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Functional proteomics revealed IL-1β amplifies TNF downstream protein signals in human synoviocytes in a TNF-independent manner

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ABSTRACT

IL-1β is readily detectable in numerous joint inflammations. It can change the transcriptomic signature of fibroblast-like synoviocytes (FLS) of arthritis toward promoting migration and invasion that are relevant to arthritis progression. We hypothesize that IL-1β partially contributes to the onset of osteoarthritis (OA). We compared the tissue samples from OA and fracture subjects and found that IL-1β expression was significantly higher in the OA synovium, while TNF-α expression showed no significance. We demonstrated that IL-1β significantly increases the IL-6 and IL-8 secretions of human normal FLS; however, IL-1β does not induce TNF secretion. With metabolic labeling based proteomics and pathway analysis, we found that IL-1β significantly increases the TNF downstream protein expression in FLS even with complete absence of TNF and/or blocking of the NF-κB pathway. Among these proteins, we verified that p62 can differentiate the OA from fracture synovitis. In conclusion, we demonstrated that IL-1β can amplify the TNF downstream protein signals in human synoviocytes in a TNF-independent manner; in addition, p62 is a potential FLS biomarker for synovitis.

1. Introduction

Over the past three decades, the dogma of innate and adaptive immunology has been challenged as numerous mesenchymal cells are gradually known to play critical roles in immune responses. For example, in brain inflammation, the transformation of astrocytes from passive to inflammatory states has been identified as an essential driving force of neuronal cell death in HIV-1 associated neurodegenerative disorders [1,2] and Parkinson's and Alzheimer's diseases (reviewed in Ref. [3]). Similarly, fibroblasts have been redefined as immune cells, with recognition of their unique role in the inflaming tumor microenvironment (reviewed in Ref. [4]) and numerous immune disorders. Specifically with regard to joint inflammatory diseases, fibroblast-like synoviocytes (FLS), which share many characteristics with fibroblasts, can function as autoantigen and proinflammatory cytokine producers and antigen-presenting cells, thus highly involve in rheumatoid arthritis (RA) [5,6] (reviewed in Ref. [7]) and osteoarthritis (OA) [8,9].

Early studies have associated FLS transformation with recruited leukocytes that release cytokines and chemokines *in situ* and in plasma, such as TNF-α, IL-1β and IL-6 (reviewed in Ref. [10]). In return, activated FLS produce more inflammation mediators, including metalloproteinases, IL-6 and IL-8, to stimulate the activation of T-cells, B-cells and mononuclear phagocytes (MPs) [11]. If this crosstalk remains unrestrained, a sustainably amplified inflammation environment will be formed, leading to detrimental and progressive joint destruction. You et al. recently found key regulators of migration and invasion in RA-FLS by using an IL-1β stimulation model and transcriptomics analysis. Indeed, there is quite limited information available to date regarding how normal FLS respond to IL-1β, leading us to hypothesize that IL-1β serves as a causal factor of the onset of OA. To this end, using human normal FLS and employing a functional proteomic strategy should be useful to move the field forward.

In this study, we obtained clinical synovium samples from subjects with either OA or fracture synovitis and compared their IL-1β and TNF expression. We then established and characterized an *in vitro* investigative model by treating human normal FLS with

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IL-1β, followed by metabolic labeling based comparative proteomics, pathway analyses as well as biological and clinical verifications. We determined a novel role of IL-1β in the activation of FLS and identified p62 as a potential biomarker of FLS to differentiate the OA and fracture synovitis.

2. Materials and methods

2.1. Human synovial tissue samples

The scientific and ethics review committees of the Sun Yat-Sen University approved this study, and written informed consents were obtained from all of the study participants. Synovial tissue samples from 10 subjects were obtained from the Sun Yat-Sen University Hospital between 2012 and 2013. Among them, 5 subjects were diagnosed with OA and sampled upon synovectomy. For comparison, another 5 subjects, who had no history of RA, OA or other arthropathies diagnosed by arthroscopy, were subjected to synovial tissue isolation during surgical operation for their condylar fracture therapy.

