TNF-α differentially regulates cell cycle genes in promyelocytic and granulocytic HL-60/S4 cells.

Elsie C. Jacobson¹, Jo K. Perry¹, Mark H. Vickers¹, Ada L. Olins², Donald E. Olins², Justin M. O’Sullivan*<br><br>¹Liggins Institute, University of Auckland, Auckland, New Zealand<br>²University of New England, Portland, ME, USA<br><br>* corresponding author: justin.osullivan@auckland.ac.nz<br><br>Author email addresses: ECJ <e.jacobson@auckland.ac.nz>, JKP <j.perry@auckland.ac.nz>, ALO <aolins@une.edu>, DEO <долин@aolins.edu>, MHV <m.vickers@auckland.ac.nz>, JMO <justin.osullivan@auckland.ac.nz><br><br>Keywords<br>Tumor necrosis factor, RNA-seq, differentiation, cell cycle, hematopoiesis, innate immune, neutrophil, macrophage, HL-60, retinoic acid.<br><br>Abstract<br>Tumor necrosis factor alpha (TNF-α) is a potent cytokine involved in systemic inflammation and immune modulation. Signaling responses that involve TNF-α are context dependent and capable of stimulating pathways promoting both cell death and survival. TNF-α treatment has been investigated as part of a combined therapy for acute myeloid leukemia due to its modifying effects on all-trans retinoic acid (ATRA) mediated differentiation into granulocytes.<br><br>To investigate the interaction between cellular differentiation and TNF-α, we performed RNA-sequencing on two forms of the human HL-60/S4 promyelocytic leukemia cell line treated with TNF-α. The ATRA-differentiated granulocytic form of HL-60/S4 cells had an enhanced transcriptional response to TNF-α treatment compared to the undifferentiated promyelocytes. The observed TNF-α responses included differential expression of cell cycle gene sets, which were generally upregulated in TNF-α treated promyelocytes, and downregulated in TNF-α treated granulocytes. This is consistent with TNF-α induced cell cycle repression in granulocytes and cell cycle progression in promyelocytes. Moreover, comparisons with gene expression changes associated with differentiation into granulocytes or macrophages indicated that TNF-α treatment of granulocytes shifts the transcriptome towards that of a macrophage.<br><br>We conclude that TNF-α treatment promotes a divergent transcriptional program in promyelocytes and granulocytes, characterized by cell cycle genes increasing expression in promyelocytes, and decreasing expression in granulocytes.
Background

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytotoxic cytokine [1] which activates the innate immune response [2-4] and induces migration [5,6] and production of pro-inflammatory cytokines [7,8]. Dysregulation of TNF-α is associated with subsets of autoimmune diseases [9,10] and anti-TNF antibodies are used to treat a range of inflammatory disorders [11-13]. Initially investigated as a cancer therapeutic due to its ability to promote apoptotic cell death specifically of tumor cells [14], systemic TNF-α treatment has failed clinical trials as a solo cancer therapeutic as it induces unacceptable levels of toxicity [15].

TNF-α signaling is complex with numerous and sometimes conflicting responses being modulated by interaction with two cell surface TNF-α receptors, TNFR1 and TNFR2 [1]. TNF-α binding can have a wide range of effects via activation of signal transduction pathways, including all three groups of mitogen activated kinases (MAPK); extracellular-signal-regulated kinases (ERKs), the cJun NH2-terminal kinases (JNKs), and the p38 MAP kinases [16], which each have complex regulatory effects on the cellular phenotype [17,18]. TNF-α signaling leads to transcriptional upregulation of pro-inflammatory cytokines including IL-6 [7] and TNF itself [8], resulting in pro-inflammatory feedback loops [19]. Notably, TNFR1 and TNFR2 have individual and combinatorial effects on cell death and inflammation [1,20,21]. TNFR1 signaling induces both pro-apoptotic pathways resulting in caspase activation, and pro-survival Nuclear Factor Kappa B (NFκB) signaling [22,23]. For example, hematopoietic cells growing in log phase rapidly undergo apoptosis in response to TNF, while quiescent cells in stationary phase re-enter the cell cycle on TNF-α stimulation [24]. These apparently conflicting TNF-α responses can be explained by temporal and developmental effects that include cell type [25], receptor expression [24], priming with cytokines or inflammatory stimuli [26,27], and cell cycle stage [28].

The HL-60/S4 cell line was derived from an acute promyelocytic leukemia patient [29]. These promyelocytic cells can be differentiated into granulocytic or macrophage forms with the addition of all-trans retinoic acid (ATRA) or 12-O-tetradecanoylphorbol-13-acetate (TPA), respectively [30]. Differentiation into the granulocytic form slows cell growth [30] and ultimately leads to cell death [31]. This discovery lead to the clinical use of ATRA as a treatment for acute promyeloid leukemia [32]. Combined treatment with ATRA and TNF-α enhance differentiation of myelogenous leukemia cells, and therefore has been investigated as a synergistic therapy [33,34]. Notably, ATRA-induced differentiation activates components of the TNF-α signaling pathway [33].

A previous study demonstrated differential effects of TNF-α treatment on candidate genes in HL-60 cells before and after ATRA treatment [35]. Here, we investigate the genome-wide transcriptional response to TNF-α treatment of the promyelocytic and granulocytic forms of HL-60/S4 cells. We identify a conserved inflammatory and apoptotic response to TNF-α treatment in both promyelocytic and granulocytic cells. We also identify opposing effects of TNF-α treatment on the expression of cell cycle genes, with increased CDK1 mRNA expression in promyelocytes, whereas granulocytes show transcriptional changes associated with arrest in G2 phase. We propose that the different TNF-α mediated responses arise through sets of genes being responsive to different thresholds of total (endogenous and exogenous) TNF-α levels.

