IL4Ra signaling abrogates hypoxic neutrophil survival and limits acute lung injury responses in vivo.

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Impact
These studies demonstrate for the first time that IL-4 directly regulates neutrophil hypoxic survival in-vitro and in-vivo, supporting its role in inflammation resolution. Our findings are key to conditions such as Acute Respiratory Distress Syndrome in which hypoxia, neutrophilic inflammation and IL-4 co-exist, as our work identifies this axis
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as an attractive therapeutic target which could be manipulated to drive inflammation resolution.

**At a glance commentary**

The role for type 2 cytokines such as IL-4 and IL-13 in inflammation resolution has been extensively studied but their precise effect on neutrophils in this context remains largely unknown. Neutrophils are key innate cells adapted to working under severe hypoxic conditions, an environment which drives their survival. Such conditions are found in the lungs of Acute Respiratory Distress Syndrome patients (ARDS). In this article we identify a significant role for both IL-4 (and IL-13) as regulators of neutrophil hypoxic survival in both human and mouse cells using a combination of *in vitro* and *in vivo* studies. We demonstrate that IL-4 is present in the lungs of ARDS sufferers and in mouse models of lung injury. Furthermore, IL-4 abrogates neutrophil hypoxic survival *in vitro* in human and mouse neutrophils in a prolyl-hydroxylase 2-dependent manner. Finally, through the careful use of animal models, including chimera studies we demonstrate the therapeutic potential of manipulating this pathway in the lung during hypoxic neutrophilic lung inflammation.
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Abstract

Rationale: Acute respiratory distress syndrome (ARDS) is defined by the presence of systemic hypoxia and consequent upon disordered neutrophilic inflammation. Local mechanisms limiting the duration and magnitude of this neutrophilic response remain poorly understood. Objectives: We aimed to test the hypothesis that during acute lung inflammation tissue production of pro-resolution type 2 cytokines (IL-4 and IL-13) dampens the pro-inflammatory effects of hypoxia through suppression of Hypoxia Inducible Factor (HIF-1)α-mediated neutrophil adaptation, resulting in resolution of lung injury. Methods: Neutrophil activation of IL4Ra signaling pathways was explored ex vivo in human ARDS patient samples, in vitro following the culture of human peripheral blood neutrophils with recombinant IL-4 under conditions of hypoxia, and in vivo, through the study of IL4Ra deficient neutrophils in competitive chimera models and wild-type mice treated with IL-4. Measurements and Main Results: IL-4 was elevated in human bronchoalveolar lavage from ARDS patients and its receptor was identified on inflamed lung neutrophils. Treatment of human neutrophils with IL-4 suppressed HIF-1α dependent hypoxic survival and limited pro-inflammatory transcriptional responses. Increased neutrophil apoptosis in hypoxia, also observed with IL-13, required active STAT signaling, and was dependent upon expression of the oxygen sensing prolyl hydroxylase PHD2. In vivo, IL-4Ra-deficient neutrophils had a survival advantage within a hypoxic inflamed niche, in contrast inflamed lung treatment with IL-4 accelerated resolution through increased neutrophil apoptosis. Conclusions: We describe an important interaction whereby IL4Ra-dependent type 2 cytokine signaling can directly inhibit hypoxic neutrophil survival in tissues and promote resolution of neutrophil mediated acute lung injury.
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Introduction

Acute respiratory distress syndrome (ARDS) is a clinical syndrome defined by the presence of bilateral pulmonary infiltrates and arterial hypoxaemia. Although a number of causes are recognized including sepsis, trauma and aspiration, ARDS is the consequence of a disordered inflammatory response in which accumulation of activated neutrophils within the lung interstitium and distal airspaces is associated with lung endothelial and epithelial injury. A fine balance therefore exists between an effective innate response that enables host pathogen control and a disordered one in which inappropriate neutrophil persistence and activation drives tissue injury. Production of local factors during the course of an inflammatory response together with changes in the physiological environment, are crucial in regulating this balance.

Hypoxia frequently characterizes acute inflammatory sites \(^1\) and is an important mediator of neutrophil survival and inflammatory function \(^2\). In human disease states, hypoxia has been associated with elevated neutrophil numbers, reduced neutrophil apoptosis and increased disease severity \(^3,4,5,6\). Thus hypoxia can be regarded as a pro-inflammatory stimulus that facilitates the persistence of neutrophils at inflamed sites.

\textit{In vivo} models have shown that enhanced neutrophil survival in hypoxia is dominantly mediated by stabilization of the transcription factor HIF-1\(\alpha\) \(^7-9\). HIF-1\(\alpha\) itself is regulated by a family of dioxygenases including the prolyl hydroxylase domain containing enzymes (PHD1-3) and factor inhibiting HIF (FIH) \(^10\). Other studies have delineated oxygen-independent regulation of the HIF pathway, with bacterial activation of TLR signaling pathways leading to HIF-1\(\alpha\) stabilization \(^11,12\).
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The interaction between hypoxia and local factors in regulating HIF-1α signaling, and the consequences for inflammatory responses, are therefore of great interest. IL-4 has long been considered an important cytokine for tissue repair, counterbalancing the effects of pro-inflammatory type 1 cytokines on the macrophage compartment on the one hand \(^\text{13}\), but conversely for promoting allergic airway inflammation on the other. Interestingly, mouse neutrophils express both IL4Rα and IL13Rα \(^\text{14}\), the components of the type 2 IL-4 receptor complex, through which both IL-4 and IL-13 signal. IL4Rα is upregulated in myeloid cells following stimulation with pro-inflammatory mediators, including lipopolysaccharide (LPS), with neutrophils displaying the highest fold increase in receptor expression \(^\text{15}\). Moreover, IL-4 is itself released by macrophages following LPS simulation during the acute response \(^\text{16}\).

