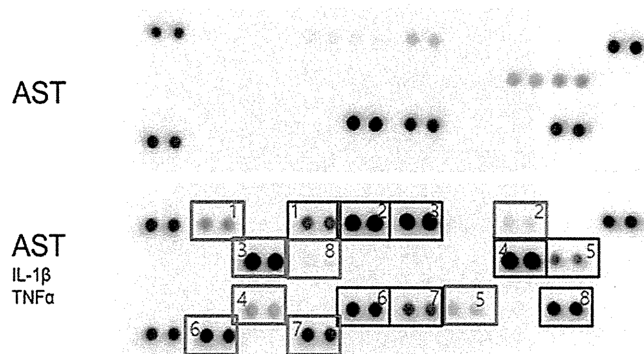

Newly Expressed cytokines/chemokines		Changed expression	
1	C5/C5a (Complement Component 5/5a)	1	G-CSF (CSFβ, CSF-3)
2	sICAM-1 (CD54)	2	GM-CSF (CSFα, CSF-2)
3	IL-1β (IL-1F2)	3	GROα (CXCL1)
4	IP-10 (CXCL10)	4	IL-6
5	MIP-1α (CCL3)	5	IL-8 (CXCL8)
6	RANTES (CCL5)	6	MCP-1 (CCL2)
7	TNF-α (TNFSF1A)	7	MIF (GIF, DER6)
8	IL-1ra (IL-1F3)	8	Serpin E1 (PAI-1)

Figure 2. Proteome profiler array of human cytokines/chmokines. The array is capable of detecting a panel of 36 cytokines, chemokines, and soluble mediators. Top panel shows expression of cytokines/chemokines in resting unstimulated human astrocyrtes, and middle panel shows expression of cytkines/chemokines in human astrocytes stimulated with IL-1β and TNF-α. Items circled in red are newly expressed cytokines in activated astrocytes and ones circled in blue are cytokines changed expression in activated aqstrocytes. In bottom panel, newly expressed cytokines and cytokines changed expression are listed separately.
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antibody (1:4, mouse; Kim Lab), a oligodendrocyte cell type-specific marker, or anti-human CD68 antibody (1:200, mouse, Millipore), a microglial marker, for 48 hrs at 4°C, followed by Alexa Fluor 594-conjugated anti-mouse IgG for 1 hr at RT. Cultures processed for immunocytochemistry were examined under an Olympus laser confocal fluorescence microscope.

Cytokine and chemokine profiling

Human astrocytes were incubated in culture medium with or without inclusion of a mixture of 10 ng/mL recombinant human IL-1β (Peprotech, Rocky Hill, NJ) and 10 ng/mL recombinant human TNF-α (Peprotech). At 24 hours after treatment, the conditioned media were harvested and processed for profiling of cytokines and chemokines on the human cytokine array panel A (R&D system, Minneapolis, MN), which is capable of detecting a panel of 36 cytokines, chemokines, and soluble mediators, such as complement 5/5a, CD40 ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNFα, and sTREM-1 (Table 1). The array membranes were reacted with the mixture of conditioned media and the antibody cocktail for 18 hrs at 4°C. After several washing, they were briefly incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Then, the membranes were exposed to HRP substrate. The intensity of the reaction was quantified on the Da vinci imaging system (Seoul, Korea).

Molecular network analysis

We imported Entrez Gene IDs corresponding to cytokine and chemokine genes into the Core Analysis tool of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems; www.ingenuity.com). IPA is a commercial knowledgebase that contains information on approximately 3,000,000 biological and chemical interactions with definite scientific evidence. By uploading the list of Gene IDs, the network-generation algorithm identifies focused genes integrated in global molecular networks. IPA calculates the score p-value that reflects the statistical significance of association between the genes and the networks by Fisher's exact test. We considered p-value <0.05 as a significant association. The information on known NF-kB target genes was collected from web accessible databases constructed by Dr. Thomas Gilmore, Boston University (www.bu.edu/nf-kb/gene-resources/target-genes) and by Bonsai Bioinformatics, Laboratoire d'Informatique Fondamentale de Lille (LIFL), Université Lille 1 (bioinfo.lifl.fr/NF-KB), as described previously [6].

Results

Cytokine and chemokine profiles of human astrocytes in culture

The purity of human astrocytes in culture exceeded 99% by GFAP labeling without any contamination of the cells expressing CD68 (microglia), galactocerebroside (oligodendrocytes) or tubulin βIII (neurons) (Fig. 1). Non-stimulated resting astrocytes incubated

Table 3. Cytokines absent in normal resting human astrocytes.

Gene	Genebank	Gene name
CD40 Ligand	NM_000074	CD40 ligand
I-309 (CCL1)	NM_002981	chemokine (C-C motif) ligand 1
IFN-γ (Type II IFN)	NM_000619	interferon, gamma
IL-1α (IL-1F1)	NM_000575	interleukin 1, alpha
IL-2	NM_000586	interleukin 2
IL-4	NM_000589	interleukin 4
IL-5	NM_000879	interleukin 5 (colony-stimulating factor, eosinophil)
IL-10	NM_000572	interleukin 10
IL-12 p70	NM_000882/	interleukin 12A (natural killer cell stimulatory factor 1)
IL-13	NM_002188	interleukin 13
IL-16	NM_172217	interleukin 16
IL-17	NM_002190	interleukin 17A
IL-17E	NM_022789	interleukin 25
IL-23	NM_016584	interleukin 23, alpha subunit p19
IL-27	NM_145659	interleukin 27
IL-32	NM_001012633	interleukin 32
I-TAC (CXCL11)	NM_005409	chemokine (C-X-C motif) ligand 11
MIP-1β (CCL4)	NM_002984	chemokine (C-C motif) ligand 4
SDF-1 (CXCL12)	NM_199168	chemokine (C-X-C motif) ligand 12
sTREM-1	NM_018643	triggering receptor expressed on myeloid cells 1