Results
In order to investigate transcriptional responses to TNF-α, the promyelocytic cell line HL-60/S4 was differentiated into a granulocytic form by treatment with ATRA for 96 hours, as described previously [36]. Both undifferentiated (promyelocytic) and differentiated (granulocytic) cells were treated with 16ng/ml TNF-α in calcium supplemented media for two hours. TNF-α treatment of promyelocytes and granulocytes were run as independent experiments; as a consequence analysis of transcriptional changes that occurred during differentiation was not appropriate. However, a recently published study investigated transcriptional changes which occurred on differentiation of promyelocytic HL-60/S4 cells into granulocytes or macrophages [30]. We reanalyzed this data for use in comparisons with the TNF-α response. All genes that were significantly differentially expressed (FDR<0.05) following differentiation or TNF-α treatment were identified (Supp table 1).

**Gene expression changes after TNF-α treatment of promyelocytic and granulocytic cells**

Triplicate non-coding RNA-seq libraries were aligned using an ultra fast universal RNA-seq aligner (STAR [37]) and assigned to genes with featureCounts [38]. DESeq2 [39] was used to filter out lowly-expressed genes before performing differential expression analysis. 14,420 genes were analyzed for TNF-α dependent differential expression in promyelocytes. 21,305 genes were analyzed for TNF-α dependent differential expression in granulocytes. Principal component analysis of variance stabilized transcripts confirmed clustering by treatment (Supp fig 1).

The promyelocytic and granulocytic forms of HL-60/S4 cells both exhibited strong transcriptional responses to TNF-α treatment (Supp table 1). In promyelocytes, 1,312 genes were significantly increased and 980 significantly decreased (FDR adjusted p value < 0.05, Fig 1A). TNF-α treatment of granulocytes significantly increased expression of 3809 genes and decreased expression of 3,597 genes (FDR adjusted p value < 0.05, Fig 1B). Notably, the granulocytic form had more than three times as many significantly differentially expressed genes (Fig 1C). Despite this, there was significant overlap in the TNF-α dependent transcriptional response between the granulocytic and promyelocytic cells (2.5 fold more than expected by chance, p<0.00001, bootstrapping). The TNF-α treatment consistently resulted in more genes being upregulated than downregulated in both the promyelocytic and granulocytic forms of the HL-60/S4 cells (p<2x10^{-6} and p=0.0005, respectively; 2-sample test for equality of proportions).

Genes that were significantly differentially expressed after TNF-α treatment in both the promyelocytic and granulocytic forms of HL-60/S4 cells exhibited strongly correlated log fold changes (p<2x10^{-8}, R²=0.65). There was a small subset of differentially expressed genes that exhibited changes in opposite directions in promyelocytes and granulocytes (Fig 1D). Notably, the effect sizes of conserved gene expression changes were similar, with a slope of 1.08. If the observed differences in the numbers of DE genes were due to a different magnitude of effect, we would expect to see a correlation in fold change between genes that were DE in one cell type but not the other. However, the fold change of non-conserved genes did not strongly correlate between cell types (Fig 1E & F, R²=0.038, R²=0.15). Collectively these results suggest that the difference in TNF-α responses is not simply due to granulocytic cells showing enhanced regulation of a conserved set of genes, resulting in a greater number that are significantly differentially expressed, but rather that additional sets of genes are changing transcriptional activity.

**Functional analysis of the TNF-α response in promyelocytes and granulocytes**
Functional analysis of gene sets is affected by the available gene function information, and the method of enrichment analysis [40]. Therefore, we used multiple annotation sources (i.e. MSigDB hallmark gene sets [41], gene ontology [42], and KEGG pathways [43]) to interrogate the TNF-α response in HL-60/S4 cells and identify robust enrichment results.

**Gene set enrichment analysis of promyelocytes and granulocytes treated with TNF-α.**

Gene set enrichment analysis (GSEA) is a method that allows analysis of an entire differential expression dataset without using arbitrary significance thresholds [44]. Instead, the entire dataset is ranked by log2 fold change, and terms can be enriched if they are over-represented at the top or bottom of the ranked list. This approach allows for a differentiation between functional categories of genes that are upregulated or downregulated after treatment [45].

To compare the overall gene expression changes in promyelocytes and granulocytes without using arbitrary significance cut-off, we performed GSEA of hallmark gene sets (Supp table 2). We found a strong upregulation response to TNF, in both the promyelocytic and granulocytic forms of the HL-60/S4 cells, that was characterized by immune and cell death pathways (Fig 2). However, the granulocytic genes that were upregulated were also enriched for terms that included notch signaling and hypoxia. Transcripts associated with WNT and beta-catenin signaling were downregulated in promyelocytes (Fig 2).

**Functional analysis of conserved, cell type-specific, and opposite TNF-α responses**

Genes that were differentially expressed in response to TNF-α treatment were divided into four groups according to the nature of the correlation between the promyelocytic and granulocytic responses: 1) conserved; 2) promyelocyte-specific; 3) granulocyte-specific; and 3) opposite responses (Supp table 1). The first group represents the conserved response, that is the genes were significantly differentially expressed in the same direction in both promyelocytic and granulocytic forms of the HL-60/S4 cells. The next two groups represent cell type-specific responses – i.e. genes that were only differentially expressed in either promyelocytes or granulocytes in response to TNF-α treatment. The final group is comprised of the 157 genes that were significantly upregulated in the first cell type and significantly downregulated in the other cell type, or vice versa. We analyzed these four groups of genes for enrichment of MSigDB, KEGG, or GO terms to investigate the conserved and differential responses to TNF-α in promyelocytes and granulocytes.

