Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells†

Neeloffer Mookherjee,‡[a] Pamela Hamill,[a] Jennifer Gardy,[a] Darren Blimkie,[b] Reza Falsafi,[a] Avinash Chikatamarla,[a] David J. Arenillas,[c] Silvana Doria,[a] Tobias R. Kollmann[b] and Robert E. W. Hancock*[a]

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The immune system is very complex, it involves the integrated regulation and expression of hundreds of proteins. To understand in greater detail how the human host defence immunomodulatory peptide LL-37 interacts with innate immunity, a systems approach was pursued. Polychromatic flow cytometry was employed to demonstrate that within human peripheral blood mononuclear cells, CD14+ monocytes, myeloid and plasmocytoid dendritic cells and T- and B-lymphocytes, all responded to LL-37, with the differential production of intracellular cytokines. Microarray analyses with CD14+ monocytes indicated the differential expression of 475 genes in response to stimulation with LL-37. To understand this complex response, bioinformatic interrogation, using InnateDB, of the gene ontology, signalling pathways and transcription factor binding sites was undertaken. Activation of the IκBα/NFκB, mitogen-activated protein kinases p38, ERK1/2 and JNK, and PI3K signalling pathways in response to LL-37 was demonstrated by pathway and ontology over-representation analyses, and confirmed experimentally by inhibitor studies. Computational analysis of the predicted transcription factor binding sites upstream of the genes that were regulated by LL-37 predicted the involvement of several transcription factors including NFκB and five novel factors, AP-1, AP-2, SP-1, E2F1, and EGR, which were experimentally confirmed to respond to LL-37 by performing transcription factor array studies on nuclear extracts from LL-37 treated mononuclear cells. These data are discussed as reflecting the integration of several responsive signalling pathways through the involvement of transcription factor complexes in gene expression activated by LL-37 in human mononuclear cells.

Introduction

Complex immune mechanisms in the mammalian host defend against exposure to potentially harmful pathogens on a daily basis. Innate immunity is the first line of host defence, and is critical in pathogen recognition and the subsequent transition, when necessary, to antigen-specific adaptive immunity. A fundamental innate defence mechanism in the mammalian host is the constitutive or inducible expression of certain host defence peptides. These peptides constitute an ancient lineage of defence molecules that exhibit both immunomodulatory and antimicrobial properties[1–5] and can be grouped into two major classes: the cathelicidins and the defensins. Cathelicidins are characterized by a conserved N-terminal "cathelin" domain of around 100 amino acid residues in the precursor protein, which is proteolytically cleaved to generate the mature active peptide from the C-terminal domain.[6] Humans have a single cathelicidin peptide—LL-37—which is an amphiphilic, α-helical, 37-amino acid cationic peptide derived from proteolytic cleavage from the C-terminal end of the CAP18 protein.[6,7] LL-37 is widely expressed in a variety of tissue types, including lung and ocular epithelium, gingival tissue, skin and synovial membranes,[8–12] in body fluids such as breast milk, sweat, saliva and bronchoalveolar fluid,[13–16] and by key immune cells including monocytic cells, neutrophils and lymphocytes.[17,18] Its expression has been shown to increase significantly during infections and inflammatory conditions[19,20] and in contrast, the lack of or altered expression of LL-37 has been linked to increased susceptibility to various conditions such as periodontal disease, chronic ulcers and atopic dermatitis.[21–23] LL-37 and its synthetic analogs have been demonstrated to protect against a variety of infections *in vivo*,[4] which may be due to selective immunomodulatory functions, since direct antimicrobial activities are strongly antagonized by physiological cation concentrations.[4,24,25]

LL-37 has been demonstrated to mediate a wide variety of immune, anti-infective and healing functions, many of which

[a] *Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, Canada. E-mail: bob@cmdr.ubc.ca; Tel: +1 604 822 2682*
[b] *Child & Family Research Institute, University of British Columbia, Vancouver, BC, Canada*
[c] *Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC, Canada*
† Electronic supplementary information (ESI) available: Details re polychromatic flow cytometry, chemokine induction, microarrays and primers. See DOI: 10.1039/b813787k
‡ *Present address*: Manitoba Centre for Proteomics and Systems Biology, University of Manitoba, MB, Canada.

have been confirmed in animal or tissue models. In addition to being a direct chemoattractant for mononuclear cells, neutrophils, eosinophils, T-cells and mast cells,[26–29] it can induce the expression of chemokines required for cell recruitment[4,30–32] and promote angiogenesis,[33] induce the degranulation of mast cells,[34] stimulate the apoptosis of epithelial cells while antagonizing neutrophil apoptosis,[35] promote wound healing and influence the transition to adaptive responses by affecting dendritic cell differentiation and the polarization of T-cells.[36] Even though several of these functions could be regarded as pro-inflammatory, LL-37 suppresses endotoxin-induced pro-inflammatory cytokine expression in human monocytic cells and mouse models, and indeed maintains or enhances anti-inflammatory responses by selectively modulating the Toll-like receptor (TLR)-to-NFκB pathway.[37,38]

There is some evidence that this complexity of action mirrors the intrinsic complexity of the innate immune system. In addition to its complex modulation of responses to TLR ligands, innate immune responses mediated by LL-37 are co-operatively enhanced in the presence of a subset of endogenous cytokines, such as IL-1β and granulocyte-macrophage colony stimulating factor (GM-CSF), that are naturally present at the site of an infection.[4,37] We recently showed that this synergistic enhancement by LL-37 and IL-1β involved the activity of a variety of key signalling molecules such as IκB-α, phosphoinositide 3-kinase (PI3K), AKT and the transcription factors CREB and NF-κB in human peripheral blood mononuclear cells.[39] The direct stimulation of phosphorylation of the mitogen-activated protein kinases (MAPK) ERK1/2 and p38 was induced by LL-37 in human monocytes and inhibitors of activation blocked chemokine induction.[40] However these studies have provided a rather fragmentary picture and we lack a detailed understanding of LL-37-mediated signal transduction and downstream responses in leukocytes that contributes to the broad variety of immune effects, from balancing inflammation to influencing the polarization of adaptive immunity.

In the present study we have identified the individual effector cell types that respond to stimulation of human PBMC with LL-37 using polychromatic flow cytometry. These results indicate that CD14+ monocytes, as well as different classes of dendritic cells and lymphocytes, respond differentially with the production of intracellular cytokines. To understand the events leading to such responses we have chosen monocytes as a model and utilized a systems biology approach to analyse gene responses to LL-37 in this cell type. Interrogation of these data with a variety of bioinformatics tools, followed up by direct biochemical confirmation, led to substantial advances in understanding the complexity of signalling pathways and transcription factors involved in the responses to LL-37.

Results

Monocytes, dendritic cells and lymphocytes respond differentially on stimulation of human PBMC with LL-37

To identify the effector immune cells targeted by LL-37 in human PBMC, these cells were treated with LL-37 in the presence or absence of the TLR4 agonist bacterial lipopolysaccharide (LPS) and analysed using a panel of antibodies to assess intracellular cytokines and cell surface markers using polychromatic flow cytometry. Gating strategies (Kollmann *et al*. unpublished data) permitted the analysis, in the PBMC population, of monocytes (identified based on FSC–SSC, high MHCII, and high CD14), myeloid dendritic cells (mDC; identified based on FSC–SSC, high MHCII, low CD14, $CD123^{-}$, and high CD11c), plasmacytoid dendritic cells (pDC; identified based on FSC–SSC, high MHCII, low CD14, $CD11c^{-}$, and high CD123), T-cells (identified based on FSC–SSC, high CD3), and B-cells (identified based on FSC–SSC, high CD19) (Fig. S1, ESI†). The induction of intracellular cytokines (TNF-α, IL-6, IL-10) and chemokines (IL-8 and MCP-1) was analysed in each individual cell type after 18 h of stimulation of PBMC with LL-37 in the presence or absence of LPS (10 ng ml^{-1}). This study demonstrated that multiple cell types within the PBMC population responded differentially to the presence of the peptide LL-37, LPS and the combined treatment (Table 1; Fig. S2, ESI†).

