Mechanisms of Signal Transduction:
A novel in vivo role for OPGL in activation of monocyte effector function and inflammatory response

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A Novel in vivo Role For OPGL in Activation Of Monocyte Effector Function and Inflammatory Response

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RUNNING TITLE: OPGL can activate monocyte effector function

Key words : RANK, cytokines, survival, endotoxic shock, inflammation

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SUMMARY

Osteoprotegerin Ligand (OPGL) is a member of the TNF ligand superfamily, and has been shown to be involved in interactions between T cell and dendritic cells. Its role in monocyte effector function, however, has not been defined. In the present study a role for OPGL in activating monocytes/macrophages has been characterized. OPGL was found to up-regulate RANK receptor expression on monocytes, regulate their effector function by inducing cytokine and chemokine secretion, activate antigen presentation through up-regulation of co-stimulatory molecule expression, and promote survival. This activation is mediated through the MAPK pathway as evidenced by activation of p38 and p42/44 MAPK, and up-regulation of bcl-xL protein levels. A physiological role for OPGL in monocyte activation and effector function was tested in a model of LPS-induced endotoxic shock. Administration of RANK-Fc to block OPGL activity in vivo was able to protect mice from death induced by sepsis, indicating a hitherto undescribed role for OPGL in monocyte function and in mediating inflammatory response. This was further tested in an animal model of inflammation-mediated arthritis. Treatment with RANK-Fc significantly ameliorated disease development and attenuated bone destruction. Thus, our study strongly suggests that administration of receptor fusion proteins to specifically block OPGL activity in vivo may result in blocking development of monocyte/macrophage mediated diseases.
INTRODUCTION

OPGL (Osteoprotegerin ligand) (also called TRANCE (TNF-related activation-induced cytokine), RANKL (receptor activator of NF-κB ligand), and ODF (osteoclast differentiation factor)) was discovered almost simultaneously by two groups during attempts to clone novel genes involved in the regulation of apoptosis and function of dendritic cells (1,2). OPGL is a member of the TNF ligand superfamily. Most TNF/TNFR superfamily proteins, including CD40L/CD40, TNF/TNFR, or LT^P/LT-β receptor, are expressed in the immune system, and are known to regulate immune response by co-coordinating homeostasis, T cell activation, dendritic cell function, or the formation of germinal centers and lymphoid organs such as Peyer’s patches and lymph nodes (3-5). Sequence analysis has shown that the extracellular domain of OPGL shares 18-28% amino acid identity with other members of the TNF superfamily and the greatest identity with CD40L(2). High levels of OPGL mRNA are detectable in T cells in the lymph nodes and bone osteoblastic cells. OPGL binds to its specific receptor, RANK (receptor activator of NF-κB), a transmembrane member of the TNFR superfamily (2). Although RANK mRNA can be detected in skeletal muscle, thymus, liver, colon, small intestine, and adrenal gland, at the protein level, RANK expression has to date been detectable only the surfaces of mature dendritic cells and osteoclasts. OPGL has also been shown to bind an alternate receptor, OPG (osteoprotegerin) (6,7). OPG acts as a soluble decoy receptor for OPGL and has been shown to neutralize the activity of
OPGL (8).

Initial functional studies on OPGL revealed its ability to activate dendritic cells and inhibit apoptosis, resulting in an increase in dendritic cell-mediated T cell proliferation in an MLR (9). This increase was likely mediated by increased expression of proinflammatory cytokines such as IL-6 and IL-1, and T cell growth factors such as IL-12 and IL-15 by dendritic cells (10). In vitro studies also revealed that OPGL, in combination with CSF-1, could activate mature osteoclasts and mediate osteoclastogenesis (6,7). Evidence for an essential role for OPGL in the immune system and bone development was provided by using OPGL−/− mice (11,12). Although DCs appeared normal, OPGL-deficient mice exhibited defects in early differentiation of T and B cells, and lacked all lymph nodes. Null mice also exhibited severe osteopetrosis and a complete lack of osteoclasts. RANK−/− mice expressed similar phenotypes suggesting that OPGL-RANK interactions provided critical signals necessary for lymph node organogenesis and osteoclast differentiation (13).

An important connection between bone and the immune system was reported by Kong et al when they observed that activated T cells could directly activate osteoclastogenesis through OPGL (14,15). This connection was further strengthened following a report that this T cell mediated regulation could be suppressed by IFN-γ produced by T cells themselves (16). These findings suggested that bone metabolism
is regulated by the immune system through complex and dynamic interactions. In addition, activation of T cells \textit{in vivo} could lead to an OPGL-mediated increase in osteoclastogenesis and bone loss, suggesting that blocking OPGL activity may serve as an efficient therapeutic approach to attenuate bone loss observed in various malignant bone disorders (8).

