Selective upregulation of the ADP-ribosyl cyclases CD38 and CD157 by TNF but not by RANK-L reveals differences in downstream signaling

Jameel Iqbal, Kevin Kumar, Li Sun, and Mone Zaidi
Department of Endocrinology and Mount Sinai Bone Program, Mount Sinai School of Medicine, New York, New York

Submitted 24 February 2006; accepted in final form 21 April 2006

Iqbal, Jameel, Kevin Kumar, Li Sun, and Mone Zaidi. Selective upregulation of the ADP-ribosyl cyclases CD38 and CD157 by TNF but not by RANK-L reveals differences in downstream signaling. Am J Physiol Renal Physiol 291: F557–F566, 2006. First published May 16, 2006; doi:10.1152/ajprenal.00066.2006.—In macrophages and osteoclast precursors, the cytokines TNF and RANK-L induce similar downstream pathways and share some of the same adaptor molecules. However, despite these similarities, no defined signaling schematic has emerged to show how each cytokine favors particular pathways. In this report, we investigate whether TNF and RANK-L differentially regulate ADP-ribosyl cyclases-enzymes that are unique in being crucial for immunological function yet detrimental to osteoclastogenesis. TNF but not RANK-L led to the sustained upregulation of both CD38 and CD157 as demonstrated by real-time PCR and flow cytometry. Further investigation demonstrated that this upregulation was a result of continuous, direct TNF signaling and involved JNK, and more critically PKC and NF-κB. Using this approach allowed us to highlight the relative importance of the PKC, NF-κB, and JNK pathways in actualizing proper outcomes of TNF signaling. Albeit speculative, we believe that differences between TNF- and RANK-1-induced activation of downstream signaling pathways, in particular PKC, are crucial for determining whether progenitor cells become geared for immunity or bone resorption.

osteoclast; osteoclast precursor; NF-κB; JNK; PKC

tnf was codiscovered as an LPS-induced protein that caused tumor necrosis and as a soluble factor that caused the cachexia of systemic infections with Trypanosoma brucei (1, 3). TNF is now recognized as the key cytokine mediating inflammation. It is produced primarily by macrophages and acts in an autocrine manner to stimulate macrophage activation and differentiation (8). Osteoprotegrin (OPG) was discovered as an agent whose overexpression led to increases in bone mineral density (26). The OPG receptor was expression cloned and identified as receptor activator of NF-κB (RANK) (14). RANK and its ligand, RANK-L, are members of the TNF receptor ligand superfamily, although they are relatively far apart evolutionarily in the TNF superfamily tree.

The similarities in the signaling pathways between TNF and RANK-L have led to the notion that the two can cooperatively increase osteoclast formation. Critically, however, TNF alone fails to induce osteoclast formation (15); consistent with separate functions, in contrast to TNF, RANK-L is a weak inflammatory agent.

One difference in signaling that leads to differential gene expression is the activation of nuclear factor of activated T cells (NFAT)2 by RANK-L (30). However, how these cyto-
which are necessary for osteoclast differentiation. Signaling to activate PI3-kinase was once thought to prevent apoptosis; however, it is now believed to modulate IKK\(\beta\) activation and may initiate the activation of the raf\(/MEK/ERK\) signaling cascade to activate Fos-related AP-1 proteins. The various combinations of NFAT and AP-1 proteins are believed to control the transcription of osteoclast-specific genes.

One distinguishing feature of TNFR signaling involves the adaptor protein RIP, which is believed to activate MKK3 and MKK6 to induce p38 MAP kinase. RIP also participates in the activation of aPKCs such as p62; this interaction appears crucial for proper IKK activation (24). Other studies have shown that increases in intracellular Ca\(^{2+}\)/11001, with PKC activation, appear necessary for full activation of NF-\(\kappa\)B (13). This is somewhat puzzling, as TNFR signaling is not thought to activate NFAT proteins; indeed, some evidence suggests that TRAF2 may act to inhibit NFAT-mediated transcription (19). Similar to the influence over differentiation that NFAT exerts, the activation of PKC can also control the differentiation of monocyte/macrophages (18).

