration of IFNAR1 degradation by melanoma-conditioned media was reversed by p38 kinase inhibitor (Figure 6B). Careful examination of Figures 4E–F indicated that phosphorylation of STAT1 is augmented in p38α-deficient cells. Conversely, inhibition of IFNα-induced activation of STAT1 by either IL-1β or melanoma-conditioned media was noticeably less evident in HeLa cells upon either knockdown of p38α (Figure 7A) or co-treatment of these cells with inhibitor of p38 SB203580 but not with MEK1 inhibitor UO126 or JNK inhibitor SP600125 (Figure 7B). We then sought to determine whether IL-1-induced p38-mediated priming phosphorylation

of IFNAR1 might play a role in response of 1205Lu melanoma cells to IFNα. To this end, we attempted to stably express either wild type IFNAR1 or IFNAR1-S532A mutant which is refractory to p38-mediated phosphorylation, and then subject the resulting cells to anti-proliferative effects of exogenously added IFNα. Regrettably, although we succeeded in establishing a number of clones that expressed wild type IFNAR1, all clones transduced with IFNAR1^{S532A} either perished or ceased to express the transgene (Figure S7). It is plausible that this result reflects the cell growth inhibitory effect of low levels of endogenous IFNα produced by the 1205Lu melanoma cells (Supplementary Table) that occurred over time of selection under conditions when secreted IL-1 and other pro-inflammatory cytokines cannot downregulate IFNAR1^{S532A}.

We next investigated the role of phosphorylation-dependent downregulation of IFNAR1 in responses of melanoma cells to Type I IFN. Upon transient co-expression of IFNAR1 with green fluorescent protein (GFP), melanoma cells that expressed IFNAR1^{S532A} were indeed significantly more sensitive to cell death induced by treatment with IFNβ (Figure 7C). Conversely, pre-treatment of 1205Lu melanoma cells with p38 kinase inhibitor VX-702 significantly augmented the inhibition of cell viability induced by IFNα (Figure 7D). Furthermore, combination of VX-702 and IFNα noticeably decreased the growth of 1205Lu cells xenotransplanted into the flanks of immunocompromised mice (Figure 7E). In all, these results suggest that p38 kinase-mediated downregulation of IFNAR1 plays an important role in dampening cellular responses to Type I IFN.

DISCUSSION

This work was prompted by our initial investigation of soluble melanoma cell-produced factors that temper IFNα/β signaling (Huangfu et al 2010). Our findings presented here suggest that pro-inflammatory cytokines (that can be secreted by melanoma cells) activate p38 kinase to promote the ligand-independent pathway of IFNAR1 degradation, and, subsequently, interfere with cellular responses to IFNα/β. These studies link tissue inflammation with decreased cell sensitivity to the effects of Type I IFN. Although mostly discussed in the context of melanoma pathogenesis and sensitivity of melanomas to IFNα/β, our data suggest that overall anti-tumorigenic defenses of endogenous IFNα/β in many target tissues that may undergo malignant transformation as well as the efficacy of many Type I IFN-based drugs used against various tumors could be negatively impacted by inflammation. Future studies will also determine whether inflammation negatively affects anti-viral defensive and immunomodulatory functions of IFNα/β, and, accordingly, may impede its use against chronic viral infections and multiple sclerosis.

Cytokine-induced activation of p38 kinase may occur not only in cancer cells but also in the tumor stroma. Accordingly, the discussed mechanism might not only contribute to refractoriness of melanoma cells to direct anti-proliferative/anti-survival effects of therapeutically administered exogenous IFNα/β but also compromise the efficacy of anti-angiogenic effects of these cytokines within the tumor microenvironment. Furthermore, the same mechanism activated in benign/pre-malignant cells of melanocytic origin could plausibly help these cells to evade anti-proliferative control from endogenous Type I IFNs. Given that ultraviolet light implicated in melanoma etiology (Leiter and Garbe 2008) is also

a potent activator of p38 kinase in human melanocytes (Jinlian et al 2007), future studies of the role of IFNAR1 stability and its regulation by p38 activators in melanoma development are warranted.

