Type I Interferon regulates cytokine-delayed neutrophil apoptosis, reactive oxygen species production and chemokine expression via activation of p38 MAPK

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ABSTRACT

Interferons (IFNs) are key regulators of a number of inflammatory conditions in which neutrophils play an important role in pathology, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), where Type I IFNs are implicated in disease pathology. However, IFNs are usually generated in vivo together with other cytokines that also have immunoregulatory functions but such interactions are poorly-defined experimentally. We measured the effects of Type-I IFN (IFNα), elevated in both RA and SLE, on the functions of healthy neutrophils incubated in vitro in the absence and presence of pro-inflammatory cytokines typically elevated in inflammatory diseases (TNFα, GM-CSF). IFNα alone had no effect on neutrophil apoptosis, however it did abrogate the anti-apoptotic effect of GM-CSF (18h, p<0.01). The enhanced stability of the anti-apoptotic protein Mcl-1 and delayed activation of caspase activation normally regulated by GM-CSF were blocked by IFNα: this effect was mediated, in part, by activation of p38 MAPK, increased turnover of the anti-apoptotic protein Mcl-1 and cleavage of caspases. IFNα alone also primed ROS production alone and maintained the transient priming effect of TNF for up to 4h: it also down-regulated GM-CSF and TNFα-activated expression of CXCL1, CXCL2, CXCL3, CXCL8, CCL3 and CCL4, but in contrast increased the expression of CXCL10. These novel data identify complex regulatory signalling networks in which Type I IFNs profoundly alter the response of neutrophils to inflammatory cytokines. This is likely to have important consequences in vivo and may explain the complexity and heterogeneity of inflammatory diseases such as RA, in which multiple cytokine cascades have been activated.

Keywords: neutrophil, interferon alpha, apoptosis, p38 MAPK
1 INTRODUCTION

Interferons (IFNs) are a family of cytokine glycoproteins that regulate the immune response to infection by bacteria, viruses and parasites and are broadly grouped into Type-I, -II and -III, based on their receptor signalling (Platanias, 2005; Li et al., 2009). Type-I IFNs, including IFNα, IFNβ, IFNκ and IFNω, signal through the IFNα receptor dimer (IFNAR1 and IFNAR2), whereas Type-II IFN, IFNγ, signals via the IFNγ receptor dimer (IFNGR1 and IFNGR2) (Platanias, 2005). Type-III IFNs, IFNλ1, IFNλ2, IFNλ3 (also known as IL29, IL28α and IL28β, respectively) signal via a receptor complex comprising IL10R2 and IFNLR1 (Li et al., 2009). Interferon receptors signal through the Janus Kinase (JAK) family of enzymes (JAK1, JAK2, JAK3, Tyk2), which, when activated, induce phosphorylation and nuclear translocation of signal transducer and activation of transcription (STAT) proteins (Platanias, 2005; Li et al., 2009; O'Shea and Plenge, 2012). Type-I interferons activate heterodimers of JAK1:Tyk2 via activation of the IFNα receptor complex leading to activation of STAT1:STAT2 heterodimers. In some instances, Type-I interferons can also activate STAT3, STAT4 and STAT5 (Platanias, 2005). Type-II interferons activate heterodimers of JAK1:JAK2 leading to activation of STAT1 homodimers.

As well as playing key roles in the host response to pathogen-mediated infections, IFNs are also implicated in the pathogenesis of a number of inflammatory disorders, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögrens syndrome and myositis (Wright et al., 2014; Higgs et al., 2012; Peck and Nguyen, 2012). In SLE, Type-I interferon signalling correlates with disease severity, and may reflect activation of plasmacytoid dendritic cells (pDCs) which contribute to auto-antibody formation in this disease (Lichtman et al., 2012; Lande et al., 2011). However, the role of IFNs in RA is less well understood and TNFα has generally been considered to be the predominant cytokine that drives disease progression, based on the success of therapeutic TNFα blockade (TNFi) (Hyrich et al., 2009, 2006).

