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**NR4A orphan nuclear receptor family members, NR4A2 and NR4A3,  
regulate neutrophil number and survival.**

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**Key points.**

1. We demonstrate an important role for NR4A receptors in regulating neutrophil lifespan and homeostasis *in vitro* and *in vivo*.
2. These findings may define targets for therapies for diseases driven by defects in neutrophil number and/or survival.

## **Abstract**

Neutrophil lifespan is plastic and highly responsive to factors that regulate cellular survival. Defects in neutrophil number and survival are common to both hematologic disorders and chronic inflammatory diseases. At sites of inflammation, neutrophils respond to multiple signals that activate protein kinase A (PKA) signaling, which positively regulates neutrophil survival. The aim of this study was to define transcriptional responses to PKA activation and to delineate the roles of these factors in neutrophil function and survival. In human neutrophil gene array studies, we show that PKA activation upregulates a significant number of apoptosis related genes, the most highly regulated of these being *NR4A2* and *NR4A3*. Direct PKA activation by the site-selective PKA agonist pair N6/8-AHA and treatment with endogenous activators of PKA, including adenosine and PGE<sub>2</sub>, results in a profound delay of neutrophil apoptosis and concomitant upregulation of *NR4A2/3* in a PKA dependent manner. *NR4A3* expression is also increased at sites of neutrophilic inflammation in a human model of intradermal inflammation. PKA activation also promotes survival of murine neutrophil progenitor cells, and siRNA to *NR4A2* decreases neutrophil production in this model. Antisense knockdown of *NR4A2* and *NR4A3* homologues in zebrafish larvae significantly reduces absolute neutrophil number without affecting cellular migration. In summary, we show that *NR4A2* and *NR4A3* are components of a downstream transcriptional response to PKA activation in the neutrophil, and that they positively regulate neutrophil survival and homeostasis.

## Introduction

Neutrophils are an essential component of the innate immune response and are the primary cellular response to tissue infection and inflammation. As the most abundant circulating leukocyte, neutrophils undergo spontaneous apoptosis in order to limit inflammation and maintain homeostasis. Ordinarily short-lived cells, inflammatory neutrophils can prolong their lifespan in order to maximize functional potential such as pathogen eradication<sup>1</sup>. As a result, neutrophils are extremely sensitive to factors that trigger cell survival, and engage transcriptional and signaling pathways that allow them to rapidly respond to their environment<sup>2</sup>. Defects in neutrophil number and survival are a common factor in hematologic conditions, including neutropenia and myeloid hyperplasia, and in chronic inflammatory diseases<sup>3</sup>. Yet, current therapeutics for these disorders are associated with long-term side effects or do not treat the underlying cellular mechanisms. Understanding the mechanisms that underpin neutrophil survival in this context will reveal targets to which novel and highly selective therapeutic approaches can be designed.

Factors that increase intracellular cAMP levels also prolong neutrophil survival<sup>4</sup>. cAMP molecules bind to and activate protein kinase A (PKA), a ubiquitous family of kinases with multiple cellular functions, including cell survival. Conversely, PKA is inactivated by depletion of cAMP, which rapidly turns off signaling, making it a candidate for the precise regulation of neutrophil survival. Although PKA has been linked to the control of neutrophil survival, as well as control of other key effector functions such as adhesion, superoxide production and matrix metalloproteinase secretion<sup>5-8</sup>, the downstream signaling of PKA in neutrophils remains unclear. This study aimed to define transcriptional responses to PKA activation and to delineate the roles of these factors in regulating neutrophil function and survival, in order to identify new therapeutic targets for conditions in which defects in neutrophil number and survival are a key component.

## Methods

### Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Adenosine, 8-(6-Aminoethyl) aminoadenosine 3':5'-cyclic monophosphate (8-AHA-cAMP), Adenosine 5'-[ $\gamma$ -thio]triphosphate tetralithium salt (ATP $\gamma$ s), dibutyryl cAMP (dbcAMP), Butaprost and LY-294002 hydrochloride were all from Sigma-Aldrich, LPS from *E. coli* serotype R515 (Enzo Life Sciences, Farmingdale, NY), N<sup>6</sup>-monobutyryladenosine-3,5'-cyclic monophosphate (N<sup>6</sup>-MB-cAMP) and 8-bromoadenosine- 3', 5-cyclic monophosphorothioate (Rp-8-Br-cAMPS) (Biolog, Bremen, Germany), prostaglandin E2 (PGE2) (Cambridge Bioscience, Cambridge, UK), Recombinant human GM-CSF (Stem Cell Technologies, Vancouver, Canada).

### Neutrophil isolation and culture

Human neutrophils were isolated by dextran sedimentation followed by plasma-Percoll gradient centrifugation from whole blood of healthy volunteers with written informed consent and ethical approval from the South Sheffield Research Ethics Committee<sup>9,10</sup>. In selected experiments, neutrophils and monocytes were further purified by negative magnetic selection, using either a custom mixture from StemCell Technologies, containing antibodies to CD36, CD2, CD3, CD9 CD19, CD56 and glycoporphin A, or the monocyte isolation kit II (Miltenyl Biotech, Bergisch Gladbach, Germany), respectively. Following negative selection neutrophil and monocyte purity was >99%.

Neutrophils were suspended at  $5 \times 10^6$ /ml in RPMI (Thermo Scientific, Waltham, MA) containing 1% penicillin/streptomycin and 10% low endotoxin FCS (PromoCell, Heidelberg, Germany) and cultured in 96-well flexiwell plates at 37°C, 5% CO<sub>2</sub>. For hypoxic culture, an *in vivo* 400 hypoxic work station (Ruskin, Bridgend, UK) with a 5% CO<sub>2</sub>/balance N<sub>2</sub> gas mix delivered an oxygen tension of 0.75 kPa into the chamber, which correlated with a culture media oxygen tension of 3 kPa. Media were allowed to equilibrate overnight prior to use. Freshly isolated neutrophils were designated as time 0. Agonists and/or inhibitors were added at time 0 and incubated as described. PKA was agonised by a combination

of 8-AHA-cAMP [100 $\mu$ M or 1mM as indicated] and N<sup>6</sup>-MB-cAMP [100 $\mu$ M or 1mM as indicated] and collectively termed N<sup>6</sup>/8-AHA. Neutrophils treated with LY294002 or Rp-8-Br-cAMPS were pre-incubated for 30 or 15 mins respectively, prior to addition of PKA agonists.

To create the monocyte conditioned supernatant (S/N) and to concentrate monocyte-derived factors, monocytes (2x10<sup>6</sup>/ml) were injected into pre-hydrated 10kDa dialysis cassettes (Thermo Scientific). Cassettes were placed inside 150cm<sup>2</sup> tissue culture flasks with re-closable lids (Helena Biosciences, Gateshead, UK) containing RPMI with 10% human serum, 1% penicillin/streptomycin, and 100ng/ml LPS. Monocytes were cultured for 20 hours, during which time autocrine factors (>10kDa) accumulated in the cassette. Monocytes were removed from cassettes and pelleted gently from the media to generate cell-free supernatant which was stored at -80°C.

### **Murine progenitor cell differentiation and culture**

Murine conditionally immortalised progenitors (mCMP, Prof. Philip Taylor, Cardiff University) expressed a *hoxb8*-estrogen receptor binding domain fusion protein and were routinely passaged in the presence of  $\beta$ -estradiol<sup>11</sup>. Upon estrogen withdrawal, mCMP were differentiated into neutrophils in the presence of murine Stem Cell Factor (SCF) and G-CSF. Briefly, mCMP were cultured at 0.1-1 x 10<sup>6</sup>/ml in base medium (OptiMEM (Invitrogen, Karlsruhe, Germany) plus 10% HI-FCS (PromoCell), 1% L-glutamine (Invitrogen), 30  $\mu$ M  $\beta$ -mercaptoethanol and 1% penicillin/streptomycin) supplemented with 10 ng/ml recombinant murine SCF (Peprotech, Rocky Hill, NJ) and 1  $\mu$ M  $\beta$ -estradiol. All experiments were performed with cells between passages 2 to 8. Differentiation of mCMP to neutrophils was carried out in base medium as above, supplemented daily with 20 ng/ml recombinant murine SCF and G-CSF for 4 days<sup>12</sup>. Hemocytometer counts and cytocentrifuge slides were made daily and the purity of mature neutrophils on day 4 was typically >90%, as assessed by cellular morphology.