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in the culture medium without addition of cytokines expressed 8 out of 36 cytokines and chemokines tested, such as G-CSF, GM-CSF, IL-6, GROα (CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), MIF and serpin E1 (Table 2, Fig. 2). Other cytokines and chemokines were undetectable in the conditioned media of non-stimulated astrocytes (Table 3). Following a 24 hr-exposure to a mixture of IL-1β and TNF-α, the expression levels of 6 cytokines, including G-CSF, GM-CSF, IL-6, GROα, IL-8 and Serpin E1, were elevated substantially (Table 4, Figs. 2 and 3), whereas the levels of both MCP-1 and MIF showed a minor reduction (Table 5, Figs. 2 and 3). In astrocytes activated with IL-1β/TNF-α for 24 hrs, there was new indcution of cytokines and chemokines including IL-1β, IL-1ra, TNF-α, IP-10 (CXCL10), MIP-1α (CCL3), RANTES (CCL5), sICAM-1 (CD54) and complement component 5 (C5a) (Table 6, Figs. 2 and 3), suggesting an existence of the positive autoregulatory feedback loop for expression of IL-1β and TNF-α. Among them, upregulated expression of RANTES was the most prominent (Fig. 3B). By database search on known NF-kB target genes, nearly all cytokines and chemokines produced by non-stimulated and activated astrocytes are direct targets of the transcription factor NF-kB, except for C5a, IL-1ra, and MIF, although the genes encoding C5a and MIF have NF-kB binding sites in the promoter regions by literature search on PubMed [7,8].

Molecular network of cytokine and chemokine secretome of human astrocytes

When the list of Entrez Gene IDs corresponding to 14 up-regulated and 2 down-regulated cytokines in IL-1β/TNF-α-activated human astrocytes (Tables 4 and 5, Figs. 2 and 3) were imported into IPA, we identified the molecular network defined by "Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking" as the most significant functional network relevant to the set of imported genes (p = 1.00E-13) (Table 7, Fig. 4). The network defined by "Cellular Movement, Hematological System Development and

Table 4. Cytokines upregulated in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
G-CSF	19.74	NM_000759	colony stimulating factor 3 (granulocyte)
GM-CSF	43.25	NM_000758	colony stimulating factor 2 (granulocyte-macrophage)
GROα (CXCL1)	10.27	NM_001511	chemokine (C-X-C motif) ligand 1
IL-6	6.09	NM_000600	interleukin 6
IL-8 (CXCL8)	1.47	NM_000584	interleukin 8
Serpin E1(PAI-1)	1.30	NM_000602	plasminogen activator inhibitor type 1

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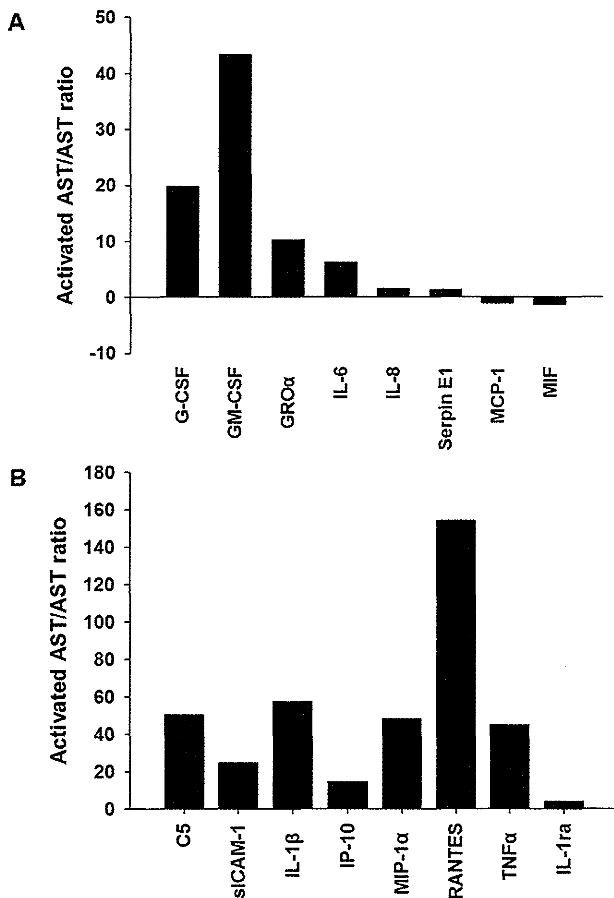

Figure 3. Cytokines/chemokines changed their secretion levels in human astrocytes stimulated with IL-1β and TNF-α. A: Forty five-fold increase in secertion of GM-CSF and twenty-fold increase in secretion of G-CSF are shown here. A minor reduction in expression of MCP-1 and MIF is also shown. B. Among the elevated levels of cytokines/chemokines in human astrocytes stimulated with IL-1β and TNF-α, upregulated expression of RANTES was the most prominent with more than 150-fold increase.
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Function, Immune Cell Trafficking" (p = 1.00E-6) was the second rank significant functional network (Table 7). IPA also indicated that nuclear factor NF-kB/RelA serves as an upstream regulator of the imported genes (p = 7.36E-24), validating the results of database search on NF-kB target genes described above. In IPA analysis, up-regulated molecules, such as MIP-1α (CCL3), RANTES (CCL5), GM-CSF, sICAM1, IL-1β, IL-6, IL-8 (CXCL8), and TNF-α, and a down-regulated molecule MCP-1 (CCL2), were categorized into NF-kB target genes located in the NF-kB signaling pathway (Fig. 4).

Discussion

Due to the limited availability of human brain tissues, only a small number of studies have previously reported the cytokine production profiles of normal human astrocytes [9–11]. In the present study, we characterized the more comprehensive profile of cytokine and chemokine named "secretome" derived from non-stimulated resting and activated human astrocytes by using a protein microarray. We found that both non-stimulated and IL-1β/TNFα-activated astrocytes produce distinct sets of cytokines and chemokines, nearly all of which represent direct targets of transcription factor NF-kB. One exception is MCP-1 (CCL2), a direct target of NF-kB [12], was down-regulated in activated astrocytes following exposure to IL-1β and TNF-α. In contrast, a previous study has indicated that the activated adult human astrocytes in culture produced increased amounts of MCP-1 [11]. The discrepancy between our results and previous findings is attributable to a difference in maturation of cultured cells, i.e. fetal versus adult astrocytes employed. These results suggest that the NF-kB signaling pathway differentially regulates gene expression of cytokines and chemokines in human astrocytes under physiological and inflammatory environments.