With cell specific consequences of IL-4 signaling, we questioned whether IL-4 could regulate neutrophilic inflammation during acute lung inflammation to promote inflammation resolution and restoration of tissue homeostasis. We provide evidence of activation of IL-4 pathways in the setting of acute lung injury and demonstrate that IL-4 prevents neutrophil hypoxic HIF-1α induction, abrogates hypoxic survival of human and mouse neutrophils, dampens pro-inflammatory cytokine expression and promotes resolution of neutrophilic inflammation \textit{in vivo}.

Methods

Blood donors for isolation of human peripheral blood neutrophils

Participants were consented in accordance with the Declaration of Helsinki principles, with AMREC approval for study of healthy human volunteers through the
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MRC / University of Edinburgh Centre for Inflammation Research blood resource (15-HV-013).

ARDS patient samples
Bronchoalveolar lavage (BAL) samples were studied from patients recruited to the HARP study (06/NIR02/77) and healthy controls undergoing research bronchoscopy (12/NI/0082), see supplementary methods.

Animals

Il4ra−/− mice on a C57BL/6 background were generated by Dr Frank Brombacher following backcross of the BALB/Il4ra−/− strain (minimum 9 generations) and a gift from Dr Cecile Benezech. Animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 with local ethical approval.

Human neutrophil isolation and culture
Human blood neutrophils, isolated by dextran sedimentation and discontinuous Percoll gradients, were cultured (5 million cells/ml) in normoxia (21% O₂, 5% CO₂) or hypoxia (1% (3kPa) O₂, 5% CO₂) ± recombinant human IL-4 (10ng/ml), IL-13 (100ng/ml) or IFNγ (100ng/ml) (Peprotech), LPS (100ng/ml)( R515 (Enzo), IL4Ra (polyclonal blocking antibody, R&D Systems), STAT3 (200nM 5,15-DPP, Sigma), or STAT6 (20nM AS 1517499, Axon Medchem).

Murine neutrophil culture
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Inflammatory neutrophils isolated by bronchoalveolar lavage (BAL) 24 hours post LPS challenge were cultured at 2 million cells/ml as detailed above.

Chimera Studies

To dissect the consequence of IL4Ra loss on bone marrow and circulating neutrophil populations in the context of hypoxia we used an organ protected chimeric approach in the liver (regional hypoxia) and a fractionated radiation strategy in the lung (systemic hypoxia). Inflammation outcomes were defined in a model of acute liver injury (CCl4) and acute lung injury (LPS), see supplementary methods.

IL4c experiment

50μl IL4c (250μg/ml recombinant murine IL-4 (Peprotech):1250μg/ml anti-mouse IL-4 (clone 11b11, Bio X Cell)) or PBS vehicle control was administered intratracheally 6 hours post LPS-nebulization and mice transferred to hypoxia.

Statistical analysis

For non-parametric data significance was determined by Mann-Whitney. Data shown as individual points with median and interquartile range values. For multiple comparisons with one variable we used one way ANOVAs with Kruskal-Wallis post test and for two variables, two-way ANOVAs with Holm-Sidak post test analysis and adjustment for multiplicity of tests. Data shown as mean +/- SEM. P<0.05 was considered significant. Individual data points represent individual mice or human samples.

Results
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**IL-4 receptor α signaling pathways are present in the airways of patients with ARDS, circulating neutrophils and in a mouse model of ARDS**

To determine whether patients with ARDS have evidence of local production of IL-4, we measured IL-4 levels in bronchoalveolar lavage (BAL) samples of ARDS patients compared to healthy controls. ARDS BAL fluid contained significantly more IL-4 than lavage samples obtained from healthy airspaces (Figure 1A). Interestingly, preliminary data suggest that neutrophils from patients with ARDS may have higher levels of IL4Ra expression than healthy controls (Figure 1B) supporting the relevance of this pathway to ARDS.

To confirm the ability of circulating neutrophils to respond to IL-4 in the context of physiological hypoxia, we examined neutrophil expression of IL4Rα. Healthy human blood neutrophils demonstrated IL4Rα surface protein expression and transcript when cultured in normoxia (21% O₂) and hypoxia (1% O₂), with loss of surface protein and induction of mRNA following stimulation with IL-4 (Figure 1C, D).

In order to determine if the IL-4 pathway is activated in an acute lung injury (ALI) mouse model we examined IL-4 BAL levels from mice exposed to nebulized lipopolysaccharide (LPS). In keeping with the data from human ARDS BAL, IL-4 is produced in the airspace of mice exposed to LPS (Figure 1E). We then determined if systemic hypoxia (10% FiO₂) altered the neutrophil inflammatory responses in vivo and found that in-vivo lung neutrophils express the IL4Ra, more so in hypoxia than in normoxia, following LPS (Figure1F). In this setting alveolar macrophages also upregulate their IL4Ra expression in hypoxia following LPS stimulation (Figure 1G) but T cells do not (Figure 1H). Importantly, BAL inflammatory neutrophils cultured ex-
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*vivo* in hypoxia with IL-4 had reduced hypoxic survival thus suggesting IL-4 can overcome the enhanced neutrophil survival observed in physiological and pathophysiological hypoxia (Figure 1I).