Interestingly, in neurons the inhibition of PKC with bisindolylmaleimide I can selectively block NF-\(\kappa\)B-induced transcription while not affecting NFAT- or AP-1-dependent transcription (21), thus highlighting the potentially important role PKC may play in differentiating downstream signal transduction.

Despite all the similarities between RANK and TNFR signaling, treatment with RANK-L and TNF preferentially lead to different outcomes on the same cell. One cell type where this distinction is crucial is the osteoclast precursor (Fig. 2). These cells have the ability to easily differentiate into osteoclasts, macrophages, and dendritic cells. How RANK-L is able to induce osteoclast formation while TNF produces activated macrophages despite both cytokines activating similar signaling pathways remains unclear.

In this report, we investigate whether TNF and RANK-L differentially regulate ADP-ribosyl cyclases-enzymes that convert NAD\(^+\) into the Ca\(^{2+}\)-releasing second messenger cyclic ADP-ribose (17). In immune cells such as lymphocytes, neu-
trophils, and dendritic cells, these enzymes augment chemotaxis (6). Thus, for example, the absence of CD38 blunts Ca$^{2+}$-mediated chemotaxis in both dendritic cells and neutrophils (23). In contrast to these beneficial effects on immune function, ADP-ribosyl cyclase activation in osteoclasts leads to inhibition of resorption (27). Similarly, genetic deletion of CD38 appears to augment osteoclast formation and function (28). Thus ADP-ribosyl cyclases appear to be a special case, where they are necessary for immunity, but inhibit osteoclast formation and bone degradation.

**EXPERIMENTAL PROCEDURES**

**Animals and cell culture.** C57 black mice (2–4 mo of age) and age-matched C57 black CD38$^{-/}$ mice (Jackson Laboratories) were killed according to institutional and National Institutes of Health guidelines, and their femurs and tibia were removed and the marrow flushed with a 23-gauge needle using α-MEM with 10% FBS (In-vitrogen) and 1% penicillin/streptomycin. The cells were grown for 1 day at 37°C in 5% CO$_2$ and then nonadherent cells were filtered through a 70-µm cell strainer (BD Biosciences). The single-cell suspension was separated using density centrifugation with Ficoll-PLUS (Amersham) by spunning at 1,500 rpm for 15 min. The interface layer was washed, counted, and plated for further experimentation.

For most experiments, macrophages were plated for 2–3 days in 10-cm dishes supplemented with 40 ng/ml murine M-CSF (R&D Systems) before experimentation commenced. For experiments involving CD11b-purified cells, whole bone marrow was purified for CD11b using antibodies coupled to magnetic beads (BD Biosciences). For most experiments, plated cells were incubated with M-CSF (40 ng/ml) for 2 days before experimentation commenced. Some experiments utilized murine TNF-α (R&D Systems), soluble GST-RANK-L (courtesy Prof. P. Ross, Washington University, St. Louis, MO), or ready-made cyclohexamide (Sigma).

**cDNA production/real-time PCR primer design.** Total RNA was purified from cells grown on a 10-cm plate using an RNeasy Mini kit (Qiagen). All media was aspirated from the cells (2 × 10$^6$ in count), and they were washed twice with PBS. The cells were disrupted by the addition of buffer RLT, scraped, and shredded using QIAshredder spin columns (Qiagen). Purification of RNA was carried out following the manufacturer’s directions.

Two micrograms of total RNA were converted into cDNA using BD Sprint PowerScript strips (BD Bioscience) following manufacturer’s directions. The reaction product was diluted and then utilized for 40-cycle two-step (95°C/60°C) PCR in an ABI Prism 7900HT (Applied Biosystems) using SYBR Green SuperMix with ROX (Bio-Rad) and 200 nM gene-specific primers (Invitrogen). Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis and melting curve analysis. Each transcript in each sample was assayed three times and the fold-change ratios between experimental and control samples for each gene used in the analysis were calculated using GAPDH or β2-microglobulin levels as a reference.

For mRNA measurements using pulsed application of 10 ng/ml TNF, following application of TNF for 1 h, the media was removed, washed once with PBS, and replaced with fresh media for the duration of the experiment.

**mRNA half-life determination.** Cells were treated with either 40 ng/ml TNF-α or control for 15 h before experimentation. Following the incubation period, 1 µM actinomycin D was added and an equal number of cells was harvested for mRNA determination by quantitative RT-PCR using SYBR green (Bio-Rad) (16).