Table 5. Cytokines downregulated in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
MCP-1 (CCL2)	−1.04	NM_002982	chemokine (C-C motif) ligand 2
MIF	−1.28	NM_002415	macrophage migration inhibitory factor

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NF-kB acts as a central regulator of innate and adaptive immune response, stress response, cell proliferation, and apoptosis [13]. The NF-kB family proteins consist of five members, such as RelA (p65), RelB, c-Rel, NF-kB1 (p105), and NF-kB2 (p100). The latter two are processed proteolytically into p50 and p52, respectively. The NF-kB family proteins constitute either homodimers or heterodimers, except for RelB that exclusively forms heterodimers. The NF-kB dimers interact with consensus DNA sequences termed the kB site located on promoters to activate or repress transcription of target genes. Only p65 and c-Rel act as a potent transcriptional activator, whereas p50 and p52 homodimers generally repress transcription, leading to differential regulation of gene expression of NF-kB targets [14]. We found that MCP-1 (CCL2), a target of NF-kB, is down-regulated in NF-kB-activated human astrocytes. Importantly, NF-kB target genes often activate NF-kB itself, providing a positive regulatory loop that amplifies and perpetuates inflammatory responses [15]. IL-1 β and TNF-α are the prototypes of NF-kB activators for the canonical NF-kB signaling pathway. We found that non-stimulated human astrocytes do not constitutively produce IL-1β or TNF-α, while activated human astrocytes could produce both, as described previously [9,11]. In contrast, a previous study has shown that IL-1β is undetectable at both mRNA and protein levels in non-stimulated or cytokine-stimulated cultured human astrocytes [10]. A different study from the same group showed that IL-8 (CXCL8) is undetectable in non-stimulated human fetal astrocytes in culture [16], being inconsistent with our results. In our study, non-stimulated astrocytes expressed a panel of NF-kB targets, such as G-CSF, GM-CSF, IL-6, GROα (CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), MIF and serpin E1, suggesting that the NF-kB signaling pathway is constitutively active to a certain extent in normal human astrocytes in culture. Notably, GM-CSF serves as an anti-apoptotic and neurotrophic factor [17].

Table 6. Cytokines newly induced in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
IL-1β	57.17	NM_000576	interleukin 1, beta
IL-1rα	3.72	NM_002182	interleukin 1 receptor accessory protein
TNFα	44.75	NM_000594	tumor necrosis factor (TNF superfamily, member 2)
sICAM-1	24.38	NM_000201	intercellular adhesion molecule 1 (CD54)
C5	50.25	NM_001735	complement component 5
IP-10 (CXCL10)	14.27	NM_001565	chemokine (C-X-C motif) ligand 10
MIP-1α (CCL3)	48.05	NM_002983	chemokine (C-C motif) ligand 3
RANTES (CCL5)	154.13	NM_002985	chemokine (C-C motif) ligand 5

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Chemokines constitutes a group of structurally related proteins that play a pivotal role in regulation of immune cell trafficking involved in inflammatory and immunoregulatory processes in the CNS [18]. In the present study, molecular network of cytokine and chemokine secretome of activated human astrocytes strongly supported the view that these soluble factors are critically involved in regulation of the cellular interaction and trafficking of immune cells. Various chemokine receptors, such as CCR2 with the ligand MCP-1 and CXCL2 with multiple ligands, such as Groα, Groβ and IL-8, are up-regulated in brain lesions of trauma, ischemia, and multiple sclerosis (MS) [19]. MIP-1 belongs to a family of C-C chemokines with a potent chemotactic activity for neutrophils and other leukocytes, composed of two members MIP-1α (CCL3) and MIP-1β (CCL4), both of which exhibit very similar but not identical proinflammatory properties through binding differentially to the receptors CCR1, CCR4 or CCR5. The upregulated expression of MIP-1α and MIP-1β was identified in acute MS lesions [20]. However, we identified MIP-1α (CCL3) but did not detect the expression of MIP-1 β (CCL4) in non-stimulated or activated human astrocytes in culture, suggesting that the gene regulatory mechanism is different between MIP-1α and MIP-1 β, although both are directly regulated by NF-kB.

RANTES is a member of C-C chemokines involved in the pathogenesis of MS and HIV-1 encephalitis by binding to the receptors CCR1, CCR3 or CCR5 [21,22]. A previous study has shown that fetal human astrocytes upon activation by IL-1β secretes a large amount of RANTES (CCL5) protein [23], supporting our observation that RANTES is the most prominently up-regulated chemokine in activated human astrocytes in culture. Importantly, RANTES plays a neuroprotective role in ischemic brain injury [24]. The expression of IP-10 (CXCL10), secreted by monocytes, endothelial cells, and fibroblasts in response to IFN-γ, shows a chemotactic activity for T cells, NK cells, dendritic cells and monocytes/macrophages through binding to the receptor CXCR3. We found that IP-10 production is greatly enhanced in activated human astrocytes in culture. Notably, the expression of IP-10 and CXCR3 is up-regulated in the brains of AD, where CXCR3 is expressed constitutively on neurons, while IP-10 expression is enhanced in a subset of reactive astrocytes surrounding senile plaques [25].

In conclusion, the comprehensive cytokine and chemokine secretome of activated human astrocytes, closely linked to NF-kB activation, suggested that astrocyte-derived cytokines and chemokines play a central role in proinflammatory (neurotoxic) and immunoregulatory (neuroprotective) responses in the CNS.

Table 7. Top 3 molecular networks of cytokine and chemokine secretome in human astrocytes.