**IL-4 abrogates neutrophil hypoxic survival and suppresses pro-inflammatory responses**

We next sought to determine whether IL-4 modified human neutrophil apoptosis in-vitro. In marked contrast to the pro-survival effects of IFNγ/LPS, IL-4 had no effect on constitutive apoptosis in normoxia, but inhibited hypoxic survival at both early (6 hour) (Figure 2A) and late (20 hour) (Figure 2B) time points, as determined by morphological appearance (chromatin condensation and loss of characteristic lobes and bridges). Culture with IL-13, which also signals through IL4Rα, similarly resulted in loss of hypoxic neutrophil survival (Figure 2C). Abrogation of hypoxic survival by IL-4 (Figure 2D, F) and IL-13 (Figure 2E, F) was further validated by flow cytometry. We confirmed that these effects were IL4Rα-dependent using an IL4Rα blocking antibody, which restored hypoxic survival in the presence of IL-4 and IL-13 (Figure 2G, H). Abrogation of enhanced neutrophil survival was not confined to hypoxic responses, with IL-4 and IL-13 also partially reversing increased survival following LPS stimulation (Figure 2I). In addition to its effects on neutrophil hypoxic survival, IL-4 suppressed LPS-induced expression of the pro-inflammatory genes CCL2, CCL3, TNF and IL1B, previously linked to HIF-1 α activity (Figure 2J) 18. Conversely, IL-4 induced CCL17 (Figure 2K), a chemokine associated with Treg recruitment, M2-polarised macrophages and tumor-associated neutrophils 19-22. IL-4 did not influence baseline reactive oxidative species (ROS) production or neutrophil respiratory burst...
IL-4 limits neutrophilic responses in hypoxia following stimulation with N-formyl-met-leu-phe (fMLP) under normoxic or hypoxic conditions (Figure 2L).

**IL-4 suppresses hypoxic induction of NF-κB protein despite preserved metabolic capacity**

Given that hypoxia upregulates glycolysis and IL-4 has been shown to affect macrophage metabolism, we sought to determine whether the effects of IL-4 on neutrophil survival were through alteration of metabolic pathways. GAPDH, PKM, PGK1 and PFKFB3 levels were however, unaltered following IL-4 treatment in-vitro (Figure 3A). Although a modest decrease in glucose uptake was observed with IL-4 (Supplementary Figure 1A), overall neutrophil glycolytic capacity was preserved (Figure 3B), in contrast to the described effects in macrophages.

In macrophages, polarization of phenotypic states has been linked to metabolic rewiring, with IL-4 favoring fatty acid oxidation. IL-4 did not affect neutrophil expression of PPARGC1B, a key regulator of β-oxidation (Supplementary Figure 1B) or flux through fatty acid oxidation (Figure 3B). Overall energy status (ATP/ADP ratio) was also not modified by IL-4 (Figure 3B), suggesting that altered metabolism and/or a lack of ATP are not responsible for the reduction in in-vitro neutrophil survival.

We have previously shown NF-κB upregulation to be critical for enhanced neutrophil survival under hypoxic conditions. Hif1a−/− neutrophils have reduced expression of the NF-κB subunit Rela and of Ikka, a kinase that targets the NF-κB inhibitor IκB for degradation. We found that IL-4 diminished NF-κB RelA protein expression under
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hypoxic conditions (Figure 3C). However, this regulation occurred at a post-transcriptional level, with the relative expression of RELA and CHUK (IKKα) mRNA unchanged by stimulation with IL-4 in the setting of hypoxia (Figure 3D).

IL-4-induced STAT signaling leads to loss of hypoxic survival by induction of PHD2 and degradation of HIF1α protein

Previous studies have linked IL-4 signaling through IL4Rα to activation of STAT6 pathways and protection in inflammatory models of arthritis. In macrophages, STAT3 and STAT6 are important mediators of IL-4 and IL-13 signaling through both the IL4Rα/γc and IL4Rα/IL13Rα. In-vitro pre-incubation with either the STAT3 inhibitor 5,15-DPP or the STAT6 inhibitor AS1517499 was sufficient to rescue hypoxia-induced survival in the presence of IL-4 or IL-13 (Figure 4A, B), suggesting both STAT3 and STAT6 are required for IL-4 to modulate neutrophil survival. Neither 5,15-DPP nor AS1517499 affected hypoxic neutrophil survival in the absence of exogenous cytokine stimulation (Supplementary Figure 2A).

STAT6-mediated enhancement of PPARγ activity and STAT3/6-mediated induction of transcripts for both PPARG and ALOX15 (encoding an enzyme that catalyses production of endogenous PPARγ agonist) has previously been observed with IL-4 stimulation in macrophages. In contrast to macrophages, neutrophil ALOX15 transcript was downregulated by hypoxia and unaffected by IL-4 (Figure 4C), but PPARG transcript was strongly upregulated by IL-4 in both normoxia and hypoxia (Figure 4D). PPARγ has been shown to induce gene expression of a “master regulator” of apoptosis, DNA damage inducible transcript 3 (DDIT3). Whilst IL-4 had an effect on DDIT3 expression, this was only in addition to the effect
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seen in hypoxia (Figure 4E). PPARγ has also been directly linked to increases in PHD transcript and protein abundance during adipocyte differentiation. We questioned whether IL-4 stimulation in-vitro could lead to a paradoxical increase in PHD activity and thereby suppress HIF. We observed that IL-4 selectively induced PHD2 (Figure 4F), but not PHD1 or PHD3 transcript (Supplementary Figure 2B). Neither hypoxic conditions nor IL-4 affected neutrophil HIF1A transcript (Figure 4G). However, in keeping with PHD-mediated degradation of HIF-1α, IL-4 dramatically reduced hypoxic stabilization of HIF1α protein (Figure 4H). To directly address the role of PHD2 in the IL-4 response, airspace neutrophils from mice deficient in myeloid PHD2 were isolated. PHD2 deficient neutrophils treated with IL-4 displayed preserved hypoxic survival confirming that PHD2 expression is essential for the IL-4-induced effects on neutrophil hypoxic survival (Figure 4I).

An overview of our proposed mechanism for IL-4/IL-13-mediated reduction in hypoxic survival is shown in Figure 4J.

