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(54) Title: LIGAND FOR RECEPTOR ACTIVATOR OF NF-κKAPPA B, LIGAND IS MEMBER OF TNF SUPERFAMILY
(57) Abstract

Isolated ligands, DNAs encoding such ligands, and pharmaceutical compositions made therefrom, are disclosed. The isolated ligands can be used to regulate an immune response. The ligands are also useful in screening for inhibitors thereof.

*(Referred to in PCT Gazette No. 3/1999, Section II)*
TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the field of cytokines, and more specifically to cytokine receptor/ligand pairs having immunoregulatory activity.

BACKGROUND OF THE INVENTION

Efficient functioning of the immune system requires a fine balance between cell proliferation and differentiation and cell death, to ensure that the immune system is capable of reacting to foreign, but not self antigens. Integral to the process of regulating the immune and inflammatory response are various members of the Tumor Necrosis Factor (TNF) Receptor/Nerve Growth Factor Receptor superfamily (Smith et al., Science 248:1019; 1990). This family of receptors includes two different TNF receptors (Type I and Type II; Smith et al., supra; and Schall et al., Cell 61:361, 1990), nerve growth factor receptor (Johnson et al., Cell 47:545, 1986), B cell antigen CD40 (Stamenkovic et al., EMBO J. 8:1403, 1989), CD27 (Camerini et al., J. Immunol. 147:3165, 1991), CD30 (Durkop et al., Cell 68:421, 1992), T cell antigen OX40 (Mallett et al., EMBO J. 9:1063, 1990), human Fas antigen (Itoh et al., Cell 66:233, 1991), murine 4-1BB receptor (Kwon et al., Proc. Natl. Acad. Sci. USA 86:1963, 1989) and a receptor referred to as Apoptosis-Inducing Receptor (AIR).

CD40 is a receptor present on B lymphocytes, epithelial cells and some carcinoma cell lines that interacts with a ligand found on activated T cells, CD40L (US patent 5,961,974). The interaction of this ligand/receptor pair is essential for both the cellular and humoral immune response. Signal transduction via CD40 is mediated through the association of the cytoplasmic domain of this molecule with members of the TNF receptor-associated factors (TRAFs; Baker and Reddy, Oncogene 12:1, 1996). It has recently been found that mice that are defective in TRAF3 expression due to a targeted disruption in the gene encoding TRAF3 appear normal at birth but develop progressive hypoglycemia and depletion of peripheral white cells, and die by about ten days of age (Xu et al., Immunity 5:407, 1996). The immune responses of chimeric mice reconstituted with TRAF3-/- fetal liver cells resemble those of CD40-deficient mice, although TRAF3-/- B cells appear to be functionally normal.

The critical role of TRAF3 in signal transduction may be in its interaction with one of the other members of the TNF receptor superfamily, for example, CD30 or CD27,
which are present on T cells. Alternatively, there may be other, as yet unidentified members of this family of receptors that interact with TRAF3 and play an important role in postnatal development as well as in the development of a competent immune system. Identifying additional members of the TNF receptor superfamily would provide an additional means of regulating the immune and inflammatory response, as well as potentially providing further insight into post-natal development in mammals.

**SUMMARY OF THE INVENTION**

The present invention provides a counterstructure, or ligand, for a novel receptor referred to as RANK (for receptor activator of NF-κB), that is a member of the TNF superfamily. The ligand, which is referred to as RANKL, is a Type 2 transmembrane protein with an intracellular domain of less than about 50 amino acids, a transmembrane domain and an extracellular domain of from about 240 to 250 amino acids. Similar to other members of the TNF family to which it belongs, RANKL has a 'spacer' region between the transmembrane domain and the receptor binding domain that is not necessary for receptor binding. Accordingly, soluble forms of RANKL can comprise the entire extracellular domain or fragments thereof that include the receptor binding region.

RANK is a Type I transmembrane protein having 616 amino acid residues that is a member of the TNFR superfamily, and interacts with TRAF3. Triggering of RANK by over-expression, co-expression of RANK and membrane bound RANKL, or by soluble RANKL or agonistic antibodies to RANK, results in the upregulation of the transcription factor
NF-κB, a ubiquitous transcription factor that is most extensively utilized in cells of the immune system.

In one aspect, there is provided an isolated receptor activator of NF-kappa B ligand (RANKL) polypeptide that binds a receptor activator of NF-kappa B (RANK), selected from the group consisting of: (a) a polypeptide comprising amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162 of SEQ ID NO:13 and y is any one of amino acids 313-317 of SEQ ID NO:13; (b) a polypeptide comprising amino acids 1-162 of SEQ ID NO:13; (c) a polypeptide comprising an amino acid sequence having at least 90% identity to amino acids 69-313 of SEQ ID NO:13; and (d) a polypeptide comprising an amino acid sequence having at least 90% identity to amino acids 138-317 of SEQ ID NO:13.

In another aspect, there is provided a fusion polypeptide comprising a first polypeptide covalently fused with a second polypeptide, wherein the first polypeptide is the RANKL polypeptide as defined above.

In another aspect, there is provided an isolated nucleic acid encoding the polypeptide as defined above.

In another aspect, there is provided an isolated nucleic acid that comprises nucleotides 1 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

In another aspect, there is provided an isolated nucleic acid that comprises nucleotides 484 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.
In another aspect, there is provided an isolated nucleic acid that comprises nucleotides 412 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

In another aspect, there is provided an isolated nucleic acid that comprises nucleotides 205 to 939 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

In another aspect, there is provided an isolated nucleic acid that comprises nucleotides 484 to 939 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

In another aspect, there is provided an isolated antibody that binds with higher affinity to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide than to a murine RANKL polypeptide as shown in SEQ ID NO:11, wherein the human RANKL polypeptide is selected from the group consisting of: (a) a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13; (b) a polypeptide that is at least 90% identical to amino acids 1-317 of SEQ ID NO:13 and that binds to a RANK polypeptide as shown in SEQ ID NO:6; (c) a polypeptide consisting of amino acids 1-162 of SEQ ID NO:13; and (d) a fragment of the polypeptide of (a), (b), or (c) that binds to a RANK polypeptide as shown in SEQ ID NO:6.
In another aspect, there is provided an isolated antibody that binds with higher affinity to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 than to a murine RANKL polypeptide as shown in SEQ ID NO:11.

In another aspect, there is provided an isolated antibody that binds to the human receptor activator of NF-kappa B ligand (RANKL) polypeptide but not to a murine RANKL polypeptide as shown in SEQ ID NO:11, wherein the human RANKL polypeptide is selected from the group consisting of: (a) a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13; (b) a polypeptide that is at least 90% identical to amino acids 1-317 of SEQ ID NO:13 and that binds to a RANK polypeptide as shown in SEQ ID NO:6; (c) a polypeptide consisting of amino acids 1-162 of SEQ ID NO:13; and (d) a fragment of the polypeptide of (a), (b), or (c) that binds to a RANK polypeptide as shown in SEQ ID NO:6.

In another aspect, there is provided an isolated antibody that binds to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13, but that does not bind to a murine RANKL polypeptide as shown in SEQ ID NO:11.

As is understood in the art, antibodies may be polyclonal or monoclonal.

In another aspect, there is provided use of the receptor activator of NF-kappa B ligand (RANKL) polypeptide as defined above as an antigen for producing an antibody.
immunoreactive to the human RANKL polypeptide as shown in SEQ ID NO:13.

In another aspect, there is provided a method for preparing an antibody to a receptor activator of NF-kappa B ligand (RANKL) polypeptide comprising immunizing a non-human animal with the RANKL polypeptide as defined above in an amount effective to produce antibodies to the RANKL polypeptide, and harvesting the antibodies.

In another aspect, there is provided use of the RANKL or the fusion polypeptide as defined above, for the preparation of an antibody that binds specifically to the polypeptide.

In another aspect, there is provided a method to screen for an inhibitor of RANK activation, the method comprising the step of providing the RANKL polypeptide as defined above under conditions where the RANKL polypeptide binds to RANK, in the presence or absence of a test compound; wherein a reduction in binding between the RANKL polypeptide and RANK in the presence of the test compound compared to its absence indicates the test compound is an inhibitor of RANK activation.

In another aspect, there is provided use of the antibody as defined above for inhibiting interaction between receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 and RANK polypeptide as shown in SEQ ID NO:6.

In another aspect, there is provided use of the antibody as defined above in the manufacture of a medicament for inhibiting interaction between receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 and RANK polypeptide as shown in SEQ ID NO:6.
In another aspect, there is provided a kit for detecting a receptor activator of NF-kappa B (RANK) or a RANK ligand (RANKL) polypeptide, or for detecting RANK activity, the kit comprising: (a) instructions for the detecting; and (b) the RANKL polypeptide or the antibody as defined above.

In another aspect, there is provided a kit comprising: (a) the soluble RANKL polypeptide as defined herein; and (b) instructions for using said RANKL polypeptide to monitor receptor activator of NF-kappa B (RANK) related activity.

These and other aspects of the present invention will become evident upon reference to the following detailed description of the invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 demonstrates the influence of RANK-Fc and hRANKL on activated T cell growth. Human peripheral blood T cells were cultured as described in Example 12; viable T cell recovery was determined by triplicate trypan blue countings.

Figure 2 illustrates the ability of RANKL to induce human DC cluster formation. Functionally mature dendritic cells (DC) were generated in vitro from CD34+ bone marrow (BM) progenitors and cultured as described in Example 13. CD1a+ DC were cultured in a cytokine cocktail alone (Figure 2A), in cocktail plus CD40L (Figure 2B), RANKL (Figure 2C), or heat inactivated (ΔH) RANKL (Figure 2D), and then photographed using an inversion microscope.
Figure 3 demonstrates that RANKL enhances DC allo-stimulatory capacity. Allogeneic T cells were incubated with varying numbers of irradiated DC cultured as
described in Example 13. The cultures were pulsed with [³H]-thymidine and the cells harvested onto glass fiber sheets for counting. Values represent the mean ± standard deviation (SD) of triplicate cultures.

Figure 4 presents an alignment of human RANK with other TNFR family members in the region of structurally conserved extracellular cysteine-rich pseudorepeats. Predicted disulfide linkages (DS1-DS3) are indicated. RANK and CD40 contain identical amino acid substitutions (C^H, C^G) eliminating DS2 in the second pseudorepeat.

Figure 5 presents an alignment of human RANKL with other TNF family members.

**DETAILED DESCRIPTION OF THE INVENTION**

A novel partial cDNA insert with a predicted open reading frame having some similarity to CD40 was identified in a database containing sequence information from cDNAs generated from human bone marrow-derived dendritic cells (DC). The insert was used to hybridize to colony blots generated from a DC cDNA library containing full-length cDNAs. Several colony hybridizations were performed, and two clones (SEQ ID NOs: 1 and 3) were isolated. SEQ ID NO: 5 shows the nucleotide and amino acid sequence of a predicted full-length protein based on alignment of the overlapping sequences of SEQ ID NOs: 1 and 3.

RANK is a member of the TNF receptor superfamily; it most closely resembles CD40 in the extracellular region. Similar to CD40, RANK associates with TRAF2 and TRAF3 (as determined by co-immunoprecipitation assays substantially as described by Rothe et al., *Cell* 83:1243, 1995). TRAFs are critically important in the regulation of the immune and inflammatory response. Through their association with various members of the TNF receptor superfamily, a signal is transduced to a cell. That signal results in the proliferation, differentiation or apoptosis of the cell, depending on which receptor(s) is/are triggered and which TRAF(s) associate with the receptor(s); different signals can be transduced to a cell via coordination of various signaling events. Thus, a signal transduced through one member of this family may be proliferative, differentiative or apoptotic, depending on other signals being transduced to the cell, and/or the state of differentiation of the cell. Such exquisite regulation of this proliferative/apoptotic pathway is necessary to develop and maintain protection against pathogens; imbalances can result in autoimmune disease.

RANK is expressed on epithelial cells, some B cell lines, and on activated T cells. However, its expression on activated T cells is late, about four days after activation. This time course of expression coincides with the expression of Fas, a known agent of apoptosis. RANK may act as an anti-apoptotic signal, rescuing cells that express RANK from apoptosis as CD40 is known to do. Alternatively, RANK may confirm an apoptotic
signal under the appropriate circumstances, again similar to CD40. RANK and its ligand
are likely to play an integral role in regulation of the immune and inflammatory response.

Moreover, the post-natal lethality of mice having a targeted disruption of the TRAF3
gene demonstrates the importance of this molecule not only in the immune response but in
development. The isolation of RANK, as a protein that associates with TRAF3, and its
ligand, RANKL, will allow further definition of this signaling pathway, and development
of diagnostic and therapeutic modalities for use in the area of autoimmune and/or
inflammatory disease.

DNAs, Proteins and Analogs

The present invention provides isolated RANKL polypeptides and analogs (or
mutesins) thereof having an activity exhibited by the native molecule (i.e., RANKL mutesins
that bind specifically to a RANK expressed on cells or immobilized on a surface or to
RANKL-specific antibodies; soluble forms thereof that inhibit RANK ligand-induced
signaling through RANK). Such proteins are substantially free of contaminating
endogenous materials and, optionally, without associated native-pattern glycosylation.
Derivatives of RANKL within the scope of the invention also include various structural
forms of the primary proteins which retain biological activity. Due to the presence of
ionizable amino and carboxyl groups, for example, a RANKL protein may be in the form
of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also
be modified by oxidation or reduction. The primary amino acid structure may be modified
by forming covalent or aggregative conjugates with other chemical moieties, such as
glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid
sequence mutants. Covalent derivatives are prepared by linking particular functional
groups to amino acid side chains or at the N- or C-termini.

Derivatives of RANKL may also be obtained by the action of cross-linking agents,
such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and
lysine residues. The inventive proteins may also be covalently bound through reactive side
groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-
activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by
adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once
bound to a substrate, the proteins may be used to selectively bind (for purposes of assay or
purification) antibodies raised against the proteins or against other proteins which are
similar to RANKL, as well as other proteins that bind RANKL or homologs thereof.

Soluble forms of RANKL are also within the scope of the invention. The
nucleotide and predicted amino acid sequence of the RANKL is shown in SEQ ID NOs:10
and 12 (murine and human, respectively). Computer analysis indicated that the RANKL is
a Type 2 transmembrane protein; murine RANKL contains a predicted 48 amino acid
intracellular domain, 21 amino acid transmembrane domain and 247 amino acid extracellular domain, and human RANKL contains a predicted 47 amino acid intracellular domain, 21 amino acid transmembrane domain and 249 amino acid extracellular domain.