The suppression by LL-37 of the LPS-induced pro-inflammatory cytokines TNF-α and IL-6 was observed in mDC, CD14+ monocytes, T-cells and B-cells (Table 1; Fig. S2a, ESI†), while LPS-induced chemokine IL-8 levels were suppressed in the presence of the peptide in mDC, CD14+ monocytes and T-cells (Table 1; Fig. S2b, ESI†). In contrast, MCP-1 production was enhanced following stimulation with LL-37 in monocytes and T-cells in the presence or absence of LPS (Table 1; Fig. S2c, ESI†). The production of the anti-inflammatory cytokine IL-10 was noticeably increased relative to unstimulated cells in mDC, pDC, monocytes, T-cells and B-cells upon stimulation with both LL-37 alone and the peptide in combination with LPS, but not with LPS alone (Table 1, and Fig. S2b, ESI†). Intracellular levels of chemokine IL-8 relative to unstimulated cells were suppressed in the presence of the LL-37 alone but only in mDC (Table 1, and Fig. S2b, ESI†).

Taken together, these qualitative evaluations of intracellular cytokine production indicate that there are multiple effector cells within the human PBMC population that respond differentially upon stimulation of PBMC with the peptide LL-37 in the absence and presence of LPS, and that the critical responder cell populations include CD14+ monocytes, mDC, and T- and B- lymphocytes.

LL-37 effects on the gene expression profile in CD14+ monocytes isolated from peptide-stimulated PBMC

Stimulation of PBMC revealed that CD14+ monocytes were among the effector cells that mounted a response to both LL-37 alone and LL-37 in the presence of LPS. Because a broad variety of LL-37-mediated immunomodulatory functions have been ascribed to human monocytes,[6,17,37,38,40–42] we selected this cell population for global transcriptional profiling. Human PBMC from 4 independent human donors were treated with LL-37 (20 μg ml^{-1}) for 4 h, followed by CD14+ monocyte isolation using positive selection with magnetic bead-based technology. RNA isolated from the CD14+ monocytes was amplified and hybridized using a

Table 1 Analysis of individual cell types for the production of cytokines upon stimulation of PBMC with LL-37 in the presence and absence of LPS. Enhanced levels of cytokines compared to unstimulated cells are indicated by ↑, while reduced levels of cytokines are indicated by ↓

Experimental conditions	IL6	TNFa	IL8	IL10	MCP-1
mDC					
LL37 (20 μg ml^{-1})	—	—	↓	—	—
LL37 (50 μg ml^{-1})	—	—	↓	↑	—
LL37 (100 μg ml^{-1})	—	—	↓	↑	—
LPS (10 ng ml^{-1})	↑	↑	↑	—	—
LPS + LL37 (20 μg ml^{-1})	↓	↓	↓	—	—
LPS + LL37 (50 μg ml^{-1})	↓	↓	↓	↑	—
LPS + LL37 (100 μg ml^{-1})	↓	↓	↓	↑	—
pDC					
LL37 (20 μg ml^{-1})	—	—	—	—	—
LL37 (50 μg ml^{-1})	—	—	—	↑	—
LL37 (100 μg ml^{-1})	—	—	—	↑	—
LPS (10 ng ml^{-1})	—	—	—	—	—
LPS + LL37 (20 μg ml^{-1})	—	—	—	—	—
LPS + LL37 (50 μg ml^{-1})	—	—	—	↑	—
LPS + LL37 (100 μg ml^{-1})	—	—	—	↑	—
CD14+ Monocytes					
LL37 (20 μg ml^{-1})	—	—	—	—	—
LL37 (50 μg ml^{-1})	—	—	—	↑	—
LL37 (100 μg ml^{-1})	—	—	—	↑	↑
LPS (10 ng ml^{-1})	↑	↑	↑	—	—
LPS + LL37 (20 μg ml^{-1})	↓	↓	↓	—	—
LPS + LL37 (50 μg ml^{-1})	↓	↓	↓	↑	—
LPS + LL37 (100 μg ml^{-1})	↓	↓	↓	↑	↑
T-cells					
LL37 (20 μg ml^{-1})	—	—	—	—	—
LL37 (50 μg ml^{-1})	—	—	—	↑	—
LL37 (100 μg ml^{-1})	—	—	—	↑	↑
LPS (10 ng ml^{-1})	↑	↑	↑	—	—
LPS + LL37 (20 μg ml^{-1})	↓	↓	↓	—	—
LPS + LL37 (50 μg ml^{-1})	↓	↓	↓	↑	—
LPS + LL37 (100 μg ml^{-1})	↓	↓	↓	↑	↑
B-cells					
LL37 (20 μg ml^{-1})	—	—	—	—	—
LL37 (50 μg ml^{-1})	—	—	—	↑	—
LL37 (100 μg ml^{-1})	—	—	—	↑	—
LPS (10 ng ml^{-1})	↑	↑	—	—	—
LPS + LL37 (20 μg ml^{-1})	↓	↓	—	—	↑
LPS + LL37 (50 μg ml^{-1})	↓	↓	—	↑	↑
LPS + LL37 (100 μg ml^{-1})	↓	↓	—	↓	↑

human 21K oligo-based microarray gene chip as previously described.[37] Differentially expressed (fold change of at least 1.5 relative to unstimulated cells) and statistically significant genes were analysed by ArrayPipe version 1.391. Upon stimulation of PBMC with LL-37 for 4 hours, 475 genes were found to be differentially expressed in CD14+ monocytes, of which 431 genes were up-regulated and 44 down-regulated (Table S1, ESI†).

Because of the uncertainty inherent in microarray data, a selection of genes of interest was subsequently selected for validation by qRT-PCR (Fig. 1). Genes encoding chemokines essential for cell recruitment to the site of infection, such as CXCL1 (Gro-α) and CCL2 (MCP-1), were significantly up-regulated between 2- and 4-fold, while CCL7 (MCP-3) gene expression was significantly up-regulated by more than 10-fold (Fig. 1a). Anti-inflammatory genes such as IL-10 and IL-19 were also up-regulated between 2- and 5-fold in response to LL-37 (Fig. 1a). These observations were consistent with the known immunomodulatory and anti-inflammatory functions of the cathelicidin.

The expression of several genes involved in key immune signalling pathways was also significantly up-regulated in the presence of LL-37. MAP2K6 (MEK6), a kinase active in the p38 pathway, was up-regulated more than 10-fold, while inositol 1,4,5-trisphosphate 3-kinase B (ITPKB), a potential regulator of calcium mobilization essential for T-cell development,[43] was up-regulated by 2-fold compared to unstimulated cells (Fig. 1b). The TIR-domain containing adaptor protein (TIRAP), a component of the Toll-like receptor pathway, and the phosphatidic acid phosphatase type 2B (PPAP2B), involved in regulation of lipid phosphate-mediated cell signalling, were up-regulated between 2- and 12-fold (Fig. 1c).

Genes encoding several membrane-associated proteins or receptors were also significantly up-regulated between 2- and 12-fold in response to LL-37 (Fig. 1d). These included the transmembrane glycoprotein neuropilin 1 (NRP1), a non-kinase endothelial growth factor receptor involved in angiogenesis,[44] the transmembrane glycoprotein CD226, involved in T-cell and NK-cell differentiation,[45] the chemokine receptor CCR1, involved in recruitment of monocytes and T-cells,[46] and the cell adhesion molecule integrin alpha 8 (ITGA8).[47] In addition, stimulation with LL-37 resulted in the up-regulation of interferon-associated genes encoding for interferon alpha 2 and interferon-induced protein IFIT1 in monocytes (Fig. 1e).