Direct IL-4 signaling reduces neutrophil survival in damaged liver

To address whether IL4Rα-deficient neutrophils have an intrinsic survival advantage in vivo, we created liver-protected competitive bone marrow-chimeras. Recipient Cd45.2+/Cd45.1+/ wild type (WT) mice were partially depleted of host bone marrow by hind-leg irradiation to retain resident liver immune cell populations, before receiving Cd45.2+/Cd45.1−/Il4ra−/ (Il4ra−/) or Cd45.2+/Cd45.1−/Il4ra+/ (WT) bone marrow (Figure 5A). We used a CCl4-mediated acute liver injury model that causes hypoxic zone 3 hepatocytes to preferentially undergo necrosis, leading to neutrophil recruitment. We also investigated the role of exogenous IL-4 in
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neutrophil survival in this system. Whilst the percentage of blood neutrophils derived from donor CD45.1- bone marrow prior to injury (Figure 5B), was marginally greater in mice that received Il4ra-/- cells compared to those that received WT (Figure 5C), chimerism in blood neutrophils at day 3 remained unchanged irrespective of treatment, demonstrating no additional competitive neutrophil survival advantage to IL4Rα expression in blood neutrophils post treatment (Figure 5D). Liver neutrophil chimerism was equivalent to blood chimerism in all groups except in CCl₄-injured mice treated with IL4c, in which Il4ra-/- donor neutrophils displayed a significant advantage compared to their WT counterparts (Figure 5E). This change in chimerism was due to an increased number of donor Il4ra-/- neutrophils in the liver of CCl₄ + IL4c-treated mice (Fig. 5F), rather than a decrease in host WT cells (Figure 5G), thereby confirming the competitive advantage conferred on Il4ra-/- tissue neutrophils when present in a necrotic environment permeated by IL-4.

IL4Rα-deficient neutrophils demonstrate a selective advantage in an LPS-induced lung inflammation model with systemic hypoxia

Having observed a competitive advantage for Il4ra-/- neutrophils in a liver injury model in the presence of exogenous IL-4, we questioned whether endogenous cytokine could be sufficient to drive a selective advantage for IL4Rα-deficient neutrophils in-vivo in the setting of systemic hypoxia combined with an LPS challenge. In keeping with previous reports of IL-4 release following LPS 33,34 (Figure 1F), we also observe IL-4 release in the setting of hypoxia in our ALI model (Figure 6A). Competitive chimeras were generated to determine if a survival advantage was conferred by neutrophil IL4Rα-deficiency in this model of ALI. Cd45.2+/− Cd45.1+/− WT recipient mice were fractionally irradiated to deplete endogenous bone marrow
cells but protect lung alveolar macrophages (Supplementary Figure 3A). The mice were subsequently reconstituted with a 50:50 mix of Cd45.2\textsuperscript{+/−} Cd45.1\textsuperscript{+/−} Il4r\textsuperscript{α+/+} WT and either Cd45.2\textsuperscript{+/+} Cd45.1\textsuperscript{−/−} Il4r\textsuperscript{α−/−} (Il4ra\textsuperscript{−/−}) or Cd45.2\textsuperscript{+/+} Cd45.1\textsuperscript{−/−} Il4r\textsuperscript{α+/+} (WT) marrow (Figure 6B) to offer a direct comparison between wildtype and knockout donor cells. Following a recovery period, mice were treated with nebulized LPS, placed in hypoxia (10% O\textsubscript{2}), and chimerism within the Ly6G\textsuperscript{+} neutrophil population determined by flow cytometry. Blood neutrophils at both time-points exhibited higher chimerism in il4ra\textsuperscript{−/−}-recipient mice (Figure 6C), an effect which was also seen in the recruited BAL neutrophils (Figure 6D). In our model, CXCR4 expression in bone marrow neutrophils was not significantly different between donor groups, suggesting that CXCR4 was not responsible for the increased chimerism of il4ra\textsuperscript{−/−} blood and BAL neutrophils in this setting (Figure 6E).

**IL-4 promotes inflammation resolution in hypoxia through increased neutrophil apoptosis**

To determine whether IL-4 could directly promote resolution of neutrophilic inflammation in the context of systemic hypoxia, we used the same model of hypoxic airway inflammation with addition of sustained local release of IL-4 using IL-4 complex (IL4c) at 6 hours post-LPS to allow for neutrophil recruitment to occur (Figure 6F). Bone marrow neutrophil expression of chemokines involved in bone marrow neutrophil retention (CXCR4) and release (CXCR2) (Figure 6G, H) was initially explored. Whilst IL-4c did appear to increase the proportion of bone marrow neutrophils expressing CXCR4 (Figure 6G), it also significantly increased the proportion of CXCR2-expressing neutrophils in-vivo to nearly 100% (Figure 6H) with an equivalent proportion of circulating neutrophils observed between treatment
IL-4 limits neutrophilic responses in hypoxia groups (Figure 6I). Furthermore, peak recruitment to the alveolar space was also equal between groups at 24 hours (Figure 6J). During the resolution phase IL-4c treatment increased 24 hour BAL neutrophil apoptosis levels, with a subsequent reduction in BAL neutrophil counts at 48 hours (Figure 6J-K), and a reduction in extracellular elastase release measured in BAL fluid samples (Figure 6L).

**Discussion**

We show that IL-4 signaling in neutrophils can regulate hypoxia-induced pro-inflammatory survival programs and can be further manipulated *in vivo* to promote restoration of constitutive rates of neutrophil apoptosis and inflammation resolution. This work extends the previously defined roles of IL-4 in type 2 mediated alternative reprogramming of macrophages during allergic, parasitic, wound healing and acute inflammatory responses to now include direct regulation of hypoxic neutrophilic inflammation.