Rank	Functional Networks	Focused Molecules	p-Value
1	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	**CCL3**, **CCL5**, **CSF2**, **CSF3**, **CXCL1**, chitinase, Collagen Alpha1, Collagen type II, Cpla2, elastase, Eotaxin, ERK1/2, ETS, Fcer1, Fcgr2, Ferritin, Fibrin, HLA-DR, IL-17f dimer, **IL-1R**, IL17a dimer, IL17R, IL1RAP, Il8r, IRAK, Lfa-1, Lymphotoxin, lymphotoxin-alpha1-beta2, NfkB-RelA, NfkB1-RelA, Nr1h, PI3K (family), Rxr, Scavenger receptor class A, VitaminD3-VDR-RXR	1.00E-13
2	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	26 s Proteasome, Akt, AMPK, BCR (complex), calpain, **CCL2**, Cdk, Collagen type IV, Cyclin A, Cyclin E, cyclooxygenase, Fibrinogen, gelatinase, **GM-CSF**, Growth hormone, HDL, Ige, Ikb, JINK1/2, Laminin, LDL, **MIF**, N-cor, NADPH oxidase, Nos, PDGF (complex), PDGF BB, PRKAA, Ptk, Rb, Rock, **Serpine1**, Smad, Sphk, TGF-beta	1.00E-06
3	Infectious Disease, Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation	Calcineurin protein(s), CD3, collagen, CYP, estrogen receptor, Hdac, hemoglobin, Histone h4, Hsp27, Hsp70, Hsp90, Icam, Iga, IgG1, Igm, **IL-6**, **IL-8**, Immunoglobulin, Interferon alpha, Ldh, Mek, Nfat (family), Notch, P38 MAPK, p70 S6k, Pro-inflammatory Cytokine, Rap1, Rsk, Serine Protease, Sod, SRC (family), STAT5a/b, **TNF**, TSH, U1 snRNP	1.00E-05

Functional networks were studied by importing Entrez Gene IDs of 14 up-regulated and 2 down-regulated cytokines in IL-1β/TNF-α-activated human astrocytes into the core analysis tool of IPA. They are listed with functional networks, focused molecules, and p-value of the Fisher's exact test. The first rank network is illustrated in Fig. 4.
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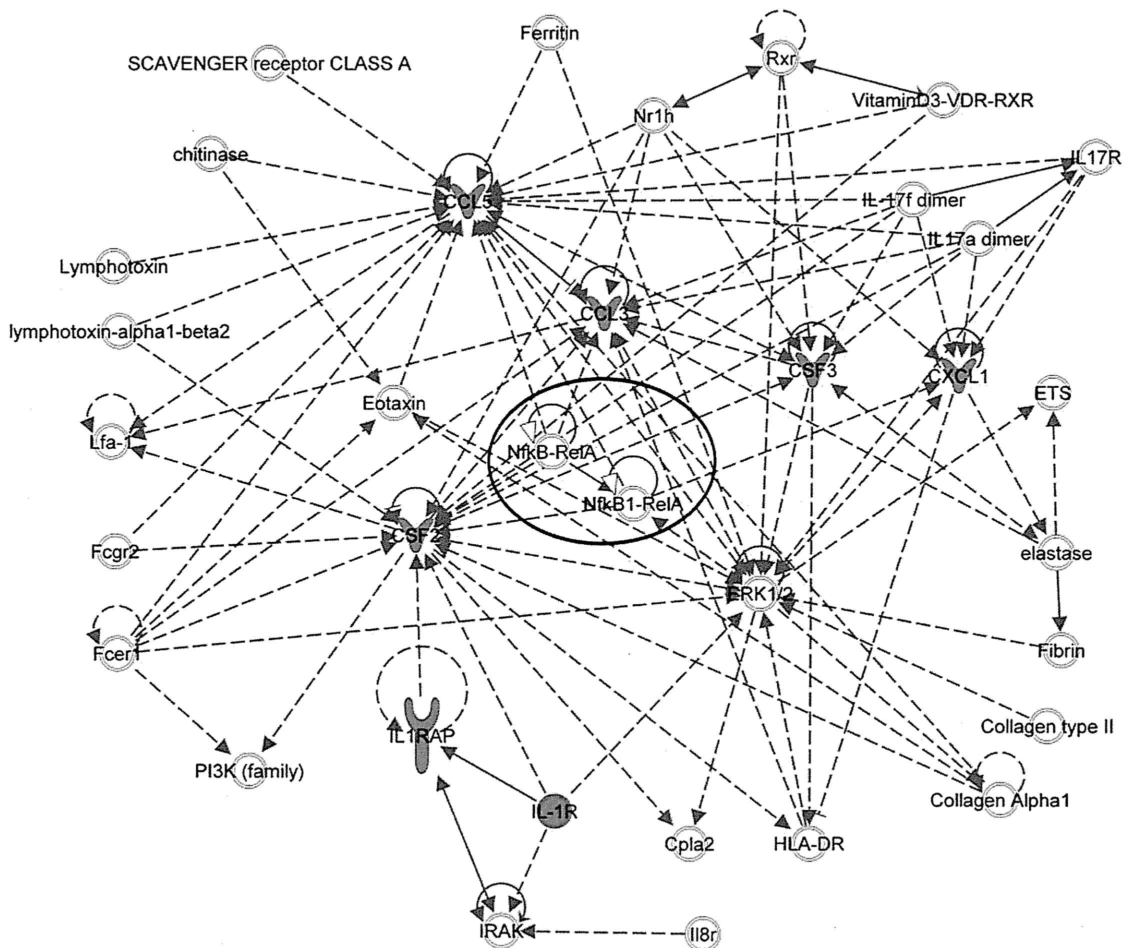

Figure 4. Molecular network showing cytokine and chemokine secretomes of human astrocytes. Entrez Gene IDs corresponding to 14 upregulated and 2 downregulated cytokines in IL-1β/TNF-α-activated human astrocytes were imported into the Ingenuity Pathways Analysis (IPA). The most significant molecular network relevant to the imported genes (red arrows) is shown. NF-kB is highlighted by blue circle. CCL5 (RANTES), CCL3 (MIP-1α), CXCL1 (GROα), CSF2 (GM-CSF), CSF3 (G-CSF), IL1R and IL1RAP are indicated by red.
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Author Contributions

Conceived and designed the experiments: HJL SUK. Performed the experiments: SSC HJL. Analyzed the data: SSC HJL JIS SUK. Contributed reagents/materials/analysis tools: IL JIS. Wrote the paper: SSC HJL JIS SUK.