Although data obtained in experiments that use Anakinra suggest that some of the effects of melanoma-conditioned media should be attributed to secreted IL-1 (Figures 2–3), the role of other cytokines cannot be excluded. Our studies pointing to secretion of IL1α are consistent with previous reports on secretion of either IL-1α or IL-1β by numerous melanoma cell lines and primary melanoma tissues (Bennicelli et al 1989, Kock et al 1989, Okamoto et al 2010). These cells and tissues also have been shown to produce other cytokines (e.g., TNFα, etc (Moretti et al 1999, Okamoto et al 2010, Sander and Boeryd 1996)) that are capable of activating p38 kinase and could plausibly contribute to inflammatory inhibition of cellular responses to IFNα/β. Furthermore, a high level of expression of soluble fragments of TNF receptors in melanoma-conditioned media (Supplementary Table) might be indicative of overall high density of TNF receptors on melanoma cells that would make them sensitive to TNFα produced by cells that constitute the tumor microenvironment. TNFα and IL-1 produced by keratinocytes (Oxholm et al 1988) may affect IFNAR1 turnover in melanomas of the radial growth phase; whereas, upon invasion beyond the basement membrane, such a role could be carried out by infiltrating macrophages that are associated with tumor progression (Torisu et al 2000). Finally, even in the absence of secreted factors, persistent activation of p38 kinase downstream of cancer-associated constitutively active oncogenic mutants of receptor tyrosine kinases (e.g., c-KIT, (Smalley et al 2009)) may also contribute to impairment of Type I IFN signaling. Future studies are warranted to investigate this possibility.

Results presented here implicate p38 kinase in the induction of IFNα/β-independent priming phosphorylation of IFNAR1. However, the identity of the priming kinase remains to be determined. Our data suggest that the priming phosphorylation of IFNAR1 on Ser532 is likely to be directly mediated by a SB203580-sensitive kinase that associates with p38 kinase and depends on p38 catalytic function. It is plausible that a yet-to-be-identified p38-dependent kinase is responsible for direct phosphorylation of Ser532. Alternatively, p38 itself might be capable of functioning as a priming kinase, although this possibility is less likely given substrate-preference of p38 for proline-directed Ser and Thr residues as phospho-acceptor sites (Roux and Blenis 2004).

Data presented here may provide a rationale to argue for combining p38 inhibitors and IFNα in treatment of patients with melanomas. However, such therapeutic considerations must be approached with a great deal of caution. First, it has been shown that IFNα/β are also capable of activating p38 kinase in some cell types and this activation contributes to expression of IFN-stimulated genes and anti-proliferative effects of IFN downstream of STAT activation (reviewed in (Platanias 2005)). Second, inhibition of p38 kinase may also negatively affect the efficacy of other promising means for induction of apoptotis in melanoma such as activation of innate immunity by double stranded RNA (Alonso-Curbelo and Soengas 2010). Third, activation of p38 kinase stimulates the internalization of the epidermal growth factor receptor (Zwang and Yarden 2006); stabilization of this receptor upon p38 kinase inhibition may increase survival of tumor cells. Genetic evidence in p38α

knockout mice indeed demonstrate an increased rate of cell proliferation and survival (Hui et al 2007). Finally, it is possible that some of proinflammatory cytokine-induced p38 kinase functions are important for activation of anti-tumor immunity (that could not be evaluated in our experiments using melanoma cells xenotransplanted into the immunocompromised animals). Indeed, high levels of proinflammatory cytokines in serum were predictive of longer relapse-free survival in melanoma patients who received IFNα treatment (Yurkovetsky et al 2007). Further molecular studies delineating specifics of IFNα signaling in melanoma and nonmelanoma tissues and oncopharmacologic investigation of inhibitors for kinases involved in regulating receptor stability in the immuno-competent models are required to improve the therapeutic efficacy of IFNα.

Materials and Methods

Plasmids and Reagents

Vectors for bacterial expression of GST-IFNAR1 and mammalian expression of human and murine Flag-IFNAR1 were described previously (Bhattacharya et al 2010). Plasmids for expression of HA-Ubc13 (wild type or catalytically inactive C87A), Flag-p38 (wild type or catalytically inactive AGF) and GST-p38α were generous gifts from Z. Ronai, R. J. Davis and M. Gaestel. ShRNA constructs for knocking down p38α kinase were purchased (Sigma). All siRNA oligos (HP Validated) were purchased from Qiagen. Lentiviral pCIG-IFNAR1-IRES-nucGFP/puro vectors for simultaneous expression of either wild type human IFNAR1 or its S532A mutant and either nuclear GFP or puromycin resistance marker (puro) were constructed on the backbone of pCIG vector (Megason and McMahon 2002). Various recombinant cytokines and chemicals were purchased from commercial sources. IKK inhibitor NBD was a generous gift of M. May. Mnk1 inhibitor CGP57380 was purchased from Tocris. Msk inhibitor HB806 (compound 13 in reference (Bregman et al 2006)) was a generous gift of E. Meggers.