We show that in ARDS, a condition characterized by systemic hypoxia and uncontrolled neutrophilic inflammation, the IL-4 axis is activated and airspace neutrophils have the capacity to respond to this stimulus. Furthermore, we observe that IL-4, and the related cytokine IL-13, directly increase apoptosis of human neutrophils under hypoxic conditions and following LPS challenge, an effect that was replicated in murine inflammatory BAL neutrophils. Abrogation of hypoxic survival by IL-4 and IL-13 was dependent on the IL4Rα-downstream signaling mediators STAT3 and STAT6. PPARγ, a known target gene of STAT3 and STAT6, and important for macrophage polarization to IL-4, was also induced by IL-4 in healthy human neutrophils, both in normoxia, as described previously, and in hypoxia.
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PPARγ upregulation has previously been linked to changes in neutrophil function in patients with glycogen storage disease type Ib \(^{38}\), where circulating neutropenia and defective respiratory burst are described, alongside increased levels of HIF-1α. In these patients’ neutrophils, treatment with a PPARγ antagonist partially rescued the functional defects. In contrast, we observe that in metabolically sufficient neutrophils, IL4Rα signaling, with induction of PPARγ, results in downregulation of HIF-1α protein expression, associated with increased PHD2 expression. These findings are in line with a previous study describing effects of PPARγ on PHD transcription and protein levels in adipocytes \(^{30}\). Importantly, we show that neutrophil PHD2 expression is essential to reproduce the inhibitory effects of IL-4 on hypoxic neutrophil survival, since PHD2\(^{-/-}\) neutrophils are resistant to IL-4-induced hypoxic apoptosis.

Additionally, we also observed a decrease in hypoxic NF-κB RelA protein levels following IL-4 treatment. We have previously shown that NF-κB acting downstream of HIF1α is crucial for neutrophil hypoxic survival \(^{8}\), while inhibition of RelA transcriptional activity by PPARγ has been implicated in apoptosis of monocye-derived macrophages \(^{39}\). NF-κB has been described as a critical transcriptional regulator of HIF-1α, but the changes in HIF-1α and NF-κB seen with the combination of hypoxia and IL-4 are limited to changes in protein abundance and thus posttranslational in nature. This is of particular relevance given the overlap in posttranscriptional regulation of HIF-1α and NF-κB by asparaginyl and prolyl hydroxylation \(^{40,41}\).

Previous in-vivo studies have demonstrated endogenous IL-4 release in the lung in response to LPS \(^{33,34}\) and upregulation of IL4Rα on myeloid cells in response to pro-
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inflammatory stimuli\(^{15}\), raising the possibility that IL-4/IL4Rα signals are generated early in the course of inflammation to ensure timely resolution. Woytschak et al. recently described a role for IL4c in limiting neutrophil recruitment during bacterial infections\(^{14}\). In contrast, our work indicates an effect of IL-4 specifically on inflammation resolution, when IL4c is administered after onset of the inflammatory response. Using competitive chimeras we show that in the inflamed liver, which is characterized by local hypoxia\(^{31}\), \(il4ra^{-/-}\) neutrophils have an advantage over their wildtype controls with increased numbers following treatment with IL-4c, demonstrating a resistance to the pro-resolution effects of this cytokine. Furthermore, in our model of hypoxic ALI, BAL neutrophil counts following IL4c treatment were similar to controls during the recruitment phase, but lower after 48 hours, while apoptosis rates were higher. In keeping with IL-4 driving apoptosis mediated inflammation resolution\(^{42}\), we observed an increase in 24 hour BAL neutrophil apoptosis levels with a subsequent reduction in BAL neutrophil counts at 48 hours following IL4c-treatment and a reduction in BAL elastase. Blood neutrophil counts as a percentage of total leukocytes were equivalent and neutrophils from IL4c-treated animals expressed high levels of CXCR2, favoring marrow release. These data suggest that, in the context of systemic hypoxia and LPS, the major effect of locally delivered exogenous IL-4 on neutrophils was not on migration.

These data thus extend our current understanding from IL-4 influencing inflammation resolution via alternative macrophage activation to a model in which IL-4 concurrently limits the acute neutrophilic response through direct inhibition of hypoxic neutrophil survival. One key limitation of this work is the lack of evidence as to whether IL-4 supplementation, in addition to promoting accelerated inflammation
IL-4 limits neutrophilic responses in hypoxia resolution, can recover organ function. These are important future studies to determine the potential of therapies targeting IL-4. Such therapies would be limited to the site of tissue injury, where both IL-4 release and tissue hypoxia occur, and not compromise systemic neutrophil function or longevity. Further understanding of the broader importance of this pathway both to allergic airway inflammation and more chronic respiratory conditions typified by neutrophilic inflammation, for example COPD, is also required particularly given the current therapeutic interest in blocking IL-4 signaling responses to limit disease progression.

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References

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Figure Legends

Figure 1: IL-4 is present in human and mouse models of ARDS with IL-4Ra expression found in both human and mouse neutrophils

(A) IL-4 levels were measured in BAL samples obtained from ARDS patients and healthy controls by ultra-sensitive ELISA. (B) Neutrophils from whole blood were identified by flow cytometry from ARDS patients and healthy controls and levels of IL-4Ra surface protein and transcript measured. (C) Representative histogram (dashed black line=normoxia isotype, dashed grey= hypoxia isotype, black solid line=normoxia, grey solid line= hypoxia) and summary data of IL4Ra expression on human peripheral blood neutrophils after 4 hours culture +/- IL-4. Cell surface protein was determined by flow cytometry, with freshly isolated (T0) cells for comparison (D) and mRNA was quantified relative to ACTB by qRT-PCR. (E) IL-4 levels from BAL from LPS-treated mice or naïve mice (C57BL/6) obtained at 24 hours was measured by multiplex assay and (F) levels of IL-4Ra expression on lung neutrophils , (G) alveolar macrophages and (H) T cells were measured by flow cytometry. (I) Apoptosis rates of inflammatory BAL neutrophils harvested from normoxic C57BL/6 mice 24 hours post-LPS nebulization then cultured ex vivo for 6 hours in hypoxia +/- 100ng/ml IL-4 as determined by morphology. Data are expressed as individual data points with median +/- IQR (A,B,E,I) or mean +/- SEM (C,D,F-H). Statistical significance was determined by Mann Whitney (A, B (IL4R mRNA), E, I), unpaired Student’s t test (B (IL4Rα gMFI)), or 2-way ANOVA with Holm-Sidak post-tests comparing each condition to unstimulated control within normoxic and hypoxic groups respectively (C, D, F-H). *p<0.05, **p<0.01, ***p<0.001
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**Figure 2: Modulation of human neutrophil hypoxic survival and inflammatory responses by IL-4**

(A) Apoptosis of cytokine-treated neutrophils was assessed by morphology at 6 and (B, C) 20 hours, or (D, E, F) by flow cytometry with annexin V at 20 hours (n=3-6).

