

T-cell receptor early signalling complex activation in response to interferon- α receptor stimulation

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Signalling through the IFN α R (interferon- α receptor) and TCR (T-cell receptor) in Jurkat T lymphocytes results in distinct immune responses. Despite this both receptors elicit ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) phosphorylation. Vav and Slp76 are shown to be required for IFN α (interferon- α)-stimulated ERK activity. These form a subset of proteins which behave identically on stimulation of both receptors. TCR deletion abrogates IFN α R-stimulated MAPK activity, whereas the canonical JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway is unaffected. Thus recruitment of the intact TCR ESC

(early signalling complex) is necessary for this downstream MAPK response. Despite using a common ESC, stimulation of the IFN α R does not produce the transcriptional response associated with TCR. Up-regulation of the MAPK pathway by IFN α R might be important to ensure that the cell responds to only one stimulant.

Key words: interferon- α receptor (IFN α R), Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT pathway), mitogen-activated protein kinase pathway (MAPK pathway), Slp76, T-cell receptor early signalling complex (TCR ESC), Vav.

INTRODUCTION

Lymphocytic class I IFNR (interferon receptor) and TCR (T-cell receptor) have distinct responses to extracellular stimulation. The former is a member of the cytokine family of receptors and signals primarily through the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway resulting in antiviral and growth inhibitory effects. In contrast, activation of the TCR through antigen presentation during an adaptive immune response leads to cell proliferation and secretion of cytokines. These discrete cellular responses are derived from the distinct downstream gene transcriptional activity. Despite the contrasting cellular outcomes arising from their activation, both receptors invoke up-regulation of the ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway [1–3].

The different cellular responses to these cytokine- and antigen-stimulated receptors have been shown to involve recruitment of a subset of common proteins. These proteins have all previously been identified as being involved in the ESC (early signalling complex) formed at the TCR within minutes of stimulation [4,5]. For example, the proto-oncogene product Vav, which is phosphorylated upon stimulation of the TCR in human peripheral blood lymphocytes and human leukaemic T-cells [6], also interacts with the IFNR-associated tyrosine kinase, Tyk2 [7,8]. Similarly, the tyrosine kinases Zap70 and Lck, and the phosphatase CD45, which are all fundamental components of the TCR signal response, are also recruited to the IFN α R (interferon- α receptor) in Jurkat cells and primary lymphocytes [9]. The MAPK responses elicited from both IFN α R- and TCR-stimulated signals are dependent on the presence of both Zap70

and Lck [2,3,10] and the resultant tyrosine phosphorylation of Zap70 occurs by the same mechanism upon IFN α R stimulation as when it is activated as part of the TCR signalling pathway [11]. Furthermore, both Lck and Zap70 were shown to play an integral role in IFN α signalling as stimulated cells where these proteins were deleted were unable to demonstrate the typical anti-proliferative effects of this cytokine [9]. The absence of Lck and Zap70 in these cells did not however affect IFN α R signalling through JAK or offer protection to cells from viral infection [9].

The utilization of common proteins in transducing two signals with distinct cellular outcomes suggests a potential cross-talk between these pathways in lymphocytes. In the present study we show further that IFN α stimulation of cells recruits the TCR ESC protein machinery, and requires a functional TCR to produce the MAPK response in both lymphoma and *ex vivo* in healthy human primary CD4+ T-cells. This response has a more limited time course than the sustained signalling at the TCR and leads to a different gene expression profile. The activation of the MAPK pathway via TCR after IFNR stimulation may be a way of committing the lymphocyte to a single course of action by occupying/blocking a subsequent TCR response to extracellular antigen presentation.

EXPERIMENTAL

DNA constructs and mutagenesis

The SLP76 construct used in this paper was cloned, in frame, into the pcDNAhygro3.1-mRFP fusion vector [3], between the NheI and XhoI sites of the multiple cloning site so that Slp76 was C-terminally tagged with monomeric RFP (red fluorescent

Abbreviations used: ERK, extracellular-signal-regulated kinase; ESC, early signalling complex; FBS, fetal bovine serum; IFN α , interferon- α ; IFN α R, IFN α receptor; IFNR, interferon receptor; IL, interleukin; JAK, Janus kinase; LAT, linker for activation of T-cells; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor κ B; NP-40, Nonidet P40; PBMC, peripheral blood mononuclear cell; RFP, red fluorescent protein; RLU, relative light unit; STAT, signal transducer and activator of transcription; TCR, T-cell receptor.

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protein). The Slp76-Y3F-RFP mutant was generated through single A→T base pair mutations using the Stratagene mutagenesis kit according to the manufacturer's instructions. These mutations resulted in replacement of tyrosine residues with phenylalanine residues at positions 112, 128 and 145 of Slp76. The integrity of all three point mutations was confirmed by gene sequencing.

Cell culture

Jurkat E6.1 cells were purchased from the ECACC (European Collection of Animal Cell Cultures), the J14 (Slp76-deficient) and PF2.4 (TCR β -reconstituted) cell lines were a gift from A. Weiss (Dept. of Medicine and HHMI, University of California San Francisco, San Francisco, CA, U.S.A.), the J.Vav1 cell line and clone 15-11 reconstituted J.Vav1 cell line were kindly provided by R. Abraham (Program in Signal Transduction Research, The Burnham Institute, La Jolla, CA, U.S.A.), the JRT3-T3.5 (TCR β -deficient) cell line was purchased from A.T.C.C. The cell lines above and primary human peripheral CD4+ T-cells (purification of these is described below) were all cultured in RPMI-1640 containing L-glutamine (Cambrex). This medium was additionally supplemented with 10% (v/v) heat-inactivated, γ -radiated FBS (fetal bovine serum) (Biosera) and antimycotic/antibiotic (BioWhittaker). Cells were maintained in a humidified incubator at 5% (v/v) CO₂ and 37°C. J.Vav1 cells and reconstituted J.Vav1 cells were cultured in the above medium, supplemented with 500 μ g/ml G418.