**A conserved TNF-α response**

Genes that exhibited significant changes in the same direction in response to TNF-α treatment in both promyelocytic and granulocytic forms of the HL-60/S4 cells were more frequently upregulated than downregulated (~3:1). Notably, this trend was not maintained for cell type-specific gene expression changes (promyelocyte-specific ~1:2:1, granulocyte-specific ~0:9:1). There were nine enriched MSigDB hallmark gene sets [41] in the conserved response gene set (Fig 3A, Supp table 3), all of which are related to well described effects of TNF-α stimulation; cytokine signaling, inflammation, and apoptosis. This was broadly consistent with the results of GSEA (Fig 2). The top three enriched pathways within KEGG were NFkB Signaling; NOD-like receptor signaling and TNF-α signaling pathways (Supp table 4). The top 10 enriched GO terms included interferon-gamma (IFNγ)-mediated signaling pathway, inflammatory response, and positive regulation of I-kappaB kinase/NFKB signaling (Supp table 5).
In summary, using three different annotation databases and four different analysis approaches, we have shown that TNF-α treatment induces a transcription profile associated with immune-like responses and NFkB signaling in both promyelocytic and granulocytic forms of HL-60/S4 cells.

Granulocyte-specific TNF-α responses

The cell type-specific promyelocytic DE genes had no significant enrichments of MSigDB gene sets, KEGG pathways, or GO terms. Cell type-specific granulocytic DE genes were enriched for MSigDB sets related to cell cycle and energetics (Fig 3B, Supp table 6). The top five enriched KEGG pathways included Cell Cycle, Protein processing in endoplasmic reticulum, and Cellular senescence (Supp table 7). The six enriched GO terms included cell division, G2/M transition of mitotic cell cycle, protein poly- and de-ubiquitination, and neutrophil degranulation (Supp table 8). Thus, granulocytes, but not promyelocytes, exhibited transcriptional changes in genes involved in cell cycle and protein processing in response to TNF-α treatment.

Opposite TNF-α responses in promyelocytes and granulocytes

Genes that were significantly differentially expressed in opposite directions in promyelocytes and granulocytes were enriched for G2M checkpoint and E2F targets within the MSigDB database (Fig 3C, Supp table 9). Differentially expressed genes in these sets were upregulated in promyelocytes and downregulated in granulocytes, with the exception of E2F2. CDK1 is included in both of these cell-cycle associated gene sets, and is considered sufficient to drive the mammalian cell cycle [46]. The set of genes that was significantly expressed in opposite directions was also enriched for three terms, including mitotic chromosome condensation, within the GO database (Supp table 10). There was no enrichment for pathways within KEGG. Collectively, these results are consistent with the conserved response to TNF-α in promyelocytes and granulocytes being characterized by inflammatory signaling pathways. However, TNF-α treatment alters genes associated with protein processing and represses cell cycle specifically in granulocytes.

Granulocytic differentiation increases transcript levels of TNF and TNF receptors

We set out to investigate how differentiation into the granulocytic form could alter the TNF-α response so profoundly, and identify similarities in the transcriptional changes that occur during differentiation and acute TNF-α treatment. We were unable to analyze transcriptional changes that occurred during differentiation, as promyelocytic and granulocytic cells were treated with TNF-α in separate experiments. In order to assess changes that occurred upon differentiation we analyzed publicly available RNA-seq data showing transcriptional changes in HL-60/S4 cells differentiated into granulocytes (ATRA) and macrophages (TPA). Principal component analysis of variance stabilized transcripts confirmed clustering by differentiation status (Supp fig 1C).

Consistent with previous reports [30], this analysis confirmed that TNF expression was upregulated during differentiation into either granulocytic or macrophage forms of HL-60/S4 cells. The magnitude of TNF transcript upregulation was different in granulocytes (log2 fold change = 1.37) and macrophages (log2 fold change = 4.08) (Supp table 1). Differentiation also induced changes in the expression levels of the TNFRs. Consistent with previous observations [30], TNFRSF1A gene expression was increased (log2 fold change = 0.87) after ATRA treatment, but there was no significant change in expression following TPA treatment. By contrast, TNFRSF1B gene expression was increased in both conditions (log2 = 1.59 and 3.30 following
ATRA and TPA treatment, respectively). This observed increase in mRNA expression of TNF-α and both TNF-α receptors may be one explanation for why granulocytes have an enhanced transcriptional response to TNF-α, compared to promyelocytes.

We wanted to know if TNF-associated genes were enriched for transcriptional changes associated with differentiation into the granulocytic or macrophage form. Therefore, we performed a GSEA of the transcriptional changes associated with differentiation into the granulocytic and macrophage form (Fig 4). As described previously, genes associated with cell-cycle terms (e.g. MYC targets and G2M checkpoint) were downregulated, while genes associated with inflammatory terms (e.g. IFN-γ response, inflammatory response, and indeed TNF-α signaling via NFκB) were upregulated in both differentiation experiments (Fig 4).