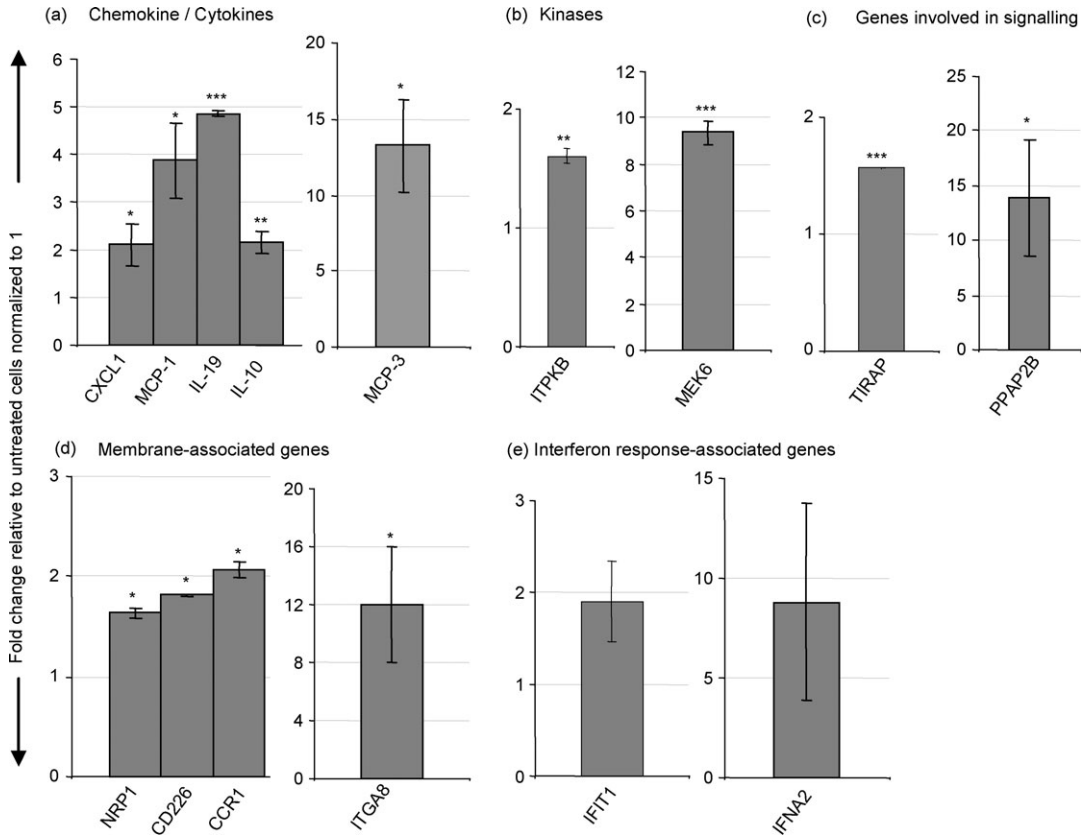

Fig. 1 LL-37 significantly up-regulated the expression of several genes in CD14+ monocytes isolated from stimulated PBMC population. Human PBMC were treated with LL-37 (20 μg ml^{-1}) for 4 h followed by positive selection of CD14+ monocytes. Transcriptional responses were evaluated in mRNA isolated from the monocytic cells by quantitative real-time PCR (qRT-PCR). Expression of genes encoding for (a) chemokines Gro-α (CXCL1), MCP-1, MCP-3 and anti-inflammatory cytokines of IL-10 family (IL-10 and IL-19), (b) kinases MEK6 and ITPKB, (c) additional signalling molecules; adaptor TIRAP and phosphatase PPAP2B, (d) membrane-associated proteins NRP-1, CD226, CCR1 and ITGA8, and (e) interferon-related proteins IFIT1 and IFNA2, were evaluated. Fold changes (*y*-axis) for each gene were normalized to housekeeping gene GAPDH, and further quantitated relative to gene expression in unstimulated cells normalized to 1 using the comparative ct method. Results represent average of five independent experiments ± standard error (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

MAPK signalling processes enriched amongst differentially expressed genes

Microarray analysis revealed the differential expression of 475 genes in response to stimulation with LL-37. To further examine the biological processes associated with the products of these genes, ontology and pathway over-representation analyses were performed. The microarray results were first uploaded to the Gene Ontology Tree Machine,[48] where they were analysed for over-represented biological process ontological terms representing specific pathways. Two pathways were identified as enriched in the up-regulated genes (Table 2), while none were enriched in down-regulated genes. Next, the microarray data were submitted to InnateDB[49] and analysed using its Pathway Over-Representation Analysis tool with default parameters. After grouping redundant pathways together, 35 pathways were found to be enriched in up-regulated genes, with 7 enriched in down-regulated genes (Table 2).

Notably, of the 37 total pathways enriched in the up-regulated gene set, 15 involve members of the MAPK family. Although only three MAPK genes were indicated as up-regulated by the microarray, and a fourth through qPCR validation, these genes clearly act as points of cross-talk between several signalling pathways. The Biocarta MAPK pathway map (http://www.biocarta.com/pathfiles/h_mapkPathway.asp) confirmed that two of the three up-regulated MAPK genes, MAP3K1 and MAP2K4, do, in fact, participate in both MAPK p38 and JNK signalling, suggesting that LL-37 may be capable of stimulating a wide range of responses.

Network analysis of additional signalling pathways and modules enriched by LL-37

Pathway over-representation analysis is limited to the discovery of pathways already annotated in public databases. In order to discover potentially novel pathways or modules that are differentially expressed in response to LL-37, we took a network-based approach. All differentially expressed genes with fold changes larger than ±1.5, regardless of *p*-value, were submitted to the InnateDB biomolecular interaction database.[49] Interactions between the protein products of these genes were retrieved and used to construct a network consisting of 220 nodes, each representing the protein product of a differentially expressed gene, and 218 edges, each representing an experimentally verified physical interaction between two proteins. The resulting graph was visualized using Cytoscape[50]

Table 2 Pathways enriched in differentially expressed genes.

Pathways enriched in up-regulated genes	Pathways enriched in down-regulated genes
AKT signalling[c]	Alpha-linolenic acid metabolism
Basal cell carcinoma	Complement activation
Beta catenin degradation	COPII-mediated vesicle transport
Botulinum neurotoxicity	Leucocyte transendothelial migration
CD40L signalling	Pantothenate and CoA biosynthesis
Cell–cell adhesion	Regulation of EIF2
Ceramide signalling[b]	Transformation of lanosterol to cholesterol
c-Met signalling[b]	—
CXCR4 signalling	—
EGF signalling[b]	—
Eicosanoid metabolism	—
EPO signalling[b]	—
FAS signalling[b]	—
Fc-epsilon receptor I signalling[b]	—
Glycan structures-degradation	—
Glycerol-3-phosphate synthesis	—
Glycosphingolipid biosynthesis-lactoseries	—
Icosanoid metabolism[b]	—
Insulin signalling	—
JNK signalling[bc]	—
Keratan sulfate biosynthesis	—
Keratinocyte differentiation[b]	—
MAPK signalling[bc]	—
Nef signalling	—
Neuronal glutamate cycle	—
NFAT signalling[b]	—
p38 signalling[bc]	—
PDGF signalling[b]	—
PECAM1 interactions	—
Prion pathway	—
Segmentation clock	—
Signalling without Wnt	—
Styrene degradation	—
T-cell receptor signalling[b]	—
TGF beta signalling[a]	—
TLR signalling[b]	—
TNF/stress related signalling[b]	—

[a] Pathways that were identified through GOTM; the remaining pathways were identified with InnateDB. [b] Pathways that involve MAPK proteins. [c] Pathways that were experimentally verified to play a role in the response to LL-37.

and was manually laid out to create a graph with as few edge crossings as possible (Fig. 2). Through manual inspection of the graph and the functional annotations associated with each node, subnetworks of interacting proteins participating in common processes were identified.

The importance of MAPK signalling was supported by this network-based analysis, which revealed the chain of interactions between MAP4K4, MAP3K1, and MAP2K4. These three genes are noted as being especially active in the JNK pathway, and when their first- and second-degree interactors were examined, many of them were also found to be involved in this particular pathway. For example, the adaptor protein Nck1 activates the JNK pathway downstream of the endothelin A receptor,[51] the enzyme p21-activated protein kinase-2 (PAK2) activates JNK pathway upstream of the MAP3K level,[52] and SPAG9 (also known as JLP) is a JNK scaffold protein that tethers MAP3K3, MAP2K4, and JNK to the transcription factors c-Myc and Max.[53]

Network-based analysis also pointed towards the likelihood of involvement of the Wnt, Notch and TGF-β pathways in LL-37 responses, pathways that have been recently recognized to cross-talk[54] and are also known to intersect with branches of the MAPK pathway. The nature of these pathways' involvement with the response to LL-37 appears to be complex, involving both potential activation and repression. An increase in β-catenin mRNA levels was observed in our dataset, and such increases have been linked at the protein level to the inhibition of TGFβ and SMAD3-mediated signalling,[55] perhaps reflecting the observed down-regulation (though not at a statistically significant level) of TGFBR1 and SMAD3 in our dataset. SMAD7, up-regulated in our dataset, is also involved in the inhibition of TGF-β signalling. In addition, increased β-catenin levels have also been linked to activation of the Notch pathway;[56] consistent with this we observed an increase in Notch1 mRNA levels in our dataset. The Notch pathway, in turn, is known to induce the expression of the HEY response genes. Consistent with this, we observed the up-regulation of HEY1 and HEY2, as well as their co-interactors ARNT and SIRT1. These proteins are known to act together as transcriptional repressors of several genes.[56,57]

A third interesting observation was the up-regulation of mRNA levels for the components of activating signal cointegrator 1 (ASC1). This complex consists of four proteins: TRIP4, ASCC1 and ASCC3, all of which were significantly up-regulated in response to LL-37; and ASCC2, which was not