However, there are several reports of an interferon gene expression signature in RA peripheral blood (Raterman et al., 2012; Reynier et al., 2011; van Baarsen et al., 2010; Mavragani et al., 2010), and we recently identified a correlation between a Type-I ‘IFN-high’ gene expression signature in peripheral blood neutrophils and good response to TNFi in a cohort of RA patients with severe disease activity (Wright et al., 2014, 2017). As TNFα inhibits the activity of pDCs and thereby the production of IFNα (Palucka et al., 2005), it appears paradoxical that patients who respond to TNFi therapy have high levels of interferon-regulated gene activity. However, whilst TNFα can often be detected at high levels in RA synovial fluid (Wright et al., 2012), concentrations of TNFα in RA serum are typically low or even undetectable, even in patients who subsequently respond well to TNFi therapy (Wright et al., 2012, 2011).

The effect of Type-II IFN (IFNγ) alone on neutrophils has been relatively well defined, and in vitro can enhance expression of the high-affinity cell-surface molecules FcγRI (CD64) (Quayle et al., 1997) and MHC-II (Cross et al., 2003), both of which can be detected on the surface of ex vivo neutrophils isolated from RA synovial fluid (Quayle et al., 1997; Cross et al., 2003). IFN-γ also delays neutrophil apoptosis and primes the respiratory burst (Sakamoto et al., 2005; Klebanoff et al., 1992). However, the effect of Type-I IFNs on neutrophil function is less clear, with studies reporting contradictory effects on both apoptosis and reactive oxygen species (ROS) production via the respiratory burst (Sakamoto et al., 2005; Koie et al., 2001; Conde et al., 1994).

The aim of this study was to characterise the functional effects of Type-I interferon (IFNα) on healthy neutrophils, in the absence and presence of GM-CSF and TNFα, cytokines typically elevated in RA. We demonstrate that co-incubation of healthy neutrophils with IFNα profoundly alters their functional responses in ways that have important consequences for understanding neutrophil phenotype in inflammatory disease. These data reveal potential complex regulatory
cytokine-signalling networks that control immune function in inflammation and inflammatory disease.

2 METHODS

2.1 Isolation of neutrophils

Participants gave written informed consent according to the declaration of Helsinki and ethics approval for this study was obtained from the University of Liverpool Committee on Research Ethics. Neutrophils (purity > 97%, viability > 98%) were isolated from healthy heparinised peripheral blood using HetaSep (Stem Cell) and Ficoll-Paque (GE Healthcare), and contaminating erythrocytes were removed by hypotonic lysis as described previously (Wright et al., 2013). Freshly-isolated neutrophils were suspended at $10^6$ or $5 \times 10^6$ cells/mL in RPMI media (containing 10% human AB serum (Sigma)) and incubated at 37°C and 5% CO$_2$ for up to 18h. Cytokines were added at the following concentrations: GM-CSF (5 ng/mL, Roche), TNF (10 ng/mL, Calbiochem), IFN-αA2 (0.1-20 ng/mL, Sigma), IFN-β1a (0.1-20 ng/mL, ImmunoTools). Preliminary experiments showed similar results using both Type-I IFNs (IFN-αA2 and IFN-β1a), therefore IFN-αA2 was used throughout and is referred to in the text as IFN-α. Inhibitors of cell signalling were added 30 min prior to cytokine stimulation: BIRB796 (10 µM, p38 MAPK inhibitor, Tocris), PD98059 (50 µM, ERK1/2 inhibitor, Merck), JNK-IN-8 (1 µM, JNK inhibitor, Selleck Chemicals). Cycloheximide (inhibitor of protein synthesis) was used at 10 µg/mL (Sigma) and added 30 min prior to cytokine treatment of neutrophils.