Soluble RANKL comprises a signal peptide and the extracellular domain or a fragment thereof. An exemplary signal peptide is that shown in SEQ ID NO:9; other signal (or leader) peptides are well-known in the art, and include that of murine Interleukin-7 or human growth hormone. RANKL is similar to other members of the TNF family in having a region of amino acids between the transmembrane domain and the receptor binding region that does not appear to be required for biological activity; this is referred to as a 'spacer' region. Amino acid sequence alignment indicates that the receptor binding region is from about amino acid 162 of human RANKL to about amino acid 317 (corresponding to amino acid 139 through 294 of murine RANKL, SEQ ID NO:10), beginning with an Ala residue that is conserved among many members of the family (amino acid 162 of SEQ ID NO:12).

Moreover, fragments of the extracellular domain will also provide soluble forms of RANKL. Those skilled in the art will recognize that the actual receptor binding region may be different than that predicted by computer analysis. Thus, the N-terminal amino acid of a soluble RANKL is expected to be within about five amino acids on either side of the conserved Ala residue. Alternatively, all or a portion of the spacer region may be included at the N-terminus of a soluble RANKL, as may be all or a portion of the transmembrane and/or intracellular domains, provided that the resulting soluble RANKL is not membrane-associated. Accordingly, a soluble RANKL will have an N-terminal amino acid selected from the group consisting of amino acids 1 through 162 of SEQ ID NO:12 (1 though 139 of SEQ ID NO:10). Preferably, the amino terminal amino acid is between amino acids 69 and 162 of SEQ ID NO:12 (human RANKL; amino acids 48 and 139 of SEQ ID NO:10). Similarly, the carboxy terminal amino acid can be between amino acid 313 and 317 of SEQ ID NO:12 (human RANKL; corresponding to amino acids 290 through 294 of SEQ ID NO:10). Those skilled in the art can prepare these and additional soluble forms through routine experimentation.

Fragments can be prepared using known techniques to isolate a desired portion of the extracellular region, and can be prepared, for example, by comparing the extracellular region with those of other members of the TNF family (of which RANKL is a member) and selecting forms similar to those prepared for other family members. Alternatively, unique restriction sites or PCR techniques that are known in the art can be used to prepare numerous truncated forms which can be expressed and analyzed for activity.

Other derivatives of the RANKL proteins within the scope of this invention include covalent or aggregative conjugates of the proteins or their fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide
sequence at the N-terminal region of the protein which co-translationally or post-
translationally directs transfer of the protein from its site of synthesis to its site of function
inside or outside of the cell membrane or wall (e.g., the yeast α-factor leader).

Protein fusions can comprise peptides added to facilitate purification or
identification of RANKL proteins and homologs (e.g., poly-His). The amino acid
sequence of the inventive proteins can also be linked to an identification peptide such as that
described by Hopp et al., *Bio/Technology* 6:1204 (1988). Such a highly antigenic peptide
provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid
assay and facile purification of expressed recombinant protein. The sequence of Hopp et
al. is also specifically cleaved by bovine mucosal enterokinase, allowing removal of the
peptide from the purified protein. Fusion proteins capped with such peptides may also be
resistant to intracellular degradation in *E. coli*.

Fusion proteins further comprise the amino acid sequence of a RANKL linked to an
immunoglobulin Fc region. An exemplary Fc region is a human IgG1, having a nucleotide
an amino acid sequence set forth in SEQ ID NO:8. Fragments of an Fc region may also be
used, as can Fc muteins. For example, certain residues within the hinge region of an Fc
region are critical for high affinity binding to FcγRI. Canfield and Morrison (J. Exp. Med.
173:1483; 1991) reported that Leu(234) and Leu(235) were critical to high affinity binding of
IgG3 to FcγRI present on U937 cells. Similar results were obtained by Lund et al. (J.
in combination, can be made in an IgG, Fc region to decrease the affinity of IgG1 for FcR.
Depending on the portion of the Fc region used, a fusion protein may be expressed as a
dimer, through formation of interchain disulfide bonds. If the fusion proteins are made
with both heavy and light chains of an antibody, it is possible to form a protein oligomer
with as many as four RANKL regions.

In another embodiment, RANKL proteins further comprise an oligomerizing
peptide such as a leucine zipper domain. Leucine zippers were originally identified in
several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988). Leucine
zipper domain is a term used to refer to a conserved peptide domain present in these (and
other) proteins, which is responsible for dimerization of the proteins. The leucine zipper
domain (also referred to herein as an oligomerizing, or oligomer-forming, domain)
comprises a repetitive heptad repeat, with four or five leucine residues interspersed with
other amino acids. Examples of leucine zipper domains are those found in the yeast
transcription factor GCN4 and a heat-stable DNA-binding protein found in rat liver
(C/EBP; Landschulz et al., *Science* 243:1681, 1989). Two nuclear transforming proteins,
*fos* and *jun*, also exhibit leucine zipper domains, as does the gene product of the murine
nuclear oncogenes *fos* and *jun* comprise leucine zipper domains preferentially form a


Leucine zipper domains fold as short, parallel coiled coils. (O'Shea et al., *Science* 254:539; 1991) The general architecture of the parallel coiled coil has been well characterized, with a "knobs-into-holes" packing as proposed by Crick in 1953 (*Acta Crystallogr.* 6:689). The dimer formed by a leucine zipper domain is stabilized by the heptad repeat, designated (abcdefg)ₘ according to the notation of McLachlan and Stewart (*J. Mol. Biol.* 98:293; 1975), in which residues a and d are generally hydrophobic residues, with d being a leucine, which line up on the same face of a helix. Oppositely-charged residues commonly occur at positions g and e. Thus, in a parallel coiled coil formed from two helical leucine zipper domains, the "knobs" formed by the hydrophobic side chains of the first helix are packed into the "holes" formed between the side chains of the second helix.

The leucine residues at position d contribute large hydrophobic stabilization energies, and are important for dimer formation (Krystek et al., *Int. J. Peptide Res.* 38:229, 1991). Lovejoy et al. recently reported the synthesis of a triple-stranded α-helical bundle in which the helices run up-up-down (*Science* 259:1288, 1993). Their studies confirmed that hydrophobic stabilization energy provides the main driving force for the formation of coiled coils from helical monomers. These studies also indicate that electrostatic interactions contribute to the stoichiometry and geometry of coiled coils.

Several studies have indicated that conservative amino acids may be substituted for individual leucine residues with minimal decrease in the ability to dimerize; multiple changes, however, usually result in loss of this ability (Landschulz et al., *Science* 243:1681, 1989; Turner and Tjian, *Science* 243:1689, 1989; Hu et al., *Science* 250:1400, 1990). van Heekeren et al. reported that a number of different amino residues can be substituted for the leucine residues in the leucine zipper domain of GCN4, and further found that some GCN4 proteins containing two leucine substitutions were weakly active.
Mutation of the first and second heptadic leucines of the leucine zipper domain of the measles virus fusion protein (MVF) did not affect syncytium formation (a measure of virally-induced cell fusion); however, mutation of all four leucine residues prevented fusion completely (Buckland et al., *J. Gen. Virol.* 73:1703, 1992). None of the mutations affected the ability of MVF to form a tetramer.

Amino acid substitutions in the $a$ and $d$ residues of a synthetic peptide representing the GCN4 leucine zipper domain have been found to change the oligomerization properties of the leucine zipper domain (Alber, Sixth Symposium of the Protein Society, San Diego, CA). When all residues at position $a$ are changed to isoleucine, the leucine zipper still forms a parallel dimer. When, in addition to this change, all leucine residues at position $d$ are also changed to isoleucine, the resultant peptide spontaneously forms a trimeric parallel coiled coil in solution. Substituting all amino acids at position $d$ with isoleucine and at position $a$ with leucine results in a peptide that tetramerizes. Peptides containing these substitutions are still referred to as leucine zipper domains.

The present invention also includes RANKL with or without associated native-pattern glycosylation. Proteins expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of DNAs encoding the inventive proteins in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of RANKL protein having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-$A_1$-$Z$, where $A_1$ is any amino acid except Pro, and $Z$ is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue $Z$, deleting Asn or $Z$, or inserting a non-$Z$ amino acid between $A_1$ and $Z$, or an amino acid other than Asn between Asn and $A_1$.

RANKL protein derivatives may also be obtained by mutations of the native RANKL or subunits thereof. A RANKL mutated protein, as referred to herein, is a polypeptide homologous to a native RANKL protein, but which has an amino acid sequence different from the native protein because of one or a plurality of deletions, insertions or substitutions. The effect of any mutation made in a DNA encoding a mutated peptide may be easily determined by analyzing the ability of the mutated peptide to bind its counterstructure in a specific manner. Moreover, activity of RANKL analogs, muteins or
derivatives can be determined by any of the assays described herein (for example, induction of NF-κB activation).

Analogs of the inventive proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present.

When a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered. Subunits of the inventive proteins may be constructed by deleting terminal or internal residues or sequences. Soluble forms of RANKL can be readily prepared and tested for their ability to induce NF-κB activation. Polypeptides corresponding to the cytoplasmic regions, and fragments thereof (for example, a death domain) can be prepared by similar techniques. Additional guidance as to the types of mutations that can be made is provided by a comparison of the sequence of RANKL to proteins that have similar structures, as well as by performing structural analysis of the inventive RANKL proteins.

Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those which do not affect the biological activity of RANKL (i.e., ability of the inventive proteins to bind antibodies to the corresponding native protein in substantially equivalent a manner, the ability to bind the counterstructure in substantially the same manner as the native protein, the ability to induce a RANKL signal, or ability to induce NF-κB activation). Examples of conservative substitutions include substitution of amino acids outside of the binding domain(s) (either ligand/receptor or antibody binding areas for the extracellular domain, or regions that interact with other, intracellular proteins for the cytoplasmic domain), and substitution of amino acids that do not alter the secondary and/or tertiary structure of the native protein. Additional examples include substituting one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known.

Mutations in nucleotide sequences constructed for expression of analog proteins or fragments thereof must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the mRNA.
Not all mutations in the nucleotide sequence which encodes a RANKL protein or fragments thereof will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A), or to provide codons that are more readily translated by the selected host, e.g., the well-known E. coli preference codons for E. coli expression.

Although a mutation site may be predetermined, it is not necessary that the nature of the mutation per se be predetermined. For example, in order to select for optimum characteristics of mutants, random mutagenesis may be conducted and the expressed mutated proteins screened for the desired activity. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Patent NOs. 4,518,584 and 4,737,462 disclose suitable techniques.

Additional embodiments of the inventive proteins include RANKL polypeptides encoded by DNAs capable of hybridizing to the DNAs of SEQ ID NO:10 or 12 under moderately stringent conditions (prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of 50°C, 5 X SSC, overnight) to the DNA sequences encoding RANKL, or more preferably under stringent conditions (for example, hybridization in 6 X SSC at 63°C overnight; washing in 3 X SSC at 55°C), and other sequences which are degenerate to those which encode the RANKL. In one embodiment, RANKL polypeptides are at least about 70% identical in amino acid sequence to the amino acid sequence of native RANKL protein as set forth in SEQ ID NOs:10 and 12. In a preferred embodiment, RANKL polypeptides are at least about 80% identical in amino acid sequence to the native form of RANKL; most preferred polypeptides are those that are at least about 90% identical to native RANKL.

Percent identity may be determined using a computer program, for example, the GAP computer program described by Devereux et al. (Nucl. Acids Res. 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). For
fragments derived from the RANKL protein, the identity is calculated based on that portion of the RANKL protein that is present in the fragment.

The biological activity of RANKL analogs or muteins can be determined by testing the ability of the analogs or muteins to induce a signal through RANK, for example, activation of transcription as described in the Examples herein. Alternatively, suitable assays, for example, an enzyme immunoassay or a dot blot, employing an antibody that binds native RANKL, or a soluble form of RANK, can be used to assess the activity of RANKL analogs or muteins. Suitable assays also include, for example, assays that measure the ability of a RANKL peptide or mutein to bind cells expressing RANK, and/or the biological effects thereon. Such methods are well known in the art.

Fragments of the RANKL nucleotide sequences are also useful. In one embodiment, such fragments comprise at least about 17 consecutive nucleotides, preferably at least about 25 nucleotides, more preferably at least 30 consecutive nucleotides, of the RANKL DNA disclosed herein. DNA and RNA complements of such fragments are provided herein, along with both single-stranded and double-stranded forms of the RANKL DNAs of SEQ ID NOs: 10 and 12, and those encoding the aforementioned polypeptides. A fragment of RANKL DNA generally comprises at least about 17 nucleotides, preferably from about 17 to about 30 nucleotides. Such nucleic acid fragments (for example, a probe corresponding to the extracellular domain of RANKL) are used as a probe or as primers in a polymerase chain reaction (PCR).

The probes also find use in detecting the presence of RANKL nucleic acids in *in vitro* assays and in such procedures as Northern and Southern blots. Cell types expressing RANKL can be identified as well. Such procedures are well known, and the skilled artisan can choose a probe of suitable length, depending on the particular intended application. For PCR, 5' and 3' primers corresponding to the termini of a desired RANKL DNA sequence are employed to amplify that sequence, using conventional techniques.

Other useful fragments of the RANKL nucleic acids are antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target RANKL mRNA (sense) or RANKL DNA (antisense) sequences. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

**Uses of DNAs, Proteins and Analogs**

The RANKL DNAs, proteins and analogs described herein will have numerous uses, including the preparation of pharmaceutical compositions. For example, soluble forms of RANKL will be useful to transduce signal via RANK. RANKL compositions (both protein and DNAs) will also be useful in development of antibodies to RANKL, both
those that inhibit binding to RANK and those that do not. The inventive DNAs are useful for the expression of recombinant proteins, and as probes for analysis (either quantitative or qualitative) of the presence or distribution of RANKL transcripts.

The inventive proteins will also be useful in preparing kits that are used to detect soluble RANK or RANKL, or monitor RANK-related activity, for example, in patient specimens. RANKL proteins will also find uses in monitoring RANK-related activity in other samples or compositions, as is necessary when screening for antagonists or mimetics of this activity (for example, peptides or small molecules that inhibit or mimic, respectively, the interaction). A variety of assay formats are useful in such kits, including (but not limited to) ELISA, dot blot, solid phase binding assays (such as those using a biosensor), rapid format assays and bioassays.