(G, H) Neutrophil apoptosis following pre-incubation with IL4Rα-neutralizing antibody (α-IL4RA) prior to culture with IL-4 or IL-13 for 20 hours in hypoxia (n=3-4). (I) Effect of IL-4 and IL-13 on LPS-induced survival in normoxia at 20 hours (n=3) as determined by morphology. (J) Effects of IL-4 on LPS-mediated pro-inflammatory gene induction in human peripheral blood neutrophils cultured for 4 hours in normoxia or hypoxia, determined by qRT-PCR (relative to ACTB) (n=4). (K) CCL17 expression relative to ACTB was determined by qRT-PCR, following 4 hours culture in normoxia or hypoxia in the presence of IFNγ, IL-4 and/or LPS (n=4). (L) Reactive oxygen species (ROS) production was determined by flow cytometry using the fluorescent oxygen sensor DCF-DA. Neutrophils were incubated with IL-4 or IFNγ+LPS for 4 hours, +/- 100nM fMLP for a further 45 minutes (n=4). Data are expressed as mean +/- SEM. Significance was determined by repeated measures 2-way ANOVA with Holm-Sidak post-test comparing cytokine treatments to unstimulated control within the normoxic and hypoxic groups respectively (A-E,K), or LPS control (J), repeated measures 1-way ANOVA with post-test for linear trend (G,H) or Holm-Sidak post-test (I) or separate 2-way ANOVAs for baseline and fMLP-stimulated ROS production, with Holm Sidak post tests comparing IFNγ+LPS and IL-4 to unstimulated control within normoxic and hypoxic groups respectively (L).

*p<0.05, **p<0.01, **** p<0.0001.
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**Figure 3: IL-4 regulates NF-κB at a post-transcriptional level without altering metabolic flux**

(A) Expression of glycolytic genes in human neutrophils after 8 hours culture in normoxia or hypoxia with IL-4 or IFNγ+LPS, determined by qRT-PCR relative to ACTB (n=4). (B) Flux through glycolysis and fatty acid oxidation (FAO) were quantified by $^3$H$_2$O release following uptake of [5-$^3$H]-glucose and [9,10-$^3$H]-palmitic acid respectively, in normoxia or hypoxia +/-IL-4. Energy status (ATP/ADP ratio) after 4 hours was determined by reverse phase HPLC (n=3). (C) NF-κB p65 (RELA) protein levels after 6 hours culture in normoxia or hypoxia +/- IL-4 were assessed by Western blot, levels quantified relative to p38 by densitometry and fold change calculated relative to normoxia (n=4). (D) Gene expression of RELA (NF-κB) and CHUK (IKKα) relative to ACTB were determined by qRT-PCR following 4 hours culture in normoxia or hypoxia +/- IL-4 (10ng/ml) (n=4). Data are expressed as mean +/- SEM. Statistical significance was determined by repeated measures 2-way ANOVA with Holm-Sidak post-test comparisons to unstimulated controls within the normoxic and hypoxic groups respectively (A, B, D) or Mann Whitney (C). *p<0.05, **p<0.01.
IL-4 limits neutrophilic responses in hypoxia

Figure 4: IL-4-mediated STAT/PPARγ signaling upregulates PHD2 transcript and reduces HIF1α protein levels, resulting in loss of hypoxic neutrophil survival

(A, B) Effect of STAT3 and STAT6 inhibitors (5,15-DPP and AS 1517499 respectively) on cytokine-induced 20 hour human neutrophil apoptosis in hypoxia (determined by morphology) (n=3-4). (C-E) Neutrophil expression of genes involved in PPARγ signaling and (F) of EGLN1/PHD2, following 4 hours cytokine/LPS treatment in normoxia or hypoxia, determined by qRT-PCR (relative to ACTB) (n=4). (G) HIF1A gene expression, following 4 hours cytokine/LPS treatment in normoxia or hypoxia, determined by qRT-PCR (relative to ACTB) (n=4). (H) HIF-1α protein levels following 6 hours culture with IL-4 in normoxia or hypoxia were assessed by Western blot and levels relative to p38 quantified by densitometry (n=4). (I) Phd2−/− mice or their littermate controls were treated with nebulized LPS and BAL neutrophils treated with IL-4 (100ng/ml) in hypoxia ex-vivo for 6 hours. Data normalized to apoptosis rates in unstimulated hypoxia control. (J) Schematic representation of IL-4 signaling pathway leading to loss of hypoxic survival in neutrophils. Data are expressed as mean +/- SEM (A-H), or median +/- IQR (I). Statistical significance was determined by 1-way ANOVA with Holm-Sidak post-test comparing cytokine only group to every other group (A,B), repeated measures 2-way ANOVA with Holm-Sidak post-test comparisons to unstimulated controls within normoxic and hypoxic groups respectively (C-H) or Mann Whitney of log-transformed data (I). *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001.
IL-4 limits neutrophilic responses in hypoxia

**Figure 5: ** *Il4ra*−/− neutrophils have a competitive advantage in bone marrow chimeras in a liver injury model

(A) *Cd45.2+/ Cd45.1−/−* mice were irradiated to deplete host BM, then reconstituted with *Cd45.2+/ Cd45.1−/−* donor marrow, either WT or *Il4ra*−/−, as depicted. Mice were exposed to a single dose of CCl₄ to induce liver damage, or olive oil vehicle control.