Cell lines and cancer samples

Primary human melanocyte cells and human melanoma cell lines (kindly provided by M. Herlyn) were maintained as previously described (Kumar et al 2007b, Liu et al 2007). Wild type and p38α-null MEF were kindly provided by A. Nebreda. 293 cells were a generous gift of J. Ninomiya-Tsuji. Proliferation assays were done with CellTiter 96 Non-Radioactive Cell proliferation Assay according to manufacture protocol (Promega). Patient-derived materials in the forms of tissue microarrays containing anonymized human melanoma tissues were provided by Thomas Jefferson University Pathology Core and NCI Skin SPORE (described in details in (Nazarian et al 2010); kindly provided by L.M. Duncan).

Antibodies and Immunotechniques

were carried out as previously described (Liu et al 2009). Polyclonal antibody against β-Trcp was described previously (Spiegelman et al., 2002). Antibodies against total (Goldman et al 1999) or phosphorylated IFNAR1 (Bhattacharya et al 2010, Kumar et al 2004) were described in detail elsewhere. Other antibodies were purchased from commercial sources and used for immunoprecipitation (IP) and immunoblotting (IB) as described previously (Bhattacharya et al 2010, Kumar et al 2004). Luminex protein array system (LabMAP® for

Multianalyte Profiling), which allows for simultaneous quantitation of muptiple analytes was used to determine the concentration of 38 selected cytokines, growth factors and soluble receptors in media conditioned by melanocytes or malignant melanoma cells as outlined in Supplementary Data and described in details elsewhere (Gu et al 2009, Linkov et al 2008). **In Vitro Kinase Assays** were carried out as previously described (Liu et al 2008, Liu et al 2009).

Immunohistochemical analysis of tissue samples

was carried out in non-identifiable coded melanoma specimens that represented two separate clinical materials. Archived materials collected by the Department of Pathology of Thomas Jefferson University contained matched sets of melanoma tissues and normal skin from nine anonymous patients. This material was used for detection of IFNAR1 levels using respective primary and secondary antibody combined with the streptavidin-biotinylated horseradish peroxidase complex reagent (Dako) and exposure to the chromagen DABplus (Dako) followed by counterstaining in hematoxylin as described elsewhere (Li et al 2006).

Automated Quantitative Analyses (AQUA) of phospho-p38 and IFNAR1 were performed on 480-sample tissue microarray built from tissues from collected by the institutions that participate in Melanoma Spore Consortium (kindly provided by Dr. Duncan and reported in (Nazarian et al 2010)). AQUA analysis was performed using AQUA/PM2000 (HistoRx).

Tumor Cell Growth Assays in vivo and in vitro

Experiments in animals (under the IUCAC protocol # 800992 of the University of Pennsylvania) was carried out in 6–8 weeks old male immunocompromised (scid) mice (Taconic). Twenty-two mice were injected subcutaneously with 1205Lu cells (3×10^6) into the right flank of lower back (day 0). The scid mice were randomly assigned to four experimental groups of 5–6 mice each: (Control) vehicle treatment; (VX) VX-702 (1 mg/kg) was given by oral gavage start on day 4 and given every other day; (IFN) IFNα2 (100 μg/mice) were injected via s.c. on day 5 into the left flank of lower back and given every other day; (IFN+VX) both VX-702 (1 mg/kg) and IFNα2 (100 μg/mice) were given over the treatment period. During the experiments, tumor volumes were measured by caliper measurement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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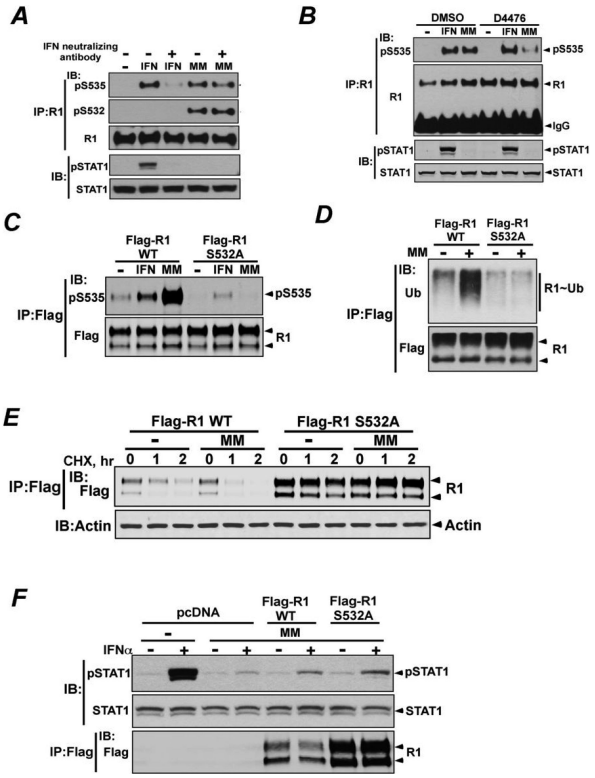