Purification of primary CD4+ T-cells

PBMCs (peripheral blood mononuclear cells) were isolated from buffy coats purchased from the Blood Bank (St. Georges Hospital, Tooting, London U.K.). The PBMCs were isolated through Ficoll density-gradient centrifugation separation according to the supplier's instructions (Amersham Biosciences). PBMCs were collected and washed and subjected to negative selection in order to isolate CD4+ T-cells. This was achieved using MACS (magnetic cell sorting) in accordance with the protocol described by the manufacturer (Miltenyi Biotec). FACS analysis showed that purity of the resultant CD4+ T-cells was above 97% in all experiments. Following purification, cells were incubated overnight in RPMI 1640 medium supplemented with 10% (v/v) FBS and antimycotic/antibiotic. The following day 5×10^6 cells per time point were placed into 2-cm-diameter dishes and stimulated with 1 mg/ml UCHT1 monoclonal antibody or 6000 units/ml IFN α (Roferon- α ; Roche). Cells were incubated for the time period required at 37°C. Where an inhibitor was used, cells were pre-incubated for 2 h with 20 nM Lck II inhibitor (Calbiochem) or 15 nM JAK inhibitor I (Calbiochem) before being stimulated with 1 mg/ml UCHT1 or 6000 units/ml IFN α . The medium was then aspirated and cells were washed with 1 ml of 1 \times Dulbecco's PBS (BioWhittaker) and centrifuged at 900 g for 5 min. Finally cells were lysed in 50–100 μ l ice-cold NP-40 (Nonidet P40)-containing lysis buffer and centrifuged at 22000 g for 20 min. Protein concentration was then determined and Western blotting was performed as described below.

Western blot analysis

Cells were grown to approx. 50% confluency and 5×10^6 cells were then used per time point. Cells were serum-starved for 2 h before being stimulated with either 1 mg/ml OKT3 monoclonal antibody (eBioscience) or 6000 units/ml of Roferon- α and incubated at 37°C for the time periods indicated. Cells were then lysed in NP-40-containing lysis buffer [50 mM Hepes, pH 7.5, 1% NP-40, 1 mM sodium pervanadate, 10 mM sodium fluoride, 10% (v/v) glycerol, 50 mM NaCl and 1 mM

EDTA] supplemented with 1% (v/v) protease inhibitor cocktail III (Calbiochem). The protein concentration of the whole-cell extracts obtained was determined through use of Bradford assay reagents (BioRad Laboratories). For Western blot analysis, 50 μ g of cell lysates were separated by SDS/PAGE (12% gels). Proteins were then transferred on to nitrocellulose membranes (Millipore). Membranes were then incubated with specific antibodies in 3% (w/v) BSA or 5% (w/v) dried low-fat milk. β -Actin was used as a loading control. Protein bands were visualized by enhanced chemiluminescent detection (Cell Signaling Technology). Images were scanned using an Alpha Innotech Densitometer.

Polyclonal antibodies specific for phospho(Tyr¹⁰⁵⁴/Tyr¹⁰⁵⁵)-Tyk2, phospho(Tyr701)-STAT1, phospho(Tyr⁷⁰⁵)-STAT3, phospho(Tyr⁶⁹⁴)-STAT3, STAT5, phospho(Ser²¹⁷/Ser²²¹)-MEK (MAPK/ERK kinase) 1/2, and phospho(Thr²⁰²/Tyr²⁰⁴)-ERK1/2 and ERK1/2 were all purchased from Cell Signaling Technology. Polyclonal antibodies against phospho(Tyr¹⁰²²/Tyr¹⁰²³)-JAK1, phospho(Tyr¹⁷⁴)-Vav, Vav (H-211), Slp76 (H-300) and pTyr (phosphotyrosine) were purchased from Santa Cruz Biotechnology.

Immunoprecipitation of protein complexes

Cells were grown at a concentration of 10×10^3 cells per ml prior to stimulation, 10×10^6 cells were then used for each time point. Cells were stimulated either with 1 mg/ml OKT3 or 6000 units/ml Roferon- α and incubated at 37°C for the amount of time indicated before being transferred on to ice and lysed immediately with 1 ml of ice-cold lysis buffer as above. Nuclei and unbroken cells were pelleted through centrifugation at 22000 g for 20 min and the supernatant was decanted into a fresh tube. The lysate concentration was determined through use of Bradford Assay reagents (BioRad Laboratories). Antibody (2 μ g) was then added to 2 mg of lysate and placed in continuous rotation at 4°C overnight. After 18 h, 80 μ l of hydrated Protein A-agarose beads (in a 1:1 slurry in 1 \times phosphate-buffered solution) were added to each sample and returned to continuous rotation for a further 4 h. Cells were then spun for 1 min at 22000 g and washed with ice-cold lysis buffer. This wash step was repeated four times. The beads were then dried and protein was eluted from the beads through addition of an equal amount of 2 \times Laemmli buffer followed by boiling at 95°C for 5 min. The supernatant was then resolved via SDS/PAGE (10% gels). The proteins were then transferred on to a nitrocellulose membrane for 2 h at 250 mA, blocked with 3% (w/v) BSA and probed with specific antibodies.

Transfection of cells and generation of stable cell lines

Cells (20×10^6 – 30×10^6) were washed with unsupplemented RPMI 1640 medium, resuspended in 350 μ l RPMI 1640 medium and transferred into an electroporation chamber. Plasmid DNA (50 μ g) was added to the cells and mixed gently. The chamber containing the cells plus DNA was then pulsed, using a BioRad gene pulser, at a charging pulse of 0.27 V and a capacitance of 960 μ F. Cells were resuspended in RPMI 1640 medium supplemented with 10% (v/v) FBS. After 48 h, 400 μ g/ml hygromycin was added and the cells were maintained, with regular medium changes, in the presence of antibiotic for 2–3 weeks in order to select for transfected cells. Antibiotic-resistant cells were then dilution-cloned in 96-well plates. Individual clones were selected for expression of the construct through Western blot analysis and on the basis of visualization of fluorescence intensity using fluorescence microscopy.

Luciferase assays

Jurkat cells stably transfected with a construct containing the gene encoding firefly luciferase under the control of a trimer of