2.2. Immunohistochemical analysis

Synovial tissue specimens were fixed with 4% paraformaldehyde in PBS, dehydrated and paraffin-embedded. For immunohistochemical analysis, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was then blocked with 3.0% hydrogen peroxide in methanol, followed by primary antibody incubation, washes and development by using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Mouse anti-IL-1β mAb (1:100; ORIGENE, Beijing, China), rabbit anti-TNF-α pAb (1:100; Sino Biological Inc., Beijing, China), mouse anti-SQSTM1 (p62) mAb (1:100; Santa Cruz, Shanghai, China) and rabbit anti-SOD2 pAb (1:800; Sino Biological Inc.) were used for these analyses. Sections were further counterstained with Meyer's hematoxylin. We then acquired images by using a Nikon 90i light microscope (Nikon, Japan). Integrated optical density (IOD) per staining area was analyzed by using the Image-Pro Plus software version 6.0 (Media Cybernetics, Silver Spring, USA).

2.3. Cell culture and treatment

Human primary normal FLS, purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), were maintained in the complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Culture surfaces were treated with poly-L-lysine (Sigma, Shanghai, China) at 2 μg/cm^2. FLS were only used between passage 3 and 8 due to the finite proliferation potential. Regarding stimulation, FLS were treated with IL-1β (Life Technologies) at 10 ng/mL for either 6 h or 24 h before subsequent analyses. Other reagents included the NF-κb inhibitor BAY 11-7082 (BAY; Beyotime, Jiangsu, China) and TNF-α (R&D, Shanghai, China).

2.4. LDH cytotoxicity detection

FLS were subjected to cytotoxicity detection by quantitating the lactate dehydrogenase (LDH) activity in culture supernatants, using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche, Shanghai, China) and following the manufacturer's instructions.

2.5. Cytometric bead array (CBA) analysis

We employed CBA to measure the supernatant concentration of IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70 by a Human Inflammatory Cytokine Kit (BD, Guangzhou, China), as previously described with minor modifications [12]. Post IL-1β stimulation, FLS were cultured in serum-free DMEM for 24 h before the supernatant was collected. Supernatants were centrifuged at 250×*g* for 5 min to remove residual cells, followed by the capture bead and detection antibody treatments. Samples were analyzed by using an Accuri C6 flow cytometer (BD) and FCAP Array™ software (BD, version 3.0.1).

2.6. Metabolic labeling based mass spectrometry

FLS were labeled by using Pierce® SILAC Protein Quantitation Kit (Pierce Biotechnology, Rockford, IL) as described previously with minor modifications [13]. Briefly, cells were cultured in "heavy" medium containing $^{13}C_6$ L-Lysine-2HCl for 6 passages prior to IL-1β stimulation, whereas untreated cells were labeled with "light" medium containing $^{12}C_6$ L-Lysine-2HCl. Cells were lysed with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM PMSF. Proteins obtained from 3 independent experiences were pooled. Equal amounts (90 μg) of heavy and light protein were mixed and subjected to SDS–PAGE separation, in-gel trypsin digestion and analysis by a Thermo LTQ-Orbitrap mass spectrometer, followed by database search and protein quantification by MaxQuant software 1.2.2.2 as we described previously [13].

2.7. Ingenuity pathway analysis (IPA)

We employed the Core Analysis module of IPA to identify the top bioprocesses and upstream activators as previously described [13,14]. In addition, we used Pathway Building module to analyze the mechanistic network and its association with IL-1β, IL-6 and IL-8.

2.8. Immunoblotting

The immunoblotting assay was performed as previously described with minor modifications [14]. Primary Abs included mouse anti-SQSTM1 (p62) mAb (1:1000, Santa Cruz), rabbit anti-NFKB2 pAb (1:1000, Sino Biological Inc.) and mouse anti-β-actin mAb (1:2000, Bioworld, St. Louis Park, MN, USA). Secondary Abs included HRP-conjugated goat anti-rabbit mAb (1:3000, Bioworld) and HRP-conjugated anti-mouse mAb (1:3000, Bioworld).

2.9. Superoxide dismutase (SOD) activity assay

We determined the SOD activity by using a detection kit (Beyotime), following the manufacturer's instructions.