Figure 1. Melanoma-secreted factors activate the ligand-independent pathway of IFNAR1 degradation

(A) HeLa cells were incubated with IFNα (1000IU/ml) or 1205Lu cell-conditioned media (MM) in the presence or absence of IFNα/β-neutralizing antibodies for 30 min. IFNAR1 proteins were purified by immunoprecipitation (IP) using IFNAR1 antibody and analyzed by immunoblot (IB) using anti-pSer532, anti-pSer535, and anti-IFNAR1 (R1) antibodies as indicated (upper panels). Phosphorylation and total levels of STAT1 in whole cell lysates are also shown.

(B) Analysis of IFNAR1 and STAT1 from HeLa cells pre-treated with CK1 inhibitor-D4476 (12.5μM) or vehicle control (DMSO) for 1 h followed by IFNα or MM for 30 min was carried out as in panel A.

(C) HeLa cells expressing Flag-IFNAR1 (WT or S532A) were treated as indicated and exogenous IFNAR1 proteins analyzed by IP-IB using indicated antibodies. Loading was normalized to achieve comparable levels of IFNAR1 in all lanes.

(D) HeLa cells transfected with Flag-IFNAR1 (WT or S532A) were treated with MM for 30 min. Ubiquitination and levels of Flag-IFNAR1 were analyzed as in Panel C.

(E) HeLa cells expressing Flag-IFNAR1 (WT or S532A) were treated with cycloheximide (CHX, 30 μg/ml) alone or together with MM for indicated times. IFNAR1 proteins were analyzed as in panel C. Levels of β-actin serving as a loading control are also shown.

(F) HeLa cells transfected with either empty vector (pcDNA3) or Flag-IFNAR1 (WT or S532A) were pre-treated with MM for 2 h and then stimulated with IFNα (60IU/ml, 30 min). Activation and levels of endogenous STAT1 as well as levels of Flag-IFNAR1 are also shown.

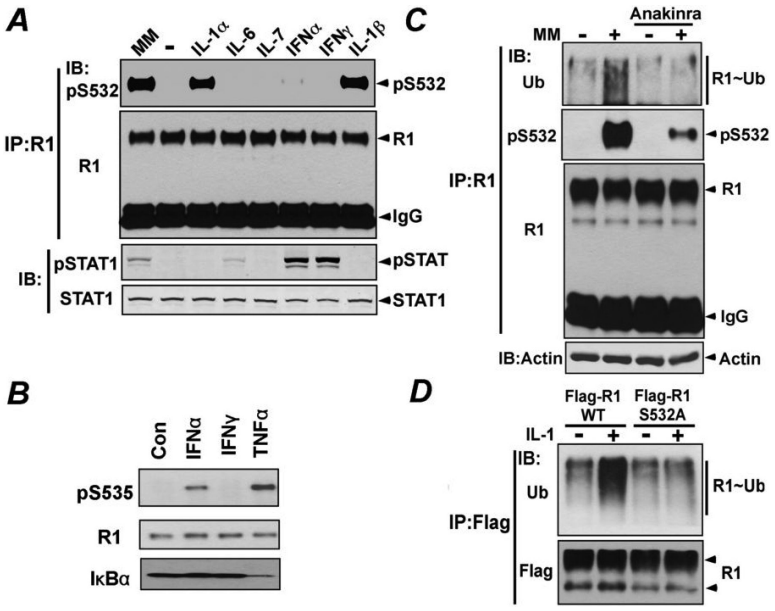

Figure 2. IL-1 stimulates priming phosphorylation and ubiquitination of IFNAR1

(A) HeLa cells were treated with MM or recombinant cytokines such as IL-6 (50ng/ml) plus shIL-6R (50ng/ml), IL-7 (10ng/ml), IFNα (1000IU/ml), IFNγ (1000IU/ml), IL-1α (10ng/ml), or IL-1β (5ng/ml) for 30 min. Phosphorylation and levels of endogenous IFNAR1 was analyzed by IP-IB using indicated antibodies. Phosphorylation and total levels of STAT1 in cell lysates are also shown.