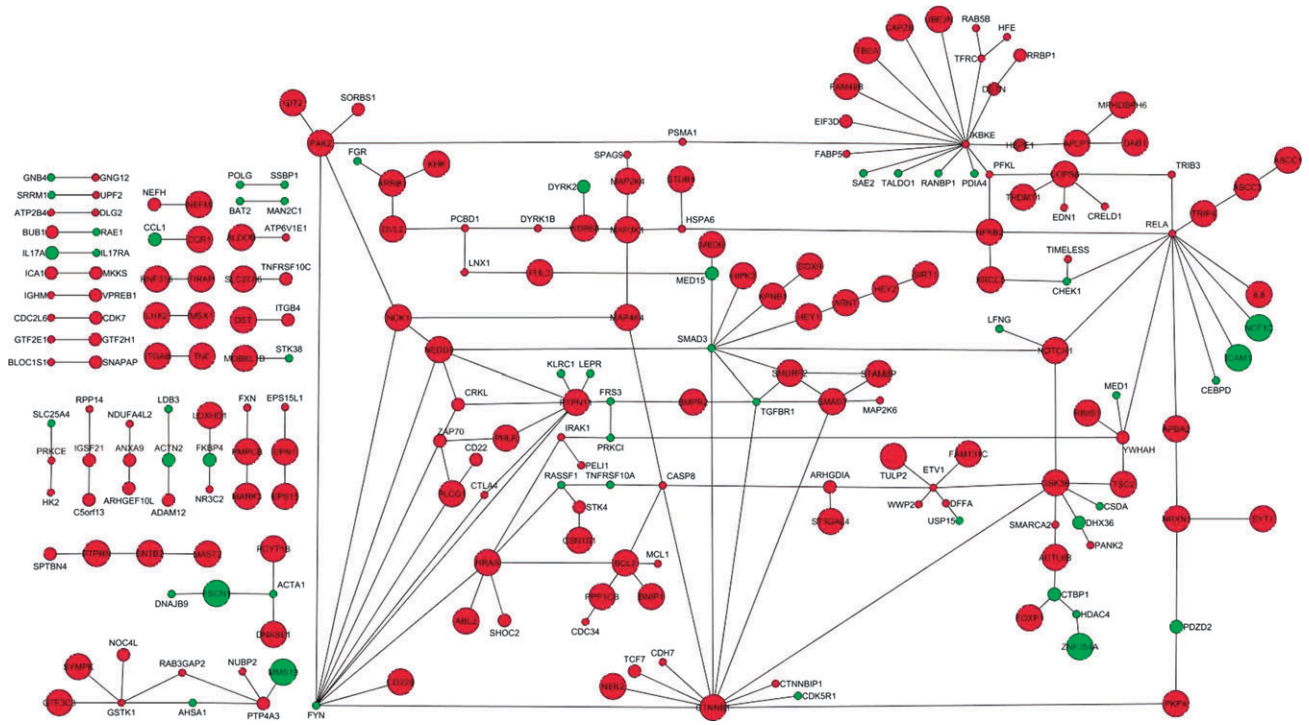

Fig. 2 Interactions between genes differentially expressed in response to LL-37. Red nodes are up-regulated genes; green nodes are down-regulated. The size of a node corresponds to the *p*-value associated with its fold change: large nodes = $p < 0.05$, medium nodes = $p < 0.1$, small nodes = $p > 0.1$.

represented on the arrays used in our experiments. ASC1 is a transcriptional coactivator of nuclear receptors and other transcription factors, including AP-1 and NF-κB,[58] though little is known beyond this. Interestingly, none of the components of the related but distinct complex ASC2 were up-regulated on our arrays.

Induction of chemokine production by LL-37 in human PBMC dependent on multiple signalling pathways

To experimentally confirm the signalling pathways involved in the LL-37 response, we examined one of the proposed primary functions of human cathelicidin LL-37, namely the recruitment of immune cells to the site of infection through the induction of chemokines.[4,5,59] Control experiments demonstrated that both CXC Type α (IL-8) and C-C Type β (MCP-1 and RANTES) chemokines were significantly induced by stimulation of human PBMC with LL-37 ($p < 0.05$; Fig. S3, ESI†). To characterize the signalling pathways involved in this induction of chemokines, the production of IL-8 and MCP-1 was evaluated in human PBMC stimulated with the peptide for 24 h, in the presence and absence of various pathway-specific pharmacological inhibitors. Both the IL-8 and MCP-1 promoters contain AP-1 and NF-κB binding sites known to be necessary for expression of these genes,[60] and thus might be expected to respond to inhibition of any pathways upstream of these factors in a similar fashion.

Pre-treatment of human PBMC with inhibitors directed against the MAPK ERK 1/2 and PI3K pathways significantly inhibited the production of both IL-8 and MCP-1 induced by LL-37 (Fig. 3), while the inhibitor of IκB-α phosphorylation virtually eliminated IL-8 and MCP-1 production in both the

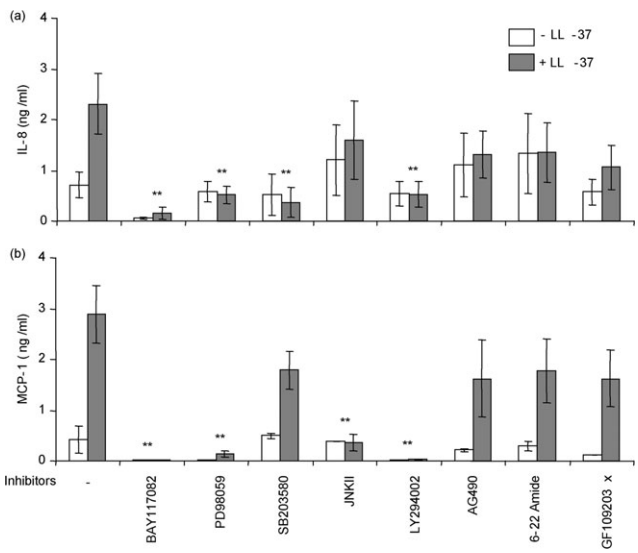

Fig. 3 LL-37-induced chemokine production in PBMC was suppressed by the selective inhibition of multiple pathways. Human PBMC were pre-treated for 1 h with 10 μM each of specific pharmacological inhibitors for IκB-α, MAPK ERK 1/2, p38 and JNK, PI3K and JAK-STAT pathways (Bay11-7085, PD98059, SB203580, JNK-II inhibitor, LY294002 and AG490, respectively), or 10 nm of PKA inhibitor (6-22 aminde) or PKC inhibitor (GF109203x), followed by stimulation with LL-37 (20 μg ml^{-1}) for 24 h. Tissue culture supernatant was monitored for production of chemokines (a) IL-8, and (b) MCP-1 by ELISA. Results represent average of four independent experiments from independent donors ± standard error (**$p < 0.01$).

presence and the absence of LL-37. These outcomes would be expected, given that both the MAPK ERK 1/2 and PI3K

Table 3 Over-represented transcription factor binding sites in LL-37-responsive genes. Binding sites for 32 transcription factors (individual factors, complexes of two factors, or families) were enriched in the promoter regions of LL-37-responsive genes. Transcription factors in italics were experimentally confirmed to display altered nuclear translocation

Transcription factor	Family	Target gene hits	*Z*-score	Fisher
AHR–ARNT	bHLH	65	2.209	4.61E-03
AP-1 family	bZIP	35	6.291	9.65E-03
BACH1	bZIP	5	11.09	4.17E-02
CDX family	Homeodomain	5	12.16	4.17E-02
CUX1	Homeodomain	11	11.4	1.40E-02
DBP	bZIP	111	8.097	9.25E-03
E2F1	Forkhead	72	2.399	8.59E-03
MYC–MAX	bHLH	68	2.636	4.17E-03
MZF1	Zinc finger	70	3.404	8.07E-03
NFKB1	Rel homology	9	10.01	1.48E-02
NF-YA	Other	54	16.81	7.14E-05
NF-YC	Other	17	12.06	2.93E-03
NR2F2	Nuclear receptor	3	12.66	6.74E-02
NR3C1 (GR)	Nuclear receptor	98	2.131	9.25E-03
NRF1	bZIP	25	9.379	4.24E-03
OCT family	Homeodomain	6	12.79	3.22E-02
POU2F1	Homeodomain	18	11.94	4.94E-03
POU3F2	Homeodomain	10	16.16	6.70E-03
RFX family	Winged helix	83	7.554	9.56E-04
RORA	Nuclear receptor	6	18.04	7.28E-03
RUNX1	Runt	34	6.427	7.90E-03
SF1	Nuclear receptor	34	9.21	3.16E-03
SP1	Nuclear receptor	75	0.8435	1.00E-02
TBP (TFIID)	Nuclear receptor	79	2.4	8.57E-03
TCF12	bHLH	83	11.21	1.32E-03
TEAD1	HTH/TEA	91	6.444	1.77E-03
TEAD2	HTH/TEA	165	0.7747	3.40E-03
TFAP2C (AP-2)	bHSH	154	5.014	6.91E-04
TFAP4	Other	67	17.1	3.37E-06
UBP1	Other	89	7.925	8.37E-03
YY1	Zinc finger	113	0.1958	6.79E-03
ZEB1	Zinc finger	16	9.413	5.68E-03

pathways are known to converge on the AP-1 and NF-κB families of transcription factors.