2.10. Statistics

Statistical significance was examined by Kolmogorov–Smirnov test (KS-test) or one-way ANOVA with Bonferroni *post hoc* multiple comparisons, using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Data were shown as mean ± s.e.m. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Synovium IL-1β and TNF-α expression

To differentiate the inflammatory characteristics of OA and fracture synovium, we immunohistochemically analyzed the IL-1β and TNF-α expression employing clinical samples (Fig. 1). Morphologically, we observed remarkably synovial stratum hyperplasia in the OA samples, while the fracture samples exhibited normal-like

synovium structures (Fig. 1A and B). Statistically, IL-1β expression in OA synovium was significantly higher than that in the fracture samples (Fig. 1C). To be noted, the two groups have no significant difference in TNF-α expression (Fig. 1D).

3.2. IL-1β mediated cytokine production in human normal FLS

With IL-1β treatment, FLS showed remarkably different cytokine secretion profile from the untreated group (Fig. 2A). Statistically, IL-1β significantly increased IL-6 and IL-8 secretions in the IL-1β 6 h group (6 h group) and 24 h groups (Fig. 2B and C). The two cytokines were undetectable in the untreated group (Fig. 2B and C). To be noted, we observed no detectable TNF secretion in all of the experimental groups. In addition, no significantly different supernatant LDH activities were detected comparing the untreated group with the 6 h and 24 h group (Fig. 2D), indicating that IL-1β treatment in this study had no cytotoxicity effects on FLS.

3.3. IL-1β-induced FLS proteome change toward TNF activation

It is interesting that IL-1β treatment does not change the TNF secretion. We next performed SILAC-based proteomics to explore the molecular signatures of IL-1β induced proteome alterations of human normal FLS.

We identified and quantified 1980 and 2053 confident proteins that were matched with at least 2 unique peptides with the protein/peptide false discovery rate less than 1% in the 6 h group (Supplementary Table 1) and the 24 h group (Supplementary Table 2), respectively. The original mass spectrometry data were deposited in the PeptideAtlase database (http://www.peptideatlas.org/) with the accession number of PASS00488. We detected 104 differentially expressed proteins (DEPs) with at least 1.5-fold changes in the 6 h group, comparing the IL-1β-treated with the untreated FLS (Supplementary Table 3). While in the 24 h group, 63 such DEPs were identified (Supplementary Table 4).

The core analysis of IPA suggested that DEPs of the 6 h group focused on 19 sub-bioprocesses of Cell Death and Differentiation as well as 18 sub-bioprocesses of Cellular Movement (Fig. 3A). In addition to the two Category I bioprocesses, DEPs of the 24 h group implicated specific activation of 10 sub-bioprocesses of Cellular Growth and Proliferation (Fig. 3B). We next used the upstream analysis of IPA to deduce the possible activators of these DEPs. To our surprise, TNF pathway was significantly activated (z-score = 2.926) in the 6 h group as indicated by 17 DEPs, while the IL-1β pathway proteins remained unchanged at this time point (Fig. 3C). In the 24 h group, we observed the IL-1β pathway activation (z-score = 2.560), in addition to the TNF pathway activation (z-score = 2.465) (Fig. 3D).

These results suggested a new role of IL-1β to activate TNF pathway in FLS even with TNF absence. We then used the pathway building tool of IPA to confirm whether this is a novel concept to the field. This tool finds any direct and indirect connections between nodes based on current knowledgebase of IPA. We input the mechanistic network of TNF pathway (10 molecules in the 24 h group, Fig. 3D), in addition to IL-6 and IL-8 that were detected by CBA as well as IL-1β (Fig. 3E). By using the Connect command for these nodes, we found that IL-1β has connections with its commonly known targets of IL-6, IL-8, TNF and SOD2 (Fig. 3E), serving as a positive control of the analysis. However, IL-1β has no direct or indirect connections with at least 7 proteins in the TNF network (Fig. 3E). This suggests that there is no report showing that IL-1β has any associations with such proteins according to the knowledgebase. We manually verified this by literature searching to avoid incomplete inclusion of the IPA knowledgebase. We noted a very recent finding showing that IL-1β induces the p62 up-regulation in human macrophages [15].