### **Assessment of neutrophil viability and apoptosis**

Neutrophil apoptosis was measured by oil immersion light microscopy (x100 objective, Nikon Eclipse TE300, Nikon, Japan). Nuclear morphology was assessed on Giemsa-stained cytocentrifuge slides by counting >300 cells per slide<sup>10</sup>. Apoptosis was also measured by Annexin V/ToPro-3 viability staining. In brief, cells were washed in PBS and stained with 2.5µl Annexin V-PE (BD Biosciences, San Jose, CA) and ToPro3 iodide (1:10,000 dilution, Molecular Probes, Eugene, OR) and samples analyzed using a FACS Calibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). Hemocytometer counts and flow cytometrical CountBright bead (ThermoFisher Scientific) assays were performed to assess cell numbers and for trypan blue exclusion.

### **RT-PCR and qPCR**

RNA was prepared from cell lysates using TRI reagent and cDNA was transcribed using high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Warrington, UK). Quantitative PCR (qPCR) was carried out using primer-probe sets from Applied Biosystems. For normalisation,  $\beta$  actin (Hs99999903) and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were used. PCR master mix was from Eurogentec (Southampton, UK), and reactions were carried out using an ABI7900 automated TaqMan system (Applied Biosystems). mRNA quantities were analysed in duplicate, normalised against GAPDH or  $\beta$  actin as an internal control gene and expressed in relation to mRNA from a media control sample as a calibrator. Results are expressed as relative gene expression/relative quantity (RQ) using the  $\Delta\Delta C_t$  method.

### **RNA Microarray**

Neutrophil RNA extracts were produced after culturing with N6/8-AHA [1mM], GM-CSF [100u/ml], LPS [1µg/ml], monocyte-conditioned media (S/N) or in hypoxic conditions for 4 hours and converted to cDNA as described. cDNA from five donors was pooled prior to microarray analysis. Stimulated neutrophils were compared to unstimulated and run on Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. To detect changes in total gene expression, data was analysed using DAVID 6.7 bioinformatics resource (<http://david.abcc.ncifcrf.gov/>).



### **siRNA gene knockdown by Amaxa Nucleofection**

Nucleofection of mCMP was carried out using a Nucleofector™ 2b device (Lonza) according to the manufacturers' protocol. Lyophilised ON-TARGET plus siRNA pools [20µM] (Thermo Scientific) for *NR4A2* (L-048281-01-0005), *NR4A3* (L-043983-01-0005) or a non-targeting control (siScrbl) were transfected with Amaxa Nucleofector Kit V (Thermo Scientific) and program D-023. All nucleofections were carried out at room temperature and recovered in 1 ml fresh growth medium. Transfected mCMP were subsequently differentiated as described. Efficiency of gene product knockdown was assessed by RT-PCR.

### **Morpholino injection and zebrafish tail injury**

Standard control [2.0 pM]; *NR4A2*-MO<sup>13</sup> [0.5-2.0 pM] or *NR4A3*-MO [0.125-0.5pM] (5'ATGGGAAAATGACTATCACACTGCT-3') (Genetools, Philomath, OR) were injected into *Tg(mpx:GFP)i114*<sup>14</sup> at the 1 cell stage. Embryos were injured at 3 days post fertilisation (dpf) by tail transection and neutrophil recruitment and resolution at the site of injury was assayed as described<sup>15</sup>. Embryos were imaged at low magnification on a TE- 2000U microscope (Nikon) and an Orca-AG camera (Hamamatsu, Japan) using a 4x NA 0.1 air objective (whole body counts) using Volocity™ and neutrophil numbers were determined.

### **Statistical analysis**

Data are expressed as mean ± standard errors of the mean (SEM). Data was analysed using analysis of variance (ANOVA) with appropriate post test using the Prism 6.0 software (GraphPad, Prism, San Diego, CA).

### **Results:**

**Transcriptional changes in human neutrophils in response to inflammatory stimuli.** Neutrophils are capable of rapid transcriptional changes that are highly tailored to environmental needs, whether this be responses to injury, inflammation or infection<sup>16,2,17</sup>. Identifying gene expression that is unique to individual stimuli will reveal potential therapeutic targets that modify specific elements of neutrophil function. In order to explore the apoptosis-related

transcriptional changes that follow PKA activation, an unbiased microarray approach was taken. PKA-dependent and independent agonists were used in order to identify genes that are unique to PKA signaling. Human neutrophils were cultured with a range of stimuli known to prolong neutrophil survival, reflecting both highly selective agonists (N6/8-AHA, GM-CSF, LPS) and more physiological stimuli (monocyte conditioned supernatant and hypoxia), for 4 hours and pooled cDNA from 5 donors was subjected to Affymetrix microarray analysis, hu133 plus 2. The complete microarray dataset (GSE94923) is available at: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94923>. Gene expression levels were compared to media treated populations and an FDR<10% and a greater than 2-fold change was considered significant. By using the web-based functional analysis tool DAVID, we then asked whether differentially expressed genes were enriched in specific functional pathways. We identified Z clusters of functional terms that were statistically significant (FDR<10%). Among these, the Gene Ontology term 'regulation of apoptosis' (GO:004281) was significant. We then focused on the specific genes represented in this term, and presented the data to show only genes that are up- or down-regulated by the PKA agonist, N6/8-AHA (Figure 1). A complete figure of all GO:004281 regulated genes and DAVID analysis files are available as supplemental data (Figure S1 & Table S1). Figure 1 shows a marked diversity in the regulation of apoptosis-related genes between survival stimuli. Only a single upregulated gene was common to all stimuli (*PSEN1* which encodes for the gamma-secretase protease complex member, presenilin-1). Five genes upregulated in common with N6/8-AHA, conditioned supernatant, GM-CSF and LPS, included the pro-inflammatory cytokines *IL-1 $\alpha$*  and *IL-1 $\beta$* . The activation of PKA by N6/8-AHA, however, also led to the initiation of a unique transcriptional apoptosis programme, with the exclusive upregulation of a wide variety of genes by greater than 2-fold, including protein kinase C, *MAPK* and *NR4A*. With respect to absolute transcript levels, *NR4A2* and *NR4A3* were the two most highly regulated genes following N6/8-AHA treatment. In addition, neutrophils were most transcriptionally responsive to PKA activation by N6/8-AHA, compared to the other stimuli, with the expression of over 70 apoptosis-related genes being induced in the curated DAVID cluster. Conditioned supernatant and GM-CSF treatment upregulated a

high number of common genes, including a number of cell death components such as *Bcl-xL*, caspase and FADD-like apoptosis regulator (*CFLAR*) and caspases 4, 5 and 10 (Figure S1). Of all stimuli, LPS and hypoxia resulted in only a limited apoptosis-related gene expression profile.

The high levels of upregulation of *NR4A2* and *NR4A3* seen in the array data sets were validated by qPCR. In parallel, three other genes associated with activation of inflammation and which showed different regulation between survival stimuli (*VEGF*, *IL-1 $\alpha$*  and *IL-1 $\beta$* ) were also validated by qPCR (Figure 2). *NR4A2* and *NR4A3* were exclusively and profoundly upregulated by N6/8-AHA treatment, linking this transcription factor family to PKA signaling in the neutrophil (Figure 2A-B). As in the microarray, specific survival factor-dependent patterns of gene expression were evident. Conditioned monocyte supernatant upregulated *VEGF*, *IL-1 $\alpha$*  and *IL-1 $\beta$*  (Figure 2C-E). LPS, GM-CSF and hypoxia had limited or no effect on target gene regulation, with the exception of upregulation of *IL-1 $\alpha$*  by GM-CSF (Figure 2A-E). These findings reveal that the human neutrophil is capable of generating unique transcriptional programmes in response to individual inflammatory stimuli, and that PKA profoundly regulates *NR4A2* and *NR4A3*, which have, as yet, no clear roles in neutrophil function.