(B) Phosphorylation of endogenous IFNAR1 from HeLa cells treated with IFNα or IFNγ (1000IU/ml), or TNFα (20ng/ml) for 30 min was analyzed by IP-IB. Levels of IκBα in whole cell lysates is also shown.

(C) HeLa cells were treated with MM alone or together with Anakinra (0.9μg/ml) for 30 min. Ubiquitination and levels of endogenous IFNAR1 phosphorylation were analyzed by IP-IB using indicated antibodies. Loading was normalized to achieve comparable levels of IFNAR1 in all lanes.

(D) HeLa cells expressing with Flag-IFNAR1 (WT or S532A) were treated with IL-1β (5ng/ml) for 30 min. Ubiquitination and levels of Flag-IFNAR1 were analyzed by IP-IB using indicated antibodies.

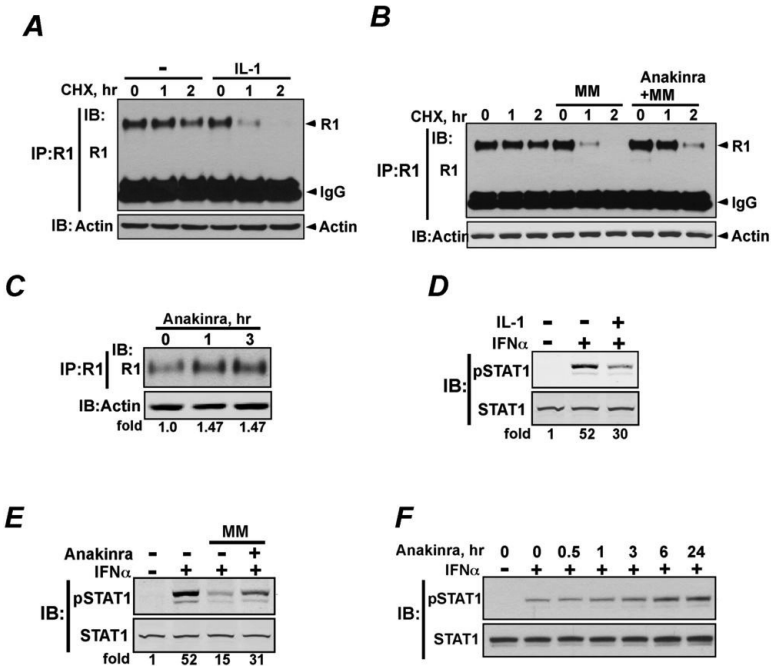

Figure 3. IL-1 promotes downregulation of IFNAR1 and inhibits IFNAR1 signaling

(A) HeLa cells were treated with CHX (30μg/ml) ± IL-1β (5ng/ml) for indicated times. Levels of IFNAR1 and β-actin were analyzed indicated antibodies.

(B) HeLa cells were treated with CHX (30μg/ml), MM, and Anakinra (0.9μg/ml) as indicated for indicated times and analyzed as in panel A.

(C) Levels of IFNAR1 in 1205Lu melanoma cells (treated as indicated) were analyzed as in panel A, quantified and normalized per the levels of β-actin (shown as fold-increase).

(D) HeLa cells were pre-treated with IL-1β (5ng/ml) for 2 h and then stimulated with human IFNα (30IU/ml) for 30 min. Signal intensity of pSTAT1 was quantified and normalized to the levels of total STAT1. Relative activation levels of STAT1 are shown as fold-increase.

(E) Analysis of STAT1 activation and levels in HeLa cells pre-treated or not with MM and treated with IFNα as indicated.

(F) Analysis of STAT1 activation in 1205Lu cells (treated as indicated) was carried out as in panel E.