2.2 Measurement of apoptosis

Neutrophils (2.5x10$^4$) were diluted in 50 µL of HBSS (Gibco) containing 0.5 µL Annexin V-FITC (Invitrogen), and incubated in the dark at room temperature for 15 min. The total volume was then made up to 250 µL with HBSS containing propidium-iodide (PI, 1 µg/mL) before analysis by flow cytometry (5,000 events analysed) using a Guava EasyCyte instrument.

2.3 qPCR

RNA was extracted using Trizol (Life Technologies) and chloroform, precipitated in isopropanol and cleaned using the RNaseasy kit (Qiagen) including a DNase digestion step. cDNA was synthesised using the Superscript III First Strand cDNA Synthesis kit (Invitrogen) using equal concentrations of RNA across samples, as per the manufacturer's instructions. Real-time PCR analysis was carried out using the QuantiTect SYBR Green PCR kit (Qiagen) as per the manufacturer’s instructions. Analysis was carried out on a Roche 480 LightCycler in a 96-well plate using a 20 µL reaction volume. Primers used are shown in Table 1.

Target gene expression was quantified against B2M as a housekeeping gene (Zhang et al., 2005; Pfaffl, 2001). We also carried out an RNA-seq experiment (n=1) to identify targets for validation by qPCR and Western blot. This was carried out using standard RNA-seq protocols on the Illumina HiSeq 2000 platform as previously described (Wright et al., 2017, 2013, 2015).

2.4 Western blotting

Neutrophils were rapidly lysed in boiling Laemmli buffer containing phosphatase inhibitors (Calbiochem). Proteins were separated by SDS-PAGE on a 12% gel and transferred onto PVDF membrane (Millipore). Primary antibodies were: Mcl-1 (1:1000, Cell Signalling), caspase-8 (1:1000, Cell Signaling), caspase-9 (1:1000, Cell Signaling), phosphorylated p38 MAPK (1:1000, Cell Signaling), total p38 MAPK (1:1000, Cell Signaling), Actin (1:10,000, Abcam). Secondary antibodies were anti-rabbit IgG (GE Healthcare) and anti-mouse IgG (Sigma) HRP-linked antibodies (1:10,000). Bound antibodies were detected using the ECL system (Millipore) on carefully-exposed film to avoid saturation (Amersham).

2.5 Measurement of respiratory burst

Neutrophils (5x10$^6$/mL) were incubated for up to 4h with IFN-α (10 ng/mL) in the absence or presence of GM-CSF (5 ng/mL), or TNF-α (10 ng/mL). Cells
(2x10^5) were diluted in HBSS containing luminol (10µM) and the respiratory burst stimulated with f-Met-Leu-Phe (fMLP) (1µM). Luminescence was measured, in duplicate, continuously for 30 min.

2.6 Statistical analysis
Statistical analysis was carried out using SPSS v20, using the Student’s t-test unless otherwise stated.

3 RESULTS
3.1 Interferon alpha abrogates GM-CSF delayed neutrophil apoptosis via p38 MAPK signalling
Blood neutrophils constitutively undergo apoptosis after around 24h but during inflammation, apoptosis can be delayed by cytokines such as TNFα and GM-CSF (Wright et al., 2013). In order to determine the effect of IFNα on neutrophil apoptosis, healthy neutrophils were incubated for 18h in the absence or presence of IFNα over a range of concentrations. IFNα alone did not significantly alter the rate of constitutive neutrophil apoptosis (Figure 1A), but it dose-dependently abrogated GM-CSF-delayed apoptosis (Figure 1A, p < 0.05). However, this effect was cytokine-specific as it had no significant effect on the rate of apoptosis in neutrophils treated for 18h with TNFα (Figure 1B). In order to explore the mechanisms responsible for the effect of IFNα on GM-CSF-delayed apoptosis, neutrophils were incubated for up to 18h with these cytokines and protein lysates were analysed by Western blot. GM-CSF has previously been shown to delay neutrophil apoptosis through stabilisation of the anti-apoptotic protein Mcl-1 (Derouet et al., 2004). We found that the levels of Mcl-1 after 6h incubation were significantly lower in neutrophils treated with GM-CSF and IFNα compared to GM-CSF alone (Figure 1C,D, p < 0.05). IFNα treatment alone had no significant effect on Mcl-1 levels compared to untreated controls (data not shown). Overnight incubations (18h) of neutrophils treated with GM-CSF and IFNα had significantly lower levels of pro-caspase-8 and pro-caspase-9 in the presence of IFNα compared to GM-CSF alone (Figure 1E-G, p < 0.05).