Thus, while a major role for OPGL in regulation of osteoclastogenesis and chondrocyte differentiation has been established, the exact nature of how OPGL regulates inflammation and the immune system has not been determined. Osteoclasts and monocytes are derived from a common myeloid progenitor and are known to utilize similar signaling pathways involving TRAFs, MAPK and NF-\(\kappa\)B, indicating that they may share a common mechanism of OPGL regulation of their function. This coupled to the fact that OPGL is most closely related in sequence to CD40L, a molecule crucial to activation of antigen presenting cells, suggests a role for OPGL in immune response.

In the present study, we have examined a novel role for OPGL in activating monocytes. RANK protein expression was detected on freshly isolated monocytes, and treatment with OPGL was shown to activate monocytes, resulting in MAPK activation, cytokine secretion and up-regulation of co-stimulatory molecule expression. OPGL was also able to protect monocytes from apoptosis and induced up-regulation of Bcl-2 pro-survival family members such as bcl-xL and bcl-2. These \textit{in vitro} findings were confirmed in an \textit{in vivo} model of LPS-induced septic shock through the use of a
receptor fusion protein approach to specifically block OPGL activity. Administration of RANK-Fc was able to protect mice from death induced by sepsis, indicating a novel role for OPGL in monocyte function \textit{in vivo}. In addition, treatment with RANK-Fc significantly ameliorated disease development in an model of inflammation-mediated arthritis, suggesting therapeutic potential in inflammatory disease. Thus, the identification of a novel immune cell population regulated by OPGL opens up the possibility that OPGL may play a key role in inflammatory immune response.
EXPERIMENTAL PROCEDURES

Cell culture- Monocytes were isolated from human peripheral blood using the Monocyte Isolation Kit (Milteny Biotec) as per manufacturer’s recommendations. Briefly, lymphocytes were isolated from human peripheral blood using Lymphocyte Separation Medium (ICN Pharmaceuticals). A magnetic labeling system (MACS MicroBeads) was used for the isolation of untouched monocytes from peripheral blood by depleting non-monocytes. Cells were maintained in complete medium (RPMI 1640 containing 10% heat-inactivated FBS, 50 U/ml penicillin and 50 µg/ml streptomycin) and cultured at 37°C with 5% CO₂.

Reagents- Monoclonal antibodies against p38 MAP kinase, phosphorylated-p38 MAP kinase, p42/44 MAP kinase, and phosphorylated-p42/44 MAP kinase were purchased from New England BioLabs Inc. (Beverly, MA). Peroxidase-conjugated α-mouse secondary antibodies were purchased from Sigma (St. Louis, MO). Monoclonal antibody to human RANK, recombinant human OPGL and CD40L were purchased from Alexis Biochemicals (San Diego, CA). PE-α-hCD80, FITC-α-hCD86, PE-α-hClass II, FITC-α-mouse IgG1 antibodies were purchased from BD-Pharmingen (New Jersey, NJ). LPS was purchased from Sigma (St. Louis, MO). Murine RANK-Fc and control IgG for in vivo studies were purchased from R&D Systems (Minneapolis, MN) and
Sigma (St. Louis, MO) respectively.

**FACS analyses**- Monocytes were adjusted to $5 \times 10^5$ cells/ml and incubated in the presence or absence of OPGL (5 µg/ml) for 24 hours. Cells were then harvested, washed with PBS containing 2% heat inactivated FBS, incubated with either of the following antibodies for 15 min at 4°C: PE-conjugated α-hCD80, FITC-conjugated α-hCD86, PE-conjugated α-Class II or α-hRANK. Cells stained with α-hRANK were washed and incubated with FITC-conjugated α-mouse IgG1 antibody for 15 min at 4°C. Following another wash step, cells were analyzed by FACS using CELLQUEST software (BD BioSciences, San Diego, CA).

**Taqman analyses**- Monocytes were adjusted to $1 \times 10^6$ cells/ml and cultured for 24 hours with (or without) various concentrations of OPGL. Total RNA was then isolated from OPGL-treated and control cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). Quantitative RT-PCR analyses were performed with 50 ng of total RNA sample and 40 µl of a reaction cocktail. The reaction cocktail in the Taqman Core Kit contained 10x buffer A, 10 Units RNase inhibitor, 200 µM dATP, dCTP, dGTP, dTTP, 4mM MgCl$_2$, 1.25 Units Taq Gold Polymerase and 25 Units MULV reverse transcriptase (Perkin Elmer, Foster City, CA). Each well contained a 10µl primer/probe
mix of 200 nM gene-specific hybridization probe, and 300 nM gene-specific amplification primers. Thermal cycling conditions: 30 min at 48°C, then 2 min at 50°C and 10 min at 95°C. The reactions then cycled 40 times with 15 sec at 95°C and 1 min at 60°C. Reactions and sequence detection was conducted with the ABI Prism 7700 Sequence Detector. Sequence of RANK/GAPDH Taqman primer/probe set used is as follows:

RANK Forward primer: 5’-AGTGGTGCGATTATAGCCCG-3’
RANK Reverse primer: 5’-GAAGGTGAGGTGGGAGGATC-3’
RANK Probe: 5’-AGCCTCTAAACTCCTGGGCTCAAGCAATC-3’
GAPDH Forward primer: 5’-TGGGCTACACTGAGCACCAG-3’
GAPDH Reverse primer: 5’-CAGCGTCAAAGGTGGAGGAG-3’
GAPDH Probe: 5’-TGGTCTCCTCTGACTTCAACAGCGACAC-3’

Detection of cytokines- Monocytes were induced with various concentrations of OPGL for 24 hours, and supernatants were harvested. Levels of secreted cytokines in the cell culture supernatants were determined using ELISAs (BD Pharmingen kits for IL-12 and IL-6, R & D Systems kits for TNF-α, MIP-1α, IL-1β).

Western blotting- Monocytes (adjusted to 1 x 10^6 cells/ml) were starved in serum-free
medium (RPMI1640 containing 50 U/ml penicillin, 50 µg/ml streptomycin) for 4 hours, and incubated with OPGL for 0, 1, 3,10, 30 and 60 mins. At each time point, cells were harvested, washed once with PBS, and lysed in buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM -glycerophosphate, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 tablet complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). Lysates (30 or 50 µg) were separated via SDS-polyacrylamide gel electrophoresis using 4-20 % Tris-glycine gels (Novex Electrophoresis) in SDS Running buffer (25 mM Tris, 0.2 M glycine and 3.5 mM SDS), and transferred onto PVDF membrane (Invitrogen Corp). The membrane was incubated in blocking buffer followed by primary antibodies for p38 and p42/44 MAPK. Antibody-antigen complexes were detected using a horseradish peroxidase-conjugated secondary antibody and ECL system (Amersham Pharmacia Biotech). Western blotting analyses of bcl-xL and bcl-2 was performed essentially as described above, with the lysis buffer being 1% SDS, 0.5% Nonidet P-40, 0.15 M NaCl, 10 mM Tris (pH 7.4), and 1 tablet complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN).

Survival assays- For in vitro assays, monocytes were adjusted to 5 x 10^5 c/ml and incubated with 0.5 mg/ml LPS, 1 µg/ml CD40 ligand, or 1 µg/ml OPGL. At the indicated
time points, cells in the respective cultures were stained with Annexin V-FITC (Clontech Labs, Palo Alto, CA) and analyzed by FACS. For in vivo assays, 8 week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were administered RANK-Fc or control IgG (100 µg intra-peritonealy) daily starting on day 0, injected with 30 mg kg-1 body weight of LPS from Escherichia coli serotype O55:B5 (Calbiochem, San Diego, CA) i.p. on day 1, and monitored for survival.

*Induction of antibody-mediated arthritis:* Arthritis was induced in two groups of 8-week-old female Balb/c mice (The Jackson Laboratory, Bar Harbor, ME) by i.v. injection of 2 mg/mouse (subarthritogenic dose) of a combination of four different monoclonal antibodies generated by the Arthrogen-CIA® mouse B-hybridoma cell lines (Chemicon, Temecula, CA). Disease development was aided by an i.p. injection of 50 µg/mouse LPS the following day. Group 1 and 2 were administered RANK-Fc and control IgG, starting the same day as the injection of the monoclonal antibodies (day 0). Mice were administered 100 µg of RANK-Fc/control IgG i.p. daily. Mice were sacrificed on day 14.

*X-ray and Micro-CT acquisition:* Planar X-ray images of the front and hind paws were acquired after sacrifice. The paws were severed with an axial cut of the distal
radius/ulna (front paw) and tibia/fibula (hind paw). The samples were then imaged with a digital planar x-ray system (MX-20, Faxitron X-ray, Inc., Wheeling, IL). X-ray images were acquired with an x-ray tube current of 300 micro-amperes (µA) and a voltage of 26 kilovolts (kV). The extracted mouse samples (front and hind paws) were also imaged with a µCT40 (SCANCO Medical, Basserdorf, Switzerland) x-ray micro-computed tomography (µCT) system. A sagittal scout image, comparable with a conventional planar x-ray, was obtained to define the start and end point for the axial acquisition of a series of CT images. The location and number of axial images were chosen to provide complete coverage of the joints of interest for each paw. The target joints of the front paw were the metacarpophalangeal (MCP) and interphalangeal joints (PIP and DIP) for digits two through five. The metatarsophalangeal (MTP 2-5), PIP (2-5), and DIP joints of the hind paw were also evaluated. The µCT images were generated by operating the x-ray tube at an energy level of 50 kV, a current of 160 µA and an integration time of 300 milliseconds. Axial images were obtained at an isotropic resolution of 16 µm.