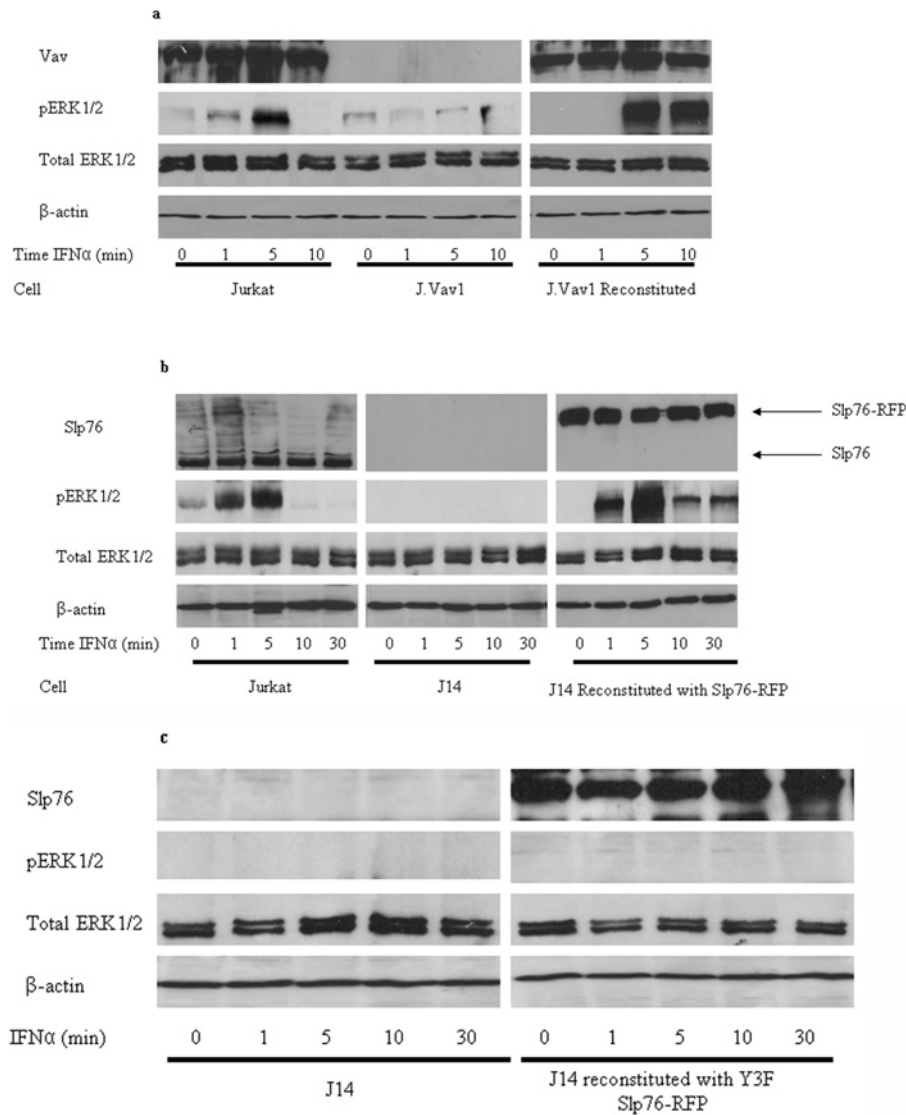


Figure 1 Absence of Vav or Slp76 abolishes downstream ERK1/2 phosphorylation in response to IFN α in Jurkat cells

Protein lysates were obtained from (a) Jurkat cells, Vav-deficient J.Vav1 cells and J.Vav1 cells reconstituted with a Vav-expressing construct, (b) Jurkat cells, Slp76-deficient J14 cells and J14 cells reconstituted with Slp76-RFP or (c) Slp76-deficient J14 cells and J14 cells reconstituted with the Slp76-Y3F-RFP mutant. Cells were stimulated with IFN α over the time course indicated. Western blotting was used to determine the expression levels of phospho(Thr²⁰²/Tyr²⁰⁴)-ERK1/2, ERK1/2 and to determine Vav and Slp76 expression levels, to ensure the presence or absence of each protein in the respective cell lines. β -Actin was used as a loading control.

the NFAT (nuclear factor of activated T-cells)-binding site from the IL (interleukin)-2 gene promoter were activated by immobilized anti-CD3 monoclonal antibody alone, Roferon- α alone (6000 units/ml) or a combination of Roferon- α and anti-CD3 antibody as described previously [12]. Cells were collected 2–24 h after activation and processed for luciferase assays as described previously [13]. Light emission was quantified in a luminometer (Berthold technologies Junior LB 9509) and expressed in RLU (relative light units).

RESULTS

Vav1 is required for MAPK activation upon IFN α R stimulation

The stimulation of Jurkat T lymphocytes by IFN α results in increased activity of the MAPK pathway shown by elevated levels of ERK1/2 phosphorylation for up to approx. 5 min [3]. This is abrogated in the absence of Lck or Zap70. We considered

whether two other known proteins of the TCR ESC, namely Vav1 and Slp76, are also implicated in an IFN α R-stimulated response. J.Vav1 cells, in which Vav expression is completely suppressed (through somatic cell gene targeting), show multiple signalling defects including the virtual abolition of ERK1/2 phosphorylation in response to TCR stimulation [14]. These cells also exhibited a significant reduction in ERK1/2 phosphorylation upon stimulation with IFN α (Figure 1a). This loss of ERK phosphorylation is reversed in reconstituted J.Vav1 cells, in which the Vav1 gene is restored (Figure 1a). The elevation of MAPK activity in Jurkat cells and reconstituted J.Vav1 cells was maximal at 5 min, mirroring the time course observed previously in IFN α -stimulated cells [3]. In resting T-cells Vav1 adopts an inactive conformation whereby the N-terminus forms an inhibitory loop that occludes the catalytic GEF (guanine-nucleotide-exchange factor) domain from accessing its substrates. TCR-stimulated phosphorylation of the key tyrosine residue,

Tyr¹⁷⁴, by upstream kinases, such as Zap70, releases this loop allowing full catalytic activation [15,16]. Western blots using an antibody against phospho(Tyr¹⁷⁴)-Vav1 showed increasing levels of phosphorylation over a 30 min time course post-stimulation by IFN α (Figures 2a and 2b). To demonstrate that this effect was based upon Zap70 kinase activity we showed that Vav1 was not phosphorylated upon stimulation by IFN α in Zap70-deficient P116 cells (Figure 2c). Thus our results suggest that the MAPK response, elicited by IFN α , requires Vav1 function in an identical manner to that reported for TCR activation.

Slp76 is recruited to a TCR-like ESC on IFN α R stimulation

On stimulation of the TCR, Vav1 and Slp76 physically associate and co-operate to induce sustained ERK1/2 signalling [17–19]. J14 cells, derived from parental Jurkat cells that completely lack Slp76 expression, elicit diminished ERK1/2 phosphorylation in response to TCR activation [20]. Similarly, the IFN α stimulation of J14 cells, as with the Vav1-deficient cells above, showed no ERK1/2 response over a 30 min time course (Figure 1b). Wild-type and J14 cells reconstituted with RFP-tagged Slp76 show recovery of MAPK activity (Figure 1b). Slp76 is implicated in the recruitment of Vav1 to the TCR ESC prior to phosphorylation [21,22]. Figure 2(c) shows that in J14 cells lacking Slp76, no Vav1 phosphorylation was apparent on IFN α stimulation suggesting that Slp76 is behaving in a similar way on cytokine stimulation. Furthermore, it is known that as part of the TCR ESC, Slp76 is phosphorylated at three key tyrosine residues located at positions 112, 128 and 145. All three of these residues are required for optimal downstream ERK activation and NFAT activity [21,22]. J14 cells reconstituted with Slp76–RFP in which the three tyrosine sites are replaced with phenylalanine (Slp76–Y3F–RFP) failed to rescue ERK1/2 activation (Figure 1c). These results are consistent with the notion that the function of Slp76 in IFN α R signalling is similar to that observed on TCR stimulation [23–25].

