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Inflammatory Cytokines IL-32 and IL-17 Have Common Signaling Intermediates despite Differential Dependence on TNF-Receptor 1

Emily Turner-Brannen,* Ka-Yee Grace Choi,* Ryan Arsenault,[†] Hani El-Gabalawy,* Scott Napper,[†] and Neeloffer Mookherjee*

Cytokines IL-32 and IL-17 are emerging as critical players in the pathophysiology of immune-mediated chronic inflammatory diseases. It has been speculated that the molecular mechanisms governing IL-32- and IL-17-mediated cellular responses are differentially dependent on the TNF pathway. In this study, kinome analysis demonstrated that following stimulation with cytokine IL-32, but not IL-17, there was increased phosphorylation of a peptide target corresponding to TNF-R1. Consistent with this observation, blocking TNF-R1 resulted in a suppression of IL-32-induced downstream responses, indicating that IL-32-mediated activity may be dependent on TNF-R1. In contrast, blocking TNF-R1 did not affect IL-17-induced downstream responses. Kinome analysis also implicated p300 (transcriptional coactivator) and death-associated protein kinase-1 (DAPK-1) as signaling intermediates for both IL-32 and IL-17. Phosphorylation of p300 and DAPK-1 upon stimulation with either IL-32 or IL-17 was confirmed by immunoblots. The presence of common targets was supported by results demonstrating similar downstream responses induced in the presence of IL-32 and IL-17, such as transcriptional responses and the direct activation of NF- κ B. Furthermore, knockdown of p300 and DAPK-1 altered downstream responses induced by IL-32 and IL-17, and impacted certain cellular responses induced by TNF- α and IL-1 β . We hypothesize that p300 and DAPK-1 represent nodes where the inflammatory networks of IL-32 and IL-17 overlap, and that these proteins would affect both TNF-R1-dependent and -independent pathways. Therefore, p300 and DAPK-1 are viable potential therapeutic targets for chronic inflammatory diseases. *The Journal of Immunology*, 2011, 186: 7127–7135.

A wide range of disorders are characterized by chronic inflammation, including rheumatoid arthritis (RA), inflammatory bowel disease (IBD), psoriasis, asthma, and certain cancers. A major limitation in the development of effective new strategies for the treatment of chronic inflammatory diseases is an inadequate understanding of the complex molecular mechanisms underpinning these disorders. It is well established that dysregulation of the inflammatory cascade is a major contributing factor to the development of chronic inflammation. Even though critical roles of certain proinflammatory cytokines have been well established in the pathogenesis of chronic inflammatory disorders (1, 2), the genetic regulation and cellular responses mediated by inflammatory cytokines appear to be heterogeneous (3). This heterogeneity may be due to the induction of different disease-associated pathways in different patients, or it may reflect differ-

ent stages of the disease, or both (4). Heterogeneity in molecular mechanisms is further reinforced by the fact that response to pharmacologic treatment varies considerably among patients. For example, in the case of RA and IBD, up to one third of patients who receive therapies such as TNF blockers do not respond to treatment (5). Furthermore, residual inflammatory activity is often present even among “good” clinical responders of the treatment (6), and progression of chronic inflammation cannot be completely controlled by biologic therapies that target proinflammatory cytokines such as TNF and IL-1 β (5, 6). Improved understanding of the molecular processes involved in the various inflammatory networks will facilitate identification of overlapping nodes or common protein targets, which may serve as alternative therapeutic targets for chronic inflammatory diseases.

Two recently discovered proinflammatory cytokines, IL-32 and IL-17, are associated with the pathogenesis of chronic inflammatory diseases (7, 8). IL-32 (NK transcript 4), found in activated T cells, NK cells, and monocytic cells, is a potent inducer of proinflammatory mediators in diseases such as RA, atopic dermatitis, and chronic obstructive pulmonary disease (9–11). IL-32 levels are significantly elevated in RA synovial tissues and can induce joint inflammation, cartilage damage (9), and osteoclast differentiation (12). IL-17, which is primarily synthesized by T lymphocytes, is a potent inducer of TNF- α , IL-1 β , and matrix metalloproteinases and contributes to the pathogenesis of chronic inflammation through mechanisms that overlap considerably with IL-32, in the case of RA, ultimately leading to articular damage (13, 14). Recently, it has been demonstrated that IL-17 can also be secreted by innate immune cells such as macrophages, dendritic cells, and NK cells (15). The role of IL-17 in chronic destructive joint inflammation in RA has made it an attractive

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Abbreviations used in this article: DAPK-1, death-associated protein kinase-1; FLS, fibroblast-like synovocyte; IBD, inflammatory bowel disease; NSC, nonsilencing control; qRT-PCR, quantitative real-time PCR; RA, rheumatoid arthritis; siRNA, small interfering RNA; TC, tissue culture.

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therapeutic target, and inhibition of this molecule and its downstream effectors is now in the advanced stages of clinical testing (14, 16).

One important difference between IL-32 and IL-17 is that these cytokines may be differentially dependent on the TNF pathway. The role of IL-32 in inflammation may, in part, involve TNF-dependent mechanisms (17), whereas the function of IL-17 in the sustenance and escalation of inflammation in arthritic conditions may be, in part, TNF independent (13). We hypothesized that a comparative evaluation of cellular responses induced in the presence of IL-32 and IL-17 would likely permit identification of common molecular targets of these cytokines, which may play a part in both TNF-dependent and -independent processes. This study investigated cellular responses induced by IL-32 and IL-17 in macrophages and fibroblast-like synoviocytes (FLS), two critical cell types contributing to the disease process in chronic inflammatory arthritis, such as that seen in RA. Kinome analysis and subsequent immunoblots demonstrated that transcriptional coactivator p300 (EP300), as well as the death-associated protein kinase-1 (DAPK-1), had increased levels of phosphorylation in response to either IL-32 or IL-17 stimulation. Moreover, knock-down of these proteins altered downstream responses induced by IL-32 and IL-17, and changed certain responses induced in the presence of TNF- α and IL-1 β . We suggest that the identified common protein targets p300 and DAPK-1 may be involved in the inflammatory networks for both TNF-dependent and -independent processes, and therefore are potential therapeutic targets for chronic inflammatory diseases.