Radiographic Analysis- X-ray images and µCT were evaluated for bone abnormalities by application of a semi-quantitative scoring system that was based on the scoring system for radiographs described by Genant et al (17). A three-dimensional (3D) surface rendering was created from the µCT data with Analyze (AnalyzeDirect Inc.,
Lenexa, KS), an image analysis software package. The x-ray image and corresponding µCT 3D rendering were simultaneously viewed and evaluated by a single reader (W.L.) who was blinded to treatment. The degree of erosions and periarticular osteoporosis were graded with a 6-point score ranging from 0 (normal) to 5 (severe). A cumulative score was determined for each paw by summing the scores of the individual joints: front paw (MCP, PIP and DIP, digits 2-5) and hind paw (MTP, PIP, DIP, digits 2-5).

Histological Analysis- Joints from all 4 feet from each animal were scored in three categories (synovial, bone, and cartilage changes) and added to achieve a final total score. Scores were derived as follows:

1 = severity is minimal and distribution is multifocal-to-diffuse OR severity is mild but distribution is focal
2 = severity mild/distribution diffuse OR severity moderate but distribution is focal
3 = severity is moderate and distribution is multifocal
4 = severity is moderate and distribution is diffuse OR severity is severe but distribution is focal
5 = severity is severe and distribution is multifocal-to-diffuse

Criteria for lesion severity scores:
Synovium, bone and cartilage lesions were scored separately. Synovial lesions were based on the amount of synovial proliferation (pannus) and inflammation. Bone lesions
were based on the amount of bone destruction/loss (occasional evidence of osteoclastic activity in areas of pannus or inflammation as minimal to segmental complete loss of bone and replacement by pannus or new/noncortical bone as severe. Cartilage lesions were based on the amount of cartilage destruction/loss (loss of nuclei and preservation of smooth cartilage surface as minimal to complete fragmentation and/or loss as severe).
RESULTS

*RANK is expressed on monocytes and is up-regulated upon stimulation with OPGL.*

To investigate potential roles for OPGL in monocyte and macrophage effector function, we first wanted to determine if RANK is expressed on monocytes. Towards this end, monocytes were freshly isolated from peripheral blood, stained with a monoclonal antibody to RANK and analyzed by FACS. As shown in Figure 1A, RANK expression on the cell surface of resting monocytes was clearly detected over control isotype staining. To determine if OPGL can regulate expression of RANK, monocytes were treated with various concentrations of OPGL (0, 0.3, 0.6, 1.25 and 2.5 µg/ml) for 24 hours. Total RNA was isolated and quantitative RT-PCR analyses were performed (Figure 1B). Upon stimulation with OPGL, a dose-dependent increase in RANK mRNA expression was observed. The highest concentration of OPGL used, 2.5 µg/ml, stimulated a four-fold increase in RANK expression over un-stimulated monocytes after 24 hours. This up-regulation in RANK expression was specific to OPGL and was not observed when monocytes were stimulated with LPS or CD40L (data not shown). Consistent with an increase in mRNA levels, up-regulation of RANK protein cell surface expression was also observed in FACS analyses upon OPGL treatment after 24 hours (Figure 1C).

*OPGL induces effector function of monocytes by up-regulating secretion of cytokines*
and chemokines- Activation of monocytes by physiological stimuli such as LPS and TNF family members like CD40L has been shown to induce their effector function resulting in secretion of pro-inflammatory cytokines such as IL-1β and TNF-α. To test if OPGL could functionally activate monocytes, we stimulated monocytes for 24 hours with incremental doses of OPGL and looked for production of pro-inflammatory cytokines such as TNF-α and IL-1β, T cell-activation cytokines including IL-12 and IL-6, and chemokines such as MIP-1α. As shown in Figure 2A-E, OPGL was able to induce secretion of these cytokines from freshly isolated monocytes in a dose-dependent manner. At the highest concentration of OPGL used (5 μg/ml), levels of cytokine concentration in the supernatant were 213 pg/ml for IL-12, 7704 pg/ml for IL-6, 13.4 pg/ml for TNF-α, 803 pg/ml for IL-1β and 8740 pg/ml for MIP-1α. These levels were comparable to those induced by LPS at 5 μg/ml (373 pg/ml for IL-12, 6193 pg/ml for IL-6, 21.3 pg/ml for TNF-α, 658 pg/ml for IL-1β and 10395 pg/ml for MIP-1α).