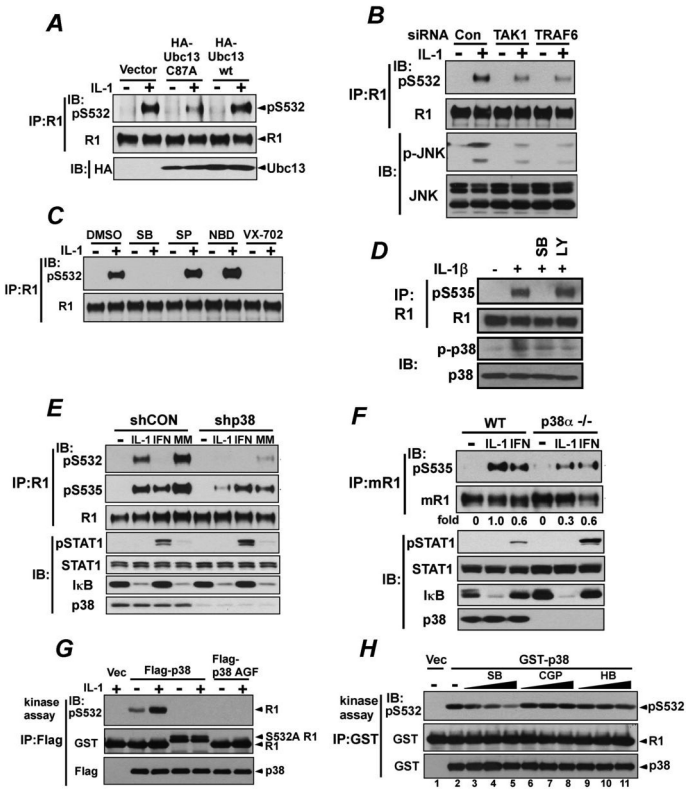

Figure 4. p38 kinase mediates priming phosphorylation of IFNAR1

(A) HeLa cells were transfected with either empty vector (pcDNA3) or HA-Ubc13 (WT or C87A) and treated with IL-1β (5ng/ml for 30 min) as indicated. Endogenous IFNAR1 was analyzed by IP-IB using indicated antibodies. Levels of HA-Ubc13 in the lysates are also shown.

(B) Phosphorylation/levels of IFNAR1 and of JNK in HeLa cells transfected with indicated siRNA and treated or not with IL-1β (5ng/ml for 30 min) were analyzed using indicated antibodies.

(C) HeLa cells were pre-treated with inhibitors of p38 kinase (SB203580, 10 μg/ml or VX-702, 1 μg/ml), or of JNK (SP600125, 20μg/ml), or IKK (NBD, 100μg/ml) or with DMSO (control) followed by treatment with IL-1β (5ng/ml) for 30 min. IFNAR1 was analyzed as in panel A.

(D) HeLa cells were pre-treated with inhibitors of p38 kinase (SB203580, 10 μg/ml), or of PI3K (LY294002, 20 μM) or DMSO for 1 h and then stimulated with IL-1β (10 ng/ml) for 30 min. Phosphorylation and levels of IFNAR1 and p38 kinase were analyzed using indicated antibodies.

(E) IFNAR1 and STAT1 proteins from HeLa cells transduced with indicated shRNA constructs and treated as indicated were analyzed as in Figure 3. Levels of p38α kinase and IκBα are also shown.

(F) Analysis of mouse IFNAR1 from MEFs (WT or p38α−/−) was carried out using indicated antibodies. Relative fold of induction of the ratio (phospho-IFNAR1/IFNAR1) was quantified and depicted. Analyses of STAT1, p38α and IκBα were carried out as in panel E.

(G) HeLa cells were transfected with either empty vector (pcDNA3) or Flag-p38α (WT or AGF mutant) and treated with IL-1β as indicated. p38α was immunopurified (using Flag antibody), and subjected to an *in vitro* kinase assay using GST-IFNAR1 ("GST-R1", WT or S532A mutant) as substrates and monitored using pS532 antibody (top panel). The amounts of GST-IFNAR1 and p38 in the IP reactions are shown.

(H) HeLa cells were transfected with either empty vector (pcDNA3) or GST-p38α as indicated. All cells were treated with IL-1β (5ng/ml) for 30 min. p38α was purified (using glutathione sepharose beads) and used as a source of kinase in an in vitro phosphorylation of GST-IFNAR1 analyzed by pS532 antibody (upper panel). These reactions were also carried out in the presence of 1×, 2×, or 10× of IC50 of the p38 inhibitor SB203580 (lanes 3–5), or Mnk1 inhibitor CGP57380 (lanes 6–8), or MSK1 inhibitor HB806 (lanes 9–11) as indicated. Levels of IFNAR1 and p38α were analyzed by IB using GST antibody (lower panels).