**TNF-α treatment has more shared responses with TPA treatment than ATRA treatment**

Gene sets upregulated following TNF-α treatment of granulocytes and differentiation of promyelocytes into granulocytes or macrophages were enriched for ontological terms associated with hypoxia and xenobiotic metabolism. Notably, genes that were upregulated in promyelocytes treated with TNF-α did not show enrichment for these ontological terms. Notch signaling was upregulated only after TNF-treatment of promyelocytes or differentiation into macrophages. Intriguingly, both early and late estrogen response genes were upregulated in granulocytes treated with TNF-α, and macrophages, despite reports that TNF-α acts to oppose estrogen signaling in breast cancer [47]. Four terms were upregulated after TNF-α treatment of granulocytes and differentiation into macrophages: IL2 stat5 signaling, epithelial mesenchymal transition, apical junction, and KRAS signaling (genes downregulated by KRAS activation).

To further compare the transcriptional changes that occur during differentiation into different cell types with the effects of TNF-α treatment, we assigned differentiation associated changes into four groups: 1) conserved; 2) granulocyte-specific; 3) macrophage-specific; and 4) opposite, i.e. significantly upregulated in granulocytes and significantly downregulated in macrophages, or vice versa (Supp table 1). The first group consists of genes that significantly change expression levels in the same direction after differentiation into either the granulocytic form with ATRA, or the macrophage form with TPA. The second and third groups contain genes that are differentially expressed only after differentiation into granulocytes or macrophages, but not both. The fourth group represents genes that are differentially expressed after differentiation into granulocytes and macrophages, but are upregulated in granulocytes and downregulated in macrophages, or vice versa. We analyzed these groups of genes for enrichment of MSigDB gene sets, KEGG pathways, and GO terms. GSEA identified the response that is conserved during differentiation into granulocytes or macrophages as being characterized by pro-inflammatory cytokine associated gene sets (i.e. IFNα, response, IFNγ response, TNF-α signaling via NFκB), and cell cycle associated gene sets (i.e. G2M checkpoint, MYC targets, and mitotic spindle) (Fig 5A). This finding was consistent with what was observed as being enriched within the KEGG pathways and GO terms (Supp table 12,13).

Genes that changed expression after differentiation into granulocytes, but not macrophages, were not enriched for MSigDB gene sets or GO terms, but they were enriched for three KEGG pathways related to protein processing (Protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, and proteasome; Supp table 14). In contrast, genes that changed expression after differentiation into macrophages, but not granulocytes, were enriched for several MSigDB gene sets related to cell cycle (Fig 5B, Supp table 15). Enriched KEGG pathways included metabolism and protein processing (i.e. carbon metabolism and protein processing in
endoplasmic reticulum), and the TNF-α signaling pathway (Supp table 16). Enriched GO terms included cell division, and terms related to RNA and protein regulation (i.e. regulation of transcription, DNA-templated, translational initiation).

**Convergence of transcriptional programs in macrophages and TNF-treated granulocytes**

There are many similarities between the behaviors of HL-60/S4 cells differentiated into granulocytes and macrophages. However, there are also notable behavioral differences that include macrophages exhibiting a decreased cell cycle rate, increased survival, and adhesion to both surfaces and other cells [30].

We investigated the genes that were significantly differentially expressed with negatively correlated log2 fold changes after differentiation into granulocytes or macrophages. This gene set was enriched for the IL2/STAT5 signaling, p53 pathway, and TNF-α signaling MSigDB hallmark terms (Fig 5C). 91% of these genes (102/112) increased expression after macrophage differentiation, and decreased expression after granulocytic differentiation. 65% of these genes (70/112) significantly increased expression in granulocytes treated with TNF-α, while only 10% (12/112) significantly decreased expression (Fig 5C). Six transcription factors (ATF3, BCL6, FOSL1, KLF4, KLF9, NR4A1) increased expression after macrophage differentiation and granulocytes treated with TNF-α, but decreased expression after granulocytic differentiation, which indicates a shared change in transcriptional programming. CD83, which is a marker of transdifferentiation of neutrophils into a dendritic-type cell, increased in both TNF-α treated cell types [48,49]. Given that macrophages and dendritic cells are phenotypically similar [50,51], CD83 could be considered a marker of macrophage transdifferentiation. Collectively our analyses suggest that TNF-α alters the transcriptional profile in HL-60/S4 cells consistent with a transition from a granulocytic to macrophage phenotype.

**Conclusion**

HL-60/S4 cells show a conserved core of responses to TNF-α treatment irrespective of the differentiation state of the cell. This is expected, since functional annotations represent canonical pathways and responses. However, granulocytes are more responsive to TNF-α, possibly due to priming from increased endogenous TNF expression, and increased levels of TNFRs. Transcriptional changes indicate that TNF-α treatment leads to an increased proportion of promyelocytic cells progressing through mitosis, but decreases the proportion of granulocytic cells progressing through mitosis. This effect may be sensitive to the sum total of the exogenous and endogenous TNF-α levels. Finally, comparisons of transcriptional changes during differentiation and TNF-α treatment suggest that TNF-α treatment of granulocytes pushes them towards a macrophage transcriptional program.