(B) Chimerism (% CD45.1+ donor neutrophils) was determined by flow cytometry. (C) Blood chimerism at day 0 (pre-CCl₄) in mice receiving WT or *Il4ra*−/− marrow. (D) Blood chimerism at day 0 (pre-CCl₄) and day 3 in mice treated with CCl₄ +/- IL4c and in olive oil vehicle controls. (E) Liver chimerism normalized to blood at day 3 post CCl₄. (F,G) Total numbers of donor (F) and host (G) neutrophils per gram of liver at day 3 post-CCl₄. Statistical significance was determined by unpaired Student’s t test (C), multiple t tests with Holm Sidak correction (day 0 vs day 3 for each treatment/genotype combination) (D) or 2-way ANOVA with Holm Sidak post tests (WT vs. *Il4ra*−/−) (E-G). Data shown as individual mice with mean+/- SEM. *p<0.05, *** p<0.001, ****p<0.0001.
IL-4 limits neutrophilic responses in hypoxia

**Figure 6: Exogenous IL-4c treatment accelerates the resolution of neutrophilic lung inflammation in hypoxia**

(A) IL-4 levels from BAL from naïve mice or mice treated with nebulized LPS and placed in hypoxia (10%) for 24 hours was measured by multiplex assay. (B) 

\( \text{Cd45.2}^{+/-} \text{Cd45.1}^{+/-} \) mice were irradiated to deplete host BM, then injected with donor marrow comprising 50% WT \( \text{Cd45.2}^{+/-} \text{Cd45.1}^{+/-} \) mixed with either 50% \( \text{Il4ra}^{+/-} \text{Cd45.2}^{+/-} \text{Cd45.1}^{-/-} \) (WT) or 50% \( \text{Il4ra}^{-/-} \text{Cd45.2}^{+/-} \text{Cd45.1}^{-/-} \) (Il4ra\(^{-/-}\)), as depicted. Mice were nebulized with LPS and placed in hypoxia 25 days post-transplant. Neutrophil chimerism (% of Ly6G\(^+\) cells lacking CD45.1) was determined by flow cytometry. Chimerism was normalized to WT control chimerism at each timepoint. (C) Blood and (D) BAL neutrophil chimerism post-LPS and hypoxia. (E) Proportion of donor (\( \text{Cd45.2}^{+/-} \text{Cd45.1}^{-/-} \)) bone marrow (BM) CXCR4\(^+\) neutrophils. (F) C57BL/6 mice were treated with intratracheal IL-4c or PBS 6 hours post-LPS nebulization and returned to hypoxia for a further 18 hours. (G) Bone marrow neutrophil expression of CXCR4 and (H) CXCR2 (H) was determined by flow cytometry (representative histograms shown- light grey FMO control, dark grey (PBS) and dotted line (IL-4c)). (I) Proportion of circulating neutrophils in whole blood leukocytes. (J) Total BAL neutrophils were determined by flow cytometry. (K) Proportion of BAL apoptotic (Annexin V\(^+\)) neutrophils was measured by flow cytometry. (L) Total BAL IgM release was measured at 24 hours. Data shown as individual points with median (A,E,G-I,L) +/- IQR or mean +/- (C,D,J,K). Statistical significance was determined by 2-way ANOVA with Holm Sidak post test (WT vs \( \text{Il4ra}^{-/-} \) for each timepoint) (C, D, J, K), Mann-Whitney (A, E, L) or unpaired Student’s t test (G, H, I). *p<0.05, **p<0.01, ***p<0.001.
Figure 1
Figure 2
Figure 3

173x153mm (300 x 300 DPI)
Figure 4

164x224mm (300 x 300 DPI)
Figure 5
Figure 6
Supplementary Methods

ARDS and healthy control BAL sampling

The diagnosis of ARDS was in accordance with the North American-European Consensus definition. Bronchoalveolar lavage (BAL) fluid was obtained from ARDS patients recruited to the HARP study (testing efficacy of simvastatin in patients with ALI/ARDS) at baseline, as previously described\(^1\). Ethical approvals for measurement of BAL cytokines in this study were obtained from the Office for Research Ethics Committee Northern Ireland (ORECNI), reference 06/NIR02/77 and Queen’s University of Belfast School Research Ethics Committee (QUB SREC), reference 18.08. Healthy non-smoking normal volunteers underwent research bronchoscopy as previously described\(^2\), with ethical approval from ORECNI, reference 12/NI/0082. Patient and healthy control demographic details are included in supplementary table 1.

Generation of lung-protected bone marrow chimeras (lung injury model)

C57BL/6J Cd45.1\(^{+/−}\) Cd45.2\(^{+/−}\) mice were irradiated with a total of 12Gy over 4 days in thrice-daily fractions prior to receiving \(1 \times 10^6\) BM cells in 100μL PBS by tail vein injection (50% Cd45.2\(^{+/+}\) C57BL/6Jor Il4ra\(^{−/−}\) and 50% Cd45.1\(^{+/−}\) Cd45.2\(^{+/−}\)).

Mouse LPS acute lung injury model

Mice were nebulized with 3mg LPS from *Pseudomonas aeruginosa* 10 (Sigma), housed in normoxia (room oxygen, 21% O\(_2\)) or hypoxia (10% O\(_2\)). Blood, BAL, lungs and bone marrow were harvested for analysis. BAL samples underwent red cell lysis (Biolegend) prior to flow cytometry staining. Single cell suspensions were obtained by mechanical
dissociation and enzymatic digestion (RPMI with 0.625 mg/ml collagenase D (Roche), 0.85 mg/ml collagenase V (Sigma-Aldrich), 1 mg/ml dispase (Gibco, Invitrogen) and 30 U/ml DNase (Roche Diagnostics GmbH).

**Generation of tissue-protected bone marrow chimeras (liver injury model)**

C57BL/6J Cd45.1+/- Cd45.2+/- mice were anaesthetized and exposed to a single dose of 9.5Gy irradiation, with hind legs and lower abdomen protected by a 2-inch lead shield. Mice were injected via the tail vein with 5x10^6 BM cells from Cd45.2+++ WT or Il4ra-/- donor mice the following day, then housed in IVC cages for 8 weeks, with baseline chimerism established by tail vein bleed (20μl).