**FACS analysis.** Cells were lifted with 5 mM EDTA, spun down, and resuspended in tubes to give 10$^6$ cells/200 µl. Cells in each tube were stained for 30 min on ice with the antibodies as indicated in the figures (all antibodies were from BD Biosciences). Cells were washed with and resuspended in PBS containing 0.1% BSA. FACS analysis was performed on a FACSscalibur (Becton Dickinson Immunocytometry System) and 10,000–30,000 total events were collected. Analysis of collected data was carried out using FlowJo software (Treestar Software) on a G5 iMAC according to program directions.

**Signal inhibitor experiments.** The following inhibitors were added 30 min before the addition of 40 ng/ml TNF: the p38 inhibitor SB-203580 (10 µM), the JNK inhibitor SP-600125 (10 µM), the MEK1/2 inhibitor UO-126 (10 µM), the JAK inhibitor 2-(1,1-dimethylcylohexyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one, and the PKC inhibitor bisindolylmaleimide I (all inhibitors from EMD Biosciences). For inhibition of NF-κB signaling, 20 ng/ml TNF-α was used as the stimulus and a cell-permeable ReLA (p65) nuclear translocation inhibitor (Santa Cruz Biotechnology) was used according to the manufacturer’s recommendations. The ability of signal inhibitors to block CD38 production was analyzed by both real-time PCR and FACS analysis, as described above.

**DNA binding and ChIP assays.** The murine CD38 promoter was analyzed for potential NF-κB-binding sites using the program Match and P-Match that utilize the TRANSFAC 6.0 database. Two potential sites were found, one at the beginning of the first exon, and another further upstream. This second site was close to an AP-1 site and we chose to examine its ability to bind NF-κB proteins in vitro and in vivo. DNA binding assays were carried out using the TRANSfactor NF-κB kit (Clonetech) according to the manufacturer’s directions. Oligos used for competitive inhibition were synthesized by Invitrogen.

**RESULTS**

We hypothesized that one possible difference in action between TNF and RANK-L could be in regulating the proinflammatory but antiosteoclastogenic enzyme CD38. CD38 is critical for proper immune cell migration; however, it inhibits bone resorption and negatively impacted osteoclast differentiation. We first examined whether there was differential regulation of ADP-ribosyl cyclases in osteoclasts and bone marrow macrophages (i.e., osteoclast precursors) by treating them with 40 ng/ml TNF for 0, 0.5, 1, 3, 6, and 12 h. Figure 3A shows that TNF induced the transcription of both ADP-ribosyl cyclases, CD38 and CD157, first detectable about 3 h and reaching a plateau by 6–12 h poststimulation.

We next compared the effects of TNF to those of RANK-L. Surprisingly, the addition of RANK-L only failed to significantly upregulate either ADP-ribosyl cyclase (Fig. 3B and data not shown). This was in contrast to the identical upregulation
Fig. 3. TNF but not RANK-L induces the transcription of the ADP-ribosyl cyclases CD38 and CD157. The mRNA expression levels of the ADP-ribosyl cyclases CD38 and CD157 were quantified in triplicate from murine bone marrow-derived macrophages following stimulation with 40 ng/ml TNF for 0, 0.5, 1, 3, 6, and 12 h. Error bars represent standard deviations. TNF (40 ng/ml) but not RANK-L (100 ng/ml) increases the mRNA levels of CD38 as quantified by real-time PCR. Murine bone marrow macrophages were TNF or RANK-L treated for 0, 3, 12, and 24 h. In contrast to CD38, both cytokines were able to equally upregulate the transient expression of interferon-γ. CD38 upregulation required the continuous presence of TNF and was not diminished by the protein synthesis inhibitor cyclohexamide. Filled bars represent groups treated for 6 h with 10 ng/ml TNF, whereas open bars represent non-TNF-treated controls. The expression levels for the inflammatory gene iNOS are shown as a comparison control. Half-lives of CD38 and CD157 were determined following mRNA upregulation induced by 40 ng/ml TNF for 12 h. The mRNA synthesis inhibitor actinomycin D was added and the mRNA levels analyzed at 0, 1, 3, and 5 h according to the protocol of Ref. 16.
of other genes; one such example was the transient induction of interferon-γ (Fig. 3B).