LL-37-induced activity also required the p38, ERK1/2 and JNK pathways (Fig. 3), supporting our previous observations that MAP kinases integral to the p38 and ERK1/2 pathways were up-regulated in response to the peptide. However, this study showed the involvement of the JNK pathway and the chemokine readouts for these pathways varied unexpectedly. The JNK inhibitor affected MCP-1 expression but did not influence IL-8 release (Fig. 3a), while the p38 inhibitor affected IL-8 expression, as previously described,[40] but did not affect MCP-1 release (Fig. 3b) upon LL-37 stimulation in PBMC.

Computational analysis of transcription factor binding sites

In order to determine which transcription factors might be active in response to LL-37 stimulation, genes identified as being significantly differentially expressed according to the microarray analysis were analysed for the over-representation of transcription factor binding sites (TFBSs). The complete set of 475 differentially expressed genes was submitted to an in-house version of the oPOSSUM tool,[61] implementing a custom set of all human matrices from the JASPAR[62] and TRANSFAC[63] databases. oPOSSUM excluded 91 genes from the dataset; in the remaining genes, it scanned 500 bp upstream and 100 bp downstream of the transcription start site for all 8-bit or higher matrices, using a match threshold of 85%. Ultimately, 32 transcription factor binding sites were found to be enriched within the promoter regions of LL-37-responsive promoters (Table 3), suggesting that the transcription factors that bind these sites may play an important role in regulating the cellular response to LL-37. This list includes several factors known to be active downstream of MAPK signalling pathways (AP1, MYC–MAX, NFKB1, SP1), as well as factors known to participate in the immune response, including CUX1, which inhibits cytokine-induced NF-κB-regulated chemokine transcription;[64] the NF-Y complex, important for MHC II expression[65] and YY1, implicated in the suppression of several immune genes.[66,67] The list contained several transcription factors with potential roles that could be implied from pathway-based analysis, including the MYC–MAX complex downstream of SPAG9-scaffolded MAPKs and the nuclear receptors and other transcription factors known to be activated by ASC1.

LL-37-induced nuclear translocation of multiple transcription factors

To validate the results of the TFBS over-representation analysis, the activation patterns of 54 transcription factors were compared between unstimulated and LL-37-treated human monocytic THP-1 cells using protein–DNA arrays (Fig. 4). Increased activity in nuclear extracts (as demonstrated by an increase in spot signal intensity relative to the nuclear extracts from untreated cells; Fig. 4b) was observed for activator proteins-1 and -2 (AP-1 and AP-2), cAMP response

element-binding (CREB) protein, E2F1, early growth response factor (EGR), nuclear factor-kappa B (NF-κB), the zinc finger protein motif containing transcription factor SP-1 (Fig. 4), and the androgen and glucocorticoid response elements ARE and GRE (data not shown). Examples of TF activities that remained unchanged following LL-37 stimulation, as evaluated by the arrays, were RXR (retinoid X-receptor) and SIE (Sis-inducible element), and in contrast TFIID (transcription factor II D) activity was decreased after LL-37 treatment. Results shown are representative of results from two independent biological replicates (Fig. 4b). The transcription factor array analysis was consistent with the computational TFBS analysis in demonstrating the activity of AP-1, AP-2, E2F1, GRE, NF-κB and SP1 (Fig. 4 and Table 2) on stimulation with LL-37 in human monocytic cells; and the activation of the nuclear receptors AR and GR as well as AP-1 and NF-κB further supported our observation that ASC1 may mediate the LL-37 response.

NF-κB is known to be central in the induction and regulation of inflammatory responses, and the results of our pathway inhibitor on transcription factor array studies demonstrated the importance of this transcription factor in the LL-37 response. Therefore, the nuclear translocation of different

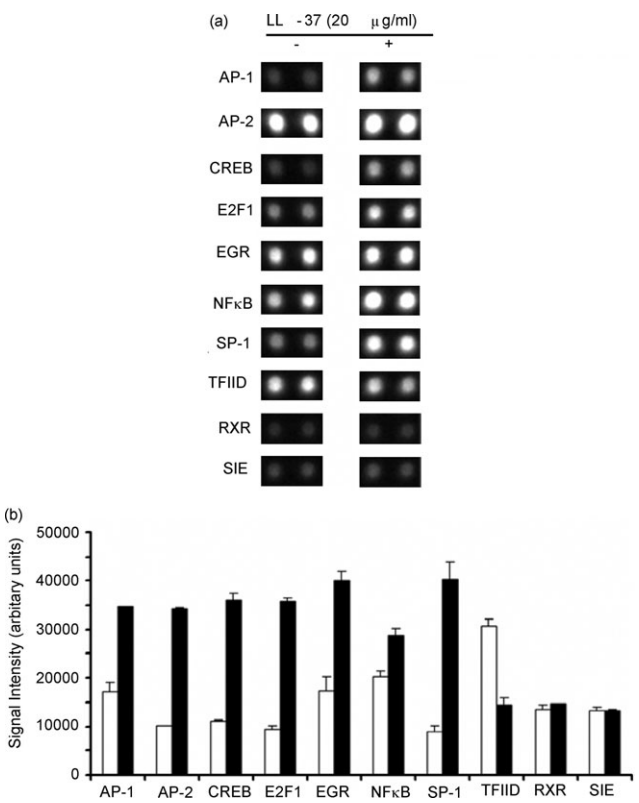

Fig. 4 LL-37 induced activation of several transcription factors in human monocytic cells. Nuclear extracts from human monocytic THP-1 cells after stimulation with LL-37 (20 μg ml^{-1}) for 1 h were analysed for nuclear translocation of transcription factors using Protein/DNA Array 1 (Panomics, Inc., CA, USA). (a) Membrane images were captured using a ChemiGenius Imaging system (Syngene), and (b) the relative signals from each spot on the membranes were quantified using Imagene software (Biodiscovery). Results shown are representative of two independent biological replicates.

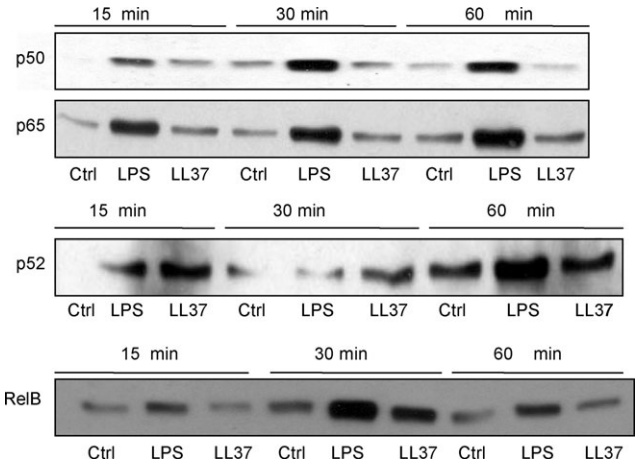

Fig. 5 LL-37-induced nuclear translocation of p50, p65, p52, and Rel B NF-κB subunits. Human monocytic THP-1 cells were stimulated with LPS (100 ng ml^{-1}) or LL-37 (20 μg ml^{-1}) for 15, 30 and 60 min. Nuclear extracts were analysed for nuclear translocation of various NF-κB subunits by Western blots using anti-p105/p50, anti-p65, anti-p100/p52, or anti-RelB antibodies. Results shown are representative of four independent experiments.

NF-κB subunits was evaluated by immunoblots in human monocytic cells stimulated with LL-37. Nuclear extracts from human monocytic THP-1 cells treated with LL-37 (20 μg ml^{-1}) for 15, 30 and 60 min were analysed for increased nuclear translocation of the NF-κB subunits p50, p65, p52, and RelB using their respective antibodies in Western blots. Consistent with our previous observations,[39] LL-37 resulted in the transiently increased nuclear translocation of NF-κB subunits p50 and p65 (Fig. 5). In addition, this study demonstrated that LL-37 induced the nuclear translocation of NF-κB subunit p52 between 15 and 30 min, and the transient nuclear translocation of RelB after 30 min of stimulation of human monocytes (Fig. 5). Thus transient increases of nuclear localization occurred with all four of the tested NF-κB subunits, albeit with varying kinetics.