**Discussion**

TNF-α treatment causes dramatic changes in the transcriptional programs of both promyelocytic and granulocytic HL-60/S4 cells. In this study we found that ATRA and TPA directed differentiation of TNF-α treatment of differentiated HL-60/S4 cells resulted in canonical TNF-α responses involving NFkB signaling, inflammatory signaling, p53 and apoptosis. Due to the reduced proliferation of granulocytic HL-60/S4 cells [30] we expected differential cell cycle effects of TNF-α treatment [24,28]. Consistent with previous reports, granulocytes had reduced expression of G2/M checkpoint and mitotic spindle genes, indicating
TNF-α-induced G2 cell cycle arrest [28]. However, previous work has shown increased proliferation in quiescent cells, while proliferating cells exhibited increased apoptosis after TNF-α treatment [24]. In contrast, we saw evidence of cell cycle repression in granulocytes, particularly at mitotic entry, while proliferating promyelocytes had an increase in cell cycle progression markers such as CDK1 [46].

There are several factors that could explain the different responses of promyelocytes and granulocytes to TNF-α. Granulocytes have increased levels of endogenous TNF-α production. Not only does this increase the total TNF-α the cells are exposed to, but endogenously produced TNF-α is membrane-bound prior to processing [52]. The transmembrane and soluble forms of TNF-α have different effects [53], possibly due to the activation of different TNFRs. Membrane-bound TNF-α can activate both TNFR1 (TNFRSF1A) and TNFR2 (TNFRSF1B), but soluble TNF-α can only activate TNFR1 [54]. Not only does the granulocytic form of HL-60/S4 cells have higher levels of endogenous TNF expression, and therefore likely higher levels of transmembrane TNF-α, it also has higher levels of both TNFRSF1A and TNFRSF1B gene expression. It has been previously proposed that increased levels of TNFR2 in granulocytic HL-60 cells explain their resistance to TNF-α-induced apoptosis [35]. Thus, it may be not just the increased levels of receptors, but the ratio of TNFR1 to TNFR2 that determines the ultimate response to TNF-α.

The TNFR1 and TNFR2 receptors have uniquely stimulated pathways [55,56], however the two receptors also interact to produce a context specific TNF-α response [57,58]. Therefore, manipulating exogenous and endogenous levels of TNF-α or changing the ratio of the TNFR1 and TNFR2 receptors will provide greater insight into the phenotypic consequences of TNF-α treatment. Based on our data, we predict a dose dependent effect of total TNF-α on gene expression, whereby a low level of TNF-α is sufficient to induce regulation of the set of genes seen in the promyelocyte response, while higher levels of TNF-α are required to repress cell cycle. However, cell cycle repression likely also requires the presence of additional factors (e.g. TNFR-associated factors [59]) that are expressed during differentiation of the HL-60/S4 cells into the granulocytic form.

A reduction in the expression of cell cycle genes at the population level indicates a change in the proportion of cells at different stages of the cell cycle. Due to the massive transcriptional changes that occur during the progression through cell cycle [60–63], this may obscure other gene regulatory programs that are associated with alterations to cell function. Despite this limitation, we found evidence of a subset of TNF-α-regulated genes that were upregulated in TNF-treated granulocytes and following macrophage differentiation, but downregulated after granulocytic differentiation. These genes encoded a suite of transcription factors, and the dendritic cell surface marker CD83. Neutrophils that take on characteristics of antigen-presenting cells are often characterized by increased levels of CD83 [48,49], suggesting that TNF-α may be stimulating transdifferentiation. This is not unprecedented, as there is evidence that neutrophils have phenotypic plasticity [64], and that TNF-α can stimulate transdifferentiation [65–67]. However, further functional characterization of TNF-α-treated granulocytic HL-60/S4 cells is required to confirm this hypothesis.

A large-scale TNF-α response experiment treating many different cell types at various stages of differentiation would allow a network analysis of the transcriptional responses and annotation-free pathway discovery [68,69]. This would provide data to test our hypothesis that total TNF-α
exposure correlates with repressive effects on the cell cycle. If this experiment were performed with single cell RNA-sequencing it would also enable the elucidation of how individual TNF-α responses result in population-wide changes in cell cycle gene expression. For instance, do all cells respond to TNF-α stimulation with cytokine production, G2/M arrest, and apoptosis, or is there a heterogeneous response between different cells in a population? Moreover, single cell data would allow us to correct for the cell cycle transcriptional effects, and identify differences that were previously masked by the population structure of unsynchronized cells.

Despite being one of the most highly studied genes in the human genome [70], the complex signaling [1] and context dependent effects [20,28,55] of TNF-α mean that much of its biology remains unknown. With the increasing accessibility of modern sequencing technologies and gene editing, high-throughput investigations of the TNF-α response and signaling will yield new results that enable the contextualization of TNF-α in cancer and immunology.

**Methods**

**Cell culture**

HL-60/S4 cells (available from ATCC #CCL-3306) were cultured at 37°C, 5% CO₂ in RPMI 1640 (ThermoFisher) supplemented with 10% fetal bovine serum (Moregate Biotech), 1% penicillin/streptomycin (ThermoFisher), 1% GlutaMAXTM (ThermoFisher) and 2mM CaCl₂. As required, cells were differentiated into a granulocytic phenotype with 1 μM all trans retinoic acid (ATRA) dissolved in ethanol (Sigma Aldrich) for four days as previously described ([30,36,71]). Cells were centrifuged (200xg, 5 min., room temp.) and suspended (undifferentiated = 1x10⁶ cells/mL; or differentiated = 5x10⁵ cells/mL) in fresh media (with 1μM ATRA for differentiated cells). Cells (9 x 10⁶) were incubated at 37°C for 2 hours before addition of 16ng/mL TNF-α or vehicle. After 2 hours treatment, cells were lysed with TRIzol LS, phase separated with chloroform, and RNA extracted with the Qiagen RNeasy micro kit (Qiagen, 74004).