Materials and Methods

Cell culture and isolation

Human FLS were isolated from synovial tissues obtained from patients with osteoarthritis who had given informed consent (in accordance with a protocol by the Institutional Review Board at the University of Manitoba, Winnipeg, MB, Canada). Briefly, the tissues were digested with 1 mg/ml collagenase and 0.05 mg/ml hyaluronidase (both obtained from Sigma-Aldrich, Oakville, ON, Canada) in HBSS (Life Technologies, Invitrogen Canada, Burlington, ON, Canada) for 2–3 h at 37°C. The cells were cultured in DMEM media containing 1 mM L-glutamine (Life Technologies) supplemented with 1 mM sodium pyruvate and 0.1 mM nonessential amino acids (referred to as complete DMEM henceforth), containing 10% (v/v) FBS in a humidified incubator at 37°C and 10% CO₂. A rabbit synoviocyte cell line, HIG-82 (ATCC CRL-1832), was cultured in Ham's F-12 growth medium containing 1 mM L-glutamine (Life Technologies) supplemented with sodium pyruvate (referred to as complete F-12 media henceforth), containing 10% (v/v) FBS in a humidified incubator at 37°C and 5% CO₂. Confluent HIG-82 or human FLS were trypsinized with 1:3 dilution of 0.5% trypsin-EDTA (Invitrogen) in HBSS. The synoviocytes (either HIG-82 or human FLS) were seeded at 2×10^4 cells/ml, either 1 ml per well in 24-well tissue culture (TC) plates, 0.5 ml per well in 48-well TC plates, or 3 ml per well in 6-well TC plates, as required. The synoviocytes were propagated overnight in their respective complete media containing 10% (v/v) FBS. The culture media were changed the next day to complete media containing 1% (v/v) FBS before addition of the various stimulants.

Human monocytic THP-1 (ATCC TIB-202) cells were cultured in RPMI 1640 media containing 2 mM L-glutamine and 1 mM sodium pyruvate (referred to as complete RPMI media henceforth), supplemented with 10% (v/v) FBS, and maintained in a humidified incubator at 37°C and 5% CO₂. The THP-1 cells were differentiated to plastic-adherent macrophage-like cells by treatment with PMA (Sigma-Aldrich Canada), as previously described (18). Cellular cytotoxicity was evaluated after 24-h stimulation with the various stimulants for all cell types used in this study by monitoring the release of lactate dehydrogenase with a colorimetric detection kit (Roche Diagnostics, Laval, QC, Canada).

Stimulants, reagents, and Abs

Recombinant human cytokines TNF- α , IL-1 β , IL-17A/F (referred to as IL-17 hereafter), and IL-32 γ (referred to as IL-32 hereafter) were all obtained

from eBioscience (San Diego, CA). Bacterial LPS from *E. coli* was obtained from Sigma-Aldrich. Anti-human mAb directed against TNF-R1 (MAB625) and p300 Ab was obtained from R&D Systems, (Minneapolis, MN). Ab directed to phospho-p300 (Ser¹⁸³⁴) and mAb to total DAPK-1 were obtained from MJS Biolynx (Brockville, ON, Canada). mAb directed against phospho-DAPK-1 (Ser³⁰⁸) was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Anti-HDAC1 polyclonal Ab was obtained from Thermo Scientific (Pierce Biotechnology, Rockford, IL). mAb to GAPDH, Abs to human NF- κ B subunits p50 and p65, and HRP-linked purified anti-rabbit IgG and anti-mouse IgG secondary Abs were all obtained from Cell Signaling Technology, distributed by New England Biolabs (Pickering, ON, Canada). Cycloheximide was obtained from EMD chemicals (Newark, NJ).

ELISA immunoassay

TC supernatants were centrifuged at $1500 \times g$ for 5–7 min to obtain cell-free samples, and aliquots were stored at -20°C until further use. Production of chemokine Gro- α was monitored in the TC supernatants by ELISA employing human Gro- α DuoSet (R&D Systems) per the manufacturer's instructions. Production of cytokine TNF- α was monitored in the TC supernatants, using specific Ab pairs from eBioscience, and production of IL-8 was monitored using specific Ab pairs from R&D Systems, according to the manufacturer's instructions. The concentration of cytokines or chemokines in the TC supernatants was evaluated by establishing a standard curve with serial dilutions of the recombinant human cytokines or chemokines.

Kinome analysis

Human monocytic THP-1 cells were differentiated to plastic-adherent macrophage-like cells, as described above, and the cells were rested for 24 h. Following that, the media were changed to RPMI complete media containing 1% (v/v) FBS, and the cells were stimulated with either IL-32 (20 ng/ml) or IL-17 (20 ng/ml) for 15 min. Subsequently, cellular lysates were prepared in lysis buffer containing 20 mM Tris-HCl with pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, protease inhibitor mixture (Sigma-Aldrich), and 1% (v/v) Triton X-100, as previously described (19). An array of peptides representing 300 selected phosphorylation events were incubated with the cellular lysates for quantifying global kinase activity, as explained previously (19). Patterns of differential phosphorylation of the peptides upon stimulation with IL-32 and IL-17 were comprehensively analyzed after normalization and background correction of signal strength, as noted earlier (19).

Immunoblots

Total cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl with pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, protease inhibitor mixture (Sigma-Aldrich), and 1% (v/v) Triton X-100. The lysates were electrophoretically resolved on 4–12% NuPAGE Bis-Tris gels (Invitrogen), followed by transfer to nitrocellulose membranes (Millipore Canada). The membranes were subsequently blocked with TBST containing 5% skimmed milk powder, and probed with various Abs, as indicated, in TBST containing 3% skimmed milk powder. Affinity purified HRP-linked secondary Abs were used for detection, as required. The membranes were developed with the Amersham ECL detection system (GE Healthcare, Baie d'Urfe, QC, Canada) according to the manufacturer's instructions.

TNF-R blocking assay

Human monocytic THP-1 cells were differentiated to plastic-adherent macrophage-like cells by treatment with PMA for 24 h (Sigma-Aldrich), as described above. The cells were rested for an additional 24 h in complete RPMI media containing 10% (v/v) FBS. Subsequently, the media were changed to complete RPMI containing 1% (v/v) FBS. Neutralization or blocking of TNF-R1 was performed using a specific mAb, MAB625 (R&D Systems), as previously described (20). Briefly, the cells were preincubated with the MAB625 for TNF-R1 (20 $\mu\text{g/ml}$) for 1 h, followed by treatment with the various stimulants, as indicated. RNA was isolated from these cells after 4 h of stimulation for the evaluation of transcriptional responses of chemokine CXCL-1 (Gro- α) and proinflammatory cytokine IL-23 employing quantitative real-time PCR (qRT-PCR). The TC supernatants were monitored for chemokine Gro- α and IL-8 production after 24 h of stimulation by ELISA.

qRT-PCR

Human FLS or plastic-adherent THP-1 macrophage-like cells were stimulated with various stimulants, as indicated, for 4 h. RNA was isolated and subsequently analyzed for gene expression by qRT-PCR, using SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen), according to the manufacturer's instructions, in the ABI Prism 7000 sequence detection system (Applied Biosystems). Fold changes were calculated using the comparative Ct method (21), after normalization with 18S RNA primers. The primers are listed in Table II.