The purified RANKL according to the invention will facilitate the discovery of inhibitors of RANK, and thus, inhibitors of an inflammatory response (via inhibition of NF-κB activation). The use of a purified RANKL polypeptide in the screening for potential inhibitors is important and can virtually eliminate the possibility of interfering reactions with contaminants. Such a screening assay can utilize either the extracellular domain of RANKL, or a fragment thereof. Detecting the inhibiting activity of a molecule would typically involve use of a soluble form of RANKL derived from the extracellular domain in a screening assay to detect molecules capable of binding RANK and inhibiting binding of the RANKL.

In addition, RANKL polypeptides can also be used for structure-based design of RANKL-inhibitors. Such structure-based design is also known as "rational drug design." The RANKL polypeptides can be three-dimensionally analyzed by, for example, X-ray crystallography, nuclear magnetic resonance or homology modeling, all of which are well-known methods. The use of RANKL structural information in molecular modeling software systems to assist in inhibitor design is also encompassed by the invention. Such computer-assisted modeling and drug design may utilize information such as chemical conformational analysis, electrostatic potential of the molecules, protein folding, etc. A particular method of the invention comprises analyzing the three dimensional structure of RANKL for likely binding sites of substrates, synthesizing a new molecule that incorporates a predictive reactive site, and assaying the new molecule as described above.

Moreover, as shown in the Examples herein, soluble forms of RANKL will be useful to induce maturation of dendritic cells (DC), and to enhance their allo-stimulatory capacity. Accordingly, RANKL proteins will be useful in augmenting an immune response, and can be used for these purposes either ex vivo (i.e., in obtaining cells such as DC from an individual, exposing them to antigen and cytokines ex vivo, and re-administering them to the individual) or in vivo (i.e., as a vaccine adjuvant that will augment humoral and/or cellular immunity). RANKL will also be useful promoting
viability of T cells in the presence of TGFβ, which will also be helpful in regulating an immune response.

Expression of Recombinant RANKL

The proteins of the present invention are preferably produced by recombinant DNA methods by inserting a DNA sequence encoding RANKL protein or an analog thereof into a recombinant expression vector and expressing the DNA sequence in a recombinant expression system under conditions promoting expression. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being inserted in a recombinant expression vector and expressed in a recombinant transcriptional unit.

Recombinant expression vectors include synthetic or cDNA-derived DNA fragments encoding RANKL, or homologs, muteins or bioequivalent analogs thereof, operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation, as described in detail below. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated.

DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame. DNA sequences encoding RANKL, or homologs or analogs thereof which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. E. coli is typically transformed using derivatives of pBR322, a plasmid derived
from an *E. coli* species (Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β-lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful bacterial expression system employs the phage λ PL promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ PL promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate dehydroxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* (Amp<sup>F</sup> gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α-factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast α-factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. See, e.g., Kurjan et al., *Cell* 30:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. The leader sequence may be modified to contain, near its 3′ end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly
useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* II site located in the viral origin of replication is included. Further, viral genomic promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A preferred eukaryotic vector for expression of RANKL DNA is referred to as pDC406 (McMahan et al., *EMBO J.* 10:2821, 1991), and includes regulatory sequences derived from SV40, human immunodeficiency virus (HIV), and Epstein-Barr virus (EBV). Other preferred vectors include pDC409 and pDC410, which are derived from pDC406. pDC410 was derived from pDC406 by substituting the EBV origin of replication with sequences encoding the SV40 large T antigen. pDC409 differs from pDC406 in that a *Bgl* II restriction site outside of the multiple cloning site has been deleted, making the *Bgl* II site within the multiple cloning site unique.

A useful cell line that allows for episomal replication of expression vectors, such as pDC406 and pDC409, which contain the EBV origin of replication, is CV-1/EBNA (ATCC CRL 10478). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) and constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter.

**Host Cells**

Transformed host cells are cells which have been transformed or transfected with expression vectors constructed using recombinant DNA techniques and which contain sequences encoding the proteins of the present invention. Transformed host cells may express the desired protein (RANKL, or homologs or analogs thereof), but host cells transformed for purposes of cloning or amplifying the inventive DNA do not need to express the protein. Expressed proteins will preferably be secreted into the culture supernatant, depending on the DNA selected, but may be deposited in the cell membrane.

Suitable host cells for expression of proteins include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *Bacillus* spp. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce proteins using RNAs.
derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (Cloning Vectors: A Laboratory Manual, Elsevier, New York, 1985).

Prokaryotic expression hosts may be used for expression of RANKL, or homologs or analogs thereof that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium, and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

Recombinant RANKL may also be expressed in yeast hosts, preferably from the Saccharomyces species, such as S. cerevisiae. Yeast of other genera, such as Pichia or Kluyveromyces may also be employed. Yeast vectors will generally contain an origin of replication from the 2μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding the protein, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and E. coli, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae trp1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929, 1978, selecting for Trp+ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil. Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems can be employed to express recombinant protein. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, Bio/Technology 6:47 (1988). Examples of
suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, CV-1/EBNA (ATCC CRL 10478), L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Purification of Recombinant RANKL

Purified RANKL, and homologs or analogs thereof are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts. For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a counter structure protein or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Gel filtration chromatography also provides a means of purifying the inventive proteins.

Affinity chromatography is a particularly preferred method of purifying RANKL and homologs thereof. For example, a RANKL expressed as a fusion protein comprising an immunoglobulin Fc region can be purified using Protein A or Protein G affinity chromatography. Moreover, a RANKL protein comprising an oligomerizing zipper domain may be purified on a resin comprising an antibody specific to the oligomerizing zipper domain. Monoclonal antibodies against the RANKL protein may also be useful in affinity chromatography purification, by utilizing methods that are well-known in the art. A ligand may also be used to prepare an affinity matrix for affinity purification of RANKL.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a RANKL composition.
Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express the inventive protein as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (J. Chromatog. 296:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Protein synthesized in recombinant culture is characterized by the presence of cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover the inventive protein from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of the inventive proteins free of other proteins which may be normally associated with the proteins as they are found in nature in the species of origin.

Uses and Administration of RANKL Compositions

The present invention provides methods of using therapeutic compositions comprising an effective amount of a protein and a suitable diluent and carrier, and methods for regulating an immune or inflammatory response. The use of RANKL in conjunction with soluble cytokine receptors or cytokines, or other immunoregulatory molecules is also contemplated.

For therapeutic use, purified protein is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, RANKL protein compositions administered to regulate immune function can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a therapeutic agent will be administered in the form of a composition comprising purified RANKL, in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed.
Ordinarily, the preparation of such protein compositions entails combining the inventive protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

As shown herein, RANKL has beneficial effects on various cells important in the immune system. Accordingly, RANKL may be adminstered to an individual as a vaccine adjuvant, or as a therapeutic agent to upregulate an immune response, for example, infectious disease. Moreover, NF-κB has been found to play a protective role in preventing apoptotic death of cells induced by TNF-α or chemotherapy. Accordingly, agonists of RANK (i.e., RANKL and agonistic antibodies) will be useful in protecting RANK-expressing cells from the negative effects of chemotherapy or the presence of high levels of TNF-α such as occur in sepsis (see, i.e., Barinaga, Science 274:724, 1996, and the articles by Beg and Baltimore and Wang et al., pages 782 and 784 of that same issue of Science).

The following examples are offered by way of illustration, and not by way of limitation. Those skilled in the art will recognize that variations of the invention embodied in the examples can be made, especially in light of the teachings of the various references cited herein.

**EXAMPLE 1**

The example describes the identification and isolation of a DNA encoding a novel member of the TNF receptor superfamily. A partial cDNA insert with a predicted open reading frame having some similarity to CD40 (a cell-surface antigen present on the surface of both normal and neoplastic human B cells that has been shown to play an important role in B-cell proliferation and differentiation; Stamenkovic et al., EMBO J. 8:1403, 1989), was identified in a database containing sequence information from cDNAs generated from human bone marrow-derived dendritic cells (DC). The insert was excised from the vector by restriction endonuclease digestion, gel purified, labeled with $^{32}$P, and used to hybridize to colony blots generated from a DC cDNA library containing larger cDNA inserts using
high stringency hybridization and washing techniques (hybridization in 5xSSC, 50% formamide at 42°C overnight, washing in 0.5xSSC at 63°C); other suitable high stringency conditions are disclosed in Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; 1989), 9.52-9.55. Initial experiments yielded a clone referred to as 9D-8A (SEQ ID NO:1); subsequent analysis indicated that this clone contained all but the extreme 5’ end of a novel cDNA, with predicted intron sequence at the extreme 5’ end (nucleotides 1-92 of SEQ ID NO:1). Additional colony hybridizations were performed, and a second clone was isolated. The second clone, referred to as 9D-15C (SEQ ID NO:3), contained the 5’ end without intron interruption but not the full 3’ end. SEQ ID NO:5 shows the nucleotide and amino acid sequence of a predicted full-length protein based on alignment of the overlapping sequences of SEQ ID NOs:1 and 3.

The encoded protein was designated RANK, for receptor activator of NF-κB. The cDNA encodes a predicted Type 1 transmembrane protein having 616 amino acid residues, with a predicted 24 amino acid signal sequence (the computer predicted cleavage site is after Leu24), a 188 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 383 amino acid cytoplasmic tail. The extracellular region of RANK displayed significant amino acid homology (38.5% identity, 52.3% similarity) to CD40. A cloning vector (pBluescriptSK-) containing human RANK sequence, designated pBluescript:huRANK (in E. coli DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 20, 1996, under terms of the Budapest Treaty, and given accession number 98285.

EXAMPLE 2

This example describes construction of a RANK DNA construct to express a RANK/Fc fusion protein. A soluble form of RANK fused to the Fc region of human IgG, was constructed in the mammalian expression vector pDC409 (US patent 5,576,191). This expression vector encodes the leader sequence of the Cytomegalovirus (CMV) open reading frame R27080 (SEQ ID NO:9), followed by amino acids 33-213 of RANK, followed by a mutated form of the constant domain of human IgG, that exhibits reduced affinity for Fc receptors (SEQ ID NO:8; for the fusion protein, the Fc portion of the construct consisted of Arg3 through Lys232). An alternative expression vector encompassing amino acids 1-213 of RANK (using the native leader sequence) followed by the IgG, mutein was also prepared. Both expression vectors were found to induce high levels of expression of the RANK/Fc fusion protein in transfected cells.

To obtain RANK/Fc protein, a RANK/Fc expression plasmid is transfected into CV-1/EBNA cells, and supernatants are collected for about one week. The RANK/Fc fusion protein is purified by means well-known in the art for purification of Fc fusion
proteins, for example, by protein A sepharose* column chromatography according to manufacturer's recommendations (i.e., Pharmacia, Uppsala, Sweden). SDS-polyacrylamide gel electrophoresis analysis indicated that the purified RANK/Fc protein migrated with a molecular weight of ~55kDa in the presence of a reducing agent, and at a molecular weight of ~110kDa in the absence of a reducing agent.

N-terminal amino acid sequencing of the purified protein made using the CMV R27080 leader showed 60% cleavage after Ala20, 20% cleavage after Pro22 and 20% cleavage after Arg28 (which is the Furin cleavage site; amino acid residues are relative to SEQ ID NO:9); N-terminal amino acid analysis of the fusion protein expressed with the native leader showed cleavage predominantly after Gln25 (80% after Gln25 and 20% after Arg23; amino acid residues are relative to SEQ ID NO:6, full-length RANK). Both fusion proteins were able to bind a ligand for RANK is a specific manner (i.e., they bound to the surface of various cell lines such as a murine thymoma cell line, EL4), indicating that the presence of additional amino acids at the N-terminus of RANK does not interfere with its ability to bind RANKL. Moreover, the construct comprising the CMV leader encoded RANK beginning at amino acid 33; thus, a RANK peptide having an N-terminus at an amino acid between Arg23 and Pro33, inclusive, is expected to be able to bind a ligand for RANK in a specific manner.

Other members of the TNF receptor superfamily have a region of amino acids between the transmembrane domain and the ligand binding domain that is referred to as a 'spacer' region, which is not necessary for ligand binding. In RANK, the amino acids between 196 and 213 are predicted to form such a spacer region. Accordingly, a soluble form of RANK that terminates with an amino acid in this region is expected to retain the ability to bind a ligand for RANK in a specific manner. Preferred C-terminal amino acids for soluble RANK peptides are selected from the group consisting of amino acids 213 and 196 of SEQ ID NO:6, although other amino acids in the spacer region may be utilized as a C-terminus.

**EXAMPLE 3**

This example illustrates the preparation of monoclonal antibodies against RANK. Preparations of purified recombinant RANK, for example, or transfected cells expressing high levels of RANK, are employed to generate monoclonal antibodies against RANK using conventional techniques, such as those disclosed in U.S. Patent 4,411,993. DNA encoding RANK can also be used as an immunogen, for example, as reviewed by Pardoll and Beckerleg in *Immunity* 3:165, 1995. Such antibodies are likely to be useful in interfering with RANK-induced signaling (antagonistic or blocking antibodies) or in inducing a signal by cross-linking RANK (agonistic antibodies), as components of

*Trade-mark
diagnostic or research assays for RANK or RANK activity, or in affinity purification of RANK.

To immunize rodents, RANK immunogen is emulsified in an adjuvant (such as complete or incomplete Freund's adjuvant, alum, or another adjuvant, such as Ribi adjuvant R700 (Ribi, Hamilton, MT), and injected in amounts ranging from 10-100 μg subcutaneously into a selected rodent, for example, BALB/c mice or Lewis rats. DNA may be given intradermally (Raz et al., *Proc. Natl. Acad. Sci. USA* 91:9519, 1994) or intramuscularly (Wang et al., *Proc. Natl. Acad. Sci. USA* 90:4156, 1993); saline has been found to be a suitable diluent for DNA-based antigens. Ten days to three weeks days later, the immunized animals are boosted with additional immunogen and periodically boosted thereafter on a weekly, biweekly or every third week immunization schedule.

Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich), ELISA (enzyme-linked immunosorbent assay), immunoprecipitation, or other suitable assays, including FACS analysis. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to a murine myeloma cell line (e.g., NS1 or preferably Ag 8.653 [ATCC CRL 1580]). Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a selective medium (for example, one containing hypoxanthine, aminopterin, and thymidine, or HAT) to inhibit proliferation of non-fused cells, myeloma-myeloma hybrids, and splenocyte-splenocyte hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with RANK, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochim. 8:871* (1971) and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described by Beckman et al., *J. Immunol.* 144:4212 (1990). Positive clones are then injected into the peritoneal cavities of syngeneic rodents to produce ascites containing high concentrations (>1 mg/ml) of anti-RANK monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to RANK protein.

Monoclonal antibodies were generated using RANK/Fc fusion protein as the immunogen. These reagents were screened to confirm reactivity against the RANK protein. Using the methods described herein to monitor the activity of the mAbs, both blocking (i.e., antibodies that bind RANK and inhibit binding of a ligand to RANK) and non-blocking (i.e., antibodies that bind RANK and do not inhibit ligand binding) were isolated.
EXAMPLE 4

This example illustrates the induction of NF-κB activity by RANK in 293/EBNA cells (cell line was derived by transfection of the 293 cell line with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively express EBNA-1 driven from human CMV immediate-early enhancer/promoter). Activation of NF-κB activity was measured in 293/EBNA cells essentially as described by Yao et al. (Immunity 3:811, 1995). Nuclear extracts were prepared and analyzed for NF-κB activity by a gel retardation assay using a 25 base pair oligonucleotide spanning the NF-κB binding sites. Two million cells were seeded into 10 cm dishes two days prior to DNA transfection and cultured in DMEM-F12 media containing 2.5% FBS (fetal bovine serum). DNA transfections were performed as described herein for the IL-8 promoter/reporter assays.

Nuclear extracts were prepared by solubilization of isolated nuclei with 400 mM NaCl (Yao et al., supra). Oligonucleotides containing an NF-κB binding site were annealed and endlabeled with 32P using T4 DNA polynucleotide kinase. Mobility shift reactions contained 10 μg of nuclear extract, 4 μg of poly(dI-dC) and 15,000 cpm labeled double-stranded oligonucleotide and incubated at room temperature for 20 minutes. Resulting protein-DNA complexes were resolved on a 6% native polyacrylamide gel in 0.25 X Tris-borate-EDTA buffer.

Overexpression of RANK resulted in induction of NF-κB activity as shown by an appropriate shift in the mobility of the radioactive probe on the gel. Similar results were observed when RANK was triggered by a ligand that binds RANK and transduces a signal to cells expressing the receptor (i.e., by co-transfecting cells with human RANK and murine RANKL DNA; see Example 7 below), and would be expected to occur when triggering is done with agonistic antibodies.

EXAMPLE 5

This example describes a gene promoter/reporter system based on the human Interleukin-8 (IL-8) promoter used to analyze the activation of gene transcription in vivo. The induction of human IL-8 gene transcription by the cytokines Interleukin-1 (IL-1) or tumor necrosis factor-alpha (TNF-α) is known to be dependent upon intact NF-κB and NF-IL-6 transcription factor binding sites. Fusion of the cytokine-responsive IL-8 promoter with a cDNA encoding the murine IL-4 receptor (mIL-4R) allows measurement of promoter activation by detection of the heterologous reporter protein (mIL-4R) on the cell surface of transfected cells.

Human kidney epithelial cells (293/EBNA) are transfected (via the DEAE/DEXTRAN method) with plasmids encoding: 1) the reporter/promoter construct (referred to as pIL-8rep), and 2) the cDNA(s) of interest. DNA concentrations are always kept constant by the addition of empty vector DNA. The 293/EBNA cells are plated at a
density of $2.5 \times 10^4$ cells/ml (3 ml/well) in a 6 well plate and incubated for two days prior to transfection. Two days after transfection, the mIL-4 receptor is detected by a radioimmunoassay (RIA) described below.

In one such experiment, the 293/EBNA cells were co-transfected with DNA encoding RANK and with DNA encoding RANKL (see Example 7 below). Co-expression of this receptor and its counterstructure by cells results in activation of the signaling process of RANK. For such co-transfection studies, the DNA concentration/well for the DEAE transfection were as follows: 40 ng of pIL-8rep [pBluescriptK+ vector (Stratagene)]; 0.4 ng CD40 (DNA encoding CD40, a control receptor; pCDM8 vector); 0.4 ng RANK (DNA encoding RANK; pDC409 vector), and either 1-50 ng CD40L (DNA encoding the ligand for CD40, which acts as a positive control when co-transfected with CD40 and as a negative control when co-transfected with RANK; in pDC304) or RANKL (DNA encoding a ligand for RANK; in pDC406). Similar experiments can be done using soluble RANKL or agonistic antibodies to RANK to trigger cells transfected with RANK.

For the mIL-4R-specific RIA, a monoclonal antibody reactive with mIL-4R is labeled with $^{125}$I via a Chloramine T conjugation method; the resulting specific activity is typically $1.5 \times 10^{16}$ cpm/nmol. After 48 hours, transfected cells are washed once with media (DMEM/F12 5% FBS). Non-specific binding sites are blocked by the addition of pre-warmed binding media containing 5% non-fat dry milk and incubation at 37°C/5% CO$_2$ in a tissue culture incubator for one hour. The blocking media is decanted and binding buffer containing $^{125}$I anti-mIL-4R (clone M1; rat IgG1) is added to the cells and incubated with rocking at room temperature for 1 hour. After incubation of the cells with the radiolabeled antibody, cells are washed extensively with binding buffer (2X) and twice with phosphate-buffered saline (PBS). Cells are lysed in 1 ml of 0.5M NaOH, and total radioactivity is measured with a gamma counter.

Using this assay, 293/EBNA co-transfected with DNAs encoding RANK demonstrated transcriptional activation, as shown by detection of muIL-4R on the cell surface. Overexpression of RANK resulted in transcription of muIL-4R, as did triggering of the RANK by RANKL. Similar results are observed when RANK is triggered by agonistic antibodies.

**EXAMPLE 6**

This example illustrates the association of RANK with TRAF proteins. Interaction of RANK with cytoplasmic TRAF proteins was demonstrated by co-immunoprecipitation assays essentially as described by Hsu et al. (*Cell* 84:299; 1996). Briefly, 293/EBNA cells were co-transfected with plasmids that direct the synthesis of RANK and epitope-tagged (FLAG®; SEQ ID NO:7) TRAF2 or TRAF3. Two days after transfection, surface proteins
were labeled with biotin-ester, and cells were lysed in a buffer containing 0.5% NP-40. RANK and proteins associated with this receptor were immunoprecipitated with anti-RANK, washed extensively, resolved by electrophoretic separation on a 6-10% SDS polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane for Western blotting. The association of TRAF2 and TRAF3 proteins with RANK was visualized by probing the membrane with an antibody that specifically recognizes the FLAG® epitope. TRAFs 2 and 3 did not immunoprecipitate with anti-RANK in the absence of RANK expression.

EXAMPLE 7

This example describes isolation of a ligand for RANK, referred to as RANKL, by direct expression cloning. The ligand was cloned essentially as described in US patent 5,961,974 for CD40L. Briefly, a library was prepared from a clone of a mouse thymoma cell line EL-4 (ATCC TIB 39), called EL-40.5, derived by sorting five times with biotinylated CD40/Fc fusion protein in a FACS (fluorescence activated cell sorter). The cDNA library was made using standard methodology; the plasmid DNA was isolated and transfected into sub-confluent CV1-EBNA cells using a DEAE-dextran method. Transfectants were screened by slide autoradiography for expression of RANKL using a two-step binding method with RANK/Fc fusion protein as prepared in Example 2 followed by radiiodinated goat anti-human IgG antibody.

A clone encoding a protein that specifically bound RANK was isolated and sequenced; the clone was referred to as 11H. An expression vector containing murine RANKL sequence, designated pDC406:muRANK-L (in E. coli DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on December 20, 1996, under terms of the Budapest Treaty, and given accession number 98284. The nucleotide sequence and predicted amino acid sequence of this clone are illustrated in SEQ ID NO:10. This clone did not contain an initiator methionine; additional, full-length clones were obtained from a 7B9 library (prepared substantially as described in US patent 5,599,905, issued February 4, 1997); the 5' region was found to be identical to that of human RANKL as shown in SEQ ID NO: 12, amino acids 1 through 22, except for substitution of a Gly for a Thr at residue 9.

This ligand is useful for assessing the ability of RANK to bind RANKL by a number of different assays. For example, transfected cells expressing RANKL can be used in a FACS assay (or similar assay) to evaluate the ability of soluble RANK to bind RANKL. Moreover, soluble forms of RANKL can be prepared and used in assays that are known in the art (i.e., ELISA or BIAcore assays essentially as described in US patent 5,961,974). RANKL is also useful in affinity purification of RANK.
and as a reagent in methods to measure the levels of RANK in a sample. Soluble RANKL is also useful in inducing NF-κB activation and thus protecting cells that express RANK from apoptosis.

**EXAMPLE 8**

This example describes the isolation of a human RANK ligand (RANKL) using a PCR-based technique. Murine RANK ligand-specific oligonucleotide primers were used in PCR reactions using human cell line-derived first strand cDNAs as templates. Primers corresponded to nucleotides 478-497 and to the complement of nucleotides 858-878 of murine RANK ligand (SEQ ID NO:10). An amplified band approximately 400 bp in length from one reaction using the human epidermoid cell line KB (ATCC CCL-17) was gel purified, and its nucleotide sequence determined; the sequence was 85% identical to the corresponding region of murine RANK ligand, confirming that the fragment was from human RANKL.

To obtain full-length human RANKL cDNAs, two human RANKL-specific oligonucleotides derived from the KB PCR product nucleotide sequence were radiolabeled and used as hybridization probes to screen a human PBL cDNA library prepared in lambda gt10 (Stratagene, La Jolla, CA), substantially as described in US patent 5,599,905, issued February 4, 1997. Several positive hybridizing plaques were identified and purified, their inserts subcloned into pBluescript SK- (Stratagene, La Jolla, CA), and their nucleotide sequence determined. One isolate, PBL3, was found to encode most of the predicted human RANKL, but appeared to be missing approximately 200 bp of 5' coding region. A second isolate, PBL5 was found to encode much of the predicted human RANKL, including the entire 5' end and an additional 200 bp of 5' untranslated sequence.

The 5' end of PBL5 and the 3' end of PBL3 were ligated together to form a full length cDNA encoding human RANKL. The nucleotide and predicted amino acid sequence of the full-length human RANK ligand is shown in SEQ ID NO:12. Human RANK ligand shares 83% nucleotide and 84% amino acid identity with murine RANK ligand. A plasmid vector containing human RANKL sequence, designated pBluescript:huRANK-L (in E. coli DH10B), was deposited with the American Type Culture Collection, Rockville, MD (ATCC) on March 11, 1997 under terms of the Budapest Treaty, and given accession number 98354.

Murine and human RANKL are Type 2 transmembrane proteins. Murine RANKL contains a predicted 48 amino acid intracellular domain, 21 amino acid transmembrane domain and 247 amino acid extracellular domain. Human RANKL contains a predicted 47 amino acid intracellular domain, 21 amino acid transmembrane domain and 249 amino acid extracellular domain.
EXAMPLE 9

This example describes the chromosomal mapping of human RANK using PCR-based mapping strategies. Initial human chromosomal assignments were made using RANK and RANKL-specific PCR primers and a BIOS Somatic Cell Hybrid PCRable DNA kit from BIOS Laboratories (New Haven, CT), following the manufacturer’s instructions. RANK mapped to human chromosome 18; RANK ligand mapped to human chromosome 13. More detailed mapping was performed using a radiation hybrid mapping panel Genebridge 4 Radiation Hybrid Panel (Research Genetics, Huntsville, AL; described in Walter, MA et al., Nature Genetics 7:22-28, 1994). Data from this analysis was then submitted electronically to the MIT Radiation Hybrid Mapper (URL: http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) following the instructions contained therein. This analysis yielded specific genetic marker names which, when submitted electronically to the NCBI Entrez browser (URL: http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=c&form=0), yielded the specific map locations. RANK mapped to chromosome 18q22.1, and RANKL mapped to chromosome 13q14.

EXAMPLE 10

This example illustrates the preparation of monoclonal antibodies against RANKL. Preparations of purified recombinant RANKL, for example, or transfixed cells expressing high levels of RANKL, are employed to generate monoclonal antibodies against RANKL using conventional techniques, such as those disclosed in US Patent 4,411,993. DNA encoding RANKL can also be used as an immunogen, for example, as reviewed by Pardoll and Beckerleg in Immunity 3:165, 1995. Such antibodies are likely to be useful in interfering with RANKL signaling (antagonistic or blocking antibodies), as components of diagnostic or research assays for RANKL or RANKL activity, or in affinity purification of RANKL.

To immunize rodents, RANKL immunogen is emulsified in an adjuvant (such as complete or incomplete Freund’s adjuvant, alum, or another adjuvant, such as Ribi adjuvant R700 (Ribi, Hamilton, MT), and injected in amounts ranging from 10-100 μg subcutaneously into a selected rodent, for example, BALB/c mice or Lewis rats. DNA may be given intradermally (Raz et al., Proc. Natl. Acad. Sci. USA 91:9519, 1994) or intramuscularly (Wang et al., Proc. Natl. Acad. Sci. USA 90:4156, 1993); saline has been found to be a suitable diluent for DNA-based antigens. Ten days to three weeks days later, the immunized animals are boosted with additional immunogen and periodically boosted thereafter on a weekly, biweekly or every third week immunization schedule.

Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich), ELISA (enzyme-linked immunosorbent
assay), immunoprecipitation, or other suitable assays, including FACS analysis. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to a murine myeloma cell line (e.g., NS1 or preferably Ag 8.653 [ATCC CRL 1580]). Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a selective medium (for example, one containing hypoxanthine, aminopterin, and thymidine, or HAT) to inhibit proliferation of non-fused cells, myeloma-myeloma hybrids, and splenocyte-splenocyte hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with RANKL, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in US Patent 4,703,004. A preferred screening technique is the antibody capture technique described by Beckman et al., *J. Immunol.* 144:4212 (1990). Positive clones are then injected into the peritoneal cavities of syngeneic rodents to produce ascites containing high concentrations (>1 mg/ml) of anti-RANK monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to RANKL protein. Using the methods described herein to monitor the activity of the mAbs, both blocking (i.e., antibodies that bind RANKL and inhibit binding to RANK) and non-blocking (i.e., antibodies that bind RANKL and do not inhibit binding) are isolated.
EXAMPLE 11

This example demonstrates that RANK expression can be up-regulated. Human peripheral blood T cells were purified by flow cytometry sorting or by negative selection using antibody coated beads, and activated with anti-CD3 (OKT3, Dako) coated plates or phytohemagglutinin in the presence or absence of various cytokines, including Interleukin-4 (IL-4), Transforming Growth Factor-β (TGF-β) and other commercially available cytokines (IL1-α, IL-2, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IFN-γ, TNF-α). Expression of RANK was evaluated by FACS in a time course experiment for day 2 to day 8, using a mouse monoclonal antibody mAb144 (prepared as described in Example 3), as shown in the table below. Results are expressed as ‘+’ to ‘++++’ referring to the relative increase in intensity of staining with anti-RANK. Double labeling experiments using both anti-RANK and anti-CD8 or anti-CD4 antibodies were also performed.

Table 1: Upregulation of RANK by Cytokines

<table>
<thead>
<tr>
<th>Cytokine (concentration)</th>
<th>Results:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (50 ng/ml)</td>
<td>+</td>
</tr>
<tr>
<td>TGF-β (5 ng/ml)</td>
<td>+ to ++</td>
</tr>
<tr>
<td>IL-4 (50 ng/ml) +TGF-β (5 ng/ml)</td>
<td>++++</td>
</tr>
<tr>
<td>IL1-α (10ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IL-2 (20ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IL-3 (25ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IL-7 (20ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IL-8 (10ng/ml)</td>
<td>-</td>
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<tr>
<td>IL-10 (50ng/ml)</td>
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<tr>
<td>IL-12 (10ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IL-15 (10ng/ml)</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ (100U/ml)</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α (10ng/ml)</td>
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</tbody>
</table>

Of the cytokines tested, IL-4 and TGF-β increased the level of RANK expression on both CD8+ cytotoxic and CD4+ helper T cells from day 4 to day 8. The combination of IL-4 and TGF-β acted synergistically to upregulate expression of this receptor on activated T cells. This particular combination of cytokines is secreted by suppresser T cells, and is believed to be important in the generation of tolerance (reviewed in Mitchison and Sieper, *Z. Rheumatol.* 54:141, 1995), implicating the interaction of RANK in regulation of an immune response towards either tolerance or induction of an active immune response.
EXAMPLE 12

This example illustrates the influence of RANK.Fc and hRANKL on activated T cell growth. The addition of TGFβ to anti-CD3 activated human peripheral blood T lymphocytes induces proliferation arrest and ultimately death of most lymphocytes within the first few days of culture. We tested the effect of RANK:RANKL interactions on TGFβ-treated T cells by adding RANK.Fc or soluble human RANKL to T cell cultures.

Human peripheral blood T cells (7 x 10⁶ PBT) were cultured for six days on anti-CD3 (OKT3, 5µg/ml) and anti-Flag (M1, 5µg/ml) coated 24 well plates in the presence of TGFβ (1ng/ml) and IL-4 (10ng/ml), with or without recombinant FLAG-tagged soluble hRANKL (1µg/ml) or RANK.Fc (10µg/ml). Viable T cell recovery was determined by triplicate trypan blue countings.

The addition of RANK.Fc significantly reduced the number of viable T cells recovered after six days, whereas soluble RANKL greatly increased the recovery of viable T cells (Figure 1). Thus, endogenous or exogenous RANKL enhances the number of viable T cells generated in the presence of TGFβ. TGFβ, along with IL-4, has been implicated in immune response regulation when secreted by the Th3/regulatory T cell subset. These T cells are believed to mediate bystander suppression of effector T cells. Accordingly, RANK and its ligand may act in an auto/paracrine fashion to influence T cell tolerance. Moreover, TGFβ is known to play a role in the evasion of the immune system effected by certain pathogenic or opportunistic organisms. In addition to playing a role in the development of tolerance, RANK may also play a role in immune system evasion by pathogens.

EXAMPLE 13

This example illustrates the influence of the interaction of RANK on CD1a⁺ dendritic cells (DC). Functionally mature dendritic cells (DC) were generated in vitro from CD34⁺ bone marrow (BM) progenitors. Briefly, human BM cells from normal healthy volunteers were density fractionated using Ficoll* medium and CD34⁺ cells immunoaffinity isolated using an anti-CD34 matrix column (Ceprate, CellPro). The CD34⁺ BM cells were then cultured in human GM-CSF (20 ng/ml), human IL-4 (20 ng/ml), human TNF-α (20 ng/ml), human CHO-derived Flt3L (FL; 100 ng/ml) in Super McCoy’s medium supplemented with 10% fetal calf serum in a fully humidified 37°C incubator (5% CO₂) for 14 days. CD1a⁺, HLA-DR⁺ DC were then sorted using a FACStar Plus™, and used for biological evaluation of RANK.

On human CD1a⁺ DC derived from CD34⁺ bone marrow cells, only a subset (20-30%) of CD1a⁺ DC expressed RANK at the cell surface as assessed by flow cytometric

*Trade-mark
analysis. However, addition of CD40L to the DC cultures resulted in RANK surface expression on the majority of CD1a+ DC. CD40L has been shown to activate DC by enhancing in vitro cluster formation, inducing DC morphological changes and upregulating HLA-DR, CD54, CD58, CD80 and CD86 expression.

Addition of RANKL to DC cultures significantly increased the degree of DC aggregation and cluster formation above control cultures, similar to the effects seen with CD40L (Figure 2). Sorted human CD1a+ DC were cultured in a cytokine cocktail (GM-CSF, IL-4, TNF-α and FL) (upper left panel), in cocktail plus CD40L (1μg/ml) (upper right), in cocktail plus RANKL (1μg/ml) (lower left), or in cocktail plus heat inactivated (AH) RANKL (1μg/ml) (lower right) in 24-well flat bottomed culture plates in 1 ml culture media for 48-72 hours and then photographed using an inversion microscope. An increase in DC aggregation and cluster formation above control cultures was not evident when heat inactivated RANKL was used, indicating that this effect was dependent on biologically active protein. However, initial phenotypic analysis of adhesion molecule expression indicated that RANKL-induced clustering was not due to increased levels of CD2, CD11a, CD54 or CD58.

The addition of RANKL to CD1a+ DC enhanced their allo-stimulatory capacity in a mixed lymphocyte reaction (MLR) by at least 3- to 10-fold, comparable to CD40L-cultured DC (Figure 3). Allogeneic T cells (1x10^5) were incubated with varying numbers of irradiated (2000 rad) DC cultured as indicated above for Figure 2 in 96-well round bottomed culture plates in 0.2 ml culture medium for four days. The cultures were pulsed with 0.5 mCi [3H]-thymidine for eight hours and the cells harvested onto glass fiber sheets for counting on a gas phase β counter. The background counts for either T cells or DC cultured alone were <100 cpm. Values represent the mean ± SD of triplicate cultures. Heat inactivated RANKL had no effect. DC allo-stimulatory activity was not further enhanced when RANKL and CD40L were used in combination, possibly due to DC functional capacity having reached a maximal level with either cytokine alone. Neither RANKL nor CD40L enhanced the in vitro growth of DC over the three day culture period. Unlike CD40L, RANKL did not significantly increase the levels of HLA-DR expression nor the expression of CD80 or CD86.

RANKL can enhance DC cluster formation and functional capacity without modulating known molecules involved in cell adhesion (CD18, CD54), antigen presentation (HLA-DR) or costimulation (CD86), all of which are regulated by CD40/CD40L signaling. The lack of an effect on the expression of these molecules suggests that RANKL may regulate DC function via an alternate pathway(s) distinct from CD40/CD40L. Given that CD40L regulates RANK surface expression on in vitro-generated DC and that CD40L is upregulated on activated T cells during DC-T cell
interactions, RANK and its ligand may form an important part of the activation cascade that is induced during DC-mediated T cell expansion. Furthermore, culture of DC in RANKL results in decreased levels of CD1b/c expression, and increased levels of CD83. Both of these molecules are similarly modulated during DC maturation by CD40L (Caux et al. J. Exp. Med. 180:1263; 1994), indicating that RANKL induces DC maturation.

Dendritic cells are referred to as "professional" antigen presenting cells, and have a high capacity for sensitizing MHC-restricted T cells. There is growing interest in using dendritic cells ex vivo as tumor or infectious disease vaccine adjuvants (see, for example, Romani, et al., J. Exp. Med., 180:83, 1994). Therefore, an agent such as RANKL that induces DC maturation and enhances the ability of dendritic cells to stimulate an immune response is likely to be useful in immunotherapy of various diseases.

**EXAMPLE 14**

This example describes the isolation of the murine homolog of RANK, referred to as muRANK. MuRANK was isolated by a combination of cross-species PCR and colony hybridization. The conservation of Cys residues in the Cys-rich pseudorepeats of the extracellular domains of TNFR superfamily member proteins was exploited to design human RANK-based PCR primers to be used on murine first strand cDNAs from various sources. Both the sense upstream primer and the antisense downstream primer were designed to have their 3' ends terminate within Cys residues.

The upstream sense primer encoded nucleotides 272-295 of SEQ ID NO:5 (region encoding amino acids 79-86); the downstream antisense primer encoded the complement of nucleotides 409-427 (region encoding amino acids 124-130). Standard PCR reactions were set up and run, using these primers and first strand cDNAs from various murine cell line or tissue sources. Thirty reaction cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 20 seconds were run. PCR products were anlyzed by electrophoresis, and specific bands were seen in several samples. The band from one sample was gel purified and DNA sequencing revealed that the sequence between the primers was approximately 85% identical to the corresponding human RANK nucleotide sequence.

A plasmid based cDNA library prepared from the murine fetal liver epithelium line FLE18 (one of the cell lines identified as positive in the PCR screen) was screened for full-length RANK cDNAs using murine RANK-specific oligonucleotide probes derived from the murine RANK sequence determined from sequencing the PCR product. Two cDNAs, one encoding the 5' end and one encoding the 3' end of full-length murine RANK (based on sequence comparison with the full-length human RANK) were recombined to generate a full-length murine RANK cDNA. The nucleotide and amino acid sequence of muRANK are shown in SEQ ID Nos:14 and 15.
The cDNA encodes a predicted Type 1 transmembrane protein having 625 amino acid residues, with a predicted 30 amino acid signal sequence, a 184 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 390 amino acid cytoplasmic tail. The extracellular region of muRANK displayed significant amino acid homology (69.7% identity, 80.8% similarity) to huRANK. Those of skill in the art will recognize that the actual cleavage site can be different from that predicted by computer; accordingly, the N-terminal of RANK may be from amino acid 25 to amino acid 35.

Other members of the TNF receptor superfamily have a region of amino acids between the transmembrane domain and the ligand binding domain that is referred to as a 'spacer' region, which is not necessary for ligand binding. In muRANK, the amino acids between 197 and 214 are predicted to form such a spacer region. Accordingly, a soluble form of RANK that terminates with an amino acid in this region is expected to retain the ability to bind a ligand for RANK in a specific manner. Preferred C-terminal amino acids for soluble RANK peptides are selected from the group consisting of amino acids 214, and 197 of SEQ ID NO:14, although other amino acids in the spacer region may be utilized as a C-terminus.

**EXAMPLE 15**

This example illustrates the preparation of several different soluble forms of RANK and RANKL. Standard techniques of restriction enzyme cutting and ligation, in combination with PCR-based isolation of fragments for which no convenient restriction sites existed, were used. When PCR was utilized, PCR products were sequenced to ascertain whether any mutations had been introduced; no such mutations were found.

In addition to the huRANK/Fc described in Example 2, another RANK/Fc fusion protein was prepared by ligating DNA encoding amino acids 1-213 of SEQ ID NO:6, to DNA encoding amino acids 3-232 of the Fc mutein described previously (SEQ ID NO:8). A similar construct was prepared for murine RANK, ligating DNA encoding amino acids 1-213 of full-length murine RANK (SEQ ID NO:15) to DNA encoding amino acids 3-232 of the Fc mutein (SEQ ID NO:8).

A soluble, tagged, poly-His version of huRANKL was prepared by ligating DNA encoding the leader peptide from the immunoglobulin kappa chain (SEQ ID NO:16) to DNA encoding a short version of the FLAG™ tag (SEQ ID NO:17), followed by codons encoding Gly Ser, then a poly-His tag (SEQ ID NO:18), followed by codons encoding Gly Thr Ser, and DNA encoding amino acids 138-317 of SEQ ID NO:13. A soluble, poly-His tagged version of murine RANKL was prepared by ligating DNA encoding the CMV leader (SEQ ID NO:9) to codons encoding Arg Thr Ser, followed by DNA encoding poly-His (SEQ ID NO:18) followed by DNA encoding amino acids 119-294 of SEQ ID NO:11.
A soluble, oligomeric form of huRANKL was prepared by ligating DNA encoding the CMV leader (SEQ ID NO:9) to a codon encoding Asp followed by DNA ending a trimer-former "leucine" zipper (SEQ ID NO:19), then by codons encoding Thr Arg Ser followed by amino acids 138-317 of SEQ ID NO:13.

These and other constructs are prepared by routine experimentation. The various DNAs are then inserted into a suitable expression vector, and expressed. Particularly preferred expression vectors are those which can be used in mammalian cells. For example, pDC409 and pDC304, described herein, are useful for transient expression. For stable transfection, the use of CHO cells is preferred; several useful vectors are described in US patent 6,027,915, for example, one of the 2A5-3 λ-derived expression vectors discussed therein.

**EXAMPLE 16**

This example demonstrates that RANKL expression can be up-regulated on murine T cells. Cells were obtained from mesenteric lymph nodes of C57BL/6 mice, and activated with anti-CD3 coated plates, Concanavalin A (ConA) or phorbol myristate acetate in combination with ionomycin (anti-CD3: 500A2; Immunex Corporation, Seattle WA; ConA, PMA, ionomycin, Sigma, St Louis, MO) substantially as described herein, and cultured from about 2 to 5 days. Expression of RANKL was evaluated in a three color analysis by FACS, using antibodies to the T cell markers CD4, CD8 and CD45RB, and RANK/Fc, prepared as described herein.