Mcl-1 is an anti-apoptotic, Bcl-2 family protein and a key regulator of neutrophil apoptosis (Moulding et al., 1998; Wardle et al., 2011). It is rapidly turned over in human neutrophils and cellular expression of Mcl-1 correlates with the level of neutrophil apoptosis. However, Mcl-1 levels can be maintained (and apoptosis delayed) either by phosphorylation and stabilisation of existing Mcl-1 protein or synthesis of new Mcl-1 protein (Derouet et al., 2004). Pre-incubation of neutrophils with cycloheximide (CHX), an inhibitor of protein synthesis, prior to addition of GM-CSF and IFNα showed that levels of Mcl-1 protein decreased more rapidly in the presence of GM-CSF and IFNα compared to GM-CSF alone (Figure 2A). This suggests that IFNα blocks the stabilisation of Mcl-1, likely via interference with post-translational modification (phosphorylation) of mature Mcl-1 protein (Thomas et al., 2010). IFNα alone had no effect on Mcl-1 levels in the presence of CHX (Figure 2A).

We next investigated the potential mechanism by which IFNα abrogates the anti-apoptotic effect of GM-CSF using signalling inhibitors of p38 MAPK, JNK and ERK1/2 which are known down-stream targets of the IFNAR1/2 complex (Platanias)
Figure 1. The effect of IFNα on neutrophil apoptosis. (A) IFNα (IFNA) alone had no effect on neutrophil apoptosis over a range of physiologically-relevant concentrations (0.1-20 ng/mL) compared to untreated neutrophils (VC, vehicle control) at 18h. However, IFNα abrogated the anti-apoptotic effect of GM-CSF (5ng/mL). (B) IFNα had no effect on the anti-apoptotic effect of TNFα (TNF). (C,D) Expression of Mcl-1 was lower in neutrophils co-incubated with GM-CSF and IFNα compared to GM-CSF alone. (E-G) The levels of pro-caspase-8 and -9 were lower after 18h culture in the presence of GM-CSF and IFNα compared to GM-CSF alone. (all experiments n=3, *p < 0.05, **p < 0.01).
Figure 2. The effect of IFNα on Mcl-1 turnover and p38 MAPK activation. (A) Pre-incubation of neutrophils with cycloheximide (CHX) prior to GM-CSF and IFNα showed increased turnover of Mcl-1 protein levels in the presence of IFNα, likely through blockade of Mcl-1 protein stabilisation. (B) p38 MAPK was rapidly phosphorylated by IFNα over 120 min in the absence and presence of GM-CSF. (C) Inhibition of p38 MAPK phosphorylation by BIRB796 significantly inhibited the abrogative effect of IFNα on GM-CSF-delayed neutrophil apoptosis over 18h (n=4, *p< 0.05, n.s. = not significant).

ROS production was significantly increased by pre-treatment with IFNα for 3h and then stimulated by fMLP (Figure 3B). Co-incubation of IFNα with GM-CSF had no significant effect on the GM-CSF-primed respiratory burst, which was sustained over a 5h incubation period (4h timepoint shown in Figure 3C). TNFα rapidly primed the neutrophil respiratory burst over short incubation times of up to 1h (data not shown), but by 4h this priming effect was lost. However, co-incubation with IFNα significantly sustained this transient priming effect of TNFα over and above the priming effect of the IFNα alone (Figure 3D).