Translocation of NF- κ B subunits p50 and p65

Nuclear extracts were prepared using NE-PER extraction reagents (Thermo Fisher Scientific), according to the manufacturer's instructions. To monitor direct NF- κ B activation, equivalent nuclear extracts (5–8 μ g) were resolved on 4–12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked with TBST containing 3% fish skin gelatin (Sigma-Aldrich) and probed with Abs specific for either NF- κ B subunit p50 or p65, in TBST containing 1% fish gelatin. The membranes were also probed with Ab to human histone protein HADC-1 to assess the equivalent protein loading. Affinity purified HRP-linked secondary Abs were used for detection, and the membranes were developed with the ECL detection system.

NF- κ B activation assay

To monitor direct NF- κ B activation in synoviocytes, a rabbit synoviocyte cell line, HIG-82 (ATCC CRL-1832), was transiently transfected with pNF κ B-MetLuc2-Reporter Vector (Clontech Laboratories, Mountain View, CA) or the provided control vector, per the manufacturer's instructions. Various stimulants were added to the transfected cells in culture media containing 1% (v/v) FBS. The cells were stimulated with either recombinant human IL-32 or IL-17, and in parallel with known activators of NF- κ B, such as proinflammatory recombinant human cytokines TNF- α and IL-1 β , for 4 or 6 h. These time points were selected according to the manufacturer's recommendations. The activation of NF- κ B was monitored by employing the Ready-To-Glow Secreted NF- κ B Luciferase Reporter Assay (Clontech), per the manufacturer's directions.

Gene silencing using small interfering RNA

Human monocytic THP-1 cells were treated with 1 μ M Accell SMARTpool small interfering RNA (siRNA) for either human p300 (EP300), human

DAPK-1, or nonsilencing control (NSC) in Accell delivery media (Dharmacon, Thermo Fisher Scientific), per the manufacturer's instructions, for 96 h. Subsequently, the cells were differentiated by PMA treatment (as described above) using complete RPMI media containing 10% (v/v) FBS and rested for 24 h before stimulation. The plastic-adherent macrophage-like THP-1 cells were stimulated with either IL-32 (20 ng/ml), IL-17 (20 ng/ml), TNF- α (10 ng/ml), IL-1 β (10 ng/ml), or bacterial LPS (10 ng/ml) for 24 h. TC supernatants were monitored for chemokine IL-8 and cytokine TNF- α production by ELISA.

Results

IL-32- and IL-17-induced protein production in human macrophages and FLS

Cytokines IL-32 and IL-17 induce the production of proinflammatory cytokines such as TNF- α and chemokines in macrophages and synovial fibroblasts under inflammatory conditions (16, 22). In this study, plastic-adherent macrophage-like THP-1 cells (in vitro) were stimulated with either IL-17 or IL-32 (5–100 ng/ml), and the TC supernatants were monitored for cytokines TNF- α and IL-1 β and for production of chemokines Gro- α and IL-8 by ELISA. Production of both TNF- α and IL-1 β following stimulation with IL-32 from 20 ng/ml onward was dose dependent and significant ($p < 0.05$) (Fig. 1A). IL-32 also induced significant ($p < 0.01$) production of chemokines Gro- α and IL-8 from 5 to 100 ng/ml (Fig. 1B). Similarly, production of IL-8 upon stimulation with 10, 20, or 50 ng/ml of IL-17 was significant ($p < 0.05$), but the amount of IL-8 produced decreased at 100 ng/ml (Fig. 1C). Taken together, these results indicated that both cytokine IL-32 and IL-17 induced downstream protein production in macrophage-like THP-1 cells, and that there was significant ($p < 0.05$) protein production upon stimulation with either 20 or 50 ng/ml of the cytokines for all the read-outs monitored in this study. We further evaluated protein production in synovial fibroblasts. Human FLS were stimulated with either IL-32 or IL-17 (10 or 20 ng/ml), and the TC supernatants were monitored for TNF- α , IL-1 β , and Gro- α

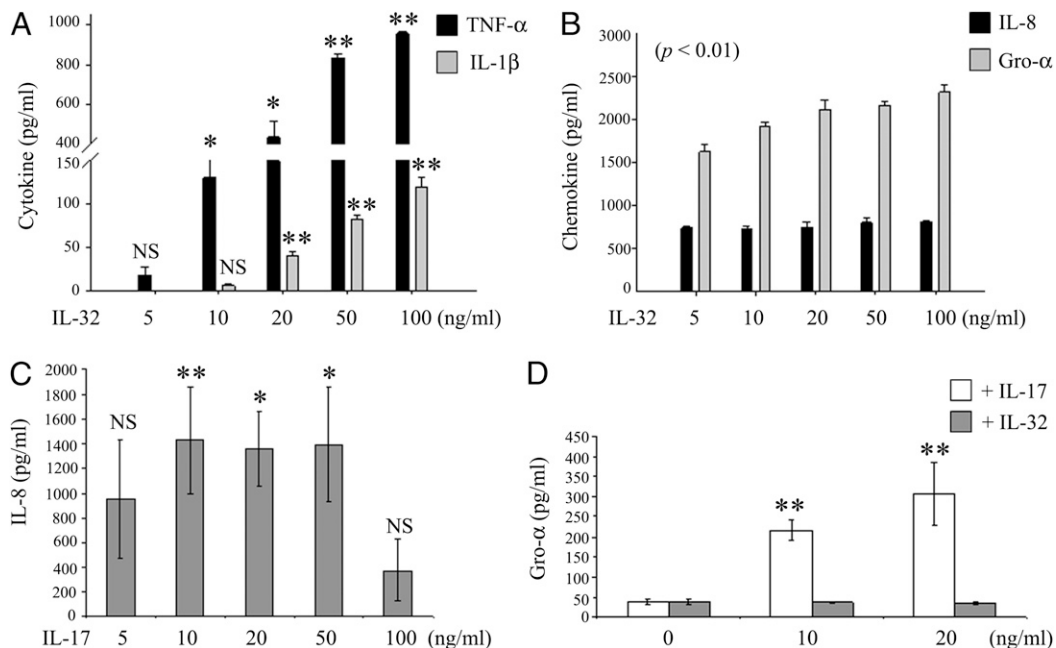


FIGURE 1. Protein production in the presence of IL-17 and IL-32. Plastic-adherent human macrophage-like THP-1 cells (in vitro) were stimulated with IL-32 (5–100 ng/ml) for 24 h. TC supernatants were monitored for the production of inflammatory cytokines TNF- α and IL-1 β (A) and chemokines Gro- α and IL-8 (B) by ELISA. C, Macrophage-like THP-1 cells were stimulated with IL-17 (5–100 ng/ml) for 24 h, and the TC supernatants were monitored for the production of IL-8 by ELISA. Results are shown after subtraction of background levels of cytokines or chemokines monitored in unstimulated cells. D, Human FLS isolated from synovial tissues (ex vivo) were stimulated with either IL-32 or IL-17 (10 or 20 ng/ml) for 24 h. The TC supernatants were monitored for chemokine Gro- α production by ELISA. All results are an average of four independent experiments (for FLS isolated from four independent donors) \pm SE. * $p < 0.05$, ** $p < 0.01$.