The relatively late onset of ADP-ribosyl cyclase upregulation by TNF (3 h see Fig. 3A) suggested that the upregulation might either be a direct consequence of continuous TNF application or might be secondary to TNF-induced products that RANK-L fails to produce. To test the former possibility, we examined CD38 upregulation using continuous or a 1-h pulsed application of TNF. Figure 3C shows that maximal upregulation was observed only with continuous application and that a pulsed dosage of TNF significantly attenuated gene induction. To examine the latter possibility, we blocked all TNF-induced protein production with cyclohexamide. Although the presence of cyclohexamide increased baseline control values for both CD38 and the inflammatory gene iNOS, it did not reverse TNF-induced increases in expression of either gene and in fact led to augmentation of gene induction in these cells (Fig. 3C). Thus it is likely that signaling downstream of TNF directly induces the upregulation of ADP-ribosyl cyclases and that crucial aspects of this signaling are absent post-RANK-L signaling.

Because TNF and RANK-L similarly led to the upregulation of many genes, we sought to examine whether the increases in CD38/CD157 mRNA were due to alterations in mRNA stability, as TNF is known to modulate mRNA stability (2). However, we found that TNF failed to foster enhancements in the mRNA stability of either CD38 or CD157 (Fig. 3D), suggesting that TNF led to increases in transcription.

As macrophages are traditionally considered heterogeneous (10), we next examined the cellular specificity of ADP-ribosyl cyclase upregulation. Specifically, CD11b+ bone marrow macrophages serve as osteoclast precursors, and we questioned whether the entire CD11b+ population increased their expression of both ADP-ribosyl cyclases or whether there were distinct cell populations responsible for the previously observed changes. To address this question, we treated Ficol1-purified cultured bone marrow cells with either TNF, RANK-L, or control and doubly stained these experimental groups with CD38, IκBα, and histone 2B promoters following stimulation with 40 ng/ml TNF for 3 h, when CD38 expression first starts to increase. We found that the amount of p65 recruited to the CD38 promoter was doubled after 3 h, with expectedly high recruitment to the IκBα promoter or no recruitment to the histone 2B promoter (Fig. 5E). This suggested that TNF directly upregulates CD38 expression by increasing the binding of NF-κB proteins, such as p65, to the CD38 gene promoter.

**DISCUSSION**

The TNF superfamily of cytokines has evolved “to coordinate the social context” of immune responses enabling cells “to maximally respond to pathogens” (20). Through evolution, one member of this superfamily has evolved to coordinate bone degradation, RANK-L. Both TNF and RANK-L induce similar downstream pathways and share some of the same adaptor molecules, such as the TRAFs, to initiate these downstream signals. One crucial difference between TNF and RANK-L signaling was the discovery that RANK-L, but not TNF, led to the sustained activation of NFAT2 (NFATc1) (12, 30). Yet despite this difference, no defined signaling schematic has emerged to show how each cytokine favors particular pathways. This is in contrast to T lymphocytes where the signaling events are downstream of the same receptor, the TCR/CD4, activation one of two pathways. Interestingly, these two pathways signal to preferentially activate either NFAT or NF-κB (25).

Although NFAT2 signaling may hold the key to RANK-L-induced osteoclastogenesis, the pathways induced exclusively by TNF to account for its dramatic inflammatory and immunoregulatory roles remain largely unknown. We hypothesized that one possible difference in action between TNF and RANK-L could be in regulating the proinflammatory but antiosteoclastogenic enzymes CD38 and CD157. This would make physiological sense as both enzymes have been shown to regulate immune cell migration, by either enhancing G protein-
mediated Ca\(^{2+}\) signals or transmitting signals upon binding to PECAM-1 (9, 22, 23). In addition to the crucial role of these enzymes in immune function, we previously showed that an agonist antibody to CD38 led to Ca\(^{2+}\) signals that inhibited bone resorption and negatively impacted osteoclast differentiation (27, 28).