3.4. Biological and clinical verification

This brought p62 into our attention. If it is the target of both IL-1β and TNF in the context of inflammation, we posit that it is an amplified biomarker in OA synovitis as compared to the

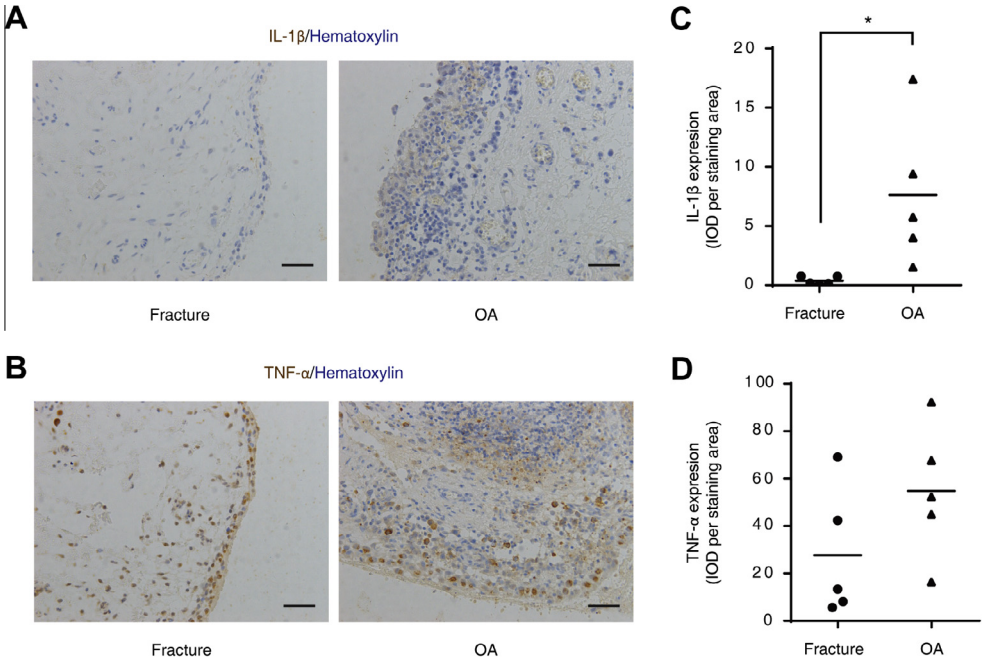


Fig. 1. IL-1β and TNF-α expression in FLS of human tissue sections. (A and B) Representative immunohistochemical images of IL-1β (A) and TNF-α (B) staining in synovial tissues from fracture and OA patients, respectively. Scale bar = 40 μm. (C and D) Statistical analyses of synovial IL-1β (C) and TNF-α (D) expression in comparison of the fracture and OA groups. The mean IOD of each group is shown with a horizontal line. *P = 0.0079, donor n = 5, two-tailed Kolmogorov–Smirnov test.