production. Gro- α production upon IL-17 stimulation in human FLS was robust, whereas IL-32 did not produce significant chemokine production (Fig. 1D). In this study, human FLS did not produce significant amounts of TNF- α or IL-1 β following stimulation with either IL-17 or IL-32 (data not shown). On the basis of these results, macrophage-like THP-1 cells were selected for a comparative analysis of induced cellular responses upon stimulation with IL-32 and IL-17 at 20 ng/ml.

Protein phosphorylations induced in the presence of IL-32 and IL-17

Phosphorylation of proteins is a critical mechanism in the regulation of cellular processes. This process is meticulously regulated by enzymes known as kinases, which are increasingly being identified as drug targets for a variety of diseases (23, 24). We interrogated kinase activities (kinome) induced in the presence of the cytokines IL-32 and IL-17, using peptide arrays representing 300 peptides, printed in triplicate, representing selected phosphorylation events, as previously described (19). Because both IL-32 and IL-17 at 20 ng/ml induced significant ($p < 0.05$) protein production in human macrophage-like THP-1 cells (Fig. 1), these cells were used for the comparative kinome analysis. Macrophage-like THP-1 cells were stimulated with either IL-32 (20 ng/ml) or IL-17 (20 ng/ml) for 15 min, and the peptide arrays were used to comprehensively analyze protein phosphorylation profiles in the presence of these cytokines, as previously explained (19). The phosphorylations of the peptides on the array were quantified in the cytokine-treated cells relative to the unstimulated control cells. Differentially phosphorylated targets were defined as ≥ 1.5 -fold increase or decrease ($p < 0.05$) in phosphorylation, compared with unstimulated control cells. The kinome data have been deposited to National Center for Biotechnology Information's Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) under series accession number GSE28649. Two key observations from the kinome analysis were 1) IL-32 significantly induced the phosphorylation of TNF-R1 by more than 4-fold, whereas the state of TNF-R1 phosphorylation was not altered in the presence of IL-17 when compared with the unstimulated control cells (Table I); and 2) two proteins, p300 and DAPK-1, were significantly phosphorylated in the presence of either IL-32 or IL-17 (Table I), indicating that these proteins are common phosphorylation targets of these cytokines.

Subsequent probing of immunoblots with specific Abs to human phospho-p300 (Ser¹⁸³⁴) and phospho-DAPK-1 (Ser³⁰⁸) conclusively demonstrated that stimulation of human macrophage-like THP-1 cells with either IL-32 or IL-17 resulted in the increased phosphorylation of both p300 and DAPK-1 when compared with unstimulated control cells after 15 min (Fig. 2).

Alteration of IL-32-induced responses on blocking of TNF-R1

Kinome analysis showed that IL-32 induced the phosphorylation of TNF-R1 (4.7-fold increase, $p < 0.01$), whereas the phosphoryla-

tion state of TNF-R1 after stimulation with IL-17 was not altered, compared with that in unstimulated cells (Table I). This observation was in agreement with previous studies hypothesizing that IL-32-mediated pathogenesis in chronic inflammatory diseases may be in part dependent on TNF- α (17). Therefore we investigated the role of TNF-R1 in IL-32-induced downstream responses by blocking receptor activity, using a specific neutralizing mAb, as previously described (20). Human macrophage-like THP-1 cells were pretreated with mAb MAB625 specific for TNF-R1 (20 μ g/ml) for 1 h, followed by stimulation with either IL-32 (20 ng/ml), IL-17 (20 ng/ml), TNF- α (10 ng/ml), or IL-1 β (10 ng/ml) for 4 or 24 h. Transcriptional responses were evaluated by qRT-PCR (primers used shown in Table II) after 4 h of stimulation. Gene expression of Gro- α (Fig. 3A) and IL-23 (Fig. 3B) induced in the presence of IL-32 and TNF- α was significantly ($p < 0.05$) suppressed, by $>70\%$, in the presence of the neutralizing mAb to TNF-R1. In contrast, transcriptional responses induced in the presence of either IL-17 or IL-1 β were not altered (Fig. 3A, 3B). A similar trend was also observed at the protein level; blocking TNF-R1 significantly ($p < 0.01$) suppressed IL-32-induced chemokine Gro- α production by $35 \pm 5\%$ and TNF- α -induced Gro- α production by 60% after 24 h of stimulation (Fig. 3C). No difference in IL-1 β -induced Gro- α production was observed (Fig. 3C). Blocking TNF-R1 significantly ($p < 0.05$) suppressed IL-32-induced IL-8 production, by $>85\%$ (Fig. 3D); however, it did not alter the production of TNF- α -induced IL-8 production (Fig. 3D).

Common downstream responses induced in the presence of cytokines IL-32 and IL-17

We showed that both IL-32 and IL-17 induced the phosphorylation of two common proteins (Table I, Fig. 2). However, phosphorylation of the common proteins p300 and DAPK-1 was not abrogated upon stimulation with either IL-32 or IL-17 in the presence of cycloheximide, an inhibitor of protein biosynthesis (data not shown). This finding indicates that these common signaling events may be indirect. Nevertheless, as the presence of common signaling intermediates would result in similar downstream cellular responses, we further interrogated transcriptional responses and activation of the key transcription factor NF- κ B in the presence of IL-32 and IL-17 in macrophages and human FLS. The cells were stimulated with either IL-32 or IL-17, and transcriptional responses were monitored after 4 h of stimulation by qRT-PCR. Both IL-32 and IL-17 induced significant ($p < 0.05$) gene expression of TNF- α , IL-23, and Gro- α , between 2- and 12-fold higher than that in unstimulated control cells in macrophages (Fig. 4A). Even though a modest—i.e., ~ 2 -fold change—increase in gene expression was produced upon stimulation with IL-17, compared with that in unstimulated control cells, this increase was consistent and statistically significant ($p < 0.05$) in macrophage-like THP-1 cells. Similarly, a significant increase in TNF- α , IL-1 β , IL-6, and IL-8 gene expression was observed in

