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

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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 discovered 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 cytokines exert their divergent roles remains largely unknown. A schematic representation of signaling from the RANK and TNF receptors is shown in Fig. 1. RANK induces osteoclastogenesis through signals from TNF receptor-associated factor (TRAF)6, although other TRAFs such as TRAF5 and TRAF3 are strongly recruited to the RANK receptor. In contrast, TNFR signals through TNFR-associated death domain (TRADD)’s association with TRAF2, although other TRAFs also bind.

Signaling from TRAF6 leads to activation of a TAB, TAK, NIK complex; this complex may also be activated via PKC. NIK is believed to activate IKK complexes, with IKKβ causing the degradation of IκBα to free NF-κB complexes. These complexes upregulate antiapoptotic/antireactive oxygen species genes such as c-FLIP and MnSOD (as has been shown for TNF, and which may hold true for RANK-L as well). Signaling from RANK to IKKα, which may also involve NIK, does not appear essential for osteoclastogenesis, but may serve as a physiological activator of p52/c-Rel-containing NF-κB complexes.

The activation of NF-κB by TNFRs is believed to occur through a TRAF-2/RIP-ubiquitin signaling complex, which activates IKKβ-containing IKK complexes. This, in turn, leads to IκBα degradation and freeing of p65/p50 complexes for nuclear translocation. Thus, there do not appear to be any major differences between RANK and TNFR with regard to what NF-κB complexes are activated, only differences in the way these complexes become activated.

An additional similarity between RANK and TNFR signaling is the activation of JNK. For the TNFR, JNK appears to be activated by a TRAF2-mediated event where MKK1 phosphorylates MKK7, which in turn phosphorylates JNKK1, both activating it and stabilizing it from degradation. This signaling pathway was recently shown to control TNF-induced apoptosis, in that JNK1 phosphorylated ITCH, which controls the degradation of the antiapoptotic protein c-FLIP. How RANK-L activates JNK remains unclear, but likely involves signaling to activate MKK7. JNK signaling is often taken as essential for osteoclastogenesis; however, it remains to be seen whether Jun protein homodimers are needed or whether this pathway is only needed to activate binding partners for fos-related proteins, which only form heterodimers.

Signaling from TRAF6 appears to activate Src kinase, which activates PLC-γ and phosophinositid (P1)3-kinase. PLC-γ is also the target of the proosteoclastogenic signaling from OSCAR/TREM2 and Syk; activated PLC-γ leads to elevations in intracellular Ca²⁺ levels, activating Ca²⁺-dependent signaling pathways such as PKC and calcineurin. These pathways play a crucial step in controlling the activation of NFAT proteins.
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\(\alpha/B\) activation and may initiate the activation of the \(\text{raf/MEK/ERK}\) 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 \(\text{Ca}^{2+}\), 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 \(\text{Ca}^{2+}\)-releasing second messenger cyclic ADP-ribose (17). In immune cells such as lymphocytes, neu-

Fig. 2. Generation and characterization of “osteoclast precursors” from a total bone marrow population. To obtain a population of “osteoclast precursors,” total bone marrow is subject to several rounds of purification. Note that in contrast to human monocyte/macrophages, the murine counterparts fail to express appreciable levels of CD38 or CD157 (top FACS). To remove already differentiated monocyte/macrophage cells, total bone marrow is plated in low-dose M-CSF for 24 h. This induces the adherence of these cells; nonadherent stem cells are then collected for purification on a ficoll-hypaque column (bottom FACS show cells loaded onto column after 24 h of plating). Cells from the ficoll media interface layer are selectively removed and plated for experimentation.
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% CO2 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 spinning 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 cycloheximide (Sigma).

dNA 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 × 10⁶ 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 10⁶ 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 FACScalibur (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-methyl ethyl)amino)-9-fluoro-3,6-dihydro-7H-benzo[1]imidazol[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 ~900 bp 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 TRANSFAC NF-κB kit (Clonetech) according to the manufacturer’s directions. Oligos used for competitive inhibition were synthesized by Invitrogen.

For ChIP analysis, cells were grown as detailed above. Following the addition of TNF (40 ng/ml), cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Cells then were rinsed with ice-cold PBS-containing protease inhibitors (COMPLETE EDTA-free, Roche). This was repeated once and the cells were scraped into a conical tube. The tubes were centrifuged for 4 min at 2,000 g at 4°C. The cell pellet formed was resuspended in 200 μl of SDS lysis buffer (Upstate Biotechnology). The lysates were sonicated to shear DNA lengths between 400 and 1,000 bps using a Misonex 3000 (PS 1). After immunoprecipitation and washing according to the manufacturer’s directions (Upstate Biotechnology), 5 M NaCl was added and the cross-links reversed at 65°C overnight. DNA was puriﬁed using QiaQuick PCR spin columns (Qiagen) according to the manufacturer’s directions. Quantitative PCR analysis of input and immunoprecipitated DNA was carried out as described for real-time PCR. The primers for CD38 ampliﬁed the region tested in DNA-binding assays above. The murine IkBα and histone H2B primer sequences, used for controls, were courtesy of G. Natoli (Switzerland).

**RESULTS**

We hypothesized that one possible difference in action between TNF and RANK-L could be in regulating the proinﬂammatory but antosteoclastogenic 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 by TNF vs. RANK-L in enzyme expression. We conducted a time course on Ficoll-puriﬁed 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, 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 Ficoll-purified cultured bone marrow cells with either TNF, RANK-L, or control and doubly stained these experimental groups with CD38, CD11b, and CD157 proteins. Using chromatin immunoprecipitation, we analyzed the CD38 promoter for potential NF-κB binding sites and found two possible sites. Because Fig. 5, A and C, suggested that the recruitment of both NF-κB protein and AP-1 proteins might be necessary for proper induction, we chose to focus on one site juxtaposed near a potential AP-1 site ~900 bp upstream of the transcriptional start site. Figure 5D shows that this site effectively bound the NF-κB protein p50 and that this binding could be competed off with either an excess quantity of the native promoter sequence or by the addition of an NF-κB consensus sequence oligomer. On the other hand, a CD38 promoter sequence containing a G-to-C bp mutation rendering effective NF-κB binding impossible failed to inhibit p50 binding (Fig. 5D).

Finally, we designed a series of primers to the murine CD38 promoter to analyze whether TNF induced the recruitment of NF-κB proteins. Using chromatin immunoprecipitation, we analyzed the 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 antosteoclastogenic 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-κB 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-κB 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, IκBα, and histone H2B 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-κB under inflammatory settings (4, 31).

Based on these reports, we hypothesized that a PKC/NF-κB 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-κB-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-κB, we were able to confirm its critical role in the induction of ADP-ribosyl 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-κB 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.

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