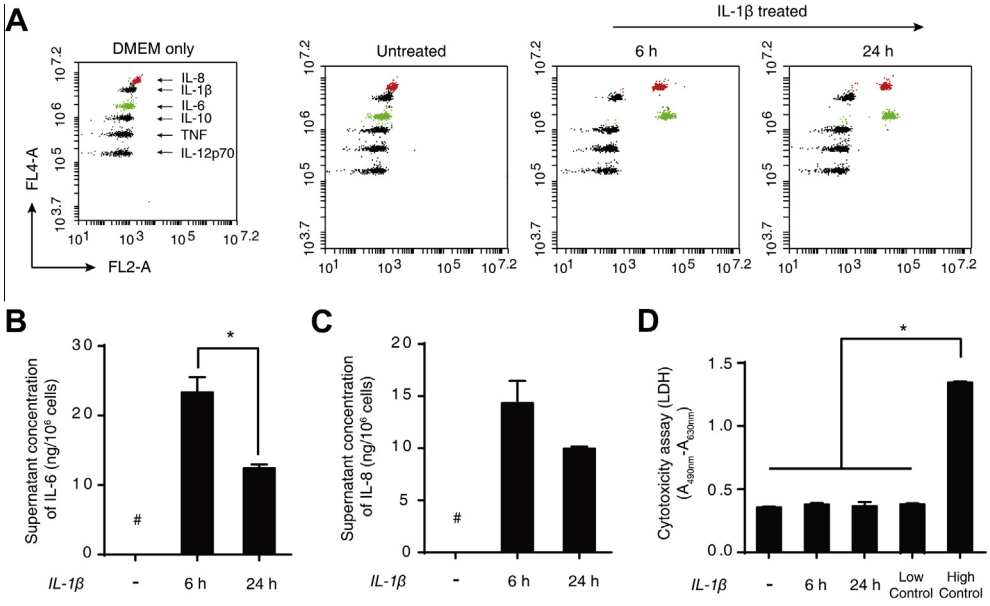


Fig. 2. Characteristics of IL-1β induced FLS activation. (A) CBA assay of human inflammatory cytokines in supernatant by flow cytometry. (B) Statistical comparison of IL-6 concentration in supernatant. $^{\#}P < 0.05$, compared with all of the other groups; $^{*}P = 0.0027$. (C) Statistics of IL-8 concentration in supernatant. $^{\#}P < 0.05$, compared with all of the other groups. (D) Cytotoxicity assay of IL-1β stimulated FLS. Low control indicates the background absorbance of the RPMI 1640 medium; high control refers to the total LDH activity in the FLS lysates. $^{*}P < 0.001$. All data shown in this figure were obtained from 3 independent experiments.

fracture synovitis. Addressing this question will deliver further evidence of IL-1β-induced TNF pathway activation with the absence of TNF.

In this regard, we included three more controls for the *in vitro* studies: BAY, neutralizing anti-TNF-α pAb (TNF pAb) and TNF-α, either used alone or in combinations (Fig. 4A). No cytotoxicity was observed for FLS in any of the experimental groups (Fig. 4A). Consistent with the proteomics results (Fig. 3E), we reproduced the IL-1β-mediated p62 and NFKB2 up-regulations (Fig. 4B), in addition to the significant SOD2 activity increase (Fig. 4C). We observed that TNF-α stimulation caused p62 and NFKB2 overexpression, which served as positive controls (Fig. 4B). Notably, simultaneously blocking NF-κB and neutralizing TNF-α did not abrogate the p62 up-regulation, favoring the IL-1β's unique role in activating TNF pathway proteins (Fig. 4B).

With clinical samples, we observed remarkably more synovial p62 expression in the OA group than in the fracture group (Fig. 4D), while the SOD2 expression remained virtually unchanged (Fig. 4E). Statistically, the p62 expression in the OA synovium was significantly higher than the fracture group, with approximately 10-fold increase comparing the mean IOD per staining area of the two groups (Fig. 4F). In addition, there was no statistical difference in SOD2 expression (Fig. 4G).

4. Discussion

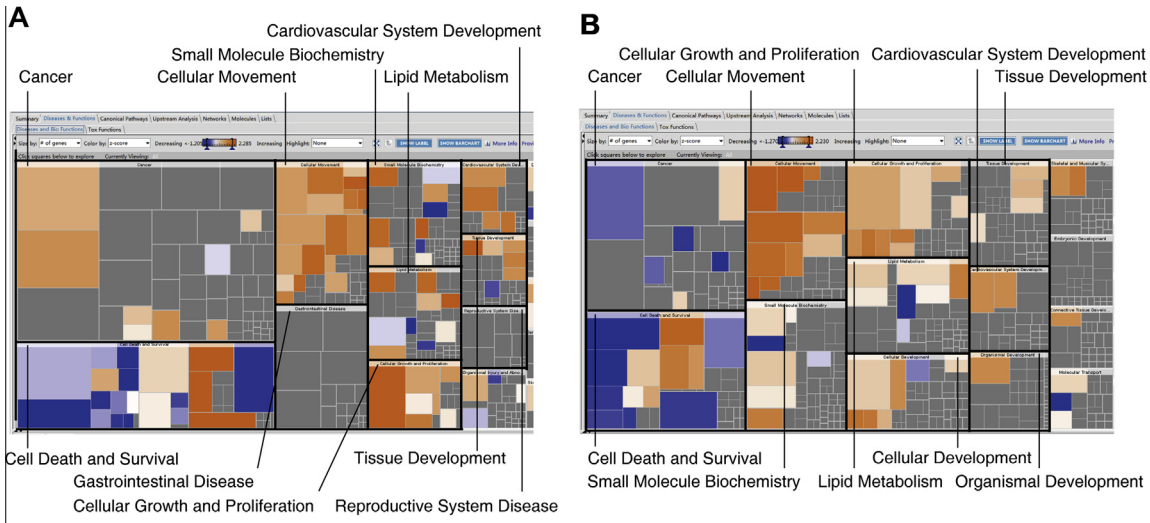
We here report for the first time that IL-1β can mediate the activation of TNF pathway in human normal synoviocytes in a TNF-independent manner. Among the TNF downstream proteins, p62 is a potential biomarker that can differentiate OA synovitis from fracture synovitis. Our findings implicate the potential benefits of anti-IL-1β therapy targeting synoviocytes in joint inflammatory disorders.