Table I. IL-32- and IL-17-induced protein phosphorylations in human monocytic cells

Phosphoprotein	ID	Kinase Target	IL-32 Relative Fold Change	$p <$	IL-17 Relative Fold Change	$p <$
TNF-R1	P19438	S274	4.7	0.01	-1.2	0.01
p300	Q09472	S1834	1.7	0.05	1.9	0.01
DAPK-1	P53355	S308	1.5	0.05	2.2	0.01

Human macrophage-like THP-1 cells were stimulated with either IL-32 or IL-17 (20 ng/ml) for 15 min. A kinome screen using peptide arrays representing 300 phosphorylation targets of kinase activity was employed for quantifying kinase activity. Differentially phosphorylated targets were defined as ≥ 1.5 -fold increase or decrease ($p < 0.05$) in phosphorylation compared with that in unstimulated control cells.

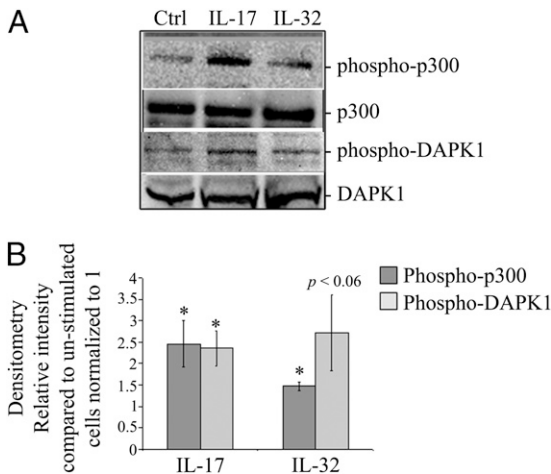


FIGURE 2. Phosphorylation of p300 and DAPK-1 in the presence of IL-32 and IL-17: Human macrophage-like THP-1 cells were stimulated with either IL-32 or IL-17 (20 ng/ml each) for 15 min, followed by probing of the cell lysates in immunoblots with specific Abs to human phospho-p300 (Ser¹⁸³⁴) or phospho-DAPK-1 (Ser³⁰⁸). Abs to the nonphosphorylated forms of p300 and DAPK-1 were used as paired loading control. *A*, An immunoblot representative of three independent experiments. *B*, Densitometric analysis; ratio of band density of cytokine-treated samples over unstimulated cells (y-axis), calculated after normalization to band density of nonphosphorylated forms of the respective proteins for each sample. Results represent an average of three independent experiments ± SE. **p* < 0.05.

human FLS (ex vivo) upon stimulation with either IL-17 or IL-32 (Fig. 4*B*).

Activation of the key inflammatory transcription factor NF-κB was evaluated by monitoring the nuclear translocation of NF-κB subunits p50 and p65 following stimulation with IL-32 and IL-17. Although NF-κB has five different subunits, inflammatory regulation is most commonly implicated by the functions of subunits p50 and p65. Thus, human FLS and macrophage-like THP-1 cells were stimulated with cytokines—either IL-32, IL-17, TNF-α, or IL-1β—for 30 min, and the nuclear extracts were probed with Abs specific to NF-κB subunits p50 and p65 by Western blots. Increased nuclear localization of NF-κB subunit p50 was demonstrated following stimulation with either IL-32 or IL-17 in both human FLS (Fig. 5*A*) or macrophage-like THP-1 cells (Fig. 5*B*), and these responses were similar to that seen after stimulation with inflammatory cytokines TNF-α and IL-1β. In contrast, even though increased nuclear localization of NF-κB subunit p65 was promoted by IL-17, this response was not robust upon stimulation with IL-32 in both cell types (Fig. 5). Furthermore, both IL-32 and IL-17 induced direct activation of NF-κB in a rabbit synovial fibroblast cell line (Fig. 5*C*). Taken together, these results indicated that both IL-32 and IL-17 can activate the inflammatory transcription factor NF-κB; however, this activity may involve different NF-κB subunit dimers.

Overall, similarities in downstream responses upon stimulation with IL-32 and IL-17 in both macrophages and synoviocytes supported the presence of common signaling intermediates for these cytokines.

Alteration of IL-32- and IL-17-induced responses upon silencing of p300 and DAPK-1 genes

To establish the functional relevance of the identified common targets, p300 and DAPK-1, we evaluated the impact of the knockdown of these proteins on cellular responses induced by IL-32 and IL-17, as well as those induced by proinflammatory cytokines TNF-α and IL-1β. Accell SMARTpool EP300 (p300) siRNA, DAPK-1 siRNA, and an NSC siRNA were used to knock down the respective proteins in human macrophage-like THP-1 cells. Transcriptional analysis by qRT-PCR showed that gene expression for p300 and DAPK-1 was significantly (*p* < 0.01) suppressed, by >60 ± 5%, on treatment with the respective siRNA and remained unaltered in cells treated with NSC, when compared with control cells not treated with siRNA (Fig. 6*A*). Immunoblots probing with specific Abs revealed a significant knockdown of both p300 (EP300) and DAPK-1 proteins in cells treated with the respective siRNAs, compared with cells treated with NSC (Fig. 6*B*). Knockdown of protein expression was higher than that observed at the mRNA level (Fig. 6*A*, 6*B*). Knockdown of either p300 or DAPK-1 was not lethal to the cells, as determined by monitoring lactate dehydrogenase release for cellular cytotoxicity (data not shown).