OPGL activates antigen presentation function of monocytes by inducing expression of co-stimulatory molecules- In addition to playing a dominant role in innate immunity by phagocytosis of microorganisms, monocytes and macrophages play a very important role in activating the adaptive immune response by activating T lymphocytes. This is accomplished by the secretion of activation cytokines such as IL-12 and IL-6, by presenting antigens, and by providing co-stimulatory signals to T cells. Antigen
presentation is accomplished by molecules such as MHC Class II, while members of the B7 family such as B7.1/CD80 and B7.2/CD86 are involved in T cell co-stimulation. To study if OPGL is involved in activation of above functions, freshly isolated monocytes were incubated with OPGL (5 µg/ml) and analyzed by FACS. As shown in Figure 3, OPGL was able to efficiently up-regulate expression of CD80 (A), CD86 (B) and MHC Class II (C) molecules on monocytes after a 24-hour stimulation. OPGL was also able to moderately up-regulate CD40 expression on monocytes (data not shown). The above results thus indicate a novel role for OPGL in activating various in vivo functions of monocytes and macrophages such as pro-inflammatory cytokine secretion and T cell activation.

OPGL activates the MAPK pathway in monocytes- To determine if monocyte activation by OPGL involves the MAPK pathway, we induced monocytes with OPGL (1 µg/ml) for 1, 3, 10, 30 and 60 mins and looked for activation of MAPK pathways (Figure 4). Phosphorylation of p38 MAPK (A) and p42/44 ERK (B) was observed at 1 min. and peaked at 10 mins for p38, while it remained at elevated levels for p42/44 MAPK even at 60 min. Interestingly, phosphorylation of p52/54 JNK/SAPK could not be detected in monocytes upon OPGL treatment (data not shown) suggesting a differential utilization of MAPK pathways.
OPGL enhances survival of monocytes- Based on previous studies suggesting a role for OPGL as a survival factor for dendritic cells, we induced apoptosis in monocytes by serum withdrawal and looked for protection from apoptosis induced by OPGL. Annexin V staining analyses revealed that OPGL protected monocytes from apoptosis at levels comparable to LPS (0.5 mg/ml) and CD40L (1 µg/ml) (Figure 5A). Similar results were also obtained for monocytes treated with α-Fas antibody and dexamethasone (data not shown). To determine possible mechanisms of OPGL-induced survival in monocytes, we looked for induction of anti-apoptotic proteins belonging to the bcl-2 family. Robust activation of bcl-xl protein expression was observed starting at 4 hours post-OPGL stimulation (1 µg/ml) in monocytes, and a moderate but reproducible up-regulation of bcl-2 protein expression was also observed (Figure 5B).

Blocking OPGL activity results in decreased susceptibility to endotoxic shock- The above results suggest an important role for OPGL in the activation of effector function of monocytes during inflammation and this was tested in an in vivo model of LPS-induced endotoxic shock (18). A soluble receptor form, RANK-Fc, comprising the extracellular domain of murine RANK was used to block OPGL function in vivo. RANK-Fc was chosen based on its ability to bind OPGL exclusively, as opposed to the soluble decoy receptor OPG, which can also bind an alternate TNF ligand, TRAIL (19). 8 week old C57/Bl6 mice were injected intra-peritoneal with 30 mg/kg LPS and 100 µg
RANK-Fc or control IgG and monitored for survival. As shown in Figure 6, administration of RANK-Fc was able to significantly block death induced by septic shock. After 72 hours, while only 20% of mice in the control group survived, 80% of RANK-Fc-treated mice were still alive. These results indicate a dominant intrinsic role for OPGL in mediating inflammatory response and potential clinical applications in combating septic shock.

 Therapeutic potential for blocking OPGL activity in inflammatory arthritis- Based on the above-observed ability of RANK-Fc to block OPGL function in activating monocytes/macrophages, we tested roles for RANK-Fc in blocking development of inflammation-mediated arthritis in vivo (20,21). This model is distinct from conventional collagen-induced arthritis (CIA) models in that inflammation induced by LPS is one of the major determinants of disease development. In this model, 8 week-old Balb/c mice were injected with a cocktail of four different monoclonal antibodies to collagen on day 0, and 50 µg of LPS on day 1 to activate inflammation. Mice were treated with 100 µg RANK-Fc or control IgG daily and monitored for inflammation in the joints and arthritis. As shown in Figure 7A, swelling in the joints of mice administered RANK-Fc was significantly lower than that in control IgG-treated mice, which was also reflected in the histology scores (Figure 7B). Mice were sacrificed on day 14 representing the end of the study, and radiographic examinations were performed. Digital planar x-rays of
fore- and hind paws revealed that the affected joints in control IgG–treated mice had severe osteolysis and osteophyte production accompanied by disfigurement and this was absent in the RANK-Fc–treated mice (Figures 7C, D). Disease was scored using conventional radiographic methods (scale of 0 to 4, with 4 representing most severe disease) with x-rays and is shown in Table 1. Individual paw scores for RANK-Fc treated mice range from 0 to 3, while those for Ig-treated mice are primarily 4 or 3. In addition, joints were also scanned by micro-CT to generate 3-dimensional rendered images. X-rays and micro-CT renderings were simultaneously viewed and visually evaluated for bone destruction by the application of a modified radiographic scoring system (17). Development of arthritis was significantly inhibited in RANK-Fc treated mice as evidenced by suppressed bone erosion and loss of bone density (Figure 8 A). The degree of erosions and periarticular osteoporosis were graded with a 6-point score ranging from 0 (normal) to 5 (severe). A cumulative score was determined for each paw by summing the scores of the individual joints: front paw (MCP, PIP and DIP, digits 2-5) and hind paw (MTP, PIP, DIP, digits 2-5), and is graphed for each mouse (five per group) in control IgG and RANK-Fc treated groups (Figure 8B). Mice treated with RANK-Fc had significantly reduced bone destruction (mean score 6 ± 2.5) compared to the control group (mean score 33.4 ± 11.3). These results thus demonstrate good efficacy for RANK-Fc in treatment of inflammation-mediated arthritis.
DISCUSSION