3.3 Interferon alpha is a key regulator of neutrophil chemokine production

Neutrophils play an important role in the crosstalk between the innate and adaptive immune system via the secretion of chemokines (Mantovani et al., 2011). We measured the expression of chemokines by healthy neutrophils in response to incubation for 1h with GM-CSF and TNFα in the absence and presence of IFNα using RNA-Seq (n=1, data not shown) and from these data, we selected a panel of genes that were subsequently analysed by qPCR (n=3). GM-CSF and TNFα significantly up-regulated the expression of CCL3, CCL4, CXCL1, CXCL2, CXCL3 and CXCL8, but not CXCL10 (Figure 4A,B). IFNα alone significantly up-regulated the expression of CXCL10 compared to untreated neutrophils (> 600-fold, data not shown). However, when neutrophils were co-incubated with IFNα and GM-CSF or TNF, the increase in expression of CCL3, CCL4, CXCL1, CXCL2, CXCL3 and CXCL8 was significantly abrogated (Figure 4C,D). Conversely, the expression of CXCL10 was significantly enhanced (Figure 4C,D).

We next investigated chemokine expression in a group of RA patients who had undergone whole transcriptome sequencing as part of a previous study (Wright et al., 2014). In this study, patients clustered...
Figure 3. Effect of IFNα on neutrophil respiratory burst. (A) IFNα (IFNA) did not prime the respiratory burst in response to fMLP (1 µM) after short incubation times of 1h, (B) but did significantly prime the respiratory burst after 3h incubation. (C) Co-incubation of IFNα with GM-CSF had no effect on neutrophil priming for up to 4h. (D) IFNα maintained TNF-induced priming for up to 4h incubation. Representative traces from n=4 experiments (*p<0.05, **p<0.01).

4 DISCUSSION

While the vast majority of in vitro experiments characterise the effects of single pro-inflammatory cytokines on immune cells, it is evident that during inflammation in vivo, multiple cytokines are involved in the regulation of immune function. In this study, we have investigated the effect of Type-I interferon (IFNα) on neutrophil functions in the absence and presence of two pro-inflammatory cytokines (GM-CSF, TNFα) that are implicated in the pathogenesis of rheumatoid arthritis (RA) (Wright et al., 2012). We have demonstrated that IFNα profoundly alters the response of neutrophils to these pro-inflammatory cytokines in ways that have important implications for understanding neutrophil phenotype in disease.

In our experiments, IFNα abrogated the anti-apoptotic effect of GM-CSF, via blocking the stabilisation of Mcl-1 protein and activation of caspases. We found that IFNα altered the phosphorylation kinetics of p38 MAPK, and that blockade of p38 MAPK with a highly selective inhibitor (BIRB796) significantly decreased the effect of IFNα on GM-CSF-delayed apoptosis. p38 MAPK is a known regulator of Mcl-1 turnover in neutrophils (Derouet et al., 2004), and inhibition...
Figure 4. Effect of IFNα on chemokine expression. Chemokine expression was upregulated in neutrophils incubated for 1h with (A) GM-CSF or (B) TNFα (fold change in expression from 0h shown, n=3 experiments). Co-incubation with IFNα (IFN-A) abrogated the expression of chemokines in the presence of (C) GM-CSF or (D) TNF, whereas expression of CXCL10 was enhanced (*p < 0.05, **p < 0.01). (E) RA patients with an ‘IFN-high’ gene expression profile expressed chemokines (CXCL1, CXCL2, CXCL3, CXCL8, CCL3, CCL4) at lower levels than ‘IFN-low’ RA patients (n=14) levels (fold difference in mean expression level between the two groups of patients shown). The expression level of CXCL10 was higher in ‘IFN-high’ RA patients than in ‘IFN-low’ patients.