**Liver injury model**

A 25% suspension of CCl₄ (Sigma-Aldrich) or olive oil (Sigma-Aldrich) (vehicle) was administered to mice intraperitoneally (i.p.) at 4μl/g. 24 hours later, 4μl/g IL4c or sterile PBS (vehicle) was administered subcutaneously. After 48 hours, mice were venaected and culled. Mice were perfused with PBS prior to liver harvest with single cell suspensions obtained as detailed above.

**Flow cytometry**

For IL4Rα expression quantification in ARDS patients, whole blood was lysed (ebioscience) and α-CD16/32 Fc block applied (ebioscience). Cells were then stained with CD45, CD11b, CD49d and IL4Rα with geometric fluorescence of neutrophil IL4Rα quantified. For healthy donor samples median fluorescence of the IL4Rα-stained
sample was divided by that of a full minus-one FMO+isotype control for the population assessed.

Mouse cells were treated with α-CD16/32 Fc block (e-bioscience) and mouse serum (Thermo Fisher) prior to staining with antibodies (see Supplementary Table 2). Relevant FMO or FMO+isotype samples were used as controls. Cells were acquired on the LSRFortessa or Calibur (Becton Dickinson). Compensation was performed using BD FACSDiva software and data analysed in FlowJo version 10.

**RNA isolation and relative quantification**

Neutrophil RNA was extracted and cDNA subjected to relative qRT-PCR quantification using TaqMan® gene expression assays (Applied Biosystems, Thermo Fisher) (Supplementary Table 3). Relative abundance was determined by interpolation from standard curve and normalized to ACTB.

**Protein extraction and immunoblotting**

Immunoblotting for blood neutrophil human HIF-1α (clone 54, BD), NF-κB p65 (polyclonal, Abcam) and p38 MAPK (polyclonal, Cell Signalling Technology), with HRP-conjugated secondaries: anti-mouse IgG (Cell Signalling Technology) and goat anti-rabbit Ig (Dako) was performed and normalized to p38 expression.

**Functional and metabolic assays**
Human neutrophils were treated with either IL-4 or IFNγ+LPS in normoxia or hypoxia for 4-6 hours for the following assays:

**ROS production:** Cells were loaded with 3 µg/ml 2′,7′-dichlorofluorescin diacetate (Molecular Probes) prior to stimulation (45 mins) with 100nM N-Formyl-Met-Leu-Phe (fMLP, Sigma). ROS levels were determined by flow cytometry (FL1 median fluorescence normalized to unstained control).

**Energy status:** Cells were washed in PBS and harvested in 100µl ice cold 5% perchloric acid supplemented with 0.5 mM EDTA. Following addition of K₂CO₃, nucleotide levels were measured using ion-pair RP-HPLC.

**Glycolytic and fatty acid flux:** For glycolysis measurement, cells were cultured in glucose-deficient RPMI 1640 medium (Gibco, Thermo Fisher) supplemented with 5.5 mM unlabelled glucose, 10% FCS and 0.4 μCi/ml [5-³H]-D-glucose (Perkin Elmer). Fatty acid oxidation was measured using complete RPMI with 10% FCS and 2 μCi/ml [9,10-³H]-palmitic acid (Perkin Elmer). Cell supernatant was added to 12% perchloric acid and ³H₂O was captured over a period of 48 hours at 37 °C. Radioactivity was determined by liquid scintillation counting.

**Human BAL IL-4 quantification**

IL-4 levels from human BAL samples from patients with ARDS or healthy donors were measured using a super-sensitive IL-4 ELISA kit as per manufacturer’s instructions (RnD).

**Mouse BAL IL-4 quantification**
IL-4 levels in BAL samples from either naïve or LPS-nebulized mice housed in normoxia or hypoxia were measured using a multiplex assay as per manufacturer's instructions (MSD).

**Supplementary References**


Supplementary Figure 1: Preserved neutrophil uptake of glucose and expression of \textit{PPARGC1B} in the presence of IL-4

(A) Human peripheral blood neutrophils were cultured for 4 hours +/- IFN\gamma+LPS or IL-4 in normoxia or hypoxia. Neutrophil uptake of the fluorescently-labelled glucose analog 2-NBDG was assessed by flow cytometry (median fluorescence intensity) (n=4). (B) Human peripheral blood neutrophils were cultured for 8 hours +/- IFN\gamma+LPS or IL-4 in normoxia or hypoxia. Relative expression of \textit{PPARGC1B} was determined by qRT-PCR (n=4). Data are expressed as mean +/- SEM. Statistical significance was determined by repeated measures 2-way ANOVA with Holm-Sidak post-test comparisons to unstimulated controls within the normoxic and hypoxic groups respectively. *p<0.05, **p<0.01, ****p<0.0001.

Supplementary Figure 2: Preserved neutrophil \textit{PHD1} and \textit{PHD3} expression with IL-4 treatment

(A) Effect of STAT3 and STAT6 inhibitors (5,15-DPP and AS 1517499 respectively) alone on human neutrophil apoptosis (measured by cell morphology) cultured in hypoxia for 20 hours. (B) Human peripheral blood neutrophils were cultured for 4 hours +/- LPS, IFN\gamma and/or IL-4 in normoxia or hypoxia. \textit{PHD1} and \textit{PHD3} gene expression relative to \textit{ACTB} were determined by qRT-PCR (n=4). Data are expressed as mean +/- SEM.
Supplementary Figure 3: Alveolar macrophages remain predominantly of host origin following fractionated irradiation

(A) Flow cytometric assessment of lung digest alveolar macrophage chimerism following fractionated irradiation as determined by Cd45.2+/Cd45.1− (donor) and Cd45.2+/Cd45.1+ expression (host).
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
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**Supplementary Table 1**
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**Supplementary Table 3**