**RNA sequencing**

Total RNA libraries were prepared by Annoroad Gene Technology Co., Ltd. (Beijing, China) using ribosomal RNA depletion with RiboZero Magnetic Gold Kit (Human/Mouse/Rat) and sequenced on an Illumina Hi-seq X 150PE.

**RNA-seq analysis**

Read quality was confirmed using FastQC v0.11.4. Reads were aligned to hg38 and gene code annotations v27 using STAR [37] v2.5.3a with default settings. FeatureCounts [38] v1.5.2 was used to aggregate transcripts for gene-level analysis and quantify the reads with GENCODE [72] annotation v27 (Additional file 3: Supp table S8). MultiQC [73] was used to summarize FastQC, STAR, and FeatureCounts outputs [73]. All subsequent analyses were performed in R [74].

Expressed genes were filtered (default settings) and differentially expressed genes (FDR<0.05) identified in DESeq2 [39] v1.16.1. Gene ontology enrichments were calculated with TopGo [42] v2.28.0 using the weight01 algorithm and the Fisher statistic. Categories containing <2 genes were removed, and p values adjusted for FDR. Kegg pathway analysis, GSEA, and enrichment
analyses were performed using clusterProfiler [75]. Intersects were displayed with Vennizable [76], subsets extracted with dplyr [77], and plots were generated with ggplot2 [78].

Data availability

TNF-α treatment RNA-seq data is available on GEO, accession GSE120579. Differentiation RNA-seq data from [80] is publicly available on NCBI, http://www.ncbi.nlm.nih.gov/bioproject/303179. Analysis of processed data is available on github, https://github.com/jacel/TNF_HL60_S4.

References

1 Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future. Cytokine Growth Factor Rev 2014;25:453–472.

2 Mizgerd JP, Spieker MR, Doerschuk CM. Early response cytokines and innate immunity: essential roles for TNF receptor 1 and type I IL-1 receptor during Escherichia coli pneumonia in mice. J Immunol 2001;166:4042–4048.

3 Francisco NM, Hsu N-J, Keeton R, et al. TNF-dependent regulation and activation of innate immune cells are essential for host protection against cerebral tuberculosis. J Neuroinflammation 2015;12:125.

4 Striz I, Brabova E, Kolesar L, et al. Cytokine networking of innate immunity cells: a potential target of therapy. Clin Sci 2014;126:593–612.

5 Vieira SM, Lemos HP, Grespan R, et al. A crucial role for TNF-alpha in mediating neutrophil influx induced by endogenously generated or exogenous chemokines, KC/CXCL1 and LIX/CXCL5. Br J Pharmacol 2009;158:779–789.

6 Smart SJ, Castle TB. TNF-alpha-induced transendothelial neutrophil migration is IL-8 dependent. Am J Physiol 1994;266:L238–45.

7 Shalaby MR, Waage A, Aarden L, et al. Endotoxin, tumor necrosis factor-alpha and interleukin 1 induce interleukin 6 production in vivo. Clin Immunol Immunopathol 1989;53:488–498.

8 Kagoya Y, Yoshimi A, Kataoka K, et al. Positive feedback between NF-xB and TNF-α promotes leukemia-initiating cell capacity. J Clin Invest 2014;124:528–542.

9 Chu CQ, Field M, Feldmann M, et al. Localization of Tumor Necrosis Factor α in Synovial Tissues and at the Cartilage–Pannus Junction in Patients With Rheumatoid Arthritis. Arthritis Rheum 1991;34:1125–1132.

10 Palucka AK, Blanck J-P, Bennett L, et al. Cross-regulation of TNF and IFN- in autoimmune diseases. Proc Natl Acad Sci 2005;102:3372–3377.

11 Maxwell JL, Zochling J, Boonen A, et al. TNF-alpha inhibitors for ankylosing spondylitis. Cochrane Database Syst Rev 2015.

12 Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol 2002;2:364–371.

13 Chowers Y, Allez M. Efficacy of anti-TNF in Crohn’s disease: how does it work? Curr Drug Targets 2010;11:138–142.

14 Ziegler-Heitbrock HW, Möller A, Linke RP, et al. Tumor necrosis factor as effector molecule in monocyte mediated cytotoxicity. Cancer Res 1986;46:5947–5952.

15 Roberts NJ, Zhou S, Diaz LA, et al. Systemic use of tumor necrosis factor alpha as an anticancer agent. Oncotarget 2011;2:739–751.

16 Sabio G, Davis RJ. TNF and MAP kinase signalling pathways. Semin Immunol 2014;26:237–245.
chromatin organisation in neutrophil-like cells. BioRxiv 2018:339085.

37 Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.

38 Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2014;30:923–930.

39 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

40 Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37:1–13.

41 Liberzon A, Birger C, Thorvaldsdottir H, et al. The Molecular Signatures Database Hallmark Gene Set Collection. Cell Syst 2015;1:417–425.

42 Alexa A, Rahnenfuhrer J. topGO: Enrichment analysis for gene ontology 2016.

43 Kanchisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28:27–30.

44 Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci 2005;102:15545–15550.

45 Mootha VK, Lindgren CM, Eriksson K-F, et al. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003;34:267–273.

46 Santamaría D, Barrière C, Cerqueira A, et al. Glk1 is sufficient to drive the mammalian cell cycle. Nature 2007;448:811–815.

47 Lee S-H, Nam H-S. TNF alpha-induced down-regulation of estrogen receptor alpha in MCF-7 breast cancer cells. Mol Cells 2008;26:285–290.