We found that indeed one crucial difference in action between TNF and RANK-L was the differential upregulation of ADP-ribosyl cyclases despite the identical upregulation of other genes. Through mechanistic studies, we confirmed that this gene induction was indeed a direct result of TNF. How TNF selectively induced these genes was the critical question, and we sought to examine the mechanism by which this occurred. Through inhibitor studies, JNK and more importantly PKC appeared to play an important role. While JNK is activated by both TNF and RANK-L, genes induced by NF-\(\kappa\)B signaling downstream of TNF signaling serve to halt JNK activation in the face of continued TNF signaling (see Fig. 1 and introduction) (32). This appears to contrast with RANK-L signaling, where continued \(c-jun\) activation appears necessary for NFAT2-mediated osteoclastogenesis (11).

Thus while JNK may play a role in the outcome of TNF vs. RANK-L signaling, it seems to serve modulatory roles for the dichotomous pair NF-\(\kappa\)B and NFAT. A previous report examining the role of PKCs in RANK-L-induced osteoclastogenesis found that PKC activation served to negatively regulate osteoclast differentiation (32). However, other reports have suggested that PKCs play an important role in mediating RANK-L...
Fig. 5. JNK, PKC, and NF-κB pathways mediate TNF-induced upregulation of ADP-ribosyl cyclases. Bone marrow macrophages were pretreated for 15 min with inhibitors to JNK, p38 MAPK, MEK1/2, JAK, or PKC signaling pathways, following which TNF (40 ng/ml) or control was added for 6 h and the levels of CD38 mRNA were analyzed by real-time PCR. Bone marrow macrophages were pretreated with either an NF-κB nuclear translocation inhibitor peptide or the chemical NF-κB inhibitor parthenolide. TNF, at 20 ng/ml, was added and the levels of CD38 mRNA were determined at 3 and 6 h following addition as described above. Bone marrow macrophages were pretreated with the indicated inhibitors as in A, and the levels of CD38 were analyzed by FACS analysis after 12 h. The murine CD38 promoter was analyzed for potential binding sites for NF-κB. The functionality of one such site was analyzed using a quantitative NF-κB p50 DNA binding assay. Both the consensus NF-κB sequence and the normal CD38, but not a G-to-C bp mutant CD38, sequence were able to compete off binding of p50. Chromatin immunoprecipitation analysis of the murine CD38, IkBa, and histone 2B promoters following stimulation with 40 ng/ml TNF for 3 h. The levels of S536-phosphorylated p65 binding to the promoters was quantified by real-time PCR and expressed as a percentage of the input amount of DNA.
action (7). These conflicting reports are in contrast to well-established PKC-mediated activation of NF-kB under inflammatory settings (4, 31).

Based on these reports, we hypothesized that a PKC/NF-kB pathway may be crucially involved in TNF-induced transcription. PKC is known to control the differentiation of monocyte/macrophages (18); interestingly, previous studies in neurons showed that the inhibition of PKC with bisindolylmaleimide I can selectively block NF-kB-induced transcription while not affecting NFAT- or AP-1-dependent transcription (21). In agreement with this, our findings suggest that PKC may be highly relevant to determining the transcription of genes necessary for either osteoclast or inflammatory differentiation.

By inhibiting NF-kB, we were able to confirm its critical role in the induction of ADP-riboosyl cyclases. We went on to show that, at the transcriptional level, TNF increased the transactivation of the rabbit CD38 promoter (29) and induced the binding of NF-kB proteins to the murine promoter in DNA binding and ChIP assays. In future studies, we will explore how TNF and RANK-L might lead to differential PKC activation and seek to further elucidate the significance of this activation in mediating inflammation vs. osteoclastogenesis.

REFERENCES


2. Zaidi M. gratefully acknowledges support from National Institutes of Health (NIH) Grants AG-14917, AG-23176, and DK-70526 and the Department of Veterans Affairs (Merit Award). J. Iqbal is supported by an NIH Grants AG-14917, AG-23176, and DK-70526 and the Department of Veterans Affairs (Merit Award). J. Iqbal is supported by an NIH Grants AG-14917, AG-23176, and DK-70526 and the Department of Veterans Affairs (Merit Award).

3. De Flora A, Zocchi E, Guida L, Franco L, and Bruzzone S.