Discussion

LL-37 is an important effector host defence peptide that is known to induce pleiotropic effects on critical immune cells, such as monocytes, dendritic cells and neutrophils, in response to infection or inflammation. Amongst these activities is the ability to chemoattract immune cells to sites of infection,[4–5,59] induce the release of chemokines that are necessary to mount an appropriate response to inflammatory stimuli and additionally, to selectively modulate the inflammatory responses in the presence of pathogen signature molecules.[37] Although it is established that LL-37 can elicit diverse biological responses from a broad variety of cells, the full range of effector cell types as well as the molecular mechanisms through which LL-37 mediates these effects is not fully understood. Here, we applied a comprehensive systems biology approach to analyse the effector cell types, signalling and transcriptional regulation events, and downstream responses induced in the presence of LL-37 in human PBMC. This approach has revealed several new insights regarding the scope of LL-37-mediated activities,

including the novel finding that LL-37 can induce chemokine induction in mDC and T- and B-lymphocytes and that it induces anti-inflammatory cytokine IL-10 expression in all the PBMC sub-populations examined (pDC, mDC, monocytes and T- and B-cells). Our findings also extend the understanding of the mechanisms through which LL-37 induces these effects, such as signalling through 5 separate pathways for the induction of cytokine expression, including all 3 MAPK pathways, and have revealed the intricacies of MAPK involvement in the expression of two different chemokines. This complexity of signalling responses was also reflected in the observation of activation of at least 10 separate transcription factors, including 4 subunits of NF-κB, based on both direct assessments of presence in the nucleus and bioinformatics analysis of gene expression responses; five of these transcription factors, AP-1, AP-2, SP-1, E2F1, and EGR had not been previously demonstrated to be involved and their up-regulation was generally consistent with the observed differentially expressed genes.

Using polychromatic FACS analysis, the broad range of responsive blood cell types was clearly revealed, indicating that monocytes, myeloid and plasmacytoid dendritic cells and B- and T-lymphocytes are all capable of responding to LL-37 differentially upon stimulation of human PBMC (Table 1, and Fig. S2, ESI†). LL-37-stimulation alone induced expression of IL-10 in all the cell populations tested: CD14+ monocytes, mDC, pDC, T- and B-cells, whereas chemokine MCP-1 production was only demonstrated in CD14+ monocytes and T-cells. LL-37 also modulated the chemokine responses to bacterial endotoxin LPS treatment differentially in the five individual cell populations. LPS-induced pro-inflammatory TNF-α and IL-6 responses were suppressed by the peptide in mDCs, CD14+ monocytes and lymphocytes, but these cytokines were not detected in pDC; IL-8 expression was suppressed in three cell types but not observed in pDC or B-cells, while in B-cells MCP-1 showed an increase only in response to the combination of LPS and LL-37.

This is the first study to provide direct evidence that LL-37 can elicit biological responses, such as cytokine induction, in mDC and lymphocyte populations (Table 1, and Fig. S2, ESI†). It has already been well established that LL-37 acts as a chemoattractant for a variety of blood cells, and that LL-37-mediated recruitment of these populations is mediated through the FPRL-1 receptor.[27] The current study extends these observations by demonstrating that LL-37 also induces differential cytokine responses in these cell types, indicating that it not only acts to recruit, but may also function as an effector molecule that can modulate the responses of these populations once they have arrived at sites of infection or inflammation. These findings support previous suggestions that LL-37 may act as a mediator in the transition between innate and adaptive immunity, based on its known ability to chemoattract 'adaptive' immune cells and, furthermore, influence the differentiation and subsequent responses of dendritic cells that are critical in the initiation of adaptive immune responses.[36,68] In this study, we observed an increase in intracellular IL-10 levels in LL-37 stimulated pDC and mDC as well as T- and B-lymphocytes at doses of 50 and 100 μg ml^{-1} and, additionally, an increase in MCP-1 induction in T-cells, albeit at high concentrations of LL-37 treatment (100 μg ml^{-1}).

IL-10 is primarily known as an anti-inflammatory cytokine which plays a key role in the regulation of inflammatory responses through inhibiting the activities of T-cells, NK cells and macrophages (reviewed in Couper *et al.*).[69] These functions are crucial in preventing immunopathology, such as tissue damage, associated with uncontrolled release of pro-inflammatory cytokines such as TNF-α and IFN-γ. It is therefore interesting that LL-37 induces the expression of IL-10 in T-, B- and pDC cells, which has not been previously shown, but also the other cell populations that were surveyed: mDCs and monocytes. The universal ability to induce expression in all of the major cell types known to be a source of IL-10 is potentially very significant as it may indicate a key role for LL-37 in initiating regulatory cascades that serve to curb excessive inflammatory responses. These data are also consistent with our previous reports examining a mixed PBMC population wherein LL-37 up-regulated the expression of anti-inflammatory IL-10 mRNA, and simultaneously maintained the expression of anti-infective, protective chemokine responses in the presence of endotoxin.[37] The present study further supports the concept of LL-37 as a multifunctional effector of immune responses and demonstrates the involvement of LL-37 in (i) both initiation and maintenance of necessary protective responses to infection (such as cell recruitment and induction of chemokines including MCP-1 and Gro-α), (ii) the regulation of pro-inflammatory responses *i.e.* the induction of IL-10 expression which exerts pleiotropic anti-inflammatory effects, and (iii) the suppression of pro-inflammatory cytokines such as TNF-α and IL-6 in response to TLR agonists, all of which result in the subsequent resolution of inflammation and avoidance of harmful immunopathology. The ability of the peptide to induce IL-10 in diverse cell types within the PBMC population also indicates that LL-37 utilizes receptors and signalling cascades that are common to all these cell populations. The SP1 transcription factor is known to be involved in the regulation of IL-10 expression, both in humans and mice,[70,71] hence it is of significance that in this study we computationally predicted and experimentally confirmed the increased activity of SP1 in LL-37-treated monocytes. It is also of interest that the p38 MAPK pathway has also been identified as a regulator of IL-10 transcription, at least in response to LPS stimulation,[71] and we further confirmed in this study that the MAPK p38 pathway is not only activated in response to LL-37 treatment but is also required for LL-37-induced chemokine induction.

The finding in the present study that LL-37 can induce chemokine expression in cell types associated with adaptive immunity, such as T- and B-cells, is novel and certainly warrants more detailed investigation. However, we chose to concentrate here on CD14+ monocytes since these are key effector cells that respond to peptide and LPS stimulation, and the observed responses were qualitatively similar to those of mDC and lymphocytes. This was further justified as a recent study demonstrated that monocytes/macrophages are critical for the anti-infective immunomodulatory properties of host defence peptides.[72]

To gain perspective on the responses induced in this critical effector cell type, we measured the global transcriptional response in CD14+ monocytes following stimulation of PBMC with LL-37 for 4 h. Microarray analysis demonstrated that LL-37 up-regulated the expression of almost 500 genes in human monocytes, many of which were validated by qRT-PCR and are genes whose products have known immune-related functions, including genes encoding chemokines (MCP-1, MCP-3, IL-8 and Gro-α), chemokine receptors, innate immune signalling molecules, genes essential for lymphocyte development/differentiation, adhesion molecules and interferon-related genes (Fig. 1). Interestingly, the IL-10 superfamily member IL-19 was also up-regulated significantly (nearly five-fold as determined by qRT-PCR) in response to LL-37 treatment. IL-19 is a recently discovered cytokine and its biological roles are still the subject of investigation; however, it is known to activate monocytes (the main producer of IL-19) in an autocrine fashion to release IL-6, TNF-α and reactive oxygen species and is implicated as contributing to the inflammatory process in psoriasis.[73] LL-37 has also been linked with the disease process in psoriasis, through its complexing with self-DNA released as a result of skin damage that subsequently triggers pDCs to produce type I IFNs that contribute to the pathogenesis of psoriasis. Whilst the induction of IL-19 by LL-37 requires further characterization, this is a further indication of the pleiotropic effects of LL-37, whereby it can possibly mediate a variety of responses that may appear to be opposing, such as anti- and pro-inflammatory effects, but may be entirely appropriate given the suitable biological context or physiological situation.