Slp76 is phosphorylated in primary CD4⁺ T-cells stimulated by IFN α

Although Jurkat cells provide an excellent model for studying signal transduction, as they are a leukaemic cell line, they bear several gene mutations and may not completely represent signalling in healthy T-cells *in vivo*. For example, Jurkat cells are known not to express the phosphatases PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIP [SH2 (Src homology 2)-domain-containing inositol phosphatase], which are involved in phospholipid metabolism (reviewed in [26]). To corroborate our Jurkat cell results peripheral primary CD4⁺ T-cells were isolated from fresh blood donors and cells were stimulated in a similar manner to Jurkat cells. Unstimulated cells, or cells stimulated with IFN α for 5 min, were lysed and immunoprecipitated with Slp76. The lysates were then subjected to Western blotting and probed with anti-phosphotyrosine antibodies. Figure 2(d) illustrates that Slp76 was tyrosine-phosphorylated following 5 min of IFN α stimulation. As a control, cells were also stimulated through the TCR with the UCHT1 antibody for the same period of time. This blot demonstrates for the first time that Slp76 undergoes IFN α -stimulated phosphorylation both in Jurkat cells and primary CD4⁺ primary T-cells in response to IFN α stimulation.

An intact TCR is required for IFN α -stimulated MAPK activity

It has been demonstrated previously that Lck, Zap70 and CD45 all play a role in IFN α signalling [3,9,27]. In the present study we

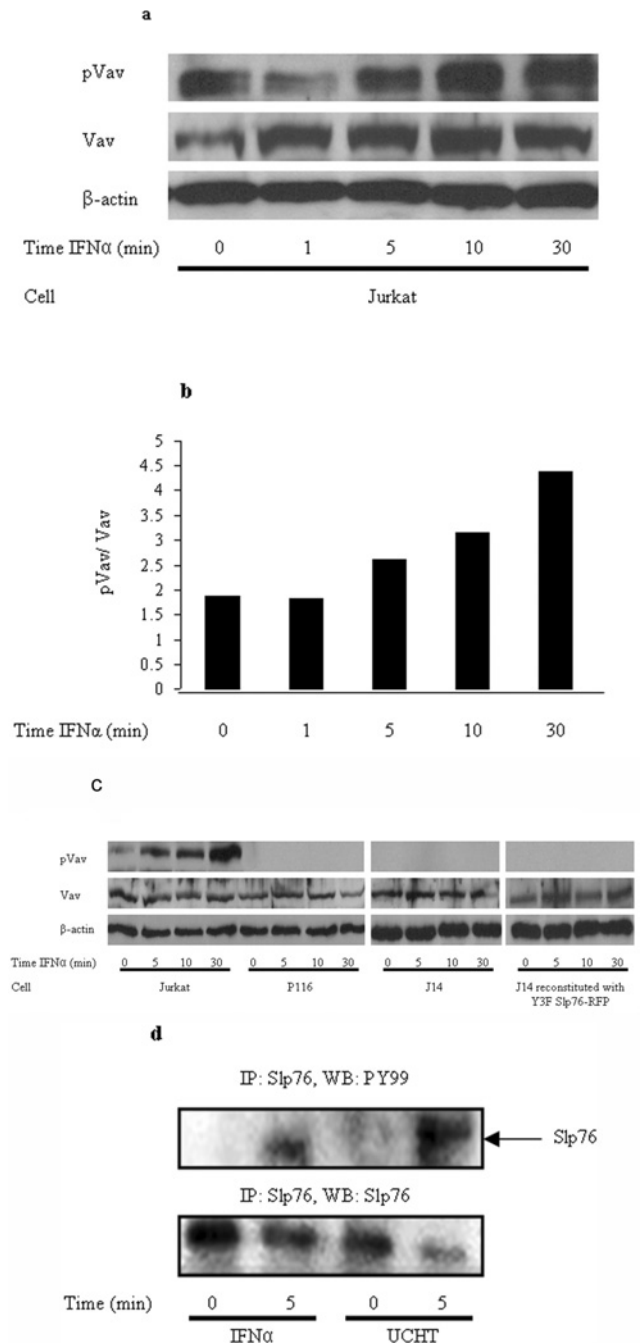


Figure 2 Activation of both Vav and Slp76 by IFN α mirrors the TCR ESC response in Jurkat and primary T-cells

(a) A Western blot was performed to determine the expression levels of phospho(Tyr¹⁷⁴)-Vav and Vav in IFN α -stimulated Jurkat cells over the indicated time course. β -Actin was used as a loading control. (b) Phospho(Tyr¹⁷⁴)-Vav levels normalized to Vav expression by densitometric analysis. (c) Western blotting was performed to determine the expression levels of phospho(Tyr¹⁷⁴)-Vav and Vav in IFN α -stimulated Jurkat, Zap70-deficient P116 cells and Slp76-deficient J14 cells over the time periods indicated. β -actin was used as a loading control. (d) Immunoprecipitation (IP) of Vav and Slp76 from primary T-cell lysate. Probing with anti-phosphotyrosine antibody (pY99) reveals that Slp76 and Vav are tyrosine-phosphorylated following 5 min of IFN α stimulation. As a control, the TCR was also stimulated for the same time period and the UCHT1 antibody was used to show the expected protein phosphorylation.

show that, in addition, both Vav1 and Slp76 are also intimately involved in cytokine-induced signalling and respond in a way that is similar to their response to TCR stimulation. Thus five

of the proteins known to be recruited to the ESC at the TCR on antigen presentation are also associated with producing a MAPK response on IFN α R stimulation. This suggests that either these proteins act as independent entities in the two distinct signalling pathways, or that IFN α R and TCR signalling are inextricably linked, i.e. the ERK1/2 activation via IFN α stimulation requires the recruitment and assembly of the TCR ESC. To test this we asked whether the TCR itself is involved in the IFN α -stimulated response. The JRT3-T3.5 cell line does not express the β -subunit of the TCR and consequently proper assembly of a complete functional receptor on the cell surface cannot occur [28]. As predicted, only basal levels of MAPK activity were exhibited upon stimulation of the cells with OKT3 compared with the parental Jurkat cell line (Figure 3a). The MAPK response was rescued in PF2.4 cells, which are reconstituted with the β -subunit and hence have a functional TCR [29]. Stimulation of JRT3-T3.5 cells with IFN α failed to produce the elevated ERK1/2 phosphorylation over the time course seen in wild-type Jurkat cells (Figure 3b). This activity can be restored in the PF2.4 cells. In addition to reduced phosphorylation of ERK1/2, IFN α stimulation of cells lacking the intact TCR also failed to effect a MEK1/2 response (Figure 3c). As MEK is the serine/threonine kinase that phosphorylates ERK this suggests that the loss of ERK1/2 activity reflects a defect in the pathway upstream of these kinases. Therefore our results show that class I interferon signalling appropriates at least part of the TCR ESC and stimulates a MAPK response via the usual mechanism associated with active TCR signalling.