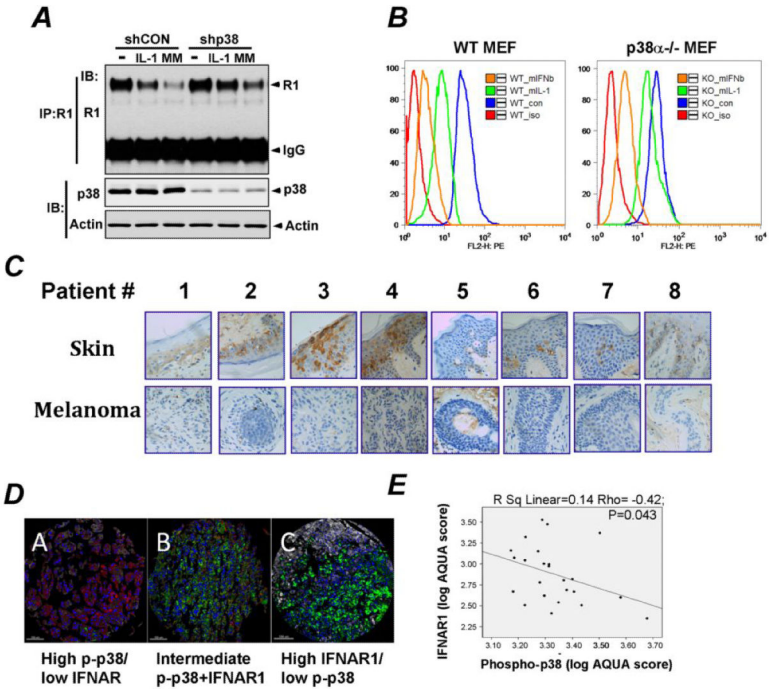

Figure 5. Activation of p38 kinase regulates IFNAR1 levels

(A) HeLa cells transduced with indicated shRNA constructs were subjected to IL-1β (5ng/ml) or MM for 2 h. IFNAR1, p38α and actin were analyzed as in Figure 4.

(B) Levels of cell surface IFNAR1 were analyzed by FACS using monoclonal anti-mouse IFNAR1 antibody in WT or p38α−/− MEF either treated with mIL-1β (5ng/ml, green) or mIFNβ (1000IU/ml, orange) for 2 h in the presence of CHX (10μg/ml). Red - isotype Ig control.

(C) Benigh human skin and metastatic melanoma tissues from the same patients were analyzed by immunohistochemistry using anti-IFNAR1 antibody.

(D) Multiplexed immunofluoresence staining of human melanoma specimens for p-p38 (red) versus IFNAR1 (green) levels in miniarray of 24 cases. Images of 3 representative cases are shown.

(E) Scatter plot of cytoplasmic p-p38 versus cytoplasmic IFNAR1 levels (log AQUA scores) in mini-array of 24 human melanoma cases analyzed using AQUA imaging platform. Negative correlation by Spearman Rank analysis is statistically significant (P=0.043). Regression line is indicated.