Ontology and pathway over-representation analysis revealed that pathways involving members of the MAPK family, as well as interrelated pathways and processes such as TGFβ signalling, cell–cell adhesion, and β-catenin degradation, were enriched amongst the differentially expressed gene set. A network-based approach, in which we looked for interactions between genes whose expression was altered in response to LL-37, further supported these observations and also revealed the potential involvement of activating signal co-integrator complex 1, a transactivator of nuclear receptors and other transcription factors known to be important to the immune response, many of which were ultimately found to be activated in response to LL-37 in this study. Identification of a potential transcriptional repression complex involving HEY1, HEY2, ARNT and SIRT was also made possible through the network-based approach, and this complex may lie downstream of the Notch pathway, predicted by pathway over-representation analysis to be enriched in LL-37 responsive genes. Only a small number of targets of hairy family repression have been identified to date;[74] however this list includes immune-relevant genes including prostaglandin D2 synthase and CD4.

Through the use of pathway inhibitors, we were able to confirm the involvement of multiple signalling pathways in the LL-37 response, including IκB-α, MAP-kinases p38, ERK1/2 and JNK, and PI3K (Fig. 3). All of these pathways are capable of activating one or both of the AP-1 and NF-κB transcription factors, both of which were confirmed in this study to respond to LL-37. Although we expected to see a similar response in LL-37-induced IL-8 and MCP-1 levels to all pathway inhibitors due to this similarity in their promoter regions, we observed conflicting data regarding p38 and JNK inhibition on IL-8 and MCP-1 induction. The failure of the p38 inhibitor to reduce MCP-1 levels may be explained by two recent studies that have revealed that while SB203580 inhibits p38 activation, it is also a potent activator of the JNK pathway.[75,76] It is this activation that might have been responsible for the normal LL-37-induced MCP-1 levels in the presence of the p38 inhibitor. The differential response of IL-8 is less readily explained, with previous reports on the use of JNK inhibitors sometimes reporting a dampening of IL-8 levels[77] and sometimes not.[78] Transcriptional control of IL-8 is complex and requires the interplay of several signalling pathways, including activation of AP-1 by JNK and stabilization of IL-8 mRNA by the p38 pathway.[79] While p38 inhibition did affect LL-37-induced IL-8 levels in our study, perhaps as a consequence of destabilization of IL-8 mRNA, inhibition of JNK activation did not. We propose that the involvement of alternative pathways and/or transcription factor complexes in lieu of JNK/AP-1 might lead to maintenance of IL-8 levels induced in the presence of the peptide despite the inhibition of JNK.

In an attempt to understand the basis for differential gene expression in response to LL-37, the profile of transcription factors activated by LL-37 in human monocytic cells was also investigated here. Computational analysis of over-represented transcription factor binding sites in the promoter regions of differentially expressed genes predicted the involvement of several immune-related transcription factors in the activity of LL-37, such as AP-1/2, NFKB1, MZF1, and SP1. Several of these predictions were validated by employing transcription factor arrays (Fig. 4) that measured the nuclear translocation of transcription factors induced by LL-37, and could be related back to observations made regarding the signalling pathways induced by the peptide. In particular, we confirmed the over-representation and increased nuclear translocation of AP-1 and SP1 which lie downstream of the JNK pathway, the importance of which was revealed by our array data, network-based analysis, and inhibitor studies, and we observed similar support for our observation that NF-κB is a major target of the LL-37 response. This result is consistent with our previous observations wherein LL-37 promoted nuclear translocation of p50 and p65 NF-κB subunits in human PBMC,[39] and also extends these observations to two other NF-κB subunits, RelB and p52.

Previous studies have shown that depending on the stimuli, pro-inflammatory responses, *e.g.* TNF-α, can be regulated by differential binding of complexes of transcription factors, and that the upstream promoter region of TNF-α includes binding sites for CREB, NF-κB, as well as EGR-1, AP-1- and AP-2-like binding sites,[80–84] all of which were shown to be induced by LL-37 in monocytic cells (Fig. 4). For example, it was demonstrated that a transcription factor complex containing NF-κB, CREB and AP-1 was essential in the production of chemokines.[85,86] As chemokine production appears to be a critical property of LL-37 (Fig. 3, and Fig. S3, ESI†), it can be hypothesized that the immunomodulatory properties of LL-37 are dependent on co-regulation mediated by transcription factor complexes. Consistent with this, the LL-37-induced

(Fig. 4) transcription factors CREB, SP-1, AP-1, AP-2 and EGR-1 have all been previously shown to interact with NF-κB,[87] and co-operative interactions of NF-κB with heterologous transcription factor complexes in the promoter/enhancer regions of immunity related genes regulate the integration of NF-κB with other signalling pathways.[87]

The current model of the activation of NF-κB includes two distinct pathways, the classical canonical pathway, mediated by inducible IκB degradation followed by the nuclear translocation of p50–p65 heterodimers, and the non-canonical pathway, mediated by tightly regulated p100 processing and nuclear translocation of p52-containing dimers.[88] LL-37 induced the nuclear translocation of both p50 and p65 subunits and the transient phosphorylation of IκB-α in human PBMC.[39] Tightly controlled induction of the canonical pathway regulated by IκB-α through transient activation of NF-κB, as implicated here (Fig. 5) in response to LL-37, results in the consequent suppression of constitutive activation of downstream inflammation.[89] This is consistent with the observations that LL-37 mediates transient phosphorylation of IκB-α,[39] and that TNFAIP3 (A20), one of the target genes for feedback inhibition of NF-κB activation, is induced by the peptide in monocytic cells.[37] Moreover, kinases that are known to be involved in the phosphorylation of p65, such as PI3K, p38 MAPK and AKT,[87] are all required for chemokine production mediated by LL-37.[39] These results, together with inhibitor studies, are consistent with a profound role for transient canonical NF-κB activation in the immunomodulatory and anti-endotoxin activities of LL-37.

This study also demonstrated that LL-37 induced the nuclear translocation of NF-κB subunits p52 and RelB in human monocytic cells (Fig. 5), consistent with the activation of the non-canonical NF-κB pathway. The non-canonical pathway has been shown to be important in the expression of genes involved in B-cell functions and in lymphocyte activation.[88,89] This is consistent with additional findings in this study indicating that both T- and B-cells are effector cells that mediate responses on stimulation of PBMC with LL-37 (Table 1). However, it is not clear if p52-RelB heterodimers, which are frequently activated by the non-canonical pathway, are activated directly by the peptide or as a result of downstream responses.

Overall, this study provides an insight into the basis for the complex downstream responses mediated by LL-37 in PBMC and monocytic cells. The intricacy of prospective transcription factor complexes activated by LL-37, the proposed integration of these complexes by multiple signalling pathways, and their role in regulation of target gene expression will require further investigation.

Material and methods

Cell isolation and cell culture

Venous blood was collected from healthy volunteers in Vacutainer® collection tubes containing sodium heparin as an anticoagulant (Becton Dickinson, Mississauga, ON) in compliance with University of British Columbia ethical approval and guidelines. RPMI-1640 media (Gibco®, Invitrogen™ Life Technologies, Burlington, ON), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen™ Life Technologies) were used to dilute the blood samples in 1 : 1 ratio; the samples were separated by centrifugation over a Ficoll-Paque® Plus (Amersham Biosciences, Piscataway, NJ, USA) density gradient. The buffy coat was washed in RPMI 1640 complete medium, and the number of PBMC was determined by trypan blue exclusion. Where indicated, CD14+ monocytes were isolated from the PBMC population by positive selection employing Dynabeads® CD14, Dynal® magnetic bead-based separation as per the manufacturer's instructions (Invitrogen™, Burlington, ON, Canada). Human monocytic cell line THP-1 (ATCC® TIB-202) was grown in RPMI-1640 complete media. 1×10^6 THP-1 cells per ml were differentiated to plastic-adherent cells by treating with 0.12 μg ml^{-1} of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Canada, Oakville, ON) for 24 h. The cells were rested for an additional 24 h in complete RPMI 1640 media prior to stimulations with various treatments. All the cell cultures were maintained at 37 °C in a humidified 5% (v/v) CO_2 incubator.