48 Ilking-Konert C, Cséko C, Wagner C, et al. Transdifferentiation of polymorphonuclear neutrophils: acquisition of CD83 and other functional characteristics of dendritic cells. J Mol Med (Berl) 2001;79:464–474.

49 Ilking-Konert C, Wagner C, Denefleh B, et al. Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease. Clin Exp Immunol 2002;130:501–508.

50 Hume DA. Macrophages as APC and the dendritic cell myth. J Immunol 2008;181:5829–5835.

51 Hashimoto D, Miller J, Merad M. Dendritic cell and macrophage heterogeneity in vivo. Immunity 2011;35:323–335.

52 Horiuchi T, Mitoma H, Harashima S, et al. Transmembrane TNF-alpha: structure, function and interaction with anti-TNF agents. Rheumatology (Oxford) 2010;49:1215–1228.

53 Amini Kadijani A, Asadzadeh Aghdai H, Sorrentino D, et al. Transmembrane TNF-α Density, but not Soluble TNF-α Level, is Associated with Primary Response to Infliximab in Inflammatory Bowel Disease. Clin Transl Gastroenterol 2017;8:e117.

54 Grell M, Douni E, Wajant H, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 1995;83:793–802.

55 Siegmund D, Kums J, Ehrenschwender M, et al. Activation of TNFR2 sensitizes macrophages for TNFR1-mediated necroptosis. Cell Death Dis 2016;7:e2375.

56 Maney NJ, Reynolds G, Krippner-Heidenreich A, et al. Dendritic Cell Maturation and Survival Are Differentially Regulated by TNFR1 and TNFR2. J Immunol 2014;193:4914–4923.

57 Defer N, Azroyan A, Pocker F, et al. TNFR1 and TNFR2 signaling interplay in cardiac myocytes. J Biol Chem 2007;282:35564–35573.

58 Naudé PJW, Den Boer JA, Luiten PGM, et al. Tumor necrosis factor receptor cross-talk.
59 Wicovsky A, Henkler F, Salzmann S, et al. Tumor necrosis factor receptor-associated factor-1 enhances proinflammatory TNF receptor-2 signaling and modifies TNFR1–TNFR2 cooperation. Oncogene 2009;28:1769–1781.

60 Boström J, Šramkova Z, Salašová A, et al. Comparative cell cycle transcriptomics reveals synchronization of developmental transcription factor networks in cancer cells. PLoS One 2017;12:e0188772.

61 Liu Y, Chen S, Wang S, et al. Transcriptional landscape of the human cell cycle. Proc Natl Acad Sci U S A 2017;114:3473–3478.

62 Peña-Díaz J, Hegre SA, Andreassen E, et al. Transcription profiling during the cell cycle shows that a subset of Polycomb-targeted genes is upregulated during DNA replication. Nucleic Acids Res 2013;41:2846–2856.

63 Liu Z, Lou H, Xie K, et al. Reconstructing cell cycle pseudo time-series via single-cell transcriptome data. Nat Commun 2017;8:22.

64 Takashima A, Yao Y. Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell. J Leukoc Biol 2015;98:489–496.

65 Nishikawa Y, Sone M, Nagahama Y, et al. Tumor necrosis factor-α promotes bile ductular transdifferentiation of mature rat hepatocytes in vitro. J Cell Biochem 2013;114:831–843.

66 Li B, Pozzi A, Young PP. TNF Accelerates Monocyte to Endothelial Transdifferentiation in Tumors by the Induction of Integrin 5 Expression and Adhesion to Fibronectin. Mol Cancer Res 2011;9:702–711.

67 Bachem MG, Sell KM, Melehior R, et al. Tumor necrosis factor alpha (TNF alpha) and transforming growth factor beta 1 (TGF beta 1) stimulate fibronectin synthesis and the transdifferentiation of fat-storing cells in the rat liver into myofibroblasts. Virchows Arch B Cell Pathol Incl Mol Pathol 1993;63:123–130.

68 Lu X, Lu J, Liao B, et al. Driver pattern identification over the gene co-expression of drug response in ovarian cancer by integrating high throughput genomics data. Sci Rep 2017;7:16188.

69 Liu Z, Meng J, Li X, et al. Identification of Hub Genes and Key Pathways Associated with Two Subtypes of Diffuse Large B-Cell Lymphoma Based on Gene Expression Profiling via Integrated Bioinformatics. Biomed Res Int 2018:2018:1–14.

70 Dolgin E. The greatest hits of the human genome: A tour through the most studied genes in biology reveals some surprises. Nature 2017;551:427–431.

71 Rowat AC, Jaalouk DE, Zwerger M, et al. Nuclear envelope composition determines the ability of neutrophil-type cells to passage through micron-scale constrictions. J Biol Chem 2013;288:8610–8618.

72 Harrow J, Frankish A, Gonzalez JM, et al. GENCODE: The reference human genome annotation for The ENCODE Project. Genome Res 2012;22:1760–1774.

73 Ewels P, Magnusson M, Lundin S, et al. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 2016;32:3047–3048.

74 Team RC. R: A language and environment for statistical computing. 2018.

75 Yu G, Wang L-G, Han Y, et al. clusterProfiler: an R Package for Computing Biological Themes Among Gene Clusters. Omi A J Integr Biol 2012;16:284–287.