Although our results reveal that the IFN α R appears to employ the TCR ESC to elicit MAPK activity, this does not result in the transcriptional response derived from anti-CD3 antibody TCR stimulation. A luciferase-based assay was used to assess whether IFN α stimulation of Jurkat cells resulted in the transcription of NFAT-dependent genes, which are characteristic of anti-CD3 antibody TCR activation. Whereas anti-CD3 antibody elicited the expected, and previously reported, transcription of NFAT, this did not occur in response to different concentrations of IFN α , over time (Figure 3d, lower panel). The effect of IFN α R stimulation in combination with anti-CD3 antibody was to moderately enhance NFAT activation at earlier time points and blunt it after 6 h (Figure 3d, upper panel). This effect on the transient NFAT activation might mean that IFN α is able to reduce the time required for anti-CD3 antibody to activate transcription. Thus although interferon stimulation elicits TCR ESC assembly and MAPK activity, the clear difference in transcriptional response suggests that the signal is subject to downstream modulation/modification.

IFN α R-induced activation of MAPK is independent of JAK/STAT signalling

Stimulation-dependent phosphorylation of JAK, and subsequent phosphorylation of the STAT proteins, is essential for mediating many of the anti-viral responses brought about by stimulation of the IFN α R. To assess whether the IFN α -stimulated signal, which uses the TCR ESC, affects the JAK/STAT pathway, cells deleted for the TCR were again used. IFN α stimulation of JRT3-T3.5 cells had no effect on Tyk2 activity (Figure 4a). This confirms that TCR ESC formation does not affect the normal JAK/STAT signalling from the IFN α R. To exemplify this further Figures 4(b)–4(d) show that STAT1, STAT3 and STAT5 respectively were tyrosine-phosphorylated in response to IFN α stimulation in wild-type Jurkat and in the JRT3-T3.5 cells. Thus one can conclude that the JAK/STAT and MAPK responses derived from IFN α R are entirely independent.

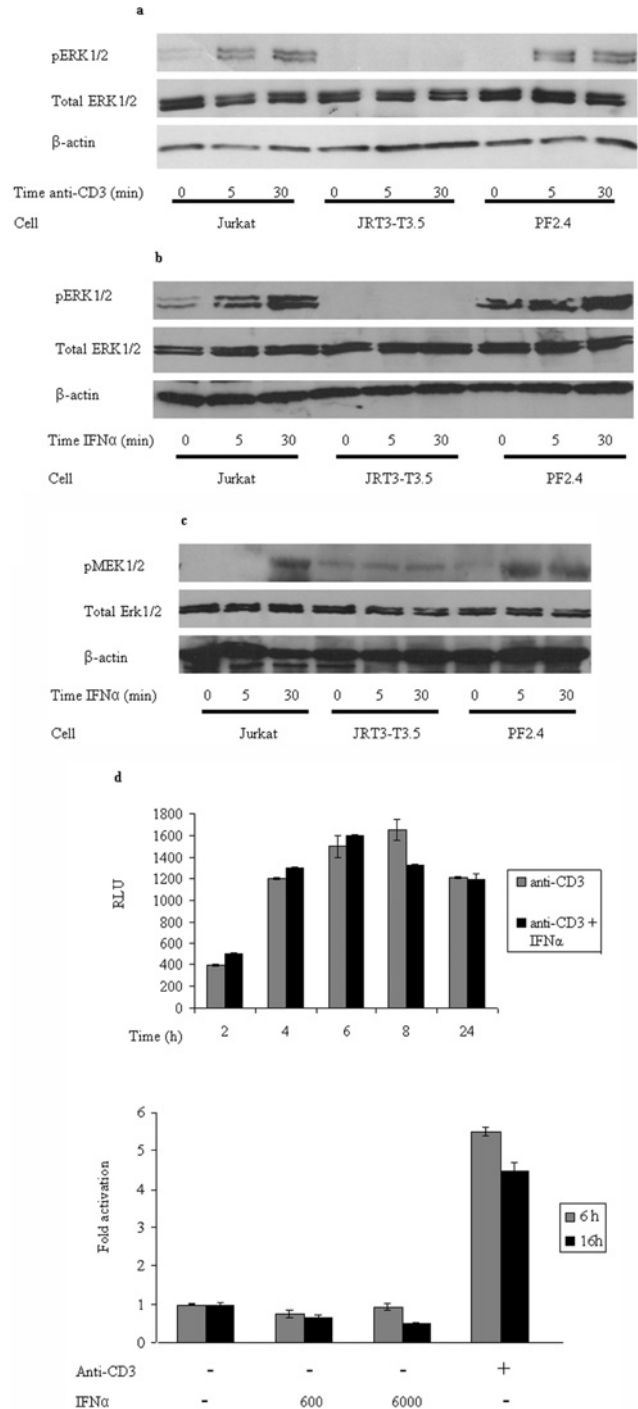


Figure 3 IFN α R-induced MAPK requires a functional TCR, but, despite this, does not induce up-regulation of the same genes as TCR-induced MAPK signalling

The Jurkat, JRT3-T3.5 (TCR $\beta^{-/-}$) and PF2.4 (stably reconstituted with the TCR β chain) cell lines were stimulated with (a) OKT3, which activates the TCR or (b) IFN α for the indicated time periods. Western blotting was then performed to determine the expression levels of phospho(Thr²⁰²/Tyr²⁰⁴)-ERK1/2 and ERK1/2. β -actin was used as a loading control. (c) Western blotting was used to determine the expression levels of phospho(Ser²¹⁷/Ser²²¹)-MEK1/2 and ERK1/2 in Jurkat, JRT3-T3.5 and PF2.4 cells that were stimulated with IFN α for the indicated time periods. β -Actin was used as a loading control. (d) Relative luciferase activity from an NFAT-luciferase reporter in Jurkat cells activated for the indicated times by immobilized anti-CD3 monoclonal antibody, either alone or in combination with IFN α . The results are expressed as RLUs (upper panel) or as relative luciferase activity in treated compared with untreated samples (fold activation; lower panel).