Interactions between the TNF superfamily ligands and their cognate receptors are essential in regulating immune response, both by promoting cell survival and proliferation and through activation-induced cell death (3). OPGL and its receptor RANK, members of the TNF superfamily, are known to be key regulators of bone metabolism and essential for the development and function of osteoclasts (6,7). Osteoclasts and monocytes are derived from a common myeloid progenitor indicating that there may be common mechanisms of regulation of their function (22). In addition, OPGL is very closely related to CD40L (1,2), which is known to be crucial for activation of monocytes/macrophages (23). This suggests a potential role for OPGL in regulation of effector function by these cells. In the present study, we have defined a novel role for OPGL in activating monocytes/macrophages. OPGL was found to regulate their effector function by inducing cytokine and chemokine secretion, activate antigen presentation through up-regulation of co-stimulatory molecule expression, and inhibit serum withdrawal-induced apoptosis. These in vitro findings were confirmed in an in vivo model of LPS-induced septic shock through the use of a receptor fusion protein approach to block OPGL activity. Administration of RANK-Fc was able to protect mice from death induced by sepsis, indicating a novel important role for OPGL in monocyte function and in mediating inflammatory response. This was further tested in an animal
model of antibody-induced arthritis. Treatment with RANK-Fc significantly ameliorated disease development and attenuated bone destruction, suggesting therapeutic potential in inflammatory disease.

OPGL and its receptor RANK were initially discovered based on their expression in T cells and dendritic cells respectively (2,9). Interactions between ligand and receptor were shown to be important for activation of dendritic cell function and in regulating cross talk between T cells and dendritic cells. Dendritic cells serve primarily to present antigens to T cells and form an important part of the antigen presenting cell population. Monocytes/macrophages represent another important component of the APC population, and in addition, also perform key effector functions such as inducing inflammation and directly killing microorganisms. They also serve as an important bridge between innate and adaptive immune responses by priming T cells through secretion of activation cytokines and presentation of antigens. To date, OPGL has not been implicated in the regulation of this key immune population and roles for OPGL in activation of monocyte/macrophages function have not been defined. Our results describe a novel function for OPGL in monocyte activation and define important roles in regulating their diverse functions including secretion of pro-inflammatory and T cell activation cytokines, antigen presentation and co-stimulation.

Results from our studies indicate that the biochemical pathways involved in OPGL signaling in monocytes appear to be mediated through p38 MAPK and p42/44
ERK. In mammalian cells, at least three MAPK pathways have been identified: ERK1/2, JNK1/2/SAPK and p38 MAPK pathways. Various cell growth and differentiation stimuli have been shown to activate the ERK1/2 pathway, leading to proliferation and differentiation responses (24). The JNK1/2 and p38 MAPK pathways are primarily activated by inflammatory cytokines and environmental stress and lead to inflammatory, apoptotic or developmental responses (24). Previous studies in monocytes have linked T cell-dependent secretion of the pro-inflammatory cytokines, IL-1β and TNF-α, to activation of the ERK1/2 pathway (25). Our studies suggest that this T cell-dependent signal may be mediated through OPGL, and the subsequent ERK1/2 activation in monocytes may play a central role in the secretion of pro-inflammatory cytokines.