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Research Article

Molecular Network of NLRP3 Inflammasome Activation-Responsive Genes in a Human Monocyte Cell Line

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Abstract

Background: Inflammasome, activated by pathogen-derived and host-derived danger signals, constitutes a multimolecular signaling complex that serves as a platform for caspase-1 (CASP1) activation and interleukin-1β (IL-1β) maturation. The activation of NLRP3 inflammasome requires two-step signals. The first "priming" signal enhances gene expression of inflammasome components. The second "activation" signal promotes the assembly of inflammasome components. Deregulated activation of NLRP3 inflammasome contributes to the pathological processes of Alzheimer's disease (AD) and multiple sclerosis (MS). However, at present, the precise mechanism regulating NLRP3 inflammasome activation and deactivation remains largely unknown.

Methods: By genome-wide gene expression profiling, we studied the molecular network of NLRP3 inflammasome activation-responsive genes in a human monocyte cell line THP-1 sequentially given two-step signals.

Results: We identified the set of 83 NLRP3 inflammasome activation-responsive genes. Among them, we found the NR4A nuclear receptor family NR4A1, NR4A2, and NR4A3, the EGR family EGR1, EGR2, and EGR3, the IκB family NFKBIZ, NFKBID, and NFKBIA as a key group of the genes that possibly constitute a negative feedback loop for shutting down inflammation following NLRP3 inflammasome activation. By molecular network analysis, we identified a complex network of NLRP3 inflammasome activation-responsive genes involved in cellular development and death, and immune and inflammatory responses, where transcription factors AP-1, NR4A, and EGR serve as a hub.

Conclusion: NLRP3 inflammasome activation-responsive genes constitute the molecular network composed of a set of negative feedback regulators for prompt resolution of inflammation.

Keywords: Inflammasome; NLRP3; NR4A1; NR4A2; NR4A3

Introduction

Inflammasome serves as a multi molecular signaling complex involved in activation of caspase-1 (CASP1) and maturation of interleukin-1β (IL-1β) and IL-18 [1,2]. A wide variety of exogenous and endogenous stimuli, characterized by microbe-derived pathogen-associated molecular patterns (PAMPs) and host- or environment-derived danger-associated molecular patterns (DAMPs), are recognized by an intracellular sensor called the NOD-like receptors (NLRs), resulting in rapid induction of inflammasome formation by ordered assembly of self-oligomerizing components.

Among various classes of inflammasome, the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) inflammasome has been most intensively studied. It is composed of NLRP3, the adaptor molecule named apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the precursor form of the cysteine protease pro-CASP1 [1,2]. NLRP3 contains a central nucleotide-binding and oligomerization (NACHT) domain essential for activation of the signaling complex via ATP-dependent oligomerization, flanked by a C-terminal leucine-rich repeat (LRR) pivotal for ligand sensing and autoregulation and a N-terminal pyrin (PYD) domain involved in a homotypic protein-protein interaction between NLRP3 and ASC. The molecular interaction of NLRP3 with ASC recruits pro-CASP1 by a homotypic interaction of caspase activation and recruitment (CARD) domains between ASC and pro-CASP1. Subsequently, the proximity-induced pro-CASP1 oligomerization causes autocatalytic activation of CASP1, resulting in processing of pro-IL-1β or pro-IL-18 into biologically active IL-1β and IL-18. Both of them act as a central regulator for induction of cytokines and chemokines that amplify inflammation by recruiting immune effector cells.

The activation of NLRP3 inflammasome requires two-step signals (Figure 1) [3,4]. The first "priming" signal termed Signal 1, such as microbe-derived lipopolysaccharide (LPS), enhances gene expression of inflammasome components and target proteins via activation of transcription factor nuclear factor-kappa B (NF-κB). The second "activation" signal termed Signal 2 promotes the organized assembly of inflammasome components. The second signal involves three major mechanisms, such as generation of reactive oxygen species (ROS), lysosomal protease leakage, and the potassium efflux [1,2]. Mitochondria often serve as the principal source of ROS. Blockade of mitophagy induces accumulation of ROS-generating mitochondria that activates NLRP3 inflammasome [5]. Furthermore, oxidized

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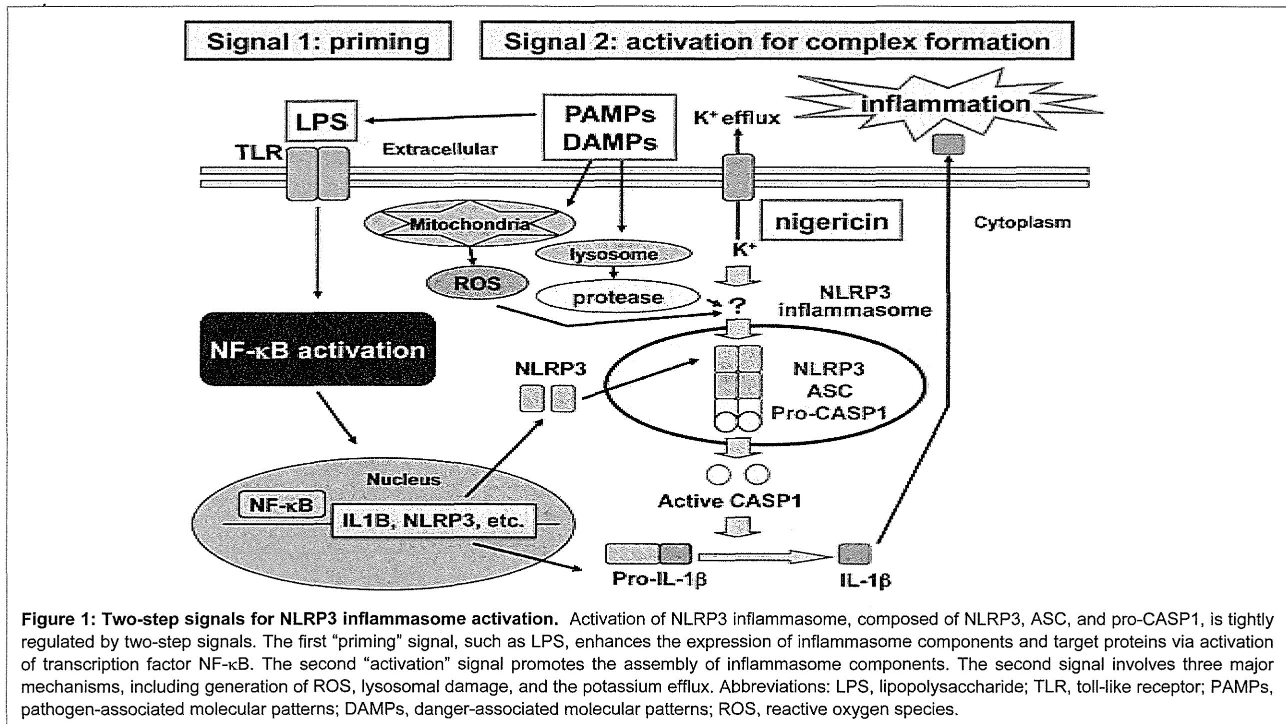