We have shown that IL-32 can induce both IL-8 message (Fig. 4*A*) and protein production (Fig. 1*B*) in macrophage-like THP-1 cells. Even though IL-17 did not significantly enhance IL-8 gene expression (Fig. 4*A*), IL-8 protein production was significantly induced following stimulation with IL-17 (Fig. 1*C*) in macrophage-like THP-1 cells. These results are consistent with previous studies demonstrating that IL-17 alone is a poor stimulus for inducing certain gene expressions, and that downstream production of certain proinflammatory cytokines and chemokines, including IL-8, is due to posttranscriptional effects exerted by IL-17, primarily by stabilizing mRNA (25, 26). Taking these findings together, we believed it valid to use IL-8 protein production as a read-out for assessing the impact of p300 and DAPK-1 knockdown on IL-32- and IL-17-induced downstream responses. Knockdown of p300 suppressed chemokine IL-8 production >80% in the presence of either IL-32 or IL-17 (Fig. 6*C*). Knockdown of DAPK-1 abrogated the production of IL-8 upon stimulation with IL-32 and significantly (*p* < 0.05) suppressed IL-17-induced IL-8 production, by >70% (Fig. 6*C*). In addition, knockdown of DAPK-1 significantly suppressed the production of IL-8, by >90%, upon stimulation with proinflammatory cytokines TNF-α and IL-1β (Fig. 6*C*). Similarly, knockdown of p300 significantly (*p* < 0.05) suppressed IL-1β-induced production of TNF-α, by >50% (Fig. 6*D*). In contrast, knockdown of either

Table II. Summary of primers used for qRT-PCR

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')
TNF-α	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGCTGATTAGAGA
CXCL-1 (Gro-α)	TCCTGCATCCCCATAGTTA	CTTCAGGAACAGCCACCAGT
IL-6	CAGGAGCCCAGCTATGAACT	GAAGGCAGCAGGCAACAC
IL-8	AGACAGCAGAGCACACAAGC	AGGAAGGCTGCCAAGAGAG
IL-23	AGCTTCATGCCCTCCCTACTG	CTGCTGAGTCTCCAGTGGT
p300	GATCTGTGTCCCTTCACCATGAG	AAACAGCCATCACAGACGAA
DAPK-1	CCCAGTTGAAGAACCCATAGC	CGAGGAACATTCATGATGTACG
18S RNA	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG

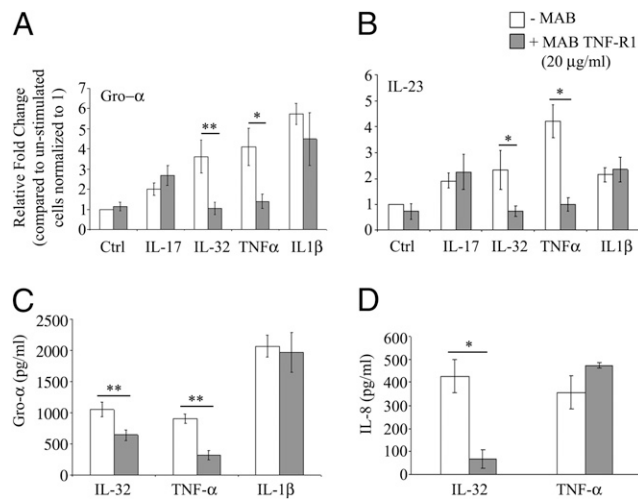


FIGURE 3. Alteration of responses in the presence of TNF-R1 mAb. Human macrophage-like THP-1 cells were pretreated with mAb specific for TNF-R1 (20 μg/ml each) for 1 h, followed by stimulation with cytokines—either IL-32 (20 ng/ml), IL-17 (20 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml). Transcriptional responses were evaluated for chemokine Gro-α (A) and proinflammatory cytokine IL-23 (B) after 4 h of stimulation by qRT-PCR. Fold changes (y-axis) for each gene was normalized to 18S RNA and is represented relative to gene expression in unstimulated cells normalized to 1, using the comparative Ct method. TC supernatants were monitored after 24 h of stimulation for the production of chemokines Gro-α (C) and IL-8 (D) by ELISA. Protein production monitored in the TC supernatants by ELISA is shown after subtraction of background levels found in unstimulated control cells. Results represent an average of three independent experiments ± SE. * $p < 0.05$, ** $p < 0.01$.

p300 or DAPK-1 did not alter LPS-induced responses in macrophage-like THP-1 cells (Fig. 6C, 6D).

Discussion

Immune-mediated chronic inflammatory diseases such as RA result from a dynamic and complex interplay of regulatory networks of signaling pathways. The complexity of these interactions can limit the efficacy of targeting a single molecule or one specific pathway for the pharmacologic management of these disorders. For example, in RA many patients do not respond to biologic therapies that target the TNF-mediated pathway, and these therapeutics do not completely control the progression of disease (5, 6). This

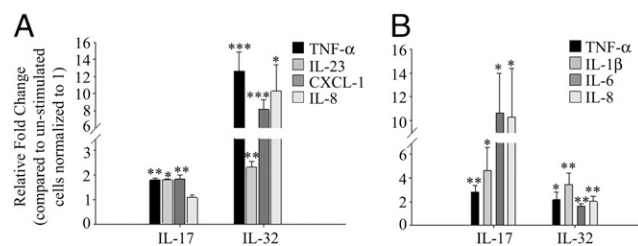


FIGURE 4. Transcriptional responses in the presence of IL-17 and IL-32. Human macrophage-like THP-1 cells (in vitro) (A) and human FLS isolated from synovial tissues (ex vivo) (B) were stimulated with either IL-17 or IL-32 (20 ng/ml each) for 4 h. RNA was isolated, and transcriptional responses were analyzed by qRT-PCR. Fold changes (y-axis) for each gene were calculated after normalization to housekeeping gene 18S RNA and quantitated relative to gene expression in unstimulated cells normalized to 1, using the comparative Ct method. Results represent an average of four independent experiments (FLS isolated from four independent donors) ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

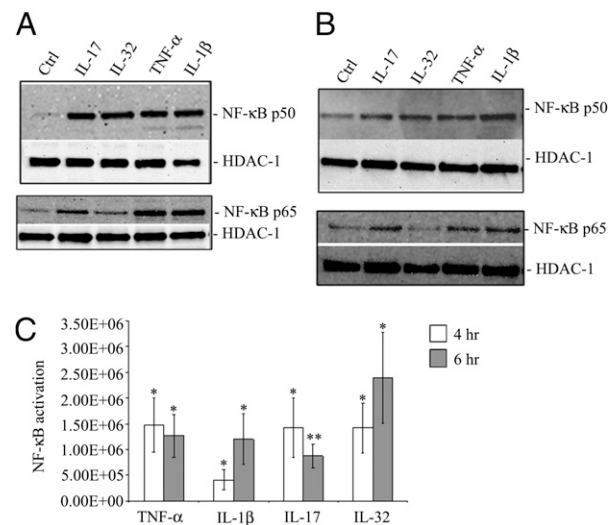


FIGURE 5. Activation of transcription factor NF-κB. Human FLS (A) or macrophage-like THP-1 cells (B) were stimulated with either IL-32 (20 ng/ml), IL-17 (20 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for 30 min. Equivalent loading of nuclear extracts (5–8 μg) was probed in immunoblots with Abs specific to either NF-κB subunits p50, p65, or HDAC-1 as input control. Immunoblot shown is representative of at least three independent experiments, for FLS independent experiments refer to cells isolated from different independent donors. C, Cells from rabbit synovial fibroblast cell line HIG-82 were transiently transfected with pNFκB-MetLuc2-Reporter Vector (Clontech). The cells were stimulated with cytokines—either TNF-α (10 ng/ml), IL-1β (10 ng/ml), IL-17 (20 ng/ml), or IL-32 (20 ng/ml). The activation of NF-κB was monitored by employing the Ready-To-Glow Secreted NF-κB Luciferase Reporter Assay (Clontech), per the manufacturer's instructions, after 4 and 6 h of stimulation. Results represent luminescence over background levels in unstimulated cells. Results are an average of at least five independent experiments ± SE. * $p < 0.05$, ** $p < 0.01$.