Circulating monocytes have a limited life span and when recruited to a site of inflammation, will undergo apoptosis in the absence of further survival stimuli. CD40L, present on CD4+ T cells has been shown to inhibit apoptosis of circulating monocytes and promote their survival (26). Our results indicate that OPGL may also be important in vivo for monocyte survival. A key parameter that determines whether a cell will respond to an apoptotic signal is the ratio of death antagonist (bcl-2, bcl-xL, bcl-w, mcl-1, bfl-1) to agonists (bax, bak, bcl-xS, bad, bid, bim) belonging to the Bcl-2 family (27). In this context, up-regulation of bcl-xL and bcl-2 induced by OPGL may well account for the decrease in the number of monocytes undergoing apoptosis at the site
of inflammation \textit{in vivo}. Enhanced survival of inflammatory cells, including monocytes, may be an important factor in the establishment of chronic inflammation that characterize both atopic and autoimmune diseases (28,29). Thus OPGL may play a role in the persistence of inflammatory responses associated with these disorders by prolonging monocyte/macrophage survival.

Classical activation signals for monocytes/macrophages include LPS, and another TNF member, CD40L. LPS is a component of cell walls of gram-negative bacteria and is a potent stimulator of inflammatory response. CD40, the receptor for CD40L is expressed on primarily on B cells, antigen presenting cells and endothelial cells (30-32). In monocytes/macrophages, activation of CD40 by its ligand, CD154, has been shown to induce secretion of pro-inflammatory cytokines and chemokines such as IL-1\textbeta, IL-6, IL-8, IL-10, IL-12, TNF-\textalpha and MIP-1\textalpha (33,34). Activation of CD40 signaling in monocytes/macrophages also results in up-regulation of co-stimulatory molecules such as B7.1/B7.2 for T cell activation (34), nitric oxide generation (35) and induction of metalloproteinase production (36) for killing of microorganisms. Thus, there are many similarities in the various physiological roles for CD40L and OPGL, including expression patterns of ligand/receptors and effects on monocytes/macrophages. Results from our and previous studies strongly indicate that they may play important roles in mediating the primary immune response, through direct killing and the induction of inflammation. However, OPGL has been implicated as an immediate early gene in T
cells (1) and may be acting prior to CD40L during innate and adaptive immune response. Rapid up-regulation of OPGL upon activation of the T cell receptor on T cells could specifically activate antigen-presenting cells and promote their survival. Both antigen-specific T cells and the antigen presenting cells would therefore depend on each other for activation and survival. Mature APCs that fail to present antigen to T cells would not receive T cell help and would therefore die of neglect. This feedback loop may play an important in the initiation and maintenance of immune response.

Previous studies have used the decoy receptor OPG as a therapeutic reagent for various malignant bone disorders (37-39). OPG however, is also known to bind another TNF ligand, TRAIL, thus complicating interpretation of results. In our study, we have used RANK-Fc to block OPGL activity since RANK binds to OPGL exclusively. Results from both in vivo models of septic shock and inflammation-mediated arthritis described in our study demonstrate the therapeutic efficacy of RANK-Fc in these applications, establishing a dominant role for OPGL in mediating sepsis and inflammatory arthritis. Furthermore, the ability of RANK-Fc to block monocyte/macrophage function strongly indicates that it may be ameliorate disease in additional clinical indications where these cell populations are known to play major roles.

Acknowledgements
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REFERENCES


### TABLE 1

**RANK-Fc Treatment ameliorates development of arthritis.**

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<td>6</td>
<td>5</td>
<td>9</td>
<td>0</td>
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Distribution of radiographic scores: Paws from control IgG and RANK-Fc treated mice were scored on a scale 0 to 4, with 4 representing most severe bone destruction.
FIGURE LEGENDS

FIG 1. RANK is expressed on monocytes and is upregulated upon OPGL treatment. A, Freshly isolated monocytes were stained for RANK expression (bold line). Isotype control staining is indicated in grey. B, Monocytes were treated with (or without) indicated concentrations of OPGL for 24 hours, and total RNA was isolated. Quantitative RT-PCR analyses were performed, and fold-increase in RANK mRNA levels of OPGL-treated over control untreated cells is shown. GAPDH levels were used to normalize loading. C, Monocytes were analyzed for RANK protein expression at 0 hrs and 24 hours after incubation in the presence or absence of OPGL (5 μg/ml). RANK cell surface expression at 0 and 24 hours is shown in gray and bold lines respectively.

FIG 2. OPGL activates effector function of monocytes by inducing cytokine and chemokine secretion. Freshly isolated monocytes were treated with the indicated concentrations of OPGL for 24 hours and supernatants were harvested. Levels of (A) IL-12, (B) IL-6, (C) TNF-α, (D) IL-1β and (E) MIP-1α in the supernatants were determined by ELISA.

FIG 3. OPGL upregulates expression of co-stimulatory molecules on monocytes. Freshly isolated monocytes were analyzed for (A) CD80, (B) CD86 and (C) MHC Class II protein expression by FACS at 0 hours and 24 hours after incubation in the presence
or absence of OPGL (5 µg/ml). Co-stimulatory molecule expression at the cell surface at 0 and 24 hours is shown in gray and bold lines respectively.