Figure 1: Two-step signals for NLRP3 inflammasome activation. Activation of NLRP3 inflammasome, composed of NLRP3, ASC, and pro-CASP1, is tightly regulated by two-step signals. The first "priming" signal, such as LPS, enhances the expression of inflammasome components and target proteins via activation of transcription factor NF-κB. The second "activation" signal promotes the assembly of inflammasome components. The second signal involves three major mechanisms, including generation of ROS, lysosomal damage, and the potassium efflux. Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ROS, reactive oxygen species.

mitochondrial DNA directly activates NLRP3 inflammasome following induction of apoptosis [6]. By serving as an inducer of two-step signals, a diverse range of danger signals armed with PAMPs, such as *Listeria monocytogenes*, *Candida albicans*, and influenza A virus and those with DAMPs, such as amyloid-β (Aβ), uric acid and cholesterol crystals, asbestos, silica, alum, hyaluronan, and adenosine 5'-triphosphate (ATP), promptly activate the NLRP3 inflammasome [7,8].

Deregulated activation of NLRP3 inflammasome contributes to the pathological processes of various diseases, such as type 2 diabetes, Alzheimer's disease (AD), and multiple sclerosis (MS) [9-11]. Lack of NLRP3 inflammasome components skews microglial cells to an anti-inflammatory M2 phenotype with an enhanced capacity of amyloid-β (Aβ) clearance in a mouse model of AD [10]. *Nlrp3*-knockout mice showed reduced severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, characterized by substantial attenuation of inflammation, demyelination and astrogliosis [12]. In active inflammatory demyelinating lesions of MS, reactive astrocytes and perivascular macrophages expressed all three components of NLRP3 inflammasome, such as NLRP3, ASC, and CASP1, along with IL-1β, suggesting that biochemical agents and monoclonal antibodies designed to block specifically NLRP3 inflammasome activation might be highly effective in treatment of active MS [11]. However, at present, the precise mechanism regulating NLRP3 inflammasome activation and deactivation remains largely unknown. In the present study, by genome-wide gene expression profiling, we attempts to clarify the comprehensive molecular network of NLRP3 inflammasome activation-responsive genes in a human monocyte cell line given consecutively two-step signals.

Materials and Methods

NLRP3 inflammasome activation

A human monocyte cell line THP-1 was obtained from RIKEN Cell Bank (Saitama, Japan). The cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 55 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (feeding medium). To load the Signal 1, the cells were incubated for 3 hours with or without 0.2 μg/ml lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA). To load the Signal 2, they were washed twice by Phosphate-Buffered Saline (PBS) and incubated further for 0.5 or 2 hours with 10 μM nigericin sodium salt (Wako Pure Chemical, Osaka, Japan) dissolved in ethanol or the equal v/v% concentration of ethanol (vehicle). Then, protein extract of the cells was processed for western blot analysis with a rabbit antibody against the C-terminal peptide of the human CASP1 p10 protein (sc-515, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit antibody against the peptide mapping at amino acid residues of 117-269 of the human IL-1β protein (sc-7884, Santa Cruz Biotechnology).

Microarray analysis

Total cellular RNA was isolated by using the TRIZOL plus RNA Purification kit (Invitrogen). The quality of total RNA was evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Three hundred ng of total RNA was processed for cDNA synthesis, fragmentation, and terminal labeling with the GeneChip Whole Transcript Sense Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). Then, the labeled cRNA was processed for hybridization at 45°C for 17 hours with Human Gene 1.0 ST Array (28,869 genes; Affymetrix). The arrays were washed in the