### **NR4A2 and NR4A3 genes are regulated by inflammation in a PKA**

**dependent manner.** Our initial studies confirmed that PKA activation potently activated a unique survival gene expression profile. Neutrophils are the most abundant circulating leukocyte and undergo spontaneous apoptosis in order to both limit inflammation and maintain homeostasis. The roles of PKA activation in granulocyte survival remain incompletely understood, and limitations in the quality of functional antagonists of this pathway have hampered exploration of the relevant signaling pathways. Functional consequences of PKA signaling on neutrophil apoptosis were studied using selective agonists of PKA. Agonising PKA with N6/8-AHA led to a concentration dependent decrease in neutrophil apoptosis at 20h (Figure 3A, Figure S2A), reaching significance at 50  $\mu$ M. The site selective type IA PKA activator, N6-MB-cAMP, also significantly delayed neutrophil apoptosis at this time (Figure 3B, Figure S2B). Consistent with a

selective pathway regulating survival, we showed N6/8-AHA-induced neutrophil survival was reversible by Rp-8-Br-cAMPS, a highly selective PKA inhibitor with no cross reactivity for EPAC (Figure 3C) but not the PI3K inhibitor, LY294002 (Figure 3D). In contrast, neutrophil survival induced by GM-CSF was prevented by LY294002. The potential relevance of *NR4A* transcripts was supported by these studies, since concomitant with the onset of spontaneous neutrophil apoptosis, both *NR4A2* and *NR4A3* transcripts rapidly decayed during neutrophil culture (Figure 3E), which was in part rescued by the survival factor dbcAMP for *NR4A3* but not *NR4A2* (Figure 3F-G).

We explored whether other candidate endogenous activators of PKA signaling commonly found at sites of inflammation, including adenosine and PGE<sub>2</sub>, also caused delays in neutrophil apoptosis. Adenosine, ATP $\gamma$ S and PGE<sub>2</sub> delayed neutrophil apoptosis, and the survival effects, with the exception of adenosine, were reversed by Rp-8-Br-cAMPS (Figure 4A, Figure S2D). In contrast, LPS- and GM-CSF-induced survival was not PKA dependent (Figure 4A, Figure S2D). To confirm that Rp-8-Br-cAMPS restored apoptosis rather than an alternative form of cell death, we show that the pan-caspase inhibitor Q-VD-OPh was able to fully reverse this effect (Figure S2B). In parallel, qPCR experiments measuring *NR4A2/3* gene expression showed adenosine, ATP $\gamma$ S and PGE<sub>2</sub> upregulated *NR4A2* and *NR4A3* transcripts, and this upregulation was blocked by Rp-8-Br-cAMPS (Figure 4B-C). Modest changes in *NR4A3* in response to LPS were not inhibited by Rp-8-Br-cAMPS (Figure 4B-C). Both PGE<sub>2</sub> and the EP2 receptor agonist, butaprost delayed neutrophil apoptosis in a concentration- and PKA-dependent manner (Figure 4D-E). PGE<sub>2</sub>-mediated upregulation of *NR4A2/3* was seen at 4 h, which was fully inhibited by Rp-8-Br-cAMPS (Figure 4F-G). These data pointed towards *NR4A2/3* expression as an important aspect of neutrophilic inflammation *in vivo* that could contribute to neutrophil persistence. The functional consequences of PKA signaling *in vivo*, where cells are also exposed to other activation signals may be different to those *in vitro*, as demonstrated by one study which observed that exposure to hypoxia reduced the survival response of neutrophils to steroids<sup>18</sup>. We therefore determined whether PKA signaling could still contribute to neutrophil survival in contexts

where more than one survival stimuli are present. Firstly, we show in an *in vivo* model of human intradermal endotoxin challenge that *NR4A3*, but not *NR4A2*, is upregulated greater than 2-fold by LPS (Figure S3), occurring at a timepoint which coincides with peak neutrophil recruitment<sup>19</sup> and indicating that PKA signalling takes place in this context. To further investigate this *in vitro*, neutrophils were co-incubated with a sub-maximal concentration of LPS and the physiological activator of PKA; PGE2. PGE2 remained able to significantly inhibit neutrophil apoptosis in the presence of LPS (Figure 5A) and in parallel, observed a significant upregulation of *NR4A2/3* when both stimuli were present (Figure 5B-C). These data suggest that PKA activation is likely to lead to *NR4A2/3* upregulation and neutrophil survival in complex environments consisting of multiple stimuli.

**NR4A2 and NR4A3 gene deletion impedes neutrophil production.** The function of NR4A in the context of neutrophil survival and inflammation was investigated by gene knockdown strategies. Since human neutrophils are genetically intractable, we adopted Hoxb8 conditionally immortalised murine myeloid progenitor cells, which allow the study of NR4A in fully functional neutrophils<sup>11</sup>. We first confirmed the importance of PKA in survival signaling in these cells by showing a decrease in percent apoptosis and increase in viable cell number following N6/8-AHA treatment (Figure 6A-C). Myeloid cells were transfected with *NR4A2* and *NR4A3* siRNA by nucleofection. Gene knockdown was verified by RT-PCR, yielding an average knockdown of 90% for *NR4A2* and 75% for *NR4A3* (Figure S4). Cell numbers were assessed over 4 days post-transfection and, in scrambled siRNA transfected control populations, cells proliferated by approximately 4 fold by day 4 (Figure 6D-E). Knockdown of *NR4A2* but not *NR4A3* resulted in a reduction in cell numbers from day 2, reaching significance at day 4 (Figure 6D-E), suggesting that NR4A proteins are involved in the regulation of neutrophil survival during differentiation and neutrophil development. The increase in cell number between day 2 and 4 may reflect a gradual regeneration of *NR4A* transcripts and therefore restoration of survival pathways. There was no impact of *NR4A2/3* knockdown on maturation

of neutrophils over time (Figure 6F) or at the end of the differentiation period (Figure 6G). *NR4A2* KD modestly increased apoptosis of neutrophils on day 4 (Figure 6G-H).

To test the possibility that *NR4A2* and *NR4A3* might influence developmental myelopoiesis *in vivo*, we used the transparent zebrafish larval model in which transgenically-labelled fluorescent neutrophils can easily be visualised during development<sup>14</sup>. To modulate expression of *NR4A* family members *in vivo*, we injected morpholino-modified antisense constructs into fertilised zebrafish eggs and observed the effect on neutrophil number and response to injury. Zebrafish larvae developed normally following morpholino injection (Figure 7A). Absolute numbers of neutrophils at 80 hpf are significantly reduced at all *NR4A2* morpholino concentrations used (Figure 7B) and at the two highest concentrations of *NR4A3* morpholino used (Figure 7C). At their maximal effects, *NR4A2/3* morpholinos reduce neutrophil number by over 50% when compared to control morpholino (Figure 7B-C). To assess the effect of *NR4A* knockdown on neutrophil function, we used a well established model of tissue injury initiated by tailfin transection in 3dpf larvae. To adjust for altered numbers of total neutrophils, we examined the proportion of total neutrophils recruited to the tailfin wound. In this system, neither *NR4A2* or *NR4A3* knockdown affected the proportion of neutrophils at the site of injury, either at 6 (Figure 7D-E) or 24 hpi (Figure 7F-G), suggesting the effects on neutrophil lifespan and development are independent of effects on neutrophil function.

## Discussion

We show that in human neutrophils, PKA activation leads to a profound upregulation of *NR4A2* and *NR4A3* mRNAs, which is paralleled by a delay in neutrophil apoptosis. PGE<sub>2</sub> treatment also delays neutrophil apoptosis and upregulates *NR4A2/3* in a PKA dependent manner. Moreover, *NR4A3* expression is increased at sites of neutrophilic inflammation in human intradermal models, and knockdown of *NR4A2/3* in murine myeloid cells and zebrafish larvae significantly reduces neutrophil number. These findings demonstrate a role for *NR4A* family members in neutrophil survival and development, and reveal the potential for new therapeutic targets for conditions in which these defects are a key component.