RANKL was not expressed on unstimulated murine T cells. T cells stimulated with either anti-CD3, ConA, or PMA/ionomycin, showed differential expression of RANKL: CD4+/CD45RBlo and CD4+/CD45RBhi cells were positive for RANKL, but CD8+ cells were not. RANKL was not observed on B cells, similar to results observed with human cells.

**EXAMPLE 17**

This example illustrates the effects of murine RANKL on cell proliferation and activation. Various cells or cell lines representative of cells that play a role in an immune response (murine spleen, thymus and lymphnode) were evaluated by culturing them under conditions promoting their viability, in the presence or absence of RANKL. RANKL did not stimulate any of the tested cells to proliferate. One cell line, a macrophage cell line referred to as RAW 264.7 (ATCC accession number TIB 71) exhibited some signs of activation.

RAW cells constitutively produce small amounts of TNF-α. Incubation with either human or murine RANKL enhanced production of TNF-α by these cells in a dose
dependent manner. The results were not due to contamination of RANKL preparations with endotoxin, since boiling RANKL for 10 minutes abrogated TNF-α production, whereas a similar treatment of purified endotoxin (LPS) did not affect the ability of the LPS to stimulate TNF-α production. Despite the fact that RANKL activated the macrophage cell line RAW T64.7 for TNF-α production, neither human RANKL nor murine RANKL stimulated nitric oxide production by these cells.

**EXAMPLE 18**

This example illustrates the effects of murine RANKL on growth and development of the thymus in fetal mice. Pregnant mice were injected with 1 mg of RANK/Fc or vehicle control protein (murine serum albumin; MSA) on days 13, 16 and 19 of gestation. After birth, the neonates continued to be injected with RANK/Fc intraperitoneally (IP) on a daily basis, beginning at a dose of 1 μg, and doubling the dose about every four days, for a final dosage of 4 μg. Neonates were taken at days 1, 8 and 15 post birth, their thymuses and spleens harvested and examined for size, cellularity and phenotypic composition.

A slight reduction in thymic size at day 1 was observed in the neonates born to the female injected with RANK/Fc; a similar decrease in size was not observed in the control neonates. At day 8, thymic size and cellularity were reduced by about 50% in the RANK/Fc-treated animals as compared to MSA treated mice. Phenotypic analysis demonstrated that the relative proportions of different T cell populations in the thymus were the same in the RANK/Fc mice as the control mice, indicating that the decreased cellularity was due to a global depression in the number of thymic T cells as opposed to a decrease in a specific population(s). The RANK/Fc-treated neonates were not significantly different from the control neonates at day 15 with respect to either size, cellularity or phenotype of thymic cells. No significant differences were observed in spleen size, cellularity or composition at any of the time points evaluated. The difference in cellularity on day 8 and not on day 15 may suggest that RANK/Fc may assert its effect early in thymic development.

**EXAMPLE 19**

This example demonstrates that the C-terminal region of the cytoplasmic domain of RANK is important for binding of several different TRAF proteins. RANK contains at least two recognizable PXQX(X)T motifs that are likely TRAF docking sites. Accordingly, the importance of various regions of the cytoplasmic domain of RANK for TRAF binding was evaluated. A RANK/GST fusion protein was prepared substantially as described in Smith and Johnson, *Gene* 67:31 (1988), and used in the preparation of various truncations as described below.
Comparison of the nucleotide sequence of murine and human RANK indicated that there were several conserved regions that could be important for TRAF binding. Accordingly, a PCR-based technique was developed to facilitate preparation of various C-terminal truncations that would retain the conserved regions. PCR primers were designed to introduce a stop codon and restriction enzyme site at selected points, yielding the truncations described in Table 1 below. Sequencing confirmed that no undesired mutations had been introduced in the constructs.

Radio-labeled (35S-Met, Cys) TRAF proteins were prepared by in vitro translation using a commercially available reticulocyte lysate kit according to manufacturer’s instructions (Promega). Truncated GST fusion proteins were purified substantially as described in Smith and Johnson (supra). Briefly, E. coli were transfected with an expression vector encoding a fusion protein, and induced to express the protein. The bacteria were lysed, insoluble material removed, and the fusion protein isolated by precipitation with glutathione-coated beads (Sepahrose 4B, Pharmacia, Uppsala Sweden).

The beads were washed, and incubated with various radiolabeled TRAF proteins. After incubation and wash steps, the fusion protein/TRAF complexes were removed from the beads by boiling in 0.1% SDS + β-mercaptoethanol, and loaded onto 12% SDS gels (Novex). The gels were subjected to autoradiography, and the presence or absence of radiolabeled material recorded. The results are shown in Table 2 below.

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These results indicate that TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 bind to the most distal portion of the RANK cytoplasmic domain (between amino-acid G544 and A616). TRAF6 also has a binding site between S339 and Y421. In this experiment, TRAF5 also bound the cytoplasmic domain of RANK.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: IMMUNEX CORPORATION

(ii) TITLE OF INVENTION: LIGAND FOR RECEPTOR ACTIVATOR OF NF-KAPPA B, LIGAND IS MEMBER OF TNF SUPERFAMILY

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: SMART & BIGGAR
(B) STREET: P.O. BOX 2999, STATION D
(C) CITY: OTTAWA
(D) STATE: ONT
(E) COUNTRY: CANADA
(F) ZIP: K1H 5Y6

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: CA 2,274,987
(B) FILING DATE: 22-DEC-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/059,978
(B) FILING DATE: 23-DEC-1996

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/813,509
(B) FILING DATE: 07-MAR-1997

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/064,671
(B) FILING DATE: 14-OCT-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: SMART & BIGGAR

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER: 72249-89

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (613)-232-2486

(B) TELEFAX: (613)-232-8440

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3115 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: BONE-MARROW DERIVED DENDRITIC CELLS
(B) CLONE: 9D-8A

(ix) FEATURE:
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(2) INFORMATION FOR SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

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(A) LIBRARY: BONE-MARROW DERIVED DENDRITIC CELLS
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(2) INFORMATION FOR SEQ ID NO:4:

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   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: HOMO SAPIENS

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: BONE-MARROW DERIVED DENDRITIC CELLS
(B) CLONE: FULL LENGTH RANK

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 39..1886

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TGACAGCTAT TTTATGACT ATCCCTGTTCT GTGCGGCGGG GGTCTATGTT TCCCCCTCAT
2226

ATTGTATTTC CTTTTCTAA CATTTCTCAG AATTTCTCTG TATCTTTTTCT CCCCTTTTTTT TAATGTTAAAG
2286

GTTTTCTCAA AAATTTCTCT AAAGTTGAGG GTCTCTTTTT TTTCTTTTTT CTCTTTTTTTT 2346
TTCTTTTTTT GGCAACCTGG CTCTGGCCCA GGCTAGAGTG CAGTGGTGCG ATTATAGCCC 2406
GGTGCGACCT CTACTCCTGG GGTCAAAGGG ACTCAATGTA TCCTCCCCACC TCAAACTTGC 2466
GAGTAGGCTGG GATCACAGCT GCGGCGACGC CCCAGCTTCC TCCCCCGAC TCCCCCCCCC 2526
CAGAGACACG GTCCCCACCAT GTTACCCGAC CTGCTCTCAA ACTCCCCAGC TAAAGCAGTC 2586
CTCCAGCCTC GGCTCTCCCA AGTACTGGGA TTACAGGCGT GAGCCCCCAG GCTGGGCTGCG 2646
TTTAGCTATT TTCTTTTTGG CCCCTGCTCA CAGTGGTTTA GAGATGGCGT TCCCCAGTGG 2706
TGTTCAATTG AAACACTTTT GGGAAAGGGC TAAACATGTC AGGCCCTGGAG ATAGTTGCTA 2766
AGTTGCTAGG AACATGTTGT GGGACTTTCG TATTCGAAA AATGTTCTAT ATCTCATTTC 2826
TTCTAAAAGA AAGAAAAAG GAAACCGAT TTATTTTCC TGAATCTTTT TAAAGGGTG 2886
TCGTTCCCTA AGCGAGACTA AGTCAGATG GTGACCTTAC CGCTAGGGT GTTAAATTAT 2946
CCATGCTGCC AGAGGCACCT AGTACTCGG TAAGCAAATTT TCTAAACTC CAAGTTGCTG 3006
CAGCTTGGCA TTCTTTCTAT TCTAGAGGTC TCTCTGGAAA AGATGGAGAA AATGAAACAGG 3066
ACATGGGGCT CCTGGAAGGA AGGGGCCGGG GAAGTTCAG GAAGATAAAA GCTGAAATT 3126
TAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 616 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Ala Pro Arg Ala Arg Arg Arg Arg Pro Leu Phe Ala Leu Leu Leu
      1  5 10 15
Leu Cys Ala Leu Leu Ala Arg Leu Gln Val Ala Leu Gln Ile Ala Pro
      20  25  30
Pro Cys Thr Ser Glu Lys His Tyr Glu His Leu Gly Arg Cys Cys Asn
     35  40  45
Lys Cys Glu Pro Gly Lys Tyr Met Ser Ser Lys Cys Thr Thr Thr Ser
     50  55  60
Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Ser Trp
     65  70  75  80
Asn Glu Glu Asp Lys Cys Leu Leu His Lys Val Cys Asp Thr Gly Lys
     85  90  95
Ala Leu Val Ala Val Val Ala Gly Asn Ser Thr Thr Pro Arg Arg Cys
    100 105 110

51
Ala Cys Thr Ala Gly Tyr His Trp Ser Gln Asp Cys Glu Cys Cys Arg
  115  120
Arg Asn Thr Glu Cys Ala Pro Gly Leu Gly Ala Gln His Pro Leu Gln
  130  135
Leu Asn Lys Asp Thr Val Cys Lys Pro Cys Leu Ala Gly Tyr Phe Ser
  145  150  155  160
Asp Ala Phe Ser Ser Thr Asp Lys Cys Arg Pro Trp Thr Asn Cys Thr
  165  170
Phe Leu Gly Lys Arg Val Glu His His Gly Thr Glu Lys Ser Asp Ala
  180  185  190
Val Cys Ser Ser Ser Leu Pro Ala Arg Lys Pro Pro Asn Glu Pro His
  195  200  205
Val Tyr Leu Pro Gly Leu Ile Ile Leu Leu Leu Phe Ala Ser Val Ala
  210  215  220
Leu Val Ala Ala Ile Ile Phe Gly Val Cys Tyr Arg Lys Lys Gly Lys
  225  230  235  240
Ala Leu Thr Ala Asn Leu Thr His Thr Trp His Ile Asn Glu Ala Cys Gly Arg
  245  250  255
Leu Ser Gly Asp Lys Glu Ser Ser Gly Asp Ser Cys Val Ser Thr His
  260  265  270
Thr Ala Asn Phe Gly Gln Gln Gly Ala Cys Glu Gly Val Leu Leu Leu
  275  280  285
Thr Leu Glu Glu Lys Thr Phe Pro Glu Asp Met Cys Tyr Pro Asp Gln
  290  295  300
Gly Gly Val Cys Gln Gly Thr Cys Val Gly Gly Gly Pro Tyr Ala Gln
  305  310  315  320
Gly Glu Asp Ala Arg Met Leu Ser Leu Val Ser Lys Thr Glu Ile Glu
  325  330  335
Glu Asp Ser Phe Arg Glu Met Pro Thr Glu Asp Glu Tyr Met Asp Arg
  340  345  350
Pro Ser Gln Pro Thr Asp Gln Leu Leu Phe Leu Thr Glu Pro Gly Ser
  355  360  365
Lys Ser Thr Pro Pro Phe Ser Glu Pro Leu Glu Val Gly Glu Asn Asp
  370  375  380
Ser Leu Ser Gln Cys Phe Thr Gly Thr Gln Ser Thr Val Gly Ser Glu
  385  390  395  400
Ser Cys Asn Cys Thr Glu Pro Leu Cys Arg Thr Asp Trp Thr Pro Met
  405  410  415
Ser Ser Glu Asn Tyr Leu Gln Lys Glu Val Asp Ser Gly His Cys Pro
  420  425  430

52
His Trp Ala Ala Ser Pro Ser Pro Asn Trp Ala Asp Val Cys Thr Gly
435
440
445
Cys Arg Asn Pro Pro Gly Glu Asp Cys Glu Pro Leu Val Gly Ser Pro
450
455
460
Lys Arg Gly Pro Leu Pro Gln Cys Ala Tyr Gly Met Gly Leu Pro Pro
465
470
475
480
Glu Glu Glu Ala Ser Arg Thr Glu Ala Arg Asp Gln Pro Glu Asp Gly
485
490
495
Ala Asp Gly Arg Leu Pro Ser Ser Ala Arg Ala Gly Ala Gly Ser Gly
500
505
510
Ser Ser Pro Gly Gly Gln Ser Pro Ala Ser Gly Asn Val Thr Gly Asn
515
520
525
Ser Asn Ser Thr Phe Ile Ser Ser Gly Gln Val Met Asn Phe Lys Gly
530
535
540
Asp Ile Ile Val Val Tyr Val Ser Gln Thr Ser Gln Glu Gly Ala Ala
545
550
555
560
Ala Ala Ala Glu Pro Met Gly Arg Pro Val Gln Glu Glu Thr Leu Ala
565
570
575
Arg Arg Asp Ser Phe Ala Gly Asn Gly Pro Arg Phe Pro Asp Pro Cys
580
585
590
Gly Gly Pro Glu Gly Leu Arg Glu Pro Glu Lys Ala Ser Arg Pro Val
595
600
605
Gln Glu Gln Gly Gly Ala Lys Ala
610
615

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: FLAG® peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
Asp Tyr Lys Asp Asp Asp Asp Lys
1
5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(vii) IMMEDIATE SOURCE:
(B) CLONE: IgG1 Fc mutein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Pro Arg Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
  1      5     10  15
Pro Glu Ala Glu Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
  20     25     30
Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
  35     40     45
Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
   50    55     60
Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
   65    70     75    80
Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
   85     90     95
Asp Trp Leu Asn Gly Lys Asp Tyr Lys Cys Lys Val Ser Asn Lys Ala
  100   105     110
Leu Pro Ala Pro Met Gln Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
  115   120    125
Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
 130   135    140
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Arg
 145   150    155    160
His Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
 165    170    175
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Leu Tyr
 180   185    190
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
 195    200    205
Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 210    215    220
Ser Leu Ser Leu Ser Pro Gly Lys
 225    230
(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 31 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: not relevant
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: CMV (R2780 Leader)