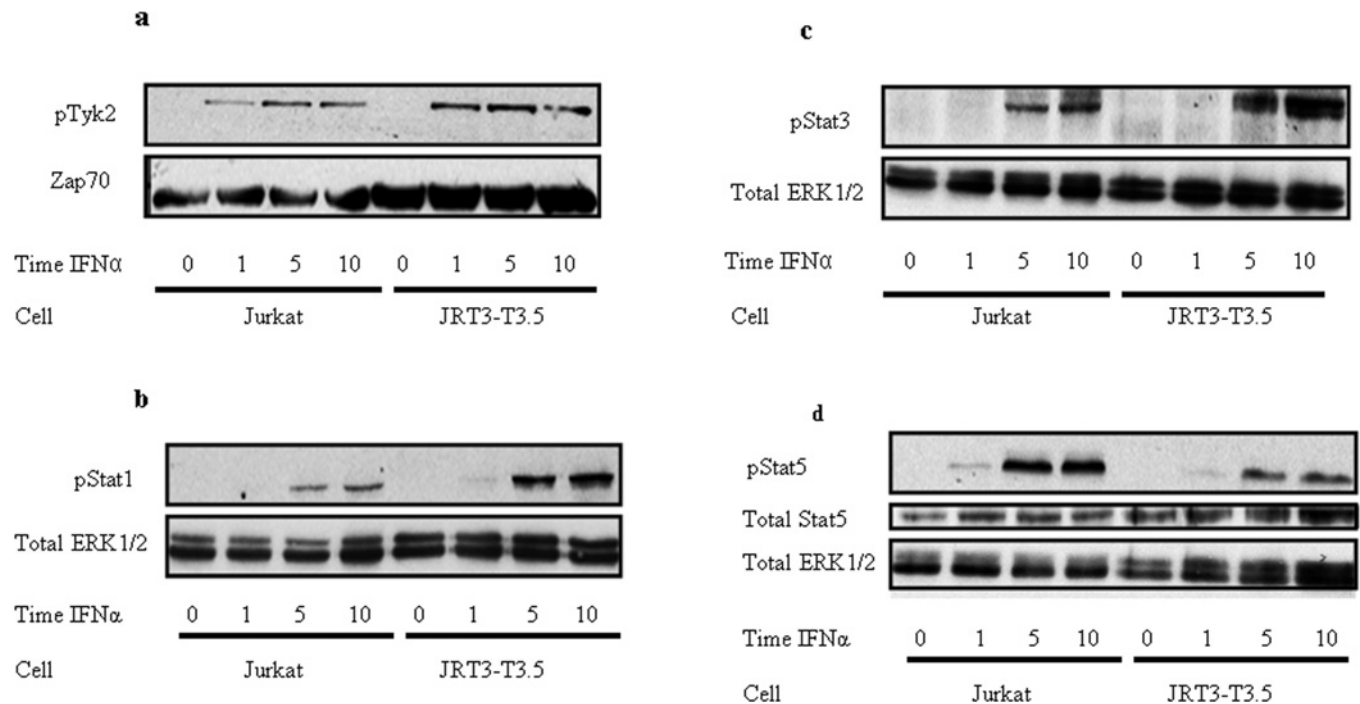


Figure 4 Absence of the TCR β chain does not affect phosphorylation of components of the JAK/STAT signalling pathway

Western blotting on cell lysates obtained from IFN α -stimulated Jurkat and JRT3-T3.5 cells using (a) anti-phospho(Tyr¹⁰⁵⁴/Tyr¹⁰⁵⁵)-Tyk2 antibody, (b) anti-phospho(Tyr⁷⁰¹)-STAT1 antibody, (c) anti-phospho(Tyr⁷⁰⁵)-STAT3 antibody and (d) anti-phospho(Tyr⁶⁹⁴)-STAT5 and anti-STAT5 antibodies. Total ERK1/2 was used to demonstrate equal loading

MAPK is phosphorylated in response to IFN α in primary human CD4⁺ cells and the TCR machinery is required

The results presented above show that the T-cell ESC machinery is mandatory for ERK MAPK signalling in response to IFN α . In order to check whether ERK phosphorylation also occurs in healthy human CD4⁺ T-cells downstream of the IFN α R, cells were stimulated *ex vivo* with IFN α . In agreement with previous findings in Jurkat cell lines [3], ERK phosphorylation was also seen over a 30 min time period (Figures 5a and 5b). The levels of ERK phosphorylation mirrored those seen in wild-type Jurkat cells (Figure 1).

As there are no TCR-deficient primary T-cells available, to assess the requirement of one of the TCR ESC proteins Lck inhibitor was utilized to pretreat CD4⁺ cells before stimulation. Lck is an essential component of the TCR ESC and signalling through ERK is blocked in its absence at the TCR. Previous studies in Jurkat cell lines have shown that Lck is also imperative for IFN α -induced ERK phosphorylation [2,3]. Figures 5(a) and 5(b) show that when peripheral CD4⁺ T-cells were stimulated in the presence of 20 nM Lck inhibitor, ERK phosphorylation was blocked downstream of the TCR when compared with CD4⁺ T-cells where no inhibitor had been added. To check that Lck was blocked by the inhibitor, the blot was also probed with anti-phospho-Lck antibody. In lanes containing Lck inhibitor-treated lysates, no phosphorylation was seen, confirming that this is an effective inhibitor. The total levels of Lck were also unaffected by this inhibitor, proving that it is effective in binding to Lck in a manner that prevents phosphorylation but does not cause degradation.

Figures 5(c) and (d) also provide confirmation that, as in Jurkat cells, ERK phosphorylation was predominantly independent of JAK/STAT signalling. When primary CD4⁺ T-cells were pretreated with the JAK1 inhibitor, ERK phosphorylation was still

seen at 5 and 10 min. STAT1, which is a downstream component of the IFN α R-induced JAK/STAT signalling pathway, was not phosphorylated in the presence of the JAK1 inhibitor. Collectively these results obtained from healthy human T-cells show that ERK phosphorylation occurs downstream of the IFN α R and that this is largely independent of JAK/STAT but dependent on functional TCR ESC.

DISCUSSION

The well characterized signalling events immediately following TCR ligation involve recruitment and assembly of a carefully orchestrated complex of enzymes and adaptor proteins. This leads to sustained signalling through the ERK/MAPK pathway, as well as a calcium flux, increased phospholipid metabolism and activation and nuclear translocation of various transcription factors such as NF- κ B (nuclear factor κ B) and NFAT. These pathways control the transcription of genes that promote cytoskeletal reorganization and polarization towards the antigen-presenting cell and cellular proliferation. On the other hand, IFN α is a cytokine that is produced by cells in response to viral dsRNA (double-stranded RNA) and bacterial lipopolysaccharides and glycoproteins as part of the innate immune response. The IFN α R signals primarily through the JAK/STAT pathway, but additional signalling through MAPKs (including p38 and c-Jun N-terminal kinase), PI3K (phosphoinositide 3-kinase)/Akt, activation of the NF- κ B transcription factor and CrkL/C3G are implicated in the overall cellular response. IFN α binding to the IFN α R induces growth inhibition and apoptosis. Thus the cellular response to TCR and IFN α R activation are fundamentally different.