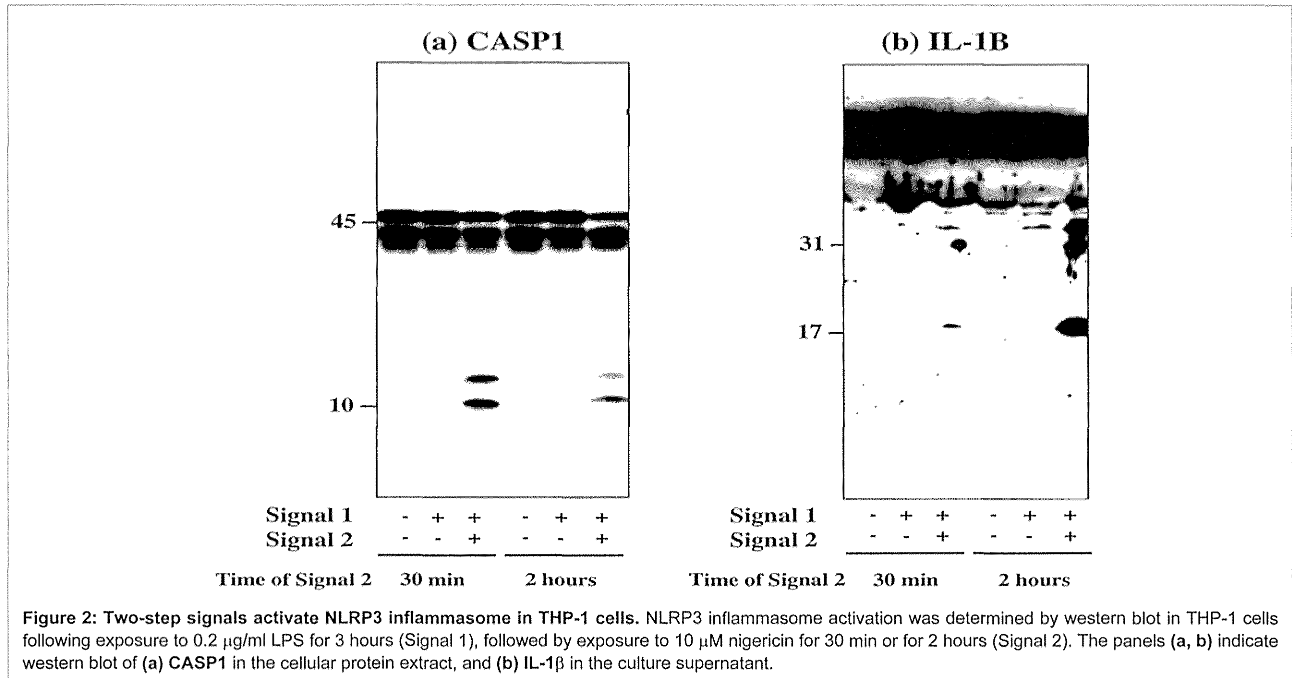

Figure 2: Two-step signals activate NLRP3 inflammasome in THP-1 cells. NLRP3 inflammasome activation was determined by western blot in THP-1 cells following exposure to 0.2 μg/ml LPS for 3 hours (Signal 1), followed by exposure to 10 μM nigericin for 30 min or for 2 hours (Signal 2). The panels **(a, b)** indicate western blot of **(a) CASP1** in the cellular protein extract, and **(b) IL-1β** in the culture supernatant.

Gene Chip Fluidic Station 450 (Affymetrix), and scanned by the Gene Chip Scanner 3000 7G (Affymetrix). The raw data were expressed as CEL files and normalized by the Robust Multi Array average (RMA) method with the Expression Console software (Affymetrix).

Quantitative reverse transcription (RT)-polymerase chain reaction (qPCR) analysis

DNase-treated total RNA isolated from THP-1 cells was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and Super Script II reverse transcriptase (Invitrogen). Then, cDNA was amplified by PCR in Light Cycler ST300 (Roche Diagnostics, Tokyo, Japan) using SYBR Green I and a panel of sense and antisense primer sets following: 5'ccagcactgccaaactggactact3' and 5' acagctcagcaaagccagggatct3' for an 162 bp product of nuclear receptor subfamily 4, group A, member 1 (NR4A1); 5'ccaaagccgaccaagacctgcttt3' and 5'ctgtgcaagaccaccccattgcaa3' for an 124 bp product of nuclear receptor subfamily 4, group A, member 2 (NR4A2); 5'gagggctgcaagggctttttcaag3' and 5' gagggctgagaaggttcctgttgt3' for a 242 bp product of nuclear receptor subfamily 4, group A, member 3 (NR4A3); and 5'ccatgttcgtcatgggtgtgaacca3' and 5'gccagtagaggcagggatgatgttc3' for a 251 bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene that serves as an endogenous control. The expression levels of target genes were standardized against the levels of G3PDH detected in the corresponding cDNA samples. All the assays were performed in triplicate.

Molecular network analysis

To identify biologically relevant molecular networks, we imported corresponding Entrez Gene IDs into Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA), KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan), or Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 9.1. STRING is an open-access database, while IPA and KeyMolnet are commercial resources.

STRING is a database that contains known and predicted, physiological and functional protein-protein interactions composed of 5,214,234 proteins from 1133 organisms [13]. STRING integrates the information from numerous resources, including experimental repositories, computational prediction methods, and public text collections. By uploading the list of UniProt IDs or Gene Symbols, STRING illustrates the union of all possible association networks.

IPA is a knowledgebase that contains approximately 3,000,000 biological and chemical interactions and functional annotations with definite scientific evidence. By uploading the list of Gene IDs and expression values, the network-generation algorithm identifies focused genes integrated in a global molecular network. IPA calculates the score p-value that reflects the statistical significance of association between the genes and the networks by the Fisher's exact test.

KeyMolnet contains knowledge-based contents on 164,000 relationships among human genes and proteins, small molecules, diseases, pathways and drugs [14]. They include the core contents collected from selected review articles with the highest reliability. By importing the list of Gene ID and expression values, KeyMolnet automatically provides corresponding molecules as nodes on the network. The neighboring network-search algorithm selected one or more molecules as starting points to generate the network of all kinds of molecular interactions around starting molecules, including direct activation/inactivation, transcriptional activation/repression, and the complex formation within one path from starting points. The generated network was compared side by side with 501 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the