(ix) FEATURE:
   (D) OTHER INFORMATION: Met1-Arg28 is the actual leader peptide; Arg29 strengthens the furin cleavage site; nucleotides encoding Thr30 and Ser31 add a SpeI site.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

   Met Ala Arg Arg Leu Trp Ile Leu Ser Leu Leu Ala Val Thr Leu Thr
   1  5  10  15

   Val Ala Leu Ala Ala Pro Ser Gln Lys Ser Lys Arg Arg Thr Ser
   20  25  30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1630 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Mus musculus

(vii) IMMEDIATE SOURCE:
   (A) LIBRARY:
   (B) CLONE: RANKL

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: 3..884

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
CC GGC TCA CAC GAG GTT CCG CTG CAC CCC GCG CCT TCT GCA CCG
Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro
1  5  10  15
GCT CGG CGG CGC CCA CCC GCC GGC TTC CTG ATG TTC CTG GCC CTC
Ala Pro Ala Pro Pro Pro Pro Ser Arg Ser Met Phe Leu Ala Leu
20  25  30
CTG GGG CTG GGA CTG GCC CAG GTG GTC TGG AGC ATC GCT CTG TCC CTG
Leu Gly Leu Gly Leu Gly Gin Val Val Cys Ser Ile Ala Leu Phe Leu
35  40  45
TAC TTT CGA GCC GCG CAG ATG GAT CCT AAC AGA ATA TCA GAA GAC AGC ACT
Tyr Phe Arg Ala Gin Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr
50  55  60
CAC TGC TTT TAT AGA ATC CTG AGA CTC CAT GAA AAC GCA GAT TTG CAG
His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Asp Leu Gin
65  70  75
GAC TCG ACT CTG GAG AGT GTA GAC ACA CTA CCT GAC TCC TGC AGG AGG
Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg
80  85  90  95
ATG AAA CAA GCC TTT CAG GGG GCC GTG CAG AAG GAA CTG CAA CAC ATT
Met Lys Gin Ala Phe Gin Gly Ala Val Gin Lys Gin Leu Gin His Ile
100 105 110
GTG GGG CCA CAG CGG TTC TCA GGA GCT CCA GCT ATG AGT GAA GCC TCA
Val Gly Pro Gin Arg Phe Ser Glu Ala Pro Ala Met Glu Gly Ser
115 120 125
TGG TTG GAT GTG GCC CAG CGG GCC TGC TGT CAG GAA TCT CAG GCC CAG CCA TTT GCA
Trp Leu Asp Val Ala Gin Arg Gly Lys Pro Glu Ala Gin Pro Phe Ala
130 135 140
CAC CTC ACC ATC AAT GCT GCC AGC ATC CCA TCG GGT TCC CAT AAA GTC
His Leu Thr Ile Asn Ala Alu Ser Ile Pro Ser Gly Ser His Lys Val
145 150 155
ACT CTG TCC TCT TGG TAC CAC GAT CGA GGC TGG GCC AAG ATC TCT AAC
Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile Ser Asn
160 165 170 175
ATG ACG TTA AGC AAC GGA AAA CTA AGG TGT AAC CAA GAT GCC TTC TAT
Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gin Asp Gly Phe Tyr
180 185 190
TAC CTG TAC GCC AAC ATT TGG TTG GAC CAT CAT GAA ACA TCG GGA AGC
Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu Thr Ser Gly Ser
195 200 205
GTA CCT ACA GAC TAT CCT CAG CTG ATG GTG TAT GTC GTT AAA ACC AGC
Val Pro Thr Asp Tyr Leu Gin Leu Met Val Tyr Val Lys Thr Ser
210 215 220
ATC AAA ATC CCA AGT TCT CAT AAC CTG ATG AAA GGA GGG AGC ACG AAA
Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser Thr Lys
225 230 235
WO 98/28426

AAC TGG TCG GCC AAT TCT GAA TCC CAC TTT TAT TCC ATA AAT GTT GGG
Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly
240 245 250 255

GGA TTC AAG CTC CGA GCT GGT GAA GAA ATT AGC ATT CAG GTC TCC
Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser
260 265 270

AAC CCT TCC CTG CGT GAT CCG GAT CAA GAT GCG AGC TAC TTT GGG GCT
Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala
275 280 285

TTC AAA GTT CAG GAC ATA GAC TGAGACTCAT TTCTGGAAC ATTAGCATGG
Phe Lys Val Gln Asp Ile Asp
290

ATGTCCCTAGA TGTTTGGAAA CTTCTTTAAA AAATGTGAT GTCTATACAT GTGTAAGACT 974
ACTAAGAGAC ATGGCCCAGC GTATGAAA AATCAAGCCC TCTCTCTTGA GCCTGTAACAG 1034
GTTGTTGTATA TGATAACTCC ATAGTTGGAT TTGATTACAT GTGATTACA CAAACGTTTTT 1094
ACAAATTCCT TAGATTTCG CAGAATTGGG AGAGATTTC CGATGCTTAT 1154
GAAAAACTTT TACCTGATGC ATGGGAAGGG GTATGACGT CTTGCTCAA CCCCTGGACA 1214
TGTGCGACTG AAGACCTTGA AATTAGAGG ATGCACTGTC ATTCGAAAGA AATGATAGTG 1274
TGAGGGTTTA AGTCTTTTGC AATGGTTTACA TTGCGCTGGG ACCTGCAAAT AAGCTTTTTT 1334
TTTCTAATGA GGAAGAAAA ATATATATAT TTTTATATAA TGCTAAAACT TATATTTACAG 1394
GTACATGTG TCTGTGCAAA AGTGTGTAA AGGTTAATGG TTGTATAGTA TTGTAGTCAA 1545
AACATTAAA AATCTTCCAC TTCTGACATA TTTATGTTG TAAATGTACA GATGATTTA 1514
ACTGTTGAC TTCGTACATC CCGTGAAGGT ACTGCTAGCT AAGGGCGCAG AATTACTTTT 1574
CTGCTGACCA CATGCTTTT ATTTCTTTAT TCTTCTTTAAC TTTAATAGAGT CCTCAG 1630

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 294 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro Ala
1  5  10  15

Pro Ala Pro Pro Ala Ala Ser Arg Ser Met Phe Leu Ala Leu Leu
20 25 30

Gly Leu Gly Leu Gly Glu Val Val Cys Ser Ile Ala Leu Phe Leu Tyr
35 40 45
| Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr His | 50 |
| Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Asp Leu Gln Asp | 65 |
| Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg Arg Met | 85 |
| Lys Gln Ala Phe Gln Gly Ala Val Gln Lys Glu Leu Gln His Ile Val | 100 |
| Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu Gly Ser Trp | 115 |
| Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro Phe Ala His | 130 |
| Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly Ser His Lys Val Thr | 145 |
| Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile Ser Asn Met | 165 |
| Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln Asp Gly Phe Tyr Tyr | 180 |
| Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu Thr Ser Gly Ser Val | 195 |
| Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val Val Lys Thr Ser Ile | 210 |
| Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser Thr Lys Asn | 225 |
| Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly | 245 |
| Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gin Val Ser Asn | 260 |
| Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe | 275 |
| Lys Val Gln Asp Ile Asp | 290 |

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 954 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE: hrANKL (full length)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..951

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATG CGC CGC GCC AGC AGA GAC TAC ACC AAG TAC CTG CTT GCC TCG GAG
Met Arg Arg Ala Ser Arg Asp Tyr Thr Lys Tyr Leu Arg Gly Ser Glu
1 5 10 15

GAG ATG GCC GCC GCC CCC GGA GCC CCG CAC GAG GCC CCC CTG CAC GCC
Glut Met Gly Gly Gly Pro Gly Ala Pro His Glu Gly Pro Leu His Ala
20 25 30

CCG CCG CCG CCT GCC CCG CAG CCC CCC GCC GCC GCC GCC TCC GCC TCC ATG
Pro Pro Pro Pro Ala Pro His Gln Pro Pro Ala Ala Ser Arg Ser Met
35 40 45

TTC GTG GCC CCT CTG GGG CTG GGG CTG GGG CAG GTT GTC TGC AGC GTG
Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Val
50 55 60

GCC CTG TTC TTC TAT TTC AGG GCC CAG ATG GAT CCT AAT AGA ATA TCA
Ala Leu Phe Phe Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser
65 70 75 80

GAA GAT GCC ACT CAC TGC ATT TAT AGA ATT TTT AGA CTC CAT GAA AAT
Glu Asp Gly Thr His Cys Ile Tyr Arg Ala Leu Arg Leu His Glu Asn
85 90 95

GCA GAT TTT CAA GAC ACA ACT CTG GAG AGT CAA GAT AGA AAA TTA ATA
Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu Ile
100 105

CCT GAT TCA TGT AGG AGA ATT AAA CAG GCC TTT CAA GGA GCT GTG CAA
Pro Asp Ser Cys Arg Arg Ile Lys Gln Ala Phe Gln Gly Ala Val Gln
115 120 125

AAG GAA TTA CAA CAT ATC GTT GGA TCA CAG CAC ATC AGA GCA GAG AAA
Lys Glu Leu Gln His Ile Val Gly Ser Gln His Ile Arg Ala Glu Lys
130 135 140

GCG ATG GTG GAT GCC TCA TGG TTA GAT CTG GCC AAG AGG AGC AAG CTT
Ala Met Val Asp Gly Ser Trp Leu Asp Ala Lys Arg Ser Lys Leu
145 150 155 160

GAA GCT CAG CCT TTT GCT CAT CTC ACT ATT AAT GCC ACC GAC ATC CCA
Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile Pro
165 170 175
(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 317 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Arg Arg Ala Ser Arg Asp Tyr Thr Lys Tyr Leu Arg Gly Ser Glu  
1      5      10       15
Glu Met Gly Gly Pro Gly Ala Pro His Gly Pro Leu His Ala  
20     25      30
Pro Pro Pro Ala Pro His Gln Pro Pro Ala Ala Ser Arg Ser Met  
35     40      45
Phe Val Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Val  
50     55      60
(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1878 base pairs  
   (B) TYPE: nucleic acid  
   (C) STRANDEDNESS: single  
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Murine

(vii) IMMEDIATE SOURCE:
   (A) LIBRARY: Murine Fetal Liver Epithelium
   (B) CLONE: muRANK

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: 1..1875

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATG GCC CCG CCG GCC CCG CCG CCG CAG CTG CCC CCG CCG CTG CTG  
Met Ala Pro Arg Ala Arg Arg Arg Arg Gln Leu Pro Ala Pro Leu Leu  
1      5      10      15

GCC CTC TGC GTG CTG CTC GTT CCA CTG CAG GTG ACT CTC CAG GTC ACT  
Ala Leu Cys Val Leu Leu Val Pro Leu Gln Val Thr Leu Gln Val Thr  
20     25     30

CCT CCA TGC ACC CAG GAG AGG CAT TAT GAG CAT CTC GGA CCG TGC TGC  
Pro Pro Cys Thr Gin Glu Arg His Tyr Glu His Leu Gly Arg Cys Cys  
35     40     45

AGC AGA TGC GAA CCA GGA AAG TAC CTG TCC TCT AAG TGC ACT CCT ACC  
Ser Arg Cys Glu Pro Gly Lys Tyr Leu Ser Ser Lys Cys Thr Pro Thr  
50     55     60

TCC GAC AGT GTG TGT CTG CCC TGT GGC CCC GAT GAG TAC TTG GAC ACC  
Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Thr  
65     70     75     80

TGG AAT GAA GAT AAA TGG TTG CTG CAT AAA GTC TGT GAT GCA GGC  
Trp Asn Glu Glu Lys Cys Leu Leu His Lys Val Cys Asp Ala Gly  
85     90

AAG GCC CTG GTG GGC GTG GAT CCT GGC AAC CAC AGC GCC CCG CCT CCC  
Lys Ala Leu Val Ala Val Asp Pro Gly Asn His Thr Ala Pro Arg Arg  
100    105    110

TGT GCT TGC AGC GCT GGC TAC CAC TGG AAC TCA GAC TGC GAG TGC TGC  
Cys Ala Cys Thr Ala Gly Tyr His Trp Asn Ser Asp Cys Glu Cys Cys  
115    120    125

CCG AAC AGG ACG GTG GCA CCT GGC TTC GGA GCT CAG CAT CCC TTG  
Arg Asn Thr Glu Cys Ala Pro Gly Phe Gly Ala Gin His Pro Leu  
130    135    140

CAG CTC AAC AAG GAT AGG GTG TGC ACA CCC TGC CTC CTG GGC TTC TGC  
Gln Leu Asn Lys Asp Thr Val Cys Thr Pro Cys Leu Leu Gly Phe Phe  
145    150    155    160

TCA GAT GTC TTT TCG TCC ACA GAC AAA TGC AAA CCT TGG ACC AAC TGC  
Ser Asp Val Phe Ser Ser Thr Asp Lys Cys Lys Pro Trp Thr Asn Cys  
165    170    175

62
ACC  CTG  GGA  AAG  CTA  GAA  GCA  CAC  CAG  GGG  ACA  ACG  GAA  TCA  GAT
Thr  Leu  Leu  Gly  Lys  Leu  Glu  Ala  His  Gln  Gly  Thr  Thr  Glu  Ser  Asp

GTG  GTC  TGC  AGG  TCT  GCC  ATG  ACA  CTG  AGG  AGA  CCA  CCC  AAG  GAG  GCC
Val  Val  Cys  Ser  Ser  Ser  Met  Thr  Leu  Arg  Arg  Pro  Pro  Lys  Glu  Ala

CAG  GCT  TAC  CTG  CCC  AGT  CTC  ATC  GTT  CTG  CTC  TCT  TTC  ATC  TCT  GTG
Gln  Ala  Tyr  Leu  Pro  Ser  Leu  Ile  Val  Leu  Val  Leu  Phe  Ile  Ser  Val

GTA  GTA  GTG  GCT  GCC  ATC  ATC  TTC  GGC  GTT  TAC  TAC  AGG  AAG  GGA  GGG
Val  Val  Val  Ala  Ala  Ile  Ile  Phe  Gly  Val  Tyr  Tyr  Arg  Lys  Gly  Gly

AAA  GCG  CTG  ACA  GCT  AAT  TTG  TGG  AAT  TGG  GTC  AAT  GAT  GCT  TGC  AGT
Lys  Ala  Leu  Thr  Ala  Asn  Leu  Trp  Asn  Trp  Val  Asn  Asp  Ala  Cys  Ser