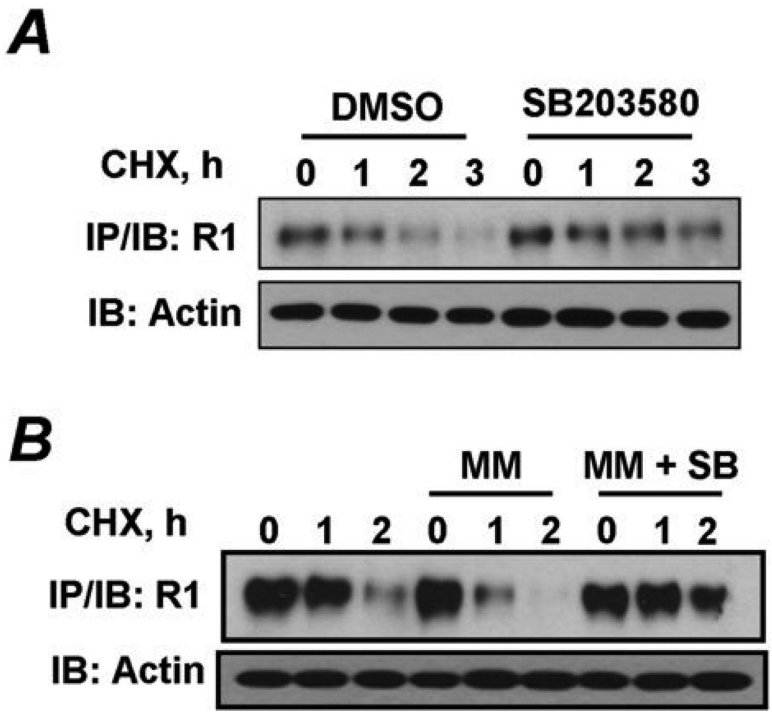

Figure 6. p38 kinase regulates turnover of IFNAR1

(A) 1205Lu cells were pre-treated with SB203580 (10 μM) or DMSO for 1 h and then treated with CHX (10 μg/ml) for indicated times. Levels of endogenous IFNAR1 were analyzed by IP/IB using anti-IFNAR1 antibody. Levels of β-actin in the lysates are shown as loading control

(B) HeLa cells were pre-treated with SB203580 (10 μM) or DMSO for 1 h and then treated with CHX (10 μg/ml) alone or together with 1205Lu metastatic melanoma conditioned media (MM) for indicated times. Levels of endogenous IFNAR1 and β-actin were analyzed as in Panel A.

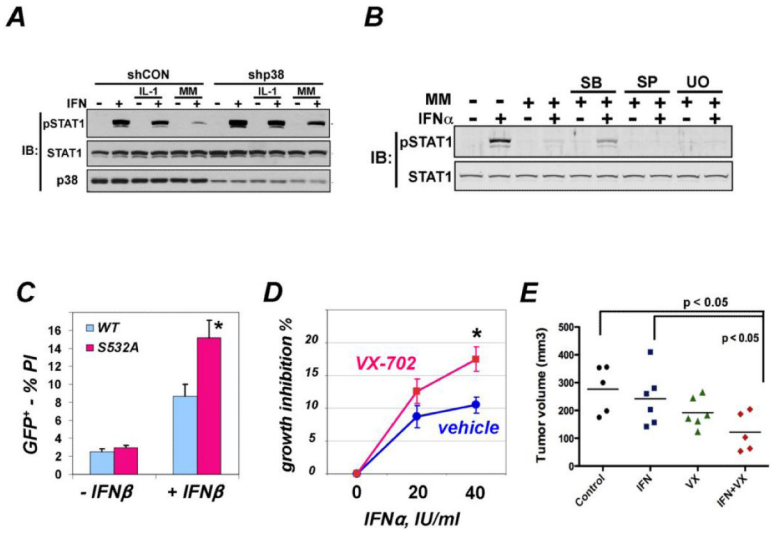

Figure 7. Role of p38 kinase in modulating cellular responses to Type I IFN

(A) HeLa cells that received the indicated shRNA constructs were pre-treated with IL-1β (5ng/ml) or 1205Lu conditioned media (MM) for 2 h and then stimulated with human IFNα (60 IU/ml) for 30 min. Activation of STAT1 was monitored by pSTAT1 antibody. The levels of STAT1 and p38 are shown.

(B) HeLa cells were pre-incubated with SB203580 (10μg/ml), SP600125 (20μg/ml), UO126 (1μg/ml), or DMSO for 1 h followed by addition of 1205Lu conditioned media (MM) for 2h and IFNα (30IU/ml for 30 min) as indicated. Activation and levels of STAT1 are shown.

(C) 1205Lu cells transduced with pCIP-Flag-IFNAR1 (WT or S532A)-IRES-nucGFP lentiviruses were treated with IFNβ (1000IU/ml for 72h). The percentage of propidium iodide- and GFP-positive cells was measured by FACS. Asterisk – $p<0.05$.

(D) Percent of growth inhibition of 1205Lu melanoma cells pre-treated or not with VX-702 (1μM for 1h) and then treated with indicated doses of IFNα for 72h. Average data from 3 independent experiments (each in triplicates) are shown as Mean ± SD. Asterisk – $p<0.05$.

(E) Volume of tumors formed by the 1205Lu cells xenotransplanted into SCID mice after six days of treatment of these mice with indicated agents.