Indeed, the anti-TNF therapy has been well-documented since 1999 to show acceptable long-term efficacy, but with concerns [16–19]. TNF-α is a potent inducer of the hyperplasia of synovium through NF-κB activation [20] (reviewed in Ref. [21]). Small molecules that block NF-κB activities, such as SB203580 [22] and SPA0355 [23], can efficiently inhibit the TNF-induced inflammation of synoviocytes *in vitro* and *in vivo*. In a ten-year clinical trial study, Keystone et al. recently found that the incorporation of TNF inhibitor adalimumab in methotrexate treatment contributes to long-term benefit with no new safety issues [24]. However, numerous reports concluded that approximately 30% RA patients are refractory or non-responsive to anti-TNF therapies [25–27]. These findings highlighted the significance of an earlier study on the collagen-induced arthritis in the TNF-deficient mouse model, demonstrating that severe arthritis can be developed even in the complete absence of TNF [28]. Here, we demonstrated that inhibiting NF-κB does not suppress the IL-1β-mediated p62 overexpression, suggesting the potential association of the synovial IL-1β with the non-responsiveness of NF-κB-oriented anti-TNF therapies.

Furthermore, we showed that OA FLS have higher IL-1β expression than fracture FLS, while FLS from both tissues have high TNF expression. These results implicated that in an inflammatory joint microenvironment with both IL-1β and TNF, augmentation of IL-1β can amplify the TNF downstream signals in FLS. This emphasizes the important role of IL-1β in initiating the onset and progression of arthritis, which is supported by Pelletier et al., showing that the elevation of IL-1β receptor is highly responsible for early experimental OA [29].

With proteomics and bioinformatics, we observed that IL-1β causes the up-regulation of cell movement and migration regulators in normal FLS, which is consistent with the RA-FLS study by You et al. [30]. In addition, we found that cellular proliferation can be promoted by IL-1β, which is comparable with numerous studies with RA- and OA-FLS [31]. Our results agreed with current knowledge and confirmed the validity of our research model. Favorable to this argument, we indeed computed IL-1β, which was experimentally added, as an upstream regulator of the DEPs identified by proteomics in this study.

The TNF downstream protein amplification theory proposed above partially explains and argues the biomarker potential of p62. Indeed, as a TNF-inducible protein, p62 is a multifunctional factor that plays important roles in the bioprocesses of autophagy



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ADD TO MY PATHWAY | ADD TO MY LIST | CUSTOMIZE TABLE | DISPLAY AS NETWORK | MECHANISTIC NETWORKS | Predicted Activ... Inhibited - (p1 of 4) | More Info

Upstream Regulator	Predicted Activation State	Activation z-score	Target molecules in dataset
IGF2BP1	Inhibited	-2.236	↓COL14A1, ↓COL1A1, ↓COL1A2, ↓COL6A2, ↓COL6A3 ...all 5
INSIG1	Inhibited	-2.236	↑ACSL4, ↑CYP51A1, ↑FADS1, ↑HMGCS1, ↑IDI1 ...all 5
TNF	Activated	2.926	↑ALDH1A3, ↑ATP2B1, ↑CAT, ↓COL1A2, ↓HMOX1, ↑ISG15, ↑KYNU, ↑MCAM, ↓MYLK, ↑NFKB2 ...all 17
AHR	Activated	2.899	↑ACOX1, ↑ATP2B1, ↓COL14A1, ↓COL1A1, ↓COL1A2, ↓COL6A1, ↓COL6A3, ↑CYP1B1, ↓FBLN2, ↑SERPINB2 ...all 10
SREBF1	Activated	2.169	↑ACSL4, ↑CYP51A1, ↑FADS1, ↑HMGCS1, ↓HMOX1, ↑IDI1, ↓PCK2 ...all 7
ESRRA	Activated	2.611	↑ACO1, ↑ACOX1, ↑CYP51A1, ↑FABP3, ↑HMGCS1, ↑IDI1, ↓PCK2 ...all 7
SPDEF	Activated	2.000	↓COL1A1, ↓COL6A1, ↓COL6A2, ↓COL6A3 ...all 4
PPARG	Activated	2.197	↑ACOX1, ↑CAT, ↓COL1A1, ↓COL1A2, ↑FABP3 ...all 5