PKA performs important signaling functions in the neutrophil, with roles in migration, adhesion, superoxide production and MMP secretion<sup>7,8,20</sup>. Roles for PKA in neutrophil lifespan have also been described, while PKA activation has been shown to have both pro-survival and pro-apoptotic outcomes<sup>21-23</sup>. In contrast to the pro-survival roles of PKA *in vitro*, PKA was found to play a role in the resolution of neutrophilic inflammation *in vivo* by driving neutrophil apoptosis<sup>23</sup>. In our study, activation of PKA by N6/8-AHA, a selective PKA type 1 agonist, results in a profound transcriptional response and upregulated more genes in neutrophils than any other pro-inflammatory stimulus tested. In addition, compared to the remaining stimuli, PKA activation induced the expression of a unique apoptosis transcriptome, including the induction of *JNK*, *IL-4*, *MMP9*, *PKC* and *NR4A2/3*. Within this unique PKA transcriptome was the down-regulation of a number of important pro-apoptotic genes including: *DEDD*, a caspase signalling molecule, *TRADD*, the multi death receptor adaptor protein and the tumor suppressor *RUNX3*. Selected qPCR validation assays showed that the targets most highly regulated by N6/8-AHA were *NR4A2/3*, linking them to PKA activation in our studies. The induction of *NR4A3* by conditioned supernatant may reflect the presence of PKA signaling agonists such as adenosine or prostaglandins.

The NR4A family of orphan nuclear receptors are emerging as important regulators of cellular function, with clear roles in inflammatory signaling<sup>24</sup>. Although the upstream agonists of NR4A receptors in neutrophils are not known, they are activated by a number of signaling pathways in other cell types, including PKA and PGE2<sup>25,26</sup>. Little is known about *NR4A2/3* gene expression in the human neutrophil and expression of NR4A/3 at the protein level has yet to be demonstrated for this cell type, due to lack of reliable antibodies. Two array studies have shown induction of NR4A family members, in particular *NR4A3* in LPS and GM-CSF/IFN $\gamma$  treated human neutrophils, although in our study, LPS did not regulate *NR4A2/3* *in vitro*<sup>27,28</sup>. This may be due to differences in cell purities since small numbers of contaminating monocytes or eosinophils may yield high gene copy numbers<sup>29</sup>. A recent murine study has shown the induction of NR4A genes in inflammatory neutrophils isolated from mice that developed serum-transfer arthritis, providing evidence for NR4A regulation at sites of inflammation *in vivo*<sup>30</sup>. Consistent with these findings, we show an increase in *NR4A3* mRNA expression at sites of neutrophilic inflammation in human intradermal endotoxin challenge models (Figure S4), although it is not possible to be certain of the cellular origin of the transcripts. The data suggest that *NR4A3* may be a suitable target to which anti-inflammatory therapeutic strategies may be designed, although this would require further study. A study by Pei *et al* shows NR4A1 protein expression in macrophages and other cells including smooth muscle cells, within human coronary artery atherosclerotic plaques, further demonstrating a clinical relevance for NR4A family expression at sites of inflammation<sup>31</sup>.

Our data link *NR4A2/3* expression to neutrophil survival, in that *NR4A2/3* transcripts are degraded at time points that precede spontaneous neutrophil apoptosis, and that agonists that induce *NR4A* expression also delay neutrophil cell death. In all cases, the upregulation of *NR4A2/3* transcripts was PKA dependent, linking NR4A regulation exclusively to upstream PKA signaling. These experiments are limited in that an NR4A inhibitor would provide definitive evidence that NR4A was essential in modulating PKA-dependent human neutrophil survival, however no such pharmacological tools existed at



the time of study. To address this further, *NR4A* gene knockdown approaches were explored. Although neutrophils are genetically intractable, siRNA strategies were possible in *hoxb8* conditionally immortalised murine myeloid progenitor cells, in which *NR4A2*, but not *NR4A3* gene knockdown reduced cell proliferation. It is not clear whether *NR4A2* knockdown murine neutrophils fail to differentiate or die prematurely by apoptosis, although the proportion of mature neutrophils was the same in all siRNA transfected populations, and the increase in Annexin V positive cells in combination with the pro-survival effect of N6/8-AHA may suggest the latter is more likely. *NR4A2/3* knockdown in a neutrophil reporter zebrafish line results in a significant reduction in total neutrophil number, suggesting the NR4A family may play a role in neutrophil differentiation *in vivo*. These findings are supported by the results obtained from studies of mCMP, and together begin to reveal a role for NR4A2/3 in myeloid cell development and differentiation. In support of this, a role for the NR4A family in regulating T cell and monocyte homeostasis has been described by others<sup>35,36</sup>. Interestingly, augmented NR4A2/3 expression has been demonstrated in bone marrow mononuclear cells and myeloid progenitors from patients with aplastic anemia and acute myeloid leukemia, further supporting roles for the NR4A family in the context of normal bone marrow development<sup>37,38</sup>. A role for NR4A2 and not NR4A3 in mCMP may reflect divergent roles for NR4A members in mice, or perhaps that NR4A3 can compensate for the absence of NR4A2. Consistent with this, distinct roles are seen in other models where NR4A3 has been shown to control proliferation of human hepatocytes and vascular smooth muscle cells<sup>32,33</sup> and NR4A2 overexpression in human synoviocytes promotes proliferation and survival<sup>34</sup>.

While we show that NR4A2/3 may play a role in neutrophil proliferation and homeostasis, we were unable to demonstrate a role for NR4A2/3 in the resolution of inflammation in zebrafish tail injury models *in vivo*. This may reflect that, aside from the induction of apoptosis, reverse migration away from injury may also play a significant part in inflammation resolution in this organism<sup>39</sup>. In support of this, PKA is thought to be a negative regulator of neutrophil migration *in vivo* which may in part explain why loss of PKA signaling

does not impede migration of neutrophils away from the site of inflammation<sup>5,40</sup>. A conditional neutrophil *NR4A* knockout strategy would reveal more about the specific role of NR4A in the neutrophil, but is beyond the scope of this study.

In conclusion, here we show an important role for the NR4A receptors in regulating neutrophil lifespan and homeostasis. Understanding the signaling underpinning these functions may help define targets for therapies for diseases driven by defects in neutrophil number and survival.

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**Contribution:** All authors contributed to writing the manuscript. SDP, JC, ECP, NO, KH, AB and AR carried out the practical experiments. IS and FF carried out bioinformatics and array analysis. MKBW and IS conceived the study and designed experiments.

**Conflict of interest disclosure:** We have no conflicts to disclose.

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## Figure legends

**Figure 1. Regulated gene clusters in stimulated neutrophils.** Neutrophils were cultured for 4h with N6/8-AHA [1mM] (PKA), monocyte conditioned supernatant (SN), GM-CSF [100u/ml], LPS [1µg/ml] and under hypoxic conditions whereafter the RNA was analysed by Affymetrix microarray (GENECHIP HU133 plus 2.0). Using DAVID web-based functional analysis application we identified several functional terms that were over-represented in the list of differentially expressed genes. This figure represents the differential expression status of genes in the gene ontology term GO:004281 (regulation of apoptosis), which was regulated by each stimuli. Greater than 2-fold upregulation is indicated in red, no change in grey and >2-fold down-regulation in green. Shows all genes upregulated (A) and downregulated (B) by N6/8-AHA. Please refer to Table S1 for DAVID analysis files and figure S1 for a complete figure of all regulated genes within GO:004281.

**Figure 2. qPCR validation of selected targets identified from microarray data.** Ultra-purified neutrophils were stimulated with N6/8-AHA (PKA) [1mM], monocyte conditioned supernatant (SN), GM-CSF [100u/ml], LPS [1µg/ml] or cultured under hypoxic conditions for 4h. cDNA was prepared and qPCR performed for the following genes: *NR4A2* (A), *NR4A3* (B), *VEGFA* (C), *IL-1α* (D) and *IL-1β* (E). Charts show mean±SEM and are generated from 5 independent experiments. Statistical analysis was carried out by one way ANOVA and Dunnett's post-test. Statistically significant comparisons are denoted by \*\* ( $P<0.01$ ) and \*\*\* ( $P<0.001$ ) where treated populations were compared to control.