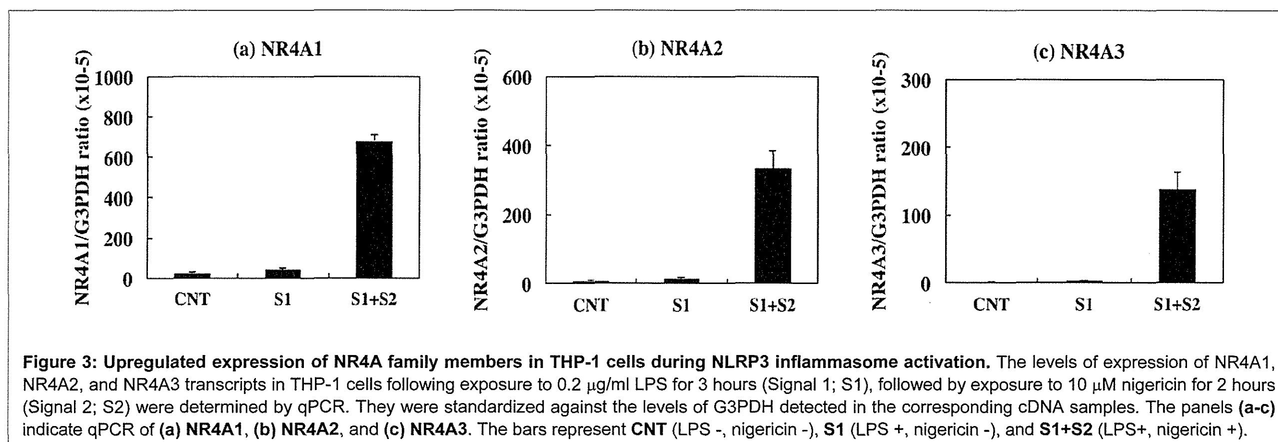

Figure 3: Upregulated expression of NR4A family members in THP-1 cells during NLRP3 inflammasome activation. The levels of expression of NR4A1, NR4A2, and NR4A3 transcripts in THP-1 cells following exposure to 0.2 µg/ml LPS for 3 hours (Signal 1; S1), followed by exposure to 10 µM nigericin for 2 hours (Signal 2; S2) were determined by qPCR. They were standardized against the levels of G3PDH detected in the corresponding cDNA samples. The panels **(a-c)** indicate qPCR of **(a) NR4A1**, **(b) NR4A2**, and **(c) NR4A3**. The bars represent **CNT** (LPS -, nigericin -), **S1** (LPS +, nigericin -), and **S1+S2** (LPS+, nigericin +).

extracted network.

Results

NLRP3 inflammasome activation in THP-1 cells following introduction of two-step signals

First, by western blot analysis, we studied NLRP3 inflammasome activation in THP-1 treated initially with exposure to 0.2 µg/ml LPS for 3 hours (Signal 1), followed by exposure to 10 µM nigericin for 30 min or 2 hours (Signal 2). The consecutive load of Signal 1 and Signal 2 markedly activated NLRP3 inflammasome in THP-1 cells, as indicated by production of cleaved products of CASP1 (Figure 2, panel a) and IL-1β (Figure 2, panel b). In contrast, the introduction of Signal 1 alone was not enough to activate NLRP3 inflammasome in THP-1 cells (Figure 2, panels a and b).

Gene expression profile during NLRP3 inflammasome activation

Next, we studied the genome-wide gene expression profile of THP-1 cells pretreated with 0.2 µg/ml LPS for 3 hours (Signal 1), washed by PBS, and exposed to 10 µM nigericin or vehicle for 2 hours (Signal 2). Then, total RNA was immediately processed for gene expression profiling on a Human Gene 1.0 ST Array. To identify NLRP3 inflammasome activation-responsive genes, we extracted the set of 83 annotated and protein-coding genes that satisfied fold change (FC) in Signal 1 (the presence of LPS versus the absence of LPS) smaller than 2-fold and FC in Signal 2 (the presence of nigericin versus the absence of nigericin) greater than 2-fold (Table 1). This gene enrichment procedure minimized the genes that were activated simply by exposure to LPS alone but not directly related to NLRP3 inflammasome activation.

Most notably, three members of NR4A nuclear receptor family, such as NR4A1 (NUR77), NR4A2 (NURR1), and NR4A3 (NOR1), were identified as those ranked within top 10 genes. Coordinated up regulation of NR4A1, NR4A2, and NR4A3 in NLRP3 inflammasome-activated THP-1 cells was validated by qPCR (Figure 3, panels a-c). Signal 1 alone mildly elevated expression of these mRNA levels, whereas introduction of Signal 2 after Signal 1 markedly elevated the levels of NR4A1, NR4A2, and NR4A3 transcripts with a 16-fold, 25-fold, or 51-fold increase, respectively. We also identified early growth response (EGR) family members, such as EGR1, EGR2, and EGR3, which belong to a family of zinc finger transcription factors involved in the regulation of cell growth, differentiation, and survival, NF-κB inhibitor (IκB) family members, such as NFKBIZ, NFKBID, and NFKBIA, along with a panel of pro inflammatory cytokines and chemokines, including CCL3, CCL3L3, IL8, CXCL2, CCL20, IL23A, and TNFSF9, as a subgroup of NLRP3 inflammasome activation-responsive genes.

Molecular network of NLRP3 inflammasome activation responsive genes

Next, by using three different bioinformatics tools for molecular network analysis based on knowledgebase, we studied biologically relevant molecular networks for the set of 83 NLRP3 inflammasome activation-responsive genes in THP-1 cells. The core analysis of IPA identified the networks defined as "Auditory and Vestibular System Development and Function, Embryonic Development, Organ Development" (p = 1.00E-32), "Cell Cycle, Cellular Development, Cell Death and Survival" (p = 1.00E-30) (Figure 4), and "Connective Tissue Disorders, Immunological Disease, Inflammatory Disease" (p = 1.00E-26) as top three most relevant functional networks. These results suggest that NLRP3 inflammasome activation-responsive genes play a pivotal role in cell development, death, and immune and inflammatory responses. KeyMolnet by the neighboring network-search algorithm operating on the core contents extracted the highly complex molecular network composed of 455 molecules and 529 molecular relations. The network showed the most statistically significant relationship with canonical pathways termed as "transcriptional regulation by AP-1" (p = 3.82E-184), "transcriptional regulation by NR4A" (p = 2.28E-105), and "transcriptional regulation by EGR" (p = 2.78E-99) (Figure 5). These results suggest a central role of transcription factors AP-1, NR4A, and EGR in regulation of expression of NLRP3 inflammasome activation-responsive genes, by acting as a hub of the molecular network.

Finally, STRING extracted a protein-protein interaction network, composed of 35 core molecules derived from the set of 83 NLRP3 inflammasome activation-responsive genes in THP-1 cells. In this network, both the set of NR4A family members NR4A1, NR4A2, and NR4A3 and EGR transcription factors EGR1, EGR2, and EGR3 constituted a close and intense protein interaction subnetwork (Figure 6).