**FIG 4. OPGL activates p38 MAPK and p42/44 ERK pathways in monocytes.** Freshly isolated monocytes were serum-starved for 6 hours, and treated with OPGL (1 µg/ml). At the indicated time points, cells lysates were harvested and Western blotting analyses performed for detection of phosphorylated (A) p38 MAPK and (B) p42/44 MAPK pathways.

**FIG 5. OPGL protects monocytes from apoptosis and induces expression of pro-survival Bcl-2 proteins.** A, Freshly isolated monocytes were incubated in serum-free medium in the presence of OPGL (1 µg/ml), CD40L (1 µg/ml), LPS (0.5 mg/ml) or no stimulus control. At the indicated time points, cells were harvested and apoptotic cells were detected by Annexin V- FITC staining. Cell stimulated with OPGL are indicated by filled squares, with CD40L and LPS by circles and triangles respectively, while control unstimulated cells are represented by open squares. B, Freshly isolated monocytes were treated with OPGL (1 µg/ml) for the indicated time points. Cells were lysed and western blotting analyses performed for detection of bcl-xL and bcl-2 protein levels. Levels of actin at each time point were assayed to control for loading.
FIG 6. Blocking OPGL activity with RANK-Fc protects mice from LPS-induced endotoxic shock. 8 week-old C57/Bl6 mice were injected i.p. with 30 mg/kg LPS serotype O55:B1 and 100 µg/day RANK-Fc (or control IgG) and monitored for survival. Group 1 administered RANK-Fc is represented by black squares and group 2 (control IgG) by white squares.

FIG 7. RANK-Fc inhibits inflammation and blocks disease development in an inflammation-mediated model of collagen-induced arthritis. 8 week-old Balb/c mice were injected with a combination of LPS (50 µg/mouse) and a cocktail of four α-collagen antibodies (4 mg/mouse). Mice were administered 100 µg/day RANK-Fc or control IgG and disease progression was monitored daily. A, LPS-mediated Inflammation per paw was scored (on a scale of 0 to 4, 4 being most severe) daily based on joint swelling. The average score per paw is plotted as a function of days-post α-collagen antibody injection. B, Inflammation was also measured by histological scoring of individual paws (on a scale of 0 to 3 representing none, mild, moderate and severe states of inflammation). C, A radiograph from a representative control mouse showing signs of arthritis, which is significantly reduced in the RANK-Fc-treated mouse. Arrowheads indicate sites of osteolysis. A blowup of this region is shown in (D).
FIG 8. RANK-Fc blocks arthritis by inhibiting bone erosion and loss of bone density in an inflammation-mediated model of arthritis. Mice in control IgG- and RANK-Fc-treated groups were sacrificed 14 days after α-collagen antibody injection and joints were scanned by micro-CT. A, Bone erosion and loss of bone density in joints of mice representative of RANK-Fc and control IgG groups are shown. The images are a 3D-surface rendering created from the μCT data using Analyze image analysis software. B, X-ray images and μCT were evaluated for bone abnormalities by application of a semi-quantitative scoring system that was based on the scoring system for radiographs described by Genant et al. The x-ray image and corresponding μCT 3D rendering were simultaneously view and evaluated by a single reader who was blinded to treatment. The degree of erosions and periarticular osteoporosis were graded with a 6-point score ranging from 0 (normal) to 5 (severe). A cumulative score was determined for each paw by summing the scores of the individual joints: front paw (MCP, PIP and DIP, digits 2-5) and hind paw (MTP, PIP, DIP, digits 2-5).
Figure 2, Seshasayee et al.

A

B

C

D

E

IL-12 (pg/ml)

IL-6 (pg/ml)

TNF-α (pg/ml)

IL-1β (pg/ml)

MIP-1α (pg/ml)

OPGL conc. (µg/ml)

OPGL conc. (µg/ml)

OPGL conc. (µg/ml)

OPGL conc. (µg/ml)
Figure 3, Seshasayee et al.

A  No Stimulation  OPGL (5 μg/ml)

CD80

B  No Stimulation  OPGL (5 μg/ml)

CD86

C  No Stimulation  OPGL (5 μg/ml)

MHC Class II
Figure 4, Seshasayee et al.

A

0 1 3 10 30 60 min.

B

0 1 3 10 30 60 min.
Figure 5, Seshasayee et al.

A

% Cell Survival

Hours-post serum withdrawal

B

0 4 8 12 20 24 30 hrs

bcl-xl

bcl-2

β-actin
Figure 6, Seshasayee et al.
Figure 7, Seshasayee et al.

A  
Mean Swelling score/paw

Day-post Arthritis Induction

Control IgG
RANK-Fc

B  
Mean Histology score/paw

Control IgG
RANK-Fc

C  
Control IgG
RANK-Fc
Figure 8, Seshasayee et al.