Peptide and inhibitors

Human peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized at The Nucleic Acid/Protein Synthesis Unit at the University of British Columbia, using Fmoc chemistry. The synthesized peptide was resuspended in endotoxin-free water (Sigma-Aldrich) and stored at −20 °C until further use. Human PBMC were pre-treated with specific pharmacological inhibitors for 1 h; subsequently the cells were treated in the presence or absence of LL-37 (20 μg ml^{-1}) for 24 h. Cells were pre-treated with 10 μM each of specific inhibitors Bay11-7085, PD98059, SB203580, JNK-II inhibitor, LY294002 and AG490 (Calbiochem, EMD Biosciences, CA, USA) for IκB-α, MAPK ERK 1/2, p38 and JNK, phosphoionositide-3 kinase (PI3K) and Janus kinase 2 (JAK) pathways, respectively. In addition, protein kinase A (PKA) and protein kinase C (PKC) inhibitors, 6-22 amide and GF109203x, respectively, were used at 10 nM concentrations, respectively (Sigma-Aldrich).

Polychromatic flow cytometry

Human PBMC (10 million cells per ml) were stimulated with LL-37 (20, 50 or 100 μg ml^{-1}) in the presence and absence of TLR4-agonist LPS (10 ng ml^{-1}) for 18 h at 37 °C in 5% CO_2 in a 96-well format. Adherent cells were detached using 2 mM EDTA (37 °C for 10 min), followed by plate centrifugation and resuspending the cells in 100 μl of FACSLyse (Becton Dickinson). The plates were stored at −80 °C until further use. The frozen plates were thawed for 15 min at 37 °C and centrifuged. The cell pellets were resuspended in 200 μl of FACS permeabilizing solution (Becton Dickinson) and incubated for 10 min at room temperature. The cells were washed with PBSAN (PBS containing 0.5% bovine serum albumin and 0.1% sodium azide), followed by staining in 100 μl of PBSAN with optimal antibody concentrations (optimized by titrating each antibody before use). The cells

were further washed × 2 with PBSAN, resuspended in PBS with 2% paraformaldehyde and analysed immediately on an FACS Aria Flow cytometer (Becton Dickinson) as previously described.[90] Staining panel for intracellular cytokines and the cell surface marker antibodies used are summarized in Table S2 (ESI†). Compensation beads (Becton Dickinson) were used to standardize voltage and used as single stain controls. At least 500 000 events were acquired uncompensated through a high-throughput sampler.

RNA extraction, amplification and hybridization of DNA microarrays

RNA was isolated and amplified as previously described.[72] Briefly, RNeasy kit (Qiagen Inc., Canada) was used for isolation of RNA from CD14+ monocytes, followed by RNase-free DNase (Qiagen) treatment and elution in RNase-free water (Ambion Inc., Austin, TX, USA). The integrity, purity and concentration of RNA were evaluated by Agilent 2100 Bioanalyzer using RNA 6000 Nano kits (Agilent Technologies, USA). RNA was (reverse) transcribed along with amino-allyl-UTP (aa-UTP) incorporation using MessageAmpII™ amplification kit, followed by labelling with mono-functional dyes, Cyanine-3 and Cyanine-5 (Amersham Biosciences). The samples were further purified using a Mega Clear kit (Ambion) according to the manufacturer's instructions. A Lambda 35 UV/VIS fluorimeter (PerkinElmer Life and Analytical Sciences, Inc., USA) was used to assess flurophore yield and incorporation. Human genome 21K Array-Ready Oligo Set™ (Qiagen Inc., USA) was used to print microarray slides at The Jack Bell Research Center (Vancouver, BC, Canada). The slides were processed, hybridized, scanned and quantified as previously described.[37]

Microarray processing and bioinformatics analysis

Processing of microarray data, including assessment of slide quality, flagging of markers and control spots, sub-gridwise background correction, data shifting, printTip LOESS normalization, averaging of biological replicates to yield overall fold changes and statistical analysis, was performed using a semi-automated, web-based software ArrayPipe version 1.391 (www.pathogenomics.ca/arraypipe) as previously described.[37,72] The microarray data were submitted to an open source MIAME compliant database Array Express under accession number E-FPMI-11. Two-sided one-sample Student's t-test on the $\log_2$-ratios within the treatment group was used for statistical analysis of the data. All genes were mapped to a single Ensembl gene using version 43.36e of the Ensembl database[91] and the ProbeLynx probe matching software.[92]

The ENSG ID was used to upload the array data to the InnateDB biomolecular interaction database[49] and the Gene Ontology Tree Machine,[93] where they were analysed for over-represented pathways and biological process ontologies, respectively, using default parameters. Interactions between differentially expressed genes were retrieved from InnateDB and visualized using Cytoscape v.2.6.[50] Over-represented transcription factor binding sites were identified using an in-house version of the oPOSSUM tool,[61] implementing a custom set of all human matrices from the JASPAR[62] and TRANSFACPro[63] databases. The analysis was performed on 500 bp upstream and 100 bp downstream of the transcription start site for all 8-bit or higher matrices, using a match threshold of 85%. All TFBSs with a Z-score >10 and/or a Fisher score <0.01 were selected as being statistically significant. TFBSs were associated with a specific transcription factor or TF family using annotation in the TRANSFAC database.

Quantitative real-time PCR

Expression of a selection of differentially expressed genes indicated from microarray analysis was further validated by qRT-PCR using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen™ Life Technologies) in the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster city, CA, USA) as previously described.[37] Briefly, 250 ng of total RNA were reverse transcribed in a 20 μl reaction volume and the qRT-PCR reaction was carried out in a 12.5 μl reaction volume containing 2.5 μl of 1/10 diluted cDNA template. Specificity of the product was evaluated by analysis of a melting curve, and the fold changes were calculated after normalization to endogenous GAPDH using the comparative ct method.[94] Primers used for qRT-PCR are listed in Table S3 (ESI†).

Nuclear and cytoplasmic extracts

THP-1 cells (3×10^6) or PBMC (5×10^6) seeded into 60 mm^2 Petri dishes (VWR International, Mississauga, ON) were stimulated with LL-37 for the indicated time points and concentrations, and subsequently washed with ice-cold phosphate buffered saline. Adherent cells were detached by treatment with Versene for 10 min at 37 °C in 5% CO_2 and then washed twice with ice-cold phosphate buffered saline. Isolation of cytoplasmic and nuclear extracts was performed using NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration of the extracts was quantified using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology) and the extracts were stored at −80 °C until further use.

Transcription factor array analysis

PMA-treated, differentiated and rested THP-1 cells (1×10^7 per 10 cm dish) were either untreated or stimulated with LL-37 (20 μg ml^{-1}) for one hour at 37 °C. Cells were harvested and nuclear extracts were isolated using a Nuclear Extraction Kit as per the manufacturer's instructions (Panomics, Inc., CA, USA). Protein concentrations in each nuclear extract were determined by BCA protein assay (Pierce, IL, USA). Equal amounts (6 μg) of extract were then hybridized to Protein/DNA Arrays 1 (Panomics, Inc., CA, USA) as per the manufacturer's instructions. Briefly, extracts were incubated with biotinylated DNA probes corresponding to transcription factor (TF) consensus binding sequences and any unbound probes were removed using a spin column. Resulting DNA–protein complexes were denatured to separate protein and bound probes. Liberated probes were then hybridized to the membrane, consisting of an array of TF binding sites. Following washing steps, bound probes were visualized using

streptavidin–HRP conjugate and chemiluminescent substrate. Membrane images were captured using a ChemiGenius Imaging system (Syngene) and the relative signals from each spot on the membranes were quantified using Imagene software (Biodiscovery).

Gel electrophoresis and immunoblots

Cytoplasmic or nuclear extracts were electrophoretically resolved on a 7.5% SDS-polyacrylamide gel (SDS-PAGE), followed by transfer to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore Canada Ltd., Mississauga, ON). Equivalent protein loading was validated by staining the PVDF membranes with Blot-Fast stain™ (Chemicon International) as per the manufacturer's instructions. The PVDF membranes were subsequently probed with antibodies for various NF-κB subunits; rabbit polyclonals anti-p105/p50, anti-p65 and anti-RelB, as well as mouse monoclonal anti-p100/p52 in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk powder. Goat anti-rabbit or anti-mouse HRP-conjugated antibodies were used for detection as appropriate, followed by development with chemiluminescence peroxidase substrate (Sigma-Aldrich) as per the manufacturer's instructions.

Detection of cytokines

Tissue culture supernatants were harvested after stimulation with peptide and/or various inhibitors by centrifugation at 2500 × *g* for 7 min to obtain cell-free samples, which were aliquoted and stored at −20 °C prior to assays for detection of cytokines. TNF-α, as well as chemokines IL-8, MCP-1 and RANTES, was detected in the tissue culture supernatants using a capture ELISA (eBioscience CA, USA) as per the manufacturer's instructions. The concentration of the cytokines/chemokines in the tissue culture supernatants was evaluated by establishing a standard curve with serial dilutions of the recombinant human cytokines as required.

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