76 Swintorn J. Vennable: Venn and Fuller area-proportional diagrams.

77 Wilham H, François R, Henry L, et al. dplyr: A Grammar of Data Manipulation 2018.

78 Wilham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.
**Figure 1.** Transcriptional changes in promyelocytic and granulocytic forms of HL-60/S4 following two hours of TNF-α treatment. A) The Log2 fold change (log2FC) and adjusted p values of all analyzed genes in promyelocytes with and without TNF-α treatment. B) The log2FC and adjusted p values of all analyzed genes in granulocytes with and without TNF-α treatment. C) There is a shared and unique transcriptional response to TNF-α treatment of HL-60/S4 promyelocytes and granulocytes. D) The log2FC of genes that were significantly differentially expressed in both HL-60/S4 and HL-60/S4+ATRA cells were positively correlated (R² = 0.65). However, there was a small subset of genes that were antico-related. E) The log2FC of genes that were only differentially expressed in HL-60/S4 cells did not correlate between HL-60/S4 and HL-60/S4+ATRA after TNF-α treatment (R² = 0.04). F) The log2FC of genes that were only differentially expressed in HL-60/S4+ATRA cells did not correlate between HL-60/S4 and HL-60/S4+ATRA after TNF-α treatment (R² = 0.15).

**Figure 2.** Transcriptional changes in promyelocytes and granulocytes treated with TNF. Gene set enrichment analysis (GSEA) of genes represented in the MSigDB Hallmark gene sets [1]. All represented genes were ranked by Log2FC, with no significance cutoff. The x axis shows significantly enriched gene sets (FDR < 0.05). The normalized enrichment score (y axis) indicates whether a given gene set was overrepresented for transcripts that exhibited large fold changes. Predicted gene sets (e.g. TNFα signaling via NFkB, p53 pathway, and IFNγ response) were enriched in both conditions. No gene sets were enriched in HL-60/S4 but not HL-60/S4+ATRA. Six gene sets (adipogenesis, estrogen response early and late, hypoxia, IFNα response, and xenobiotic metabolism) were enriched in HL-60/S4+ATRA, but not HL-60/S4.

**Figure 3.** Gene set overrepresentation in differential expression subsets of TNF-α treated promyelocytes and granulocytes. A) Genes that were significantly differentially expressed in the same direction after TNF-α treatment were overrepresented in several gene sets canonically associated with TNF-α response. B) Genes that were only significantly differentially expressed in granulocytes were overrepresented in 9 gene sets, including 4 cell cycle associated gene sets. They were also overrepresented in the reactive oxygen species pathway, a neutrophilic response to TNF-α. C) Genes that were significantly differentially expressed in opposite directions were overrepresented in two cell-cycle associated gene sets. All differentially expressed hallmark G2M checkpoint genes were upregulated in promyelocytes, and downregulated in granulocytes cells, with the exception of E2F2, a transcription factor that promotes quiescence by binding to promoters and transcriptionally repressing cell cycle genes [2]. All differentially expressed E2F2 target genes were upregulated in promyelocytes, and downregulated in granulocytes.

**Figure 4.** Transcriptional changes in promyelocytes differentiated into granulocytes with ATRA or macrophages with TPA, compared to TNF-α treatment of promyelocytes and granulocytes. Gene set enrichment analysis (GSEA) of genes represented in the MSigDB Hallmark gene sets [1]. All represented genes were ranked by Log2FC, with no significance cutoff. The x axis shows significantly enriched gene sets (FDR < 0.05), and the y axis is calculated from the proportion of genes in the leading edge out of the number of ranked genes, and out of the gene set size. All conditions were associated with upregulated inflammatory gene sets, and both differentiation conditions were associated with downregulation of cell cycle gene sets.

**Figure 5.** Gene set overrepresentation in differential expression subsets of promyelocytes differentiated into granulocytes or macrophages. A) Genes that were significantly differentially expressed in the same direction after differentiation were overrepresented in gene sets associated with inflammatory signaling and cell cycle. B) Genes that were only significantly differentially expressed in cells differentiated into macrophages with TPA were predominantly associated with
cell cycle. C) Genes that were significantly differentially expressed in opposite directions were overrepresented in 3 gene sets: IL2 STAT5 signaling, p53 pathway, and TNF-α signaling via NFKB. A majority of genes in all categories increased expression after macrophage differentiation and in granulocytes treated with TNF-α, and decrease expression after granulocytic differentiation.

Additional files

Additional file 1. Supplementary table 1. Significantly differentially expressed genes in promyelocytes treated with TNF-α, differentiated into granulocytes and macrophages, and granulocytes treated with TNF-α.

Additional file 2. Supplementary figure 1. PCA of VST-normalized transcript counts show conditions cluster together in A) promyelocytes treated with TNF-α, B) granulocytes treated with TNF-α, and C) promyelocytes differentiated into granulocytes and macrophages.

Additional file 3. Supplementary tables 2-16. Functional analysis summary tables of GSEA, GO, KEGG, and hallmark terms enriched in subsets of differentially expressed genes.

Competing interests

The authors declare that they have no competing interests

Funding

This research was supported by a Health Research Council Explorer grant (HRC 15/604) to JMO. ECJ was recipient of a University of Auckland doctoral scholarship, and Maurice Wilkins Centre travel grant. The funding bodies had no role in the study design, collection, analysis, and interpretation of the data, or preparing the manuscript.

Authors’ Contributions

ECJ performed the experiments, analyzed and interpreted the data, and wrote the manuscript with support from JMO, JKP, and MHV. All authors contributed to revisions and approved the final manuscript.

Acknowledgements

The authors would like to thank Peter Shepherd and the members of the JMO lab group for comments and discussion.