**Figure 3. PKA activation regulates neutrophil survival.** Percoll-purified neutrophils were cultured with media or N6/8-AHA (A) at concentrations of 10, 50 and 100 or N6-MB-cAMP (B) at concentrations of 500 and 1000 µM for 20h. Neutrophils were pre-treated with media (open bars) or Rp-8-Br-cAMPS [0.7 mM] (black bar) for 30 min prior to the addition of N6/8-AHA [100 µM] for a further 20h (C). Neutrophils were pre-treated with media (open bars) or LY294002 [10 µM] for 30 min (black bars) and cultured for a further 20h with GM-CSF [50 u/ml] or N6/8-AHA [100 µM] (D). Apoptosis was determined by light microscopy (A-D). Charts show mean ± SEM percentage apoptosis from 3 (C), 4 (A, B) or 5 (D) independent experiments. Statistical analyses was carried out by ANOVA with Bonferroni post-test and significant differences indicated by \* ( $P<0.05$ ), \*\* ( $P<0.01$ ) and \*\*\* ( $P<0.001$ ) where treated populations were compared to media control, or as indicated by line. Neutrophils were aged in culture and RNA was made at timepoints of 1, 4 and 6h (E). In selected experiments, neutrophils were cultured with dbcAMP for 4 and 20h and RNA made at 0, 4 and 20h. *NR4A2/3* expression was determined by qPCR (E-G). Charts show fold change from 1h media (E) or 0h control (F,G) where *NR4A* expression was normalised to *GAPDH* loading control. Each panel shows data from 3 independent experiments.

**Figure 4. PGE2 signaling regulates neutrophil apoptosis and NR4A expression in a PKA dependent manner.** Ultra-purified neutrophils were cultured in the absence (open bars) or presence (black bars) of Rp-8-Br-cAMPS (RP8) [0.7mM] for 30 min prior to the addition of the following stimuli: N6/8-

AHA [100 μM], PGE2 [10 μM], ATPys [1 μM], adenosine [100 μM], LPS [100 ng/ml] and GM-CSF [50 u/ml] for a further 5 h (A). Apoptosis was determined by light microscopy and expressed as mean ± SEM from 5 independent experiments. In parallel experiments, neutrophils were cultured in the absence (open bars) or presence (black bars) of Rp-8-Br-cAMPS [0.7 mM] for 30 min prior to the addition of the following stimuli: LPS [100 ng/ml], PGE2 [10 μM], adenosine [100 μM] or ATPys [1 μM], for a further 4 h (B, C). *NR4A2* (B) and *NR4A3* (C) expression was measured by qPCR. Charts show fold change from 0h control where *NR4A* expression was normalised to *GAPDH* loading control and are from 3 independent expts. Neutrophils were treated with media or a concentration-response of PGE2 ranging from 10 nM to 10 μM (D) or Butaprost [100 nM and 1 mM] (E) in the presence (open squares) or absence (closed circles) of Rp-8-Br-cAMPS [0.7 mM] for 4 h. Apoptosis was determined by light microscopy and expressed as mean ± SEM from 3 (D, E) independent expts. Ultra-purified neutrophils were pre-incubated with media or Rp-8-Br-cAMPS [0.7 mM] for 30 mins before the addition of media or PGE2 [10 μM] for a further 1 or 4 h. *NR4A2* (F) and *NR4A3* (G) expression was measured by qPCR. Charts show fold change from 0h control where *NR4A* expression was normalised to *GAPDH* loading control and are from 3 independent expts. Data were analysed by ANOVA with Bonferroni or Sidak post-test and statistical differences indicated by \*( $P < 0.05$ ), \*\*( $P < 0.01$ ), \*\*\*( $P < 0.001$ ), and \*\*\*\*( $P < 0.0001$ ). Comparisons were made between agonist alone or agonist plus Rp-8-Br-cAMPS treated conditions for panels D and E, or as the lines indicate for the remaining panels.

**Figure 5. PGE2 promotes neutrophil survival and increases NR4A2/3 mRNA transcripts in the presence of LPS.** Ultra-purified neutrophils were pretreated for 15 minutes with (black bars) or without (open bars) Rp-8-Br-cAMPS (RP8) [0.7 mM] before addition of 1 ng/ml LPS, 10 μM PGE2 or both LPS and PGE2 together. Apoptosis was measured by light microscopy and expressed as mean ± SEM, n=4 (A). *NR4A2* (B) and *NR4A3* (C) expression was measured by qPCR. Charts show fold change from media control where *NR4A* expression was normalised to *GAPDH* loading control and are from 5 independent expts. Statistical analysis was performed by Two-Way ANOVA with Sidak's posttests. Asterisks (\*) denote significant differences to the relevant media control. Octothorpes (#) indicate differences between control and Rp-8-Br-cAMPS treated conditions. Results were considered to be statistically significant for  $P < 0.05$  (\*, #) and  $P < 0.01$  (\*\*).

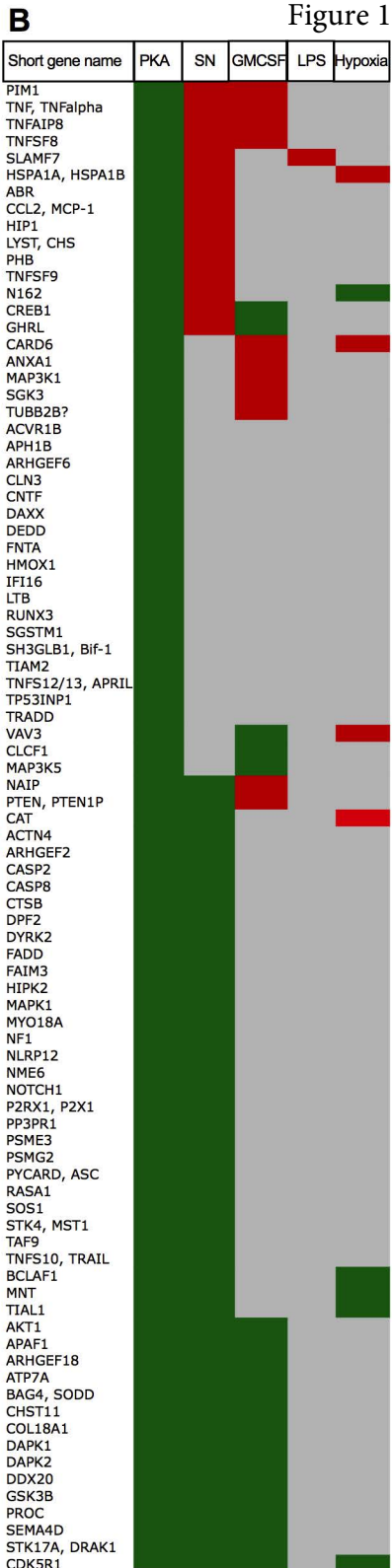
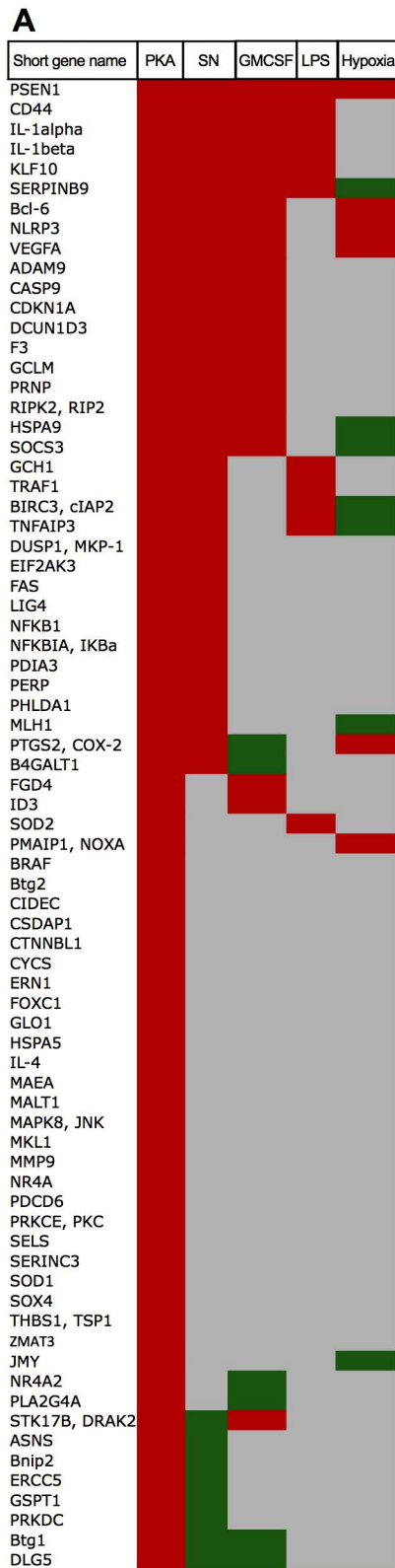
**Figure 6. NR4A2 siRNA knockdown inhibits myeloid cell proliferation.** Hoxb8 conditionally immortalised murine myeloid progenitor cells were subjected to estrogen withdrawal and differentiated to mature neutrophils (>90% maturity) in the presence of SCF and G-CSF for 4 days with daily media replenishment. Mature neutrophils were incubated with or without N6/8AHA [100 μM] for 6 hours in apoptosis medium (A-C). Cell viability and apoptosis was visualized by oil immersion light microscopy (A, where arrow denotes an apoptotic cell) and quantified by flow cytometry (B, C). RNAi transfections were conducted one day post-estrogen withdrawal using Amaxa Nucleofector technology. Cells were transfected with siRNA for *NR4A2*, *NR4A3* or a non-targeting control (siScrb1) on day 1. Total cell number was determined by haemocytometer counts (D-E) at days 1, 2, 3 and 4. Neutrophil maturity was