Despite the two receptors eliciting opposing cellular responses, it is evident that they share similarities in their modes of signalling as both receptors are able to phosphorylate ERK/MAPK. In order

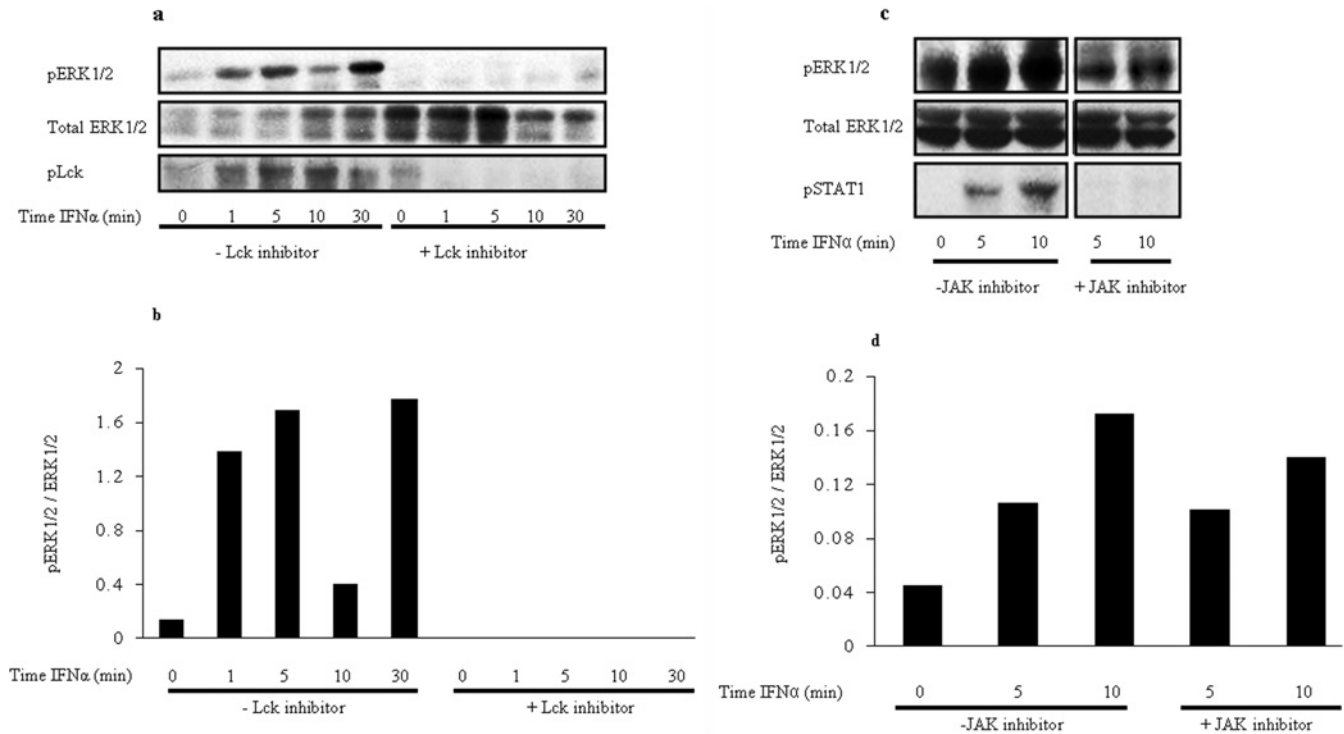


Figure 5 ERK is phosphorylated in response to IFN α in primary human CD4+ T-cells: components of the TCR machinery are required for this ERK phosphorylation

(a) Freshly isolated human peripheral CD4+ T-cells were stimulated for up to 30 min in the presence and absence of Lck inhibitor I. UCHT1 was used to stimulate cells as a control for 5 min in the presence and absence of inhibitor. Lysates were immunoblotted with anti-phospho-ERK1/2 antibody (pERK1/2; top panel), anti-phospho-Lck antibody (pLck; bottom panel) or anti-ERK1/2 antibody (total ERK 1/2; middle panel). (b) Normalized densitometric analysis of ERK phosphorylation. The level of increased ERK activity is relative to the level of total protein. (c) Human peripheral CD4+ T-cells were stimulated for the time periods indicated without (left-hand side) or with 15 nm (right-hand side) of JAK-1 inhibitor for 5 and 10 min on the same gel. The same basal time point (no IFN α added) was used, as shown in the left-most lane. Lysates were used to immunoblot with either anti-phospho-ERK1/2 antibody (top panel), anti-phospho-STAT1 antibody (bottom panel) or anti-ERK1/2 antibody (total ERK 1/2; middle panel). (d) Densitometric analysis of ERK phosphorylation taking into account level of protein present in each lane. The level of ERK phosphorylation is shown in arbitrary units.

for this pathway to ensue the two receptors require an overlapping subset of proteins. Although these proteins were initially thought to be exclusively part of the TCR signalling machinery, they have also been shown to be utilized in signalling by other T-cell-expressed receptors, e.g. the chemokine receptor CXCR4 [30–32] and integrin receptors [33,34].

Previous work has demonstrated that the IFN α R requires TCR-associated proteins including Lck, Zap70, CD45 [3,9], Vav1 [7,35] and possibly LAT (linker for activation of T-cells) [36]. In the present study we show for the first time that Vav1 is required for ERK1/2 phosphorylation in response to IFN α R stimulation. The IFN α R-stimulated Vav1 phosphorylation appears to be identical with that observed with TCR, i.e. phosphorylation occurs upon Tyr¹⁷⁴, located within the acidic region of the protein [15,16]. In addition, we have demonstrated that Slp76 is tyrosine-phosphorylated as a result of IFN α R stimulation and is important for the ensuing ERK1/2 MAPK response. IFN α -stimulated Slp76 phosphorylation appears to be important as reconstitution of J14 cells with the Slp76-Y3F mutant, did not restore MAPK signalling. The acidic region of Slp76 contains residues involved in interactions with Zap70, LAT, Nck, Vav and ITK (IL2-inducible T-cell kinase), resulting in a multimolecular signalling complex [20]. The fact that the Slp76-Y3F mutant ablated Vav1 phosphorylation suggests that in interferon signalling Vav1 functions downstream of Slp76. Thus the role of Vav1 and Slp76 on IFN α -stimulation appears to be identical with that seen when they are recruited to the

TCR on presentation of an antigen. Furthermore, we show for the first time that Slp76 is tyrosine-phosphorylated in response to IFN α stimulation in primary human peripheral CD4+ T lymphocytes. This substantiates results obtained from the Jurkat T-cell lymphoma cell line.