finding suggests that despite the central role proposed for TNF-α in the pathogenesis of RA, there exists a complex network of various cytokine-mediated regulatory pathways contributing to the inflammatory microenvironment. Identification of overlapping nodes within the different cytokine-mediated networks could be valuable as potential drug targets for diseases characterized by chronic inflammation.

In this study, we investigated the molecular mechanisms induced by two new cytokines, IL-32 and IL-17. These cytokines are emerging as critical contributors to the pathogenesis of various chronic inflammatory and autoimmune diseases. IL-32 is a newly described potent proinflammatory cytokine, elevated levels of which have been directly correlated to severity of chronic inflammatory disorders, including RA and IBD (9, 27). However, the network of regulatory signaling pathways induced by IL-32 has yet to be completely defined. In contrast, molecular regulation mediated by IL-17 has been relatively well characterized (7, 28, 29). IL-17 is known to be a potent mediator of proinflammatory cytokines, chemokines, acute phase response elements, and defensins (7, 14, 30). IL-17 is associated with the pathophysiology of autoimmune diseases, including RA, IBD, systemic lupus, psoriasis, and autoimmune encephalitis (reviewed in Ref. 30). Recently, this cytokine was also shown to be expressed in atherosclerotic plaques (31). Despite similarities in proinflammatory functions of IL-32 and IL-17, and both being described as therapeutic cytokine targets (14, 32), previous reports have speculated that responses mediated by these cytokines may be differentially dependent on the TNF pathway. The role of IL-32 in mediating influx of inflammatory cells and subsequent cartilage damage in

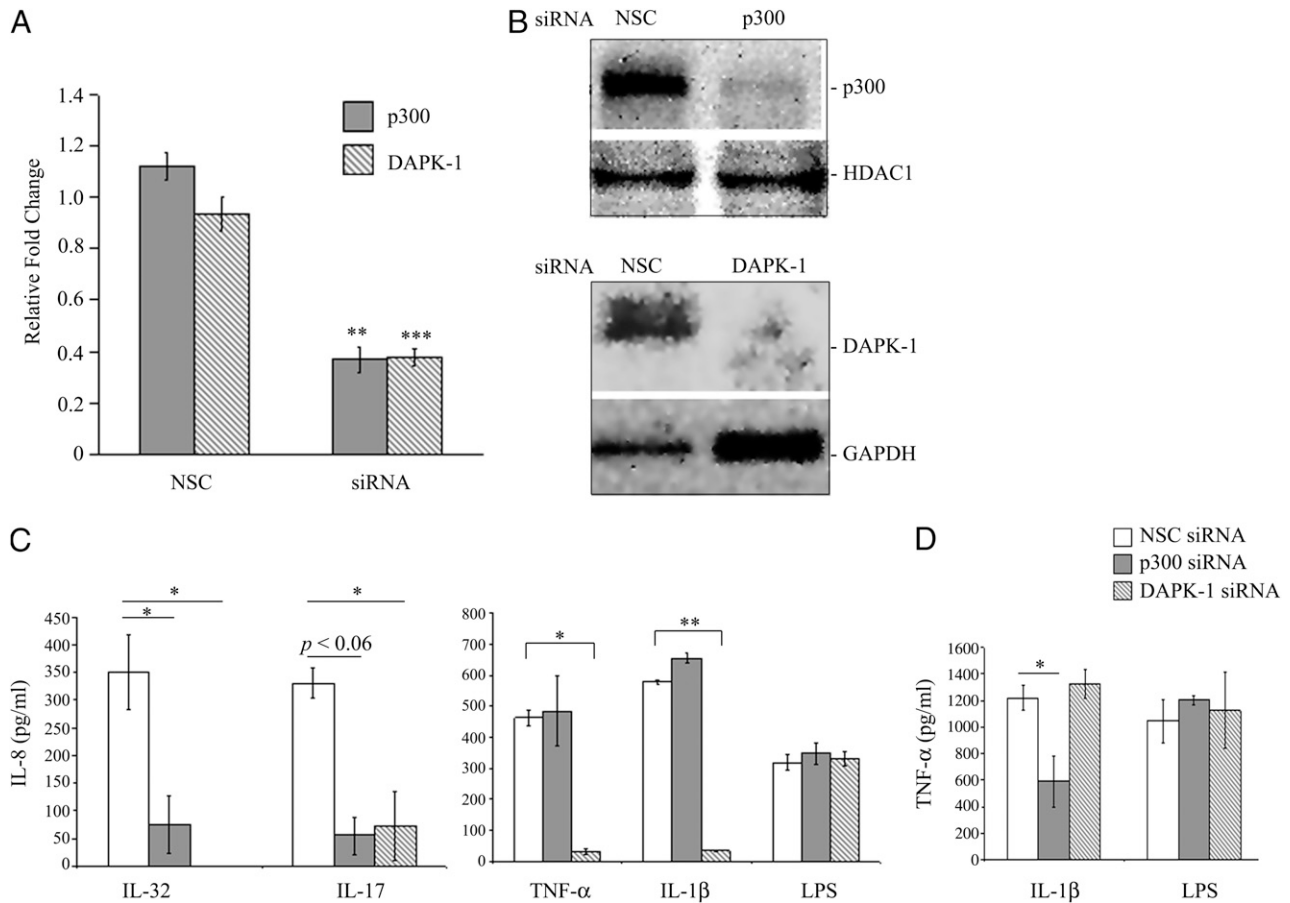


FIGURE 6. Knockdown of p300 and DAPK-1 in macrophages. Human macrophage-like THP-1 cells were treated with Accell SMARTpool siRNA for either human p300, human DAPK-1, or NSC in Accell delivery media for 96 h. Knockdown efficiency was evaluated by qRT-PCR for mRNA expression (A) and immunoblots (B) by probing the nuclear extracts with specific Ab to human p300, and total cell extracts with Ab to human DAPK-1. Abs specific to human HDAC and GAPDH were used to estimate loading controls, respectively. The knockdown cells were stimulated with either IL-32 (20 ng/ml), IL-17 (20 ng/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), or LPS (10 ng/ml) for 24 h. TC supernatants were monitored for the production of chemokine IL-8 (C) and cytokine TNF-α (D) by ELISA. Results are representative of at least three independent experiments ± SE. * $p < 0.05$, ** $p < 0.01$.