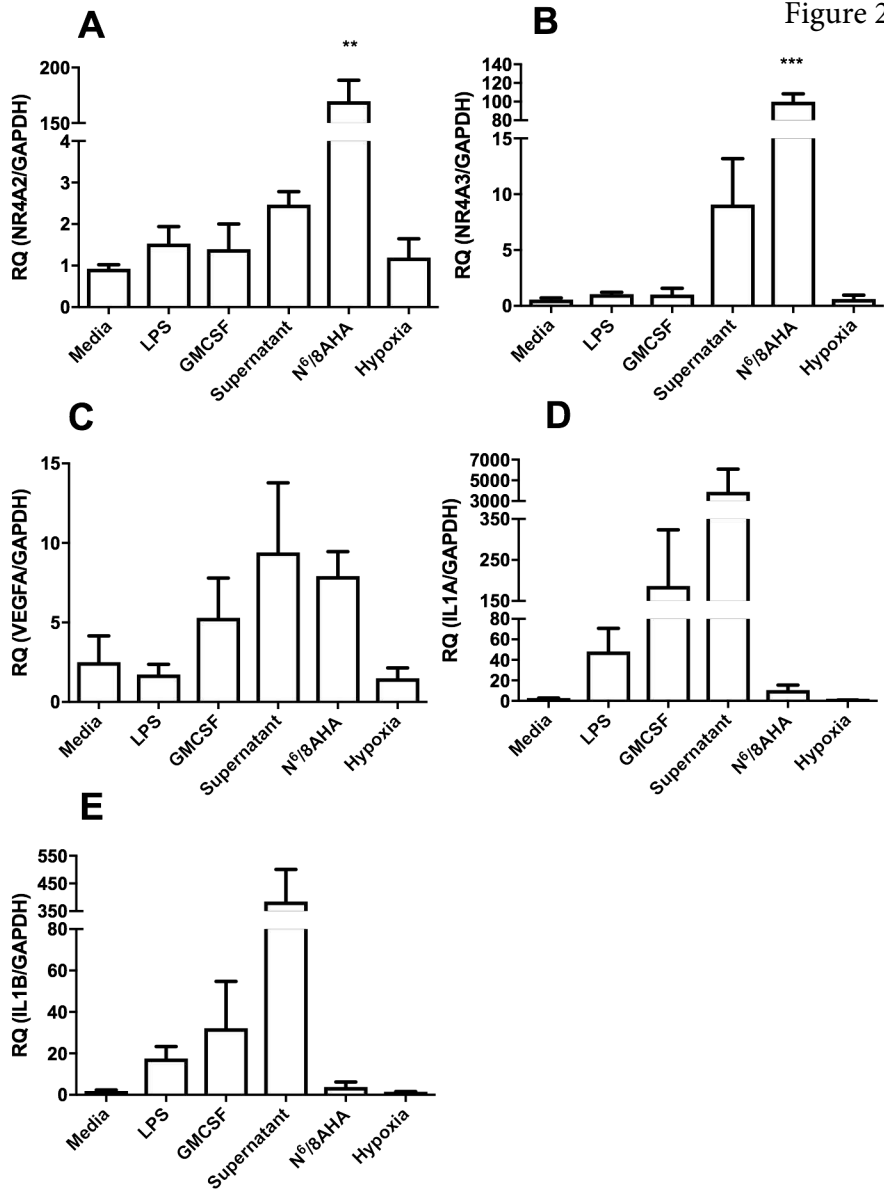
assessed by light microscopy (F-G). Apoptosis was measured on day 4 (H) and day 5 (I) by flow cytometry. Data are expressed as mean  $\pm$  SEM, n=4 (B, C, G), n=3 (D, E), n=1 (F, H, I). Statistical analysis was performed by ANOVA with Bonferroni post-test. Significant differences to media controls or siScrbl transfected cultures were denoted by \*\*( $P<0.01$ ) or \*\*\*( $P<0.001$ ) respectively.

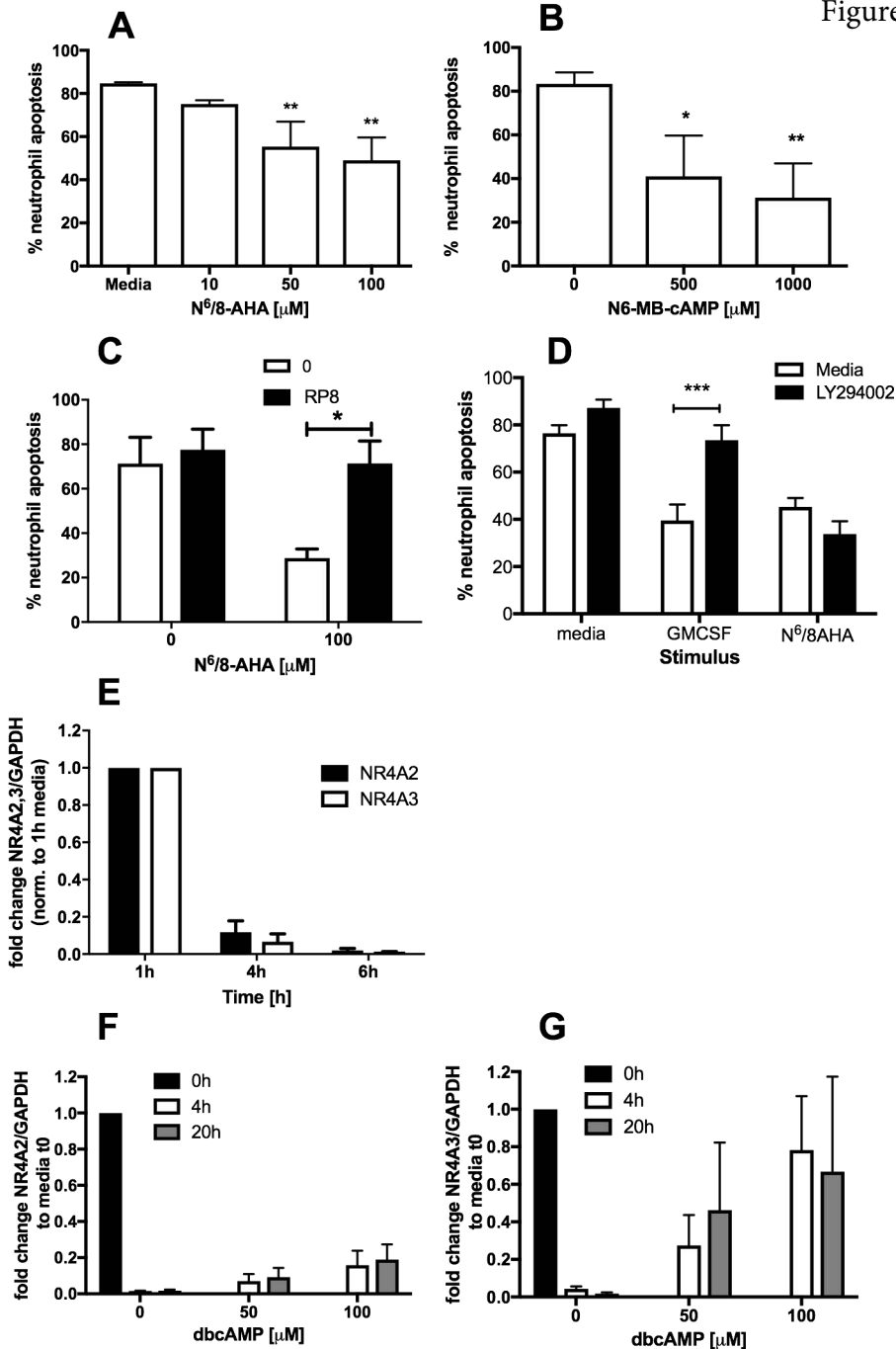
**Figure 7. NR4A2/3 regulates neutrophil number in zebrafish larvae.**