Activators of p38 MAPK signalling, such as vitamin D, have been shown to have a pro-apoptotic effect on neutrophils via regulation of expression of Bcl-2 and caspase family member genes (Tang et al., 2018; Yang et al., 2015). Whilst we saw changes in the levels of Mcl-1, caspase-8 and caspase-9 protein in our experiments, we did not see any change in expression of these genes in our RNA-seq experiment (data not shown), suggesting the effect of IFNα is post-translational. p38 MAPK has
also been shown to be anti-apoptotic in neutrophils via the regulation of caspase phosphorylation and activation (Alvarado-Kristensson et al., 2004; Aoshiba et al., 1999) and therefore there remain subtleties in the IFNα activation of p38 MAPK that have not been completely addressed in our study. It is interesting to note that IFNα did not alter the delay in apoptosis induced by TNFα in our study. TNFα-delayed apoptosis in human neutrophils is not dependent upon Mcl-1 levels or stabilisation of Mcl-1 protein; this is mediated via NF-κB activation and synthesis of the Bcl-2 family protein A1/Bfl-1 (Cross et al., 2008).

IFNγ has previously been shown to prime the neutrophil respiratory burst, but the role of IFNα in this process is less clear, with conflicting reports in the literature of the effect of IFNα on neutrophil apoptosis and ROS production (Sakamoto et al., 2005; Koie et al., 2001; Conde et al., 1994). This may be due to the IFNα isoform that was used in in vitro experiments. IFNα has 13 different isoforms (Gibbert et al., 2013), and the isoform used in our experiments was IFNαA2, which is the most abundant isoform in humans. In addition, inconsistencies in the measurement of IFNs as units/mL rather than ng/mL, often makes direct comparison of experimental conditions difficult. We comprehensively showed that over incubations of up to 1h IFNα was not able to prime the respiratory burst. However, over longer incubations > 3h, IFNα primed the respiratory burst, both alone and in combination with TNFα. This may indicate a requirement for gene expression and a secondary, autocrine signalling effect to fully initiate the primed response.

Our experiments also identified an important role for IFNα in the regulation of chemokine production by neutrophils. We showed that IFNα significantly decreased the expression of chemokine genes CXCL1, CXCL2, CXCL3, CCL3 and CCL4 in response to GM-CSF and TNFα, but enhanced the expression of CXCL10. This has important implications for neutrophil-mediated regulation of the immune response in vivo. The chemokines CXCL1, CXCL2 and CXCL3 (GRO-α, -β and -γ, respectively) and CCL3 and CCL4 (MIP-1α and -1β, respectively) predominantly function as neutrophil chemoattractants, whereas CXCL10 (IP-10) is primarily a chemoattractant for T-cells, monocytes, NK cells and dendritic cells. Therefore, the cellular signalling events regulated by combinations of inflammatory cytokines (GM-CSF and/or TNFα) and IFNs in vivo may act as a switch to direct the immune response away from neutrophil-mediated inflammation towards lymphocyte-mediated inflammation, underlying the key role of neutrophils in the cross-talk between the innate and adaptive immune systems (Mantovani et al., 2011). Importantly, we noted that the changes in chemokine expression in the presence of IFNα mirrored exactly the phenotype that is seen in RA neutrophils, underlying the important role of IFNα in the regulation and fine-tuning of neutrophil function in inflammatory disease.

In summary, our data show that the response of human neutrophils to TNFα and GM-CSF is profoundly altered in vitro by IFNα, whereby IFNα acts as a switch to alter the inflammatory neutrophil phenotype by regulating apoptosis, ROS release and chemokine production. This has important consequences for neutrophil inflammatory responses in vivo, particularly in complex and heterogeneous inflammatory diseases such as RA in which combinations of these cytokines exist, and may explain why some patients respond better to certain biologic therapies than others.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

LGA carried out the experiments and analysed the data. HLW designed the research, carried out the experiments, analysed the data, and wrote the manuscript. RJM revised the manuscript. SWE analysed the data and revised the manuscript.
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