D

Summary | Diseases & Functions | Canonical Pathways | Upstream Analysis | Networks | Molecules | Lists

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Upstream Regulator	Predicted Activation State	Activation z-score	Target molecules in dataset
IL1B	Activated	2.560	↑AKR1B1, ↓COL1A1, ↓DNMT1, ↑FGF2, ↑PLA2G4A, ↑SERPINB2, ↑SOD2 ...all 7
TNF	Activated	2.465	↑ALDH1A3, ↑ATP2B1, ↑CTSF, ↑NFKB2, ↑SCARB1, ↑SERPINB2, ↑SERPINE2, ↑SOD2, ↑SQSTM1, ↑TGM2 ...all 10
AHR	Activated	2.385	↑ATP2B1, ↓COL1A1, ↓COL6A3, ↓FBLN2, ↓PDGFRB, ↑SERPINB2 ...all 6
IL1A	Activated	2.204	↓ALDH1A1, ↑ALDH1A3, ↑FGF2, ↑PLA2G4A, ↑SOD2 ...all 5
IKBKB	Activated	2.200	↑CTSF, ↑NFKB2, ↑SERPINB2, ↑SERPINE2, ↑SOD2 ...all 5

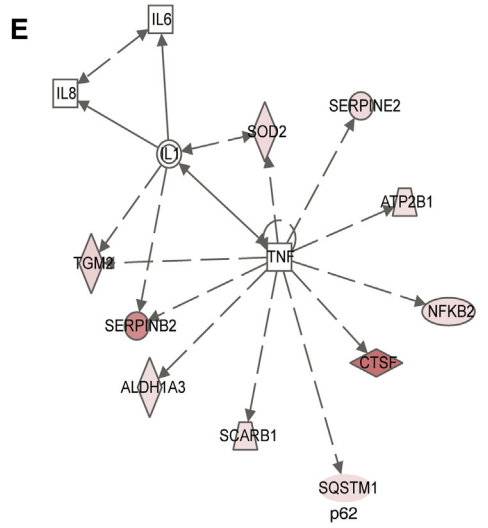

Fig. 3. IPA. (A and B) Heat maps of biological functions regulated by DEPs. FLS were treated by IL-1β for 6 h (A) and 24 h (B), respectively, followed by the proteomics identification of DEPs as compared to the untreated FLS. The top 10 Category Level I bioprocesses are indicated by black blocks. Orange squares represent up-regulated Category Level II bioprocesses with positive *z*-score, whereas down-regulated bioprocesses with negative *z*-score are shown as blue squares. A gray square shows a non-significantly regulated process. The square size is indicative to the number of genes. (C and D) Upstream analysis of DEPs. Upstream regulators were predicted by IPA based on the DEPs of the 6 h (C) and 24 h (D) groups, respectively. Activated (*z*-score > 1.96) and inhibited (*z*-score < −1.96) regulators are highlighted in orange and blue, respectively. (E) Mechanistic network analysis of the TNF pathway in FLS with 24 h IL-1β stimulation. Up-regulated proteins are highlighted in red and the color depth is correlated to the fold change. Solid and dashed lines with arrows indicate direct and indirect effects, respectively. HGNC gene names are used and complete gene games can be found in the Supplementary Tables 3 and 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