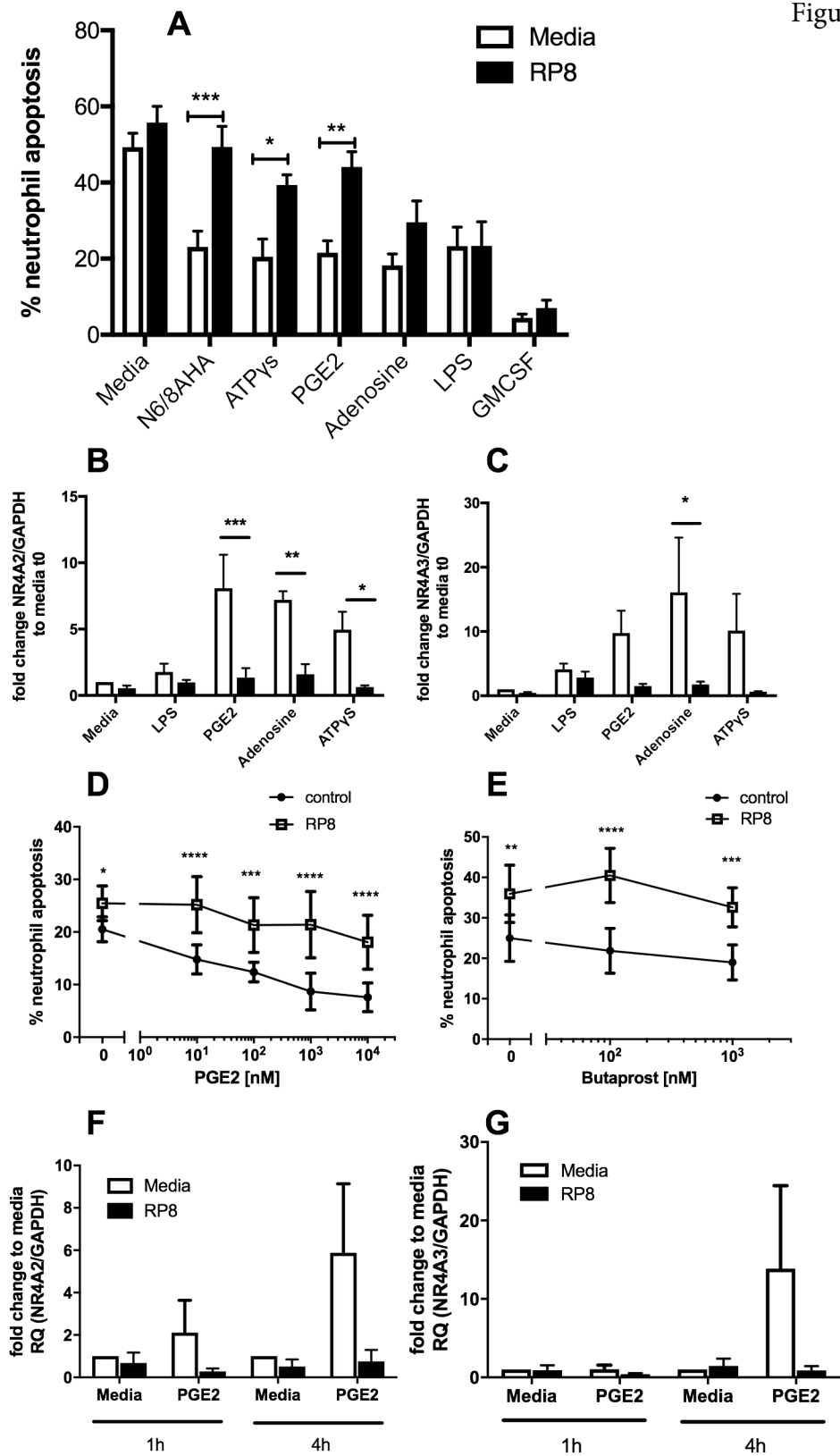
*mpx*:GFP zebrafish larvae were injected with NR4A2 [0.5 pM, 1 pM or 2 pM] (A, B, D, F), NR4A3 [0.125 pM, 0.25 pM or 0.5 pM] (A, C, E, G) or control morpholino (MO) [2 pM] (A-G) at the one cell stage. Neutrophils were visualised as GFP positive events by fluorescent microscopy (A). Total neutrophil number was assessed at 80 hpf (B-C) (n=20 performed as 3 separate experiments). Larvae were injured at 72hpf (hours post fertilisation) and neutrophil counts performed at 6 (D-E) and 24 hpi (hours post injury) (F-G) (n=16 performed as two separate experiments). Statistical analysis was performed by ANOVA with Dunnett's post-test. Significant differences compared to control MO were denoted by \*\*\*( $P<0.001$ ) and \*\*\*\* ( $P<0.0001$ )

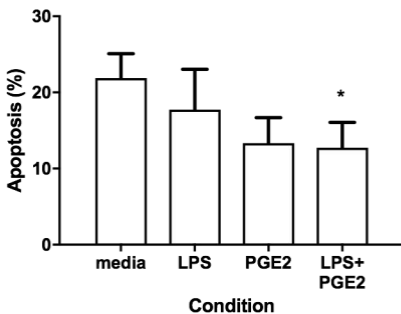
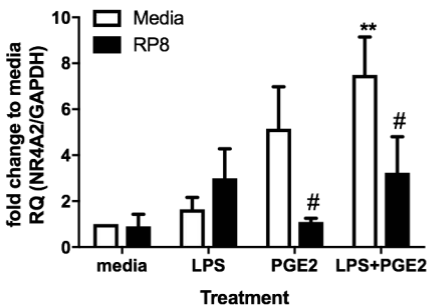
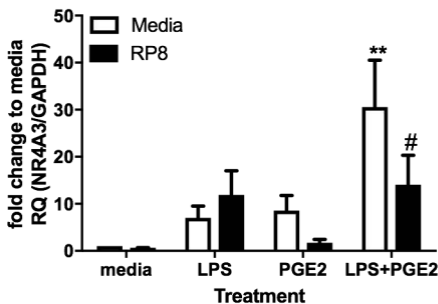