arthritis is dependent on TNF-related mechanisms (17); in contrast, IL-17-mediated inflammation under arthritic conditions is, in part, TNF independent (13). To date, no studies have been done that provide insight into the differential regulatory processes or explain the basis for the differential TNF pathway dependence in IL-32- and IL-17-mediated inflammatory responses. The key findings in this study were 1) IL-32-mediated responses, but not IL-17-mediated ones, may be dependent on TNF-R1 (Fig. 3, Table I); and 2) p300 and DAPK-1 are common signaling intermediates for both IL-32 and IL-17 (Fig. 2, Table I), and knockdown of these proteins impaired the cytokine-mediated downstream responses (Fig. 6). These results suggest that, even though IL-32 and IL-17 may have differential dependence on the TNF-pathway, their induced inflammatory networks overlap, and that there are common downstream targets of these cytokines.

We have shown that, unlike IL-17-mediated responses, cytokine IL-32-mediated downstream cellular responses were dependent on TNF-R1 (Fig. 3), which supported the kinome analysis (Table I). However, different amounts of TNF-α may be produced following stimulation with 20 ng/ml of IL-17 and IL-32 in macrophage-like THP-1 cells (as indicated by the results shown in Fig. 4A), which could also have an impact on the differential effects seen with TNF-R1 blocking in the presence of these cytokines. TNF-α stimulation was used as a positive control for the TNF-R1 neutralization assays. As expected, blocking of TNF-R1 suppressed TNF-α-induced transcriptional responses and Gro-

α production but did not inhibit IL-8 production (Fig. 3D). Two receptors with distinct signaling mechanisms have been defined for the proinflammatory events induced in the presence of the cytokine TNF-α, TNF-R1 (TNFRSF1A or p55), and TNF-R2 (TNFRSF1B or p75). TNF-α-induced IL-8 production upon blocking TNF-R1 indicates that this response is probably mediated by the TNF-R2 signaling pathway. These results also suggest that IL-32-induced responses are not a result of the TNF-α feedback loop and may be directly dependent on TNF-R1 activity. Therefore, it may be speculated that the TNF pathway-dependent role of IL-32 in chronic inflammation is due to the engagement of the cytokine with TNF-R1, which warrants further investigation. The only direct interacting protein partner demonstrated for IL-32 is a neutrophil-derived serine protease, proteinase 3 (33). To date, no other receptor has been described for IL-32. This study suggests that IL-32-mediated responses are dependent on TNF-R1, and thus provides molecular insight for the speculation that inflammatory functions of IL-32 are, in part, dependent on the TNF pathway.

We identified p300 and DAPK-1 as common protein phosphorylation targets for IL-32 and IL-17. Our results indicate that there may be overlap in the inflammatory networks induced by these two cytokines, as well as commonalities in the regulatory processes triggered by these cytokines. The functional relevance of the identified common protein targets was confirmed using knockdown studies, which showed that knockdown of either p300

or DAPK-1 altered both IL-32- and IL-17-induced downstream responses (Fig. 6C). In addition, knockdown of p300 and DAPK-1 also altered certain TNF- and IL-1 β -induced cellular responses (Fig. 6C, 6D). These results were consistent with our hypothesis that common protein targets of IL-32 and IL-17 would likely be involved in TNF-dependent and -independent cellular responses.

The transcriptional coactivator p300, in concert with other transcription factors, triggers gene expression in the presence of inflammatory stimuli. For example, p300 is essential for IL-1 β -induced PG release and for phospholipase A(2) and NF- κ B activation in human tracheal smooth muscles (34). We showed that IL-32 and IL-17 stimulation resulted in the phosphorylation of p300 at Ser¹⁸³⁴ (Fig. 2, Table I). A previous report has demonstrated that phosphorylation of p300 at Ser¹⁸³⁴ results from the nuclear translocation of AKT upon the activation of the PI3K/AKT pathway in response to proinflammatory cytokine TNF- α (35). Similarly, the involvement of activation of the AKT pathway upon stimulation with IL-32 has been recently demonstrated in osteoclasts (12), as it has for IL-17 in retinal astrocytes and bronchial epithelial cells (28, 36). With the data taken together, we can therefore speculate that the cytokines IL-32 and IL-17 induce the phosphorylation of p300 at Ser¹⁸³⁴ by engaging the PI3K/AKT pathway, resulting in the activation of NF- κ B.

The second protein target identified for cytokines IL-32 and IL-17 in this study was DAPK-1, which is an apoptotic regulator and has been implicated in the disease process of several cancers (37–39). IL-32 is known to promote apoptosis of keratinocytes in atopic dermatitis (11), which supports our results that identified an apoptosis regulator, DAPK-1, as a protein target for IL-32. Another study has shown that inflammation can lead to aberrant DNA methylation of the DAPK-1 gene in a model of IBD (40). In addition, DAPK has been recently described as a potential therapeutic target for chronic neurodegenerative diseases (41). Because dysregulation of inflammation has been correlated with several cancers, as well as with neurodegenerative disorders such as Alzheimer's disease, it may be speculated that DAPK-1 plays a regulatory role in pathways induced in the presence of chronic inflammatory stimuli, such as the cytokines investigated in this study.

In summary, this study provided molecular insight into the TNF pathway-dependent role of the IL-32-mediated inflammatory network, demonstrating that IL-32-induced cellular responses could be dependent on TNF-R1, which contrasts with the activity of IL-17. Despite differential dependence on TNF-R1 activity, we suggest that the common signaling intermediates p300 and DAPK-1 represent nodes where the inflammatory networks mediated by IL-32 and IL-17 overlap, leading to the convergence of signaling in the activation of NF- κ B and resulting in common downstream proinflammatory cellular responses. We can also speculate that p300 and DAPK-1 may be pivotal checkpoints for TNF-dependent and -independent inflammatory networks. This discovery merits further investigation to elucidate the role of p300 and DAPK-1 as potential therapeutic targets for immune-mediated chronic inflammatory diseases, especially for nonresponders of anti-TNF therapy.

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Disclosures

The authors have no financial conflicts of interest.

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