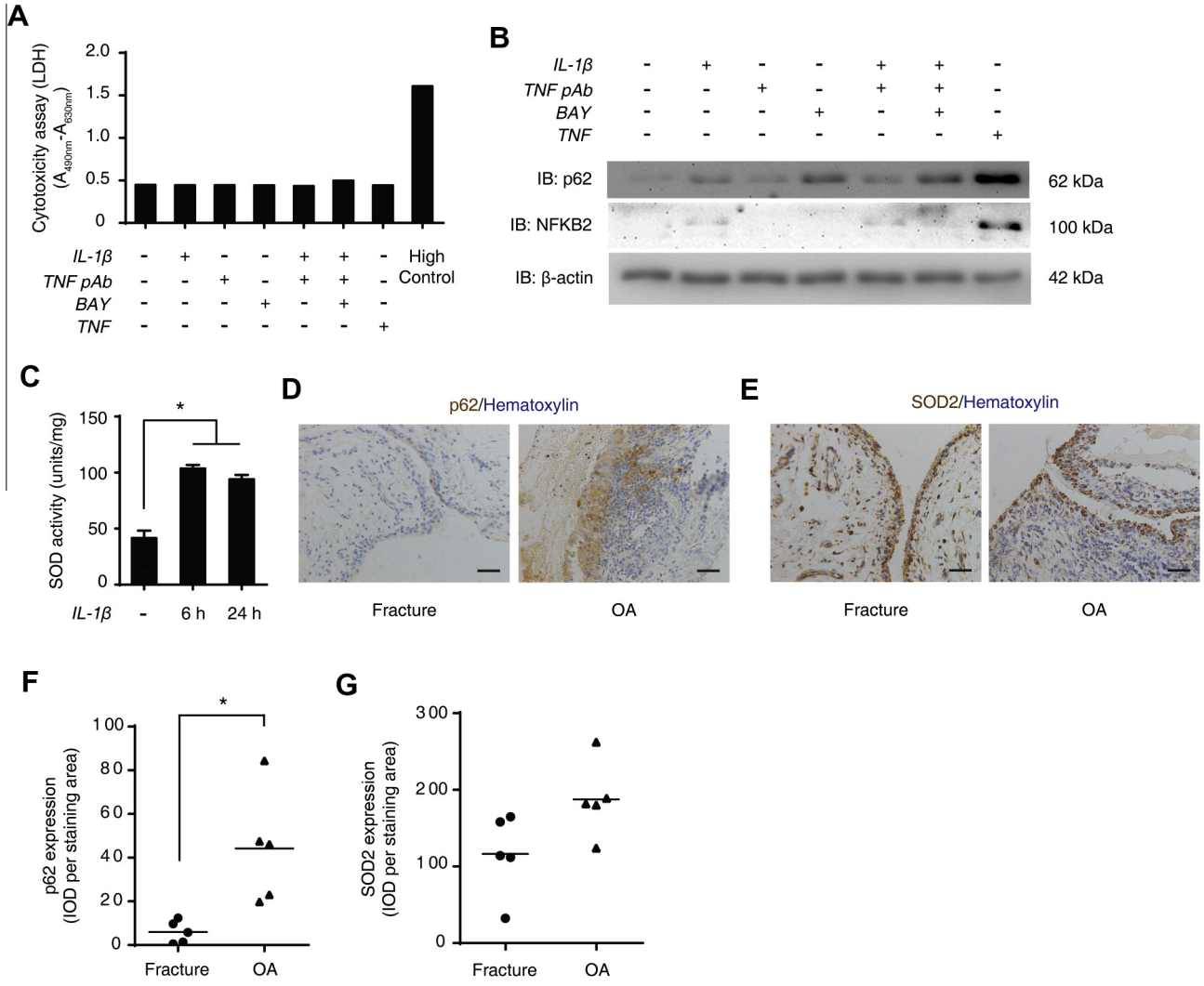

Fig. 4. Biological and clinical verification of IL-1β-amplified TNF pathway signals. (A) Cytotoxicity assay on FLS. For BAY 11-7082 (BAY) application, cells were pretreated with the drug for 1 h at 2.5 μM, followed by continuous culture for 24 h in complete media. If used, the neutralizing anti-TNF-α pAb (TNF pAb) and TNF-α were added at 0.1 ng/mL and 10 ng/mL, respectively. (B) Immunoblotting analysis on the p62 and NFKB2 expression. The drug treatment was consistent with the cytotoxicity assay. (C) SOD activity measurement. $^{*}P < 0.001$, $n = 3$. Statistical difference was tested by one-way ANOVA with Bonferroni *post hoc* multiple comparisons (two-tailed). (D and E) Representative images of p62 (D) and SOD (E) staining immunohistochemistry on human synovial tissues. Scale bar = 40 μm. (F and G) Statistics of p62 (F) and SOD (G) expression in clinical samples. The mean IOD of each group is indicated by a horizontal line. $^{*}P = 0.0079$, donor $n = 5$, two-tailed Kolmogorov–Smirnov test.

and inflammation [15,32] (reviewed in Ref. [33]). Amplification of p62 by IL-1β could lead to various outcomes that warrant further investigations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.008.

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