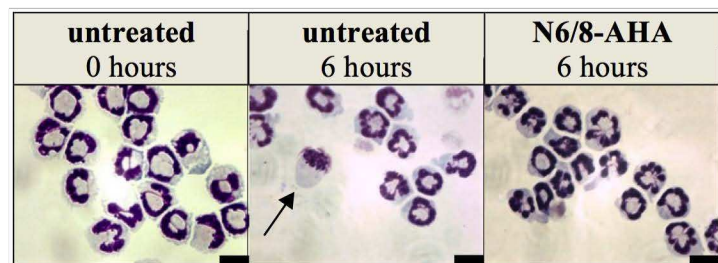
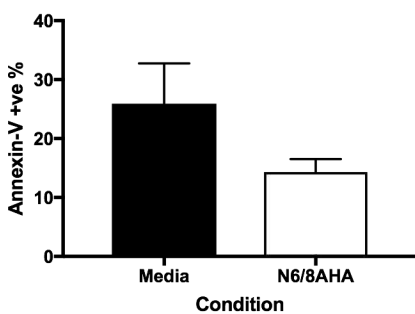
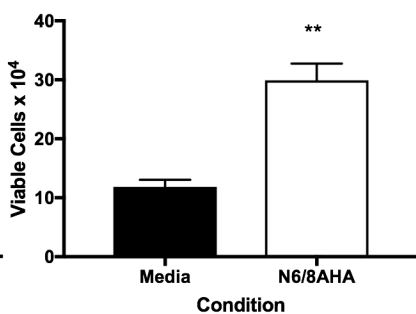
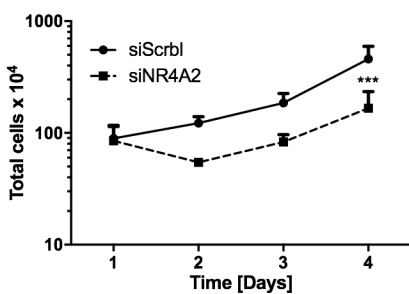
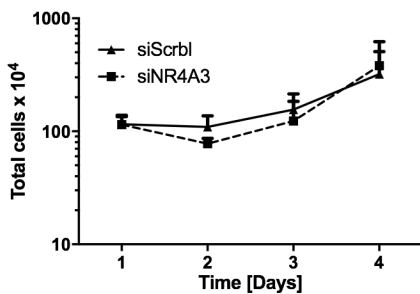
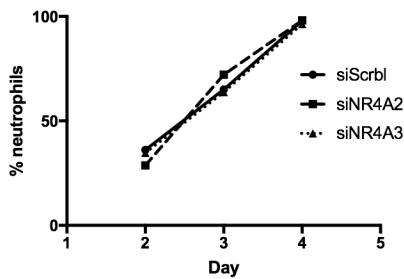
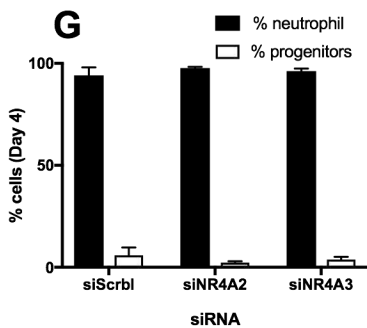
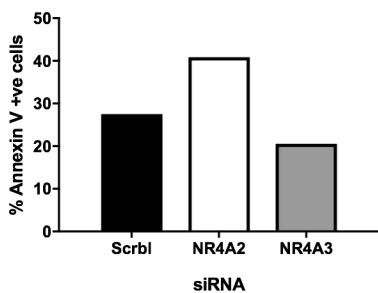
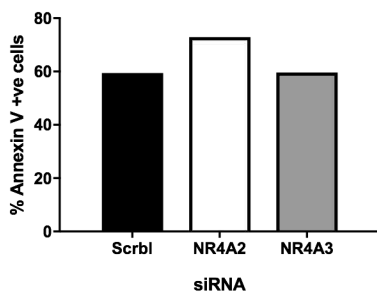






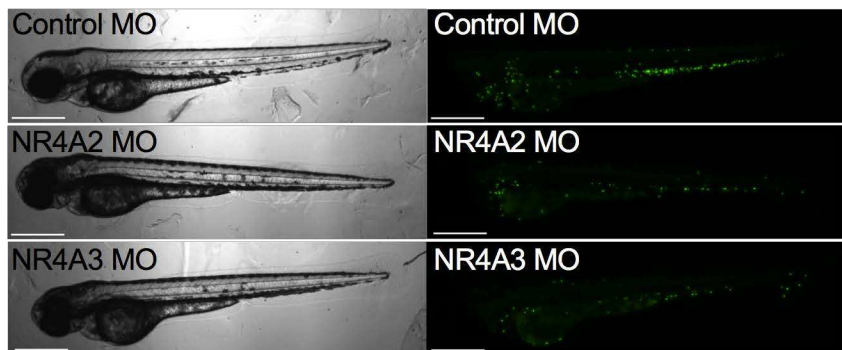
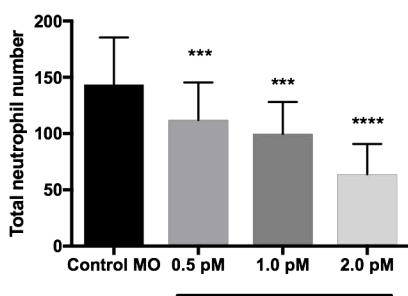
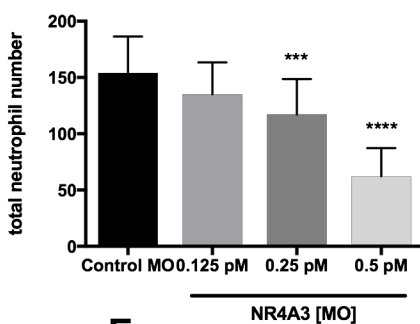
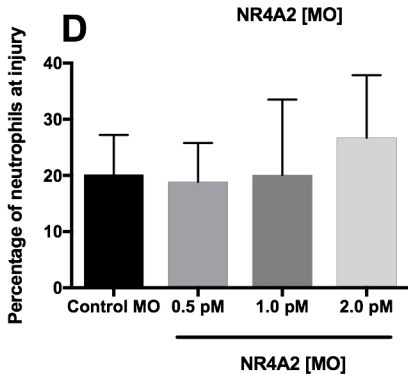
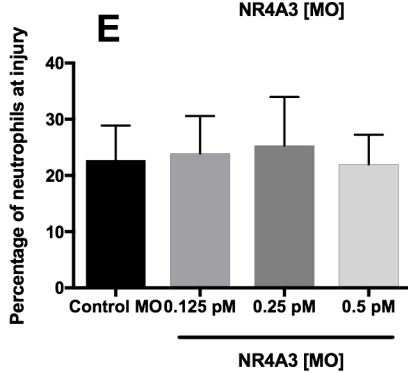
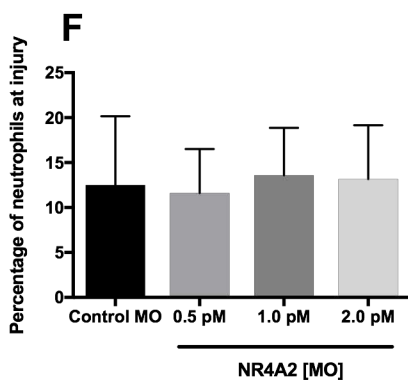
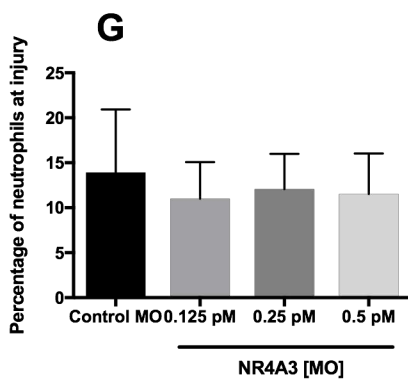


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## **NR4A orphan nuclear receptor family members, NR4A2 and NR4A3, regulate neutrophil number and survival**

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