The involvement of the TCR ESC proteins CD45, Lck, Zap70, Vav and Slp76 in IFN α -stimulated signalling via the MAPK pathway prompted investigation of whether the TCR itself was in some way involved. A requirement for the TCR would be indicative of cross-talk between the IFN α R and the TCR. Our results show that in the absence of the intact TCR, ERK1/2 phosphorylation is compromised. The JRT3-T3.5 cell line, lacking the TCR β subunit, exhibited severely diminished ERK1/2 phosphorylation, which could be restored upon reconstitution with the TCR β subunit. In addition, the JRT3-T3.5 cells displayed curtailed MEK phosphorylation and lower levels of total protein tyrosine phosphorylation, thus confirming the integrity of the TCR-activated signal through the MAPK pathway. The fact that the phosphorylation of Tyk2 and the transcription factors STAT1, STAT3 and STAT5 were detected at equivalent levels in both wild-type Jurkat and JRT3-T3.5 cells suggest that the normally observed interferon-induced JAK/STAT signalling was unaffected by the MAPK parallel pathway. However, as STAT proteins can also be serine-phosphorylated upon cytokine induction [37], our results do not exclude the possibility that activation of the parallel MAPK signalling pathway could be involved in serine phosphorylation of the IFN α -activated STAT proteins.

Further evidence that the IFN α R utilizes TCR machinery in healthy human CD4+ T-cells is shown in Figure 5. IFN α -induced MAPK signalling was seen over a 30 min time course, but this was blocked in the presence of Lck inhibitor. Lck is an integral component of the TCR ESC and its absence has been shown previously to prevent proper TCR ESC formation and halt downstream ERK/MAPK signalling at the TCR [3]. We demonstrated that Lck is phosphorylated in response to IFN α in human CD4+ T-cells. Additionally, Lck was also required for ERK/MAPK signalling at the IFN α R (Figure 5a), which demonstrates cross-talk between the TCR and IFN α R for the first time in healthy human T-cells. Inhibition of Lck did not affect phosphorylation of STAT1, which corroborates the finding in Jurkat cells that cross-talk between the TCR and IFN α R is involved in ERK/MAPK activation independently of JAK/STAT signalling and does not overlap with this pathway during early signalling events. Furthermore, inhibition of JAK activation did not affect ERK phosphorylation downstream of the TCR (Figure 5c) thus JAKs are not involved in initiation of ERK/MAPK signals at the IFN α R upon IFN α stimulation. Thus, as in Jurkat cells, JAK/STAT independent TCR-IFN α R cross-talk is evident and essential for ERK/MAPK phosphorylation.

The IL-2 gene is transcribed upon TCR stimulation. The secreted cytokine binds to its receptor to augment the G₁/S-phase transition of the cell cycle in TCR-activated T-cells resulting in clonal expansion and cellular differentiation [38,39]. It is known that at the TCR, both Vav1 and Slp76 co-operate to induce IL-2 transcription [40,41]. Given, as we have demonstrated, that both of these proteins are involved in IFN α signalling, it is plausible that they could mediate transcription of this gene. This would, however, not be consistent with the growth inhibitory events normally mediated by the IFN α R. The lack of NFAT transcriptional activity and hence absence of IL-2 up-regulation show that IFN α is unable to effect an entirely normal TCR-like transcriptional response. Thus the cell is not compromised between two distinct, opposing responses.

Although the assembly of the ESC for signalling through both IFN α R and TCR appears to be highly similar, the downstream effects of the resulting MAPK activation are different. The IFN α R signal results in maximal ERK1/2 phosphorylation at approx. 5 min (Figures 1a and 1b), whereas the TCR response is prolonged [3]. In PC12 cells it has been proposed that transient activation precludes nuclear translocation of ERK2, which occurs with a sustained signal [42,43]. It is thus possible that the IFN α -induced short-lived phosphorylation of ERK2 abrogates its translocation into the nucleus. Cytoplasmically localized ERK1/2 has been implicated in the regulatory function of IFN α signalling. Phosphorylated ERK1/2 has been shown to have serine/threonine kinase activity towards STAT1, enabling this protein to activate transcription [44]. In addition, direct binding of phosphorylated ERK2 to the IFN α R further suggests a role in modulation of this pathway.

Our results are consistent with the previously reported mechanism for the involvement of TCR ESC proteins in IFN α R signalling being based on regulation by Lck and/or CD45 [9]. The physical association of these proteins with the IFN α R is able to activate the kinase domain of membrane-localized Lck. Once activated Lck can act on the ζ -subunit of the TCR and, in a similar way to that observed on antigen presentation, the TCR is able to recruit the ESC.

In conclusion, the present study highlights that signalling from cell membrane-bound receptors is not based on isolated mutually exclusive pathways, but rather that these pathways are in some way networking to allow greater communication in the cell. We speculate that networking of this type may be essential to

allow the cell to discern which course to commit to in the case of exposure to multiple external stimuli. For example, a cell committed to an interferon-stimulated response needs to ensure that a signal from another cell-surface receptor is not able to corrupt this. Thus signalling through a common pathway, such as MAPK in the present study, may serve to alert the cell to down-regulate/block signalling from other receptors. Engaging the MAPK pathway via the TCR ESC in transducing one response (non-proliferation from IFN α R) disables this pathway and halts any possibility that the cell will transduce a simultaneous signal from the TCR invoking proliferation. Engaging the TCR machinery in order to prevent signalling through the TCR while the cell is committed to an alternative response is a fail-safe method to stop the cell from responding to two opposing instructions.

AUTHOR CONTRIBUTION

Claire Stevens performed most of the experimental work and conceived many of the experiments. Ann-Marie Simeone repeated some of the original Western blots and prepared several of the Figures. Susan John supervised Claire Stevens in the primary cell work and performed repeat blots with inhibitors in primary cells. Zamil Ahmed oversaw Claire Stevens and provided useful input for the cell-based assays and also provided useful comment at the manuscript write-up stage. Orso Lucherini performed the transcriptional analysis experiments. Tatiana Baldari supervised and provided reagents for the transcriptional analysis experiments. John Ladbury conceived the project and supervised throughout and also had a major role in manuscript writing and production.

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