AGT  CTG  AGG  AAT  AAG  GAG  TCG  TCA  GGG  GAC  CGT  TGT  GCT  GGT  TCC
Ser  Leu  Ser  Gly  Asn  Lys  Ser  Ser  Gly  Asp  Arg  Cys  Ala  Gly  Ser

CAC  TCG  GCA  ACC  TCC  AGT  CAG  CA A  GAA  GTG  TGT  GAA  GGT  ATC  TTA  CTA
His  Ser  Ala  Thr  Ser  Glu  Lys  Glu  Glu  Val  Cys  Glu  Gly  Ile  Leu  Leu

ATG  ACT  CGG  GAG  GAG  ATG  GTT  CCA  GAA  GAC  GGT  GCT  GGA  GTC  TGT
Met  Thr  Arg  Glu  Glu  Arg  Met  Val  Pro  Glu  Asp  Gly  Ala  Gly  Val  Cys

GGG  CCT  GTG  TGT  GCG  GCA  GGT  GGG  CCC  TGG  GCA  GAA  GTC  AGA  GAT  TCT
Gly  Pro  Val  Cys  Ala  Ala  Gly  Pro  Trp  Ala  Val  Arg  Asp  Ser  Pro  Ala  Val

AGG  ACG  TTG  ACA  GTA  AGG  GAG  GTT  GAG  ACG  AAA  GAA  GAC  CTC  TCG
Arg  Thr  Phe  Thr  Leu  Ser  Glu  Val  Glu  Thr  Glu  Gly  Asp  Leu  Ser

AGG  AAG  ATT  CCC  ACA  GAG  GAT  GAG  TAC  ACG  GAC  CGG  CCC  TCG  CAG  CCT
Arg  Lys  Ile  Pro  Thr  Glu  Asp  Glu  Tyr  Thr  Asp  Arg  Pro  Ser  Gln  Pro

TCG  ACT  GTG  TCA  CTG  CTC  ATC  CAG  CAG  GGA  AGC  AAG  TCT  ATA  CCC
Ser  Thr  Gly  Ser  Leu  Leu  Ile  Gln  Gln  Gly  Ser  Lys  Ser  Ile  Pro

CCA  TTC  CAG  GAG  CCC  CTG  GAA  GTG  GGG  GAG  AAC  GAC  AGT  TTA  AGC  CAG
Pro  Phe  Glu  Pro  Leu  Glu  Val  Gly  Glu  Asp  Ser  Leu  Ser  Gln

TGT  TTC  ACC  GGG  ACT  GAA  ACG  AGT  TCT  GAG  GGC  GTG  GAC  TCC
Cys  Phe  Thr  Gly  Thr  Glu  Ser  Thr  Val  Asp  Ser  Glu  Gly  Cys  Asp  Phe

ACT  GAG  CCT  CCC  AGC  AGA  ACT  GAC  TCT  ATG  CCC  GTG  TCC  CCT  GAA  AAG
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 625 amino acids

64
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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Ser Asp Ser Val Cys Leu Pro Cys Gly Pro Asp Glu Tyr Leu Asp Thr
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Lys Ala Leu Val Ala Val Asp Pro Gly Asn His Thr Ala Pro Arg Arg
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Cys Ala Cys Thr Ala Gly Tyr His Trp Asn Ser Asp Cys Glu Cys Cys
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Arg Arg Asn Thr Glu Cys Ala Pro Gly Phe Gly Ala Glu His Pro Leu
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Gln Leu Asn Lys Asp Thr Val Cys Thr Pro Cys Leu Leu Gly Phe Phe
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Ser Asp Val Phe Ser Ser Thr Asp Lys Cys Lys Pro Trp Thr Asn Cys
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Thr Leu Leu Gly Lys Leu Glu Ala His Gln Gly Thr Glu Ser Asp
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Gln Ala Tyr Leu Pro Ser Leu Ile Val Leu Leu Phe Ile Ser Val
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(2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:16:
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(2) INFORMATION FOR SEQ ID NO:17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:17:
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(2) INFORMATION FOR SEQ ID NO:18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:18:
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(2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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Arg
CLAIMS:

1. An isolated receptor activator of NF-kappa B ligand (RANKL) polypeptide that binds a receptor activator of NF-kappa B (RANK), selected from the group consisting of:

(a) a polypeptide comprising amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162 of SEQ ID NO:13 and y is any one of amino acids 313-317 of SEQ ID NO:13;

(b) a polypeptide comprising amino acids 1-162 of SEQ ID NO:13;

(c) a polypeptide comprising an amino acid sequence having at least 90% identity to amino acids 69-313 of SEQ ID NO:13; and

(d) a polypeptide comprising an amino acid sequence having at least 90% identity to amino acids 138-317 of SEQ ID NO:13.

2. The isolated polypeptide of claim 1 comprising an amino acid sequence having at least 90% identity to amino acids 1 to 317 of SEQ ID NO:13.

3. The isolated polypeptide of claim 1 comprising an amino acid sequence having at least 90% identity to amino acids 69 to 313 of SEQ ID NO:13.

4. The isolated polypeptide of claim 1 comprising an amino acid sequence having at least 90% identity to amino acids 138 to 317 of SEQ ID NO:13.

5. The isolated polypeptide of claim 1 comprising amino acids 1 to 317 of SEQ ID NO:13.
6. The isolated polypeptide of claim 1 comprising amino acids 162 to 313 of SEQ ID NO:13.

7. The isolated polypeptide of claim 1 comprising amino acids 162 to 317 of SEQ ID NO:13.

8. The isolated polypeptide of claim 1 comprising amino acids 69 to 313 of SEQ ID NO:13.

9. The isolated polypeptide of claim 1 comprising amino acids 138 to 317 of SEQ ID NO:13.

10. The isolated polypeptide of claim 1 consisting of amino acids 1 to 317 of SEQ ID NO:13.

11. The isolated polypeptide of claim 1 consisting of amino acids 162 to 313 of SEQ ID NO:13.

12. The isolated polypeptide of claim 1 consisting of amino acids 162 to 317 of SEQ ID NO:13.

13. The isolated polypeptide of claim 1 consisting of amino acids 69 to 313 of SEQ ID NO:13.

14. The isolated polypeptide of claim 1 consisting of amino acids 138 to 317 of SEQ ID NO:13.

15. The isolated polypeptide of claim 1, comprising amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 and y is any one of amino acids 313-317.

16. The isolated polypeptide of claim 1 consisting of the amino acid sequence x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 of SEQ ID NO:13 and y is any one of amino acids 313-317 of SEQ ID NO:13.

17. The isolated polypeptide of any one of claims 3, 4 and 6 to 9 which is a soluble polypeptide.
18. A fusion polypeptide comprising a first polypeptide covalently fused with a second polypeptide, wherein the first polypeptide is the RANKL polypeptide as defined in any one of claims 1 to 17.

5 19. The fusion polypeptide of claim 18 wherein the second polypeptide comprises a sequence selected from the group consisting of a CMV leader, a kappa chain leader, a signal polypeptide, an immunoglobulin Fc region, a poly-Histidine tag, an antigenic polypeptide, a FLAG™ tag, a leucine zipper, and combinations thereof.

10 20. The polypeptide of claim 19, wherein the second polypeptide comprises a CMV leader having the amino acid sequence as shown in SEQ ID NO:9, and a leucine zipper having the amino acid sequence as shown in SEQ ID NO:19.

15 21. The polypeptide of claim 19, wherein the second polypeptide comprises a kappa chain leader comprising the amino acid sequence as shown in SEQ ID NO:16, a FLAG™ tag comprising the amino acid sequence as shown in SEQ ID NO:17, and a poly-Histidine tag comprising the amino acid sequence as shown in SEQ ID NO:18.

20 22. A composition comprising the polypeptide as defined in any one of claims 1 to 21 and a pharmaceutically acceptable carrier.

23. An isolated nucleic acid encoding the polypeptide as defined in any one of claims 1 to 21.

24. An isolated nucleic acid that comprises nucleotides 1 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.
25. An isolated nucleic acid that comprises nucleotides 484 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

26. An isolated nucleic acid that comprises nucleotides 412 to 951 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

27. An isolated nucleic acid that comprises nucleotides 205 to 939 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

28. An isolated nucleic acid that comprises nucleotides 484 to 939 of SEQ ID NO:12 and encodes a polypeptide that binds a receptor activator of NF-kappa B (RANK) protein, or the complement thereof.

29. The isolated nucleic acid of claim 23 that encodes a polypeptide comprising amino acids 1-317 of SEQ ID NO:13, amino acids 162-317 of SEQ ID NO:13, amino acids 69-313 of SEQ ID NO:13, or amino acids 162-313 of SEQ ID NO:13, wherein said polypeptide binds to a receptor activator of NF-kappa B (RANK) protein.

30. A recombinant expression vector comprising the nucleic acid as defined in any one of claims 23 to 29.

31. A host cell transformed or transfected with the expression vector as defined in claim 30.

32. A process for preparing a receptor activator of NF-kappa B ligand (RANKL) polypeptide, comprising culturing the host cell as defined in claim 31 under conditions promoting expression, and recovering the RANKL polypeptide.
33. An isolated antibody that binds with higher affinity to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide than to a murine RANKL polypeptide as shown in SEQ ID NO:11, wherein the human RANKL polypeptide is selected from the group consisting of:

(a) a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13;

(b) a polypeptide that is at least 90% identical to amino acids 1-317 of SEQ ID NO:13 and that binds to a RANK polypeptide as shown in SEQ ID NO:6;

(c) a polypeptide consisting of amino acids 1-162 of SEQ ID NO:13; and

(d) a fragment of the polypeptide of (a), (b), or (c) that binds to a RANK polypeptide as shown in SEQ ID NO:6.

34. The antibody according to claim 33, wherein said human RANKL polypeptide is selected from the group consisting of:

(a) a polypeptide consisting of amino acids 1-317 of SEQ ID NO:13;

(b) a polypeptide consisting of amino acids 69-313 of SEQ ID NO:13;

(c) a polypeptide consisting of amino acids 162-313 of SEQ ID NO:13;

(d) a polypeptide consisting of amino acids 138-317 of SEQ ID NO:13; and
(e) a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13.

5 35. The antibody according to claim 34, wherein said human RANKL polypeptide is a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13.

10 36. The antibody according to any one of claims 33 to 35 that is a monoclonal antibody.

37. The antibody according to any one of claims 33 to 35 that is a polyclonal antibody.

38. An isolated antibody that binds with higher affinity to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 than to a murine RANKL polypeptide as shown in SEQ ID NO:11.

39. The antibody according to claim 38 that is a monoclonal antibody.

20 40. The antibody according to claim 38 that is a polyclonal antibody.

41. An isolated antibody that binds to the human receptor activator of NF-kappa B ligand (RANKL) polypeptide but not to a murine RANKL polypeptide as shown in SEQ ID NO:11, wherein the human RANKL polypeptide is selected from the group consisting of:

(a) a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 1-162
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of SEQ ID NO:13, and y is any one of amino acids 313-317 of
SEQ ID NO:13;

(b) a polypeptide that is at least 90% identical
to amino acids 1-317 of SEQ ID NO:13 and that binds to a
5 RANK polypeptide as shown in SEQ ID NO:6;

(c) a polypeptide consisting of amino acids 1-162
of SEQ ID NO:13; and

(d) a fragment of the polypeptide of (a), (b), or
(c) that binds to a RANK polypeptide as shown in SEQ ID
10 NO:6.

42. The antibody according to claim 41, wherein said human RANKL polypeptide is selected from the group consisting of:

(a) a polypeptide consisting of amino acids 1-317
15 of SEQ ID NO:13;

(b) a polypeptide consisting of amino acids 69-313
of SEQ ID NO:13;

(c) a polypeptide consisting of amino acids
162-313 of SEQ ID NO:13;

(d) a polypeptide consisting of amino acids
20 138-317 of SEQ ID NO:13; and

(e) a polypeptide consisting of amino acids x to y
25 of SEQ ID NO:13, wherein x is any one of amino acids 69-162
of SEQ ID NO:13, and y is any one of amino acids 313-317 of
SEQ ID NO:13.

43. The antibody according to claim 42, wherein said human RANKL polypeptide is a polypeptide consisting of amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids
acids 69-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13.

44. The antibody according to any one of claims 41 to 43 that is a monoclonal antibody.

45. The antibody according to any one of claims 41 to 43 that is a polyclonal antibody.

46. An isolated antibody that binds to a human receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13, but that does not bind to a murine RANKL polypeptide as shown in SEQ ID NO:11.

47. The antibody according to claim 46 that is a monoclonal antibody.

48. The antibody according to claim 46 that is a polyclonal antibody.

49. The antibody according to any one of claims 33 to 48 which inhibits interaction between receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 and RANK polypeptide as shown in SEQ ID NO:6.

50. Use of the receptor activator of NF-kappa B ligand (RANKL) polypeptide as defined in any one of claims 1 to 21 as an antigen for producing an antibody immunoreactive to the human RANKL polypeptide as shown in SEQ ID NO:13.

51. A method for preparing an antibody to a receptor activator of NF-kappa B ligand (RANKL) polypeptide comprising immunizing a non-human animal with the RANKL polypeptide according to any one of claims 1 to 21 in an amount effective to produce antibodies to the RANKL polypeptide, and harvesting the antibodies.
52. The method according to claim 50 or 51, wherein the RANKL polypeptide is selected from the group consisting of:

(a) a polypeptide comprising amino acids 1-317 of SEQ ID NO:13;

(b) a polypeptide comprising amino acids 69-313 of SEQ ID NO:13;

(c) a polypeptide comprising amino acids 1-162 of SEQ ID NO:13;

(d) a polypeptide comprising amino acids 162-313 of SEQ ID NO:13;

(e) a polypeptide comprising amino acids 138-317 of SEQ ID NO:13;

(f) a polypeptide comprising amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13; and

(g) a polypeptide that is at least 90% identical to amino acids 1-317 of SEQ ID NO:13, and binds to a RANK polypeptide as shown in SEQ ID NO:6.

53. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids 1-317 of SEQ ID NO:13.

54. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids 69-313 of SEQ ID NO:13.
55. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids 1-162 of SEQ ID NO:13.

56. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids 162-313 of SEQ ID NO:13.

57. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids 138-317 of SEQ ID NO:13.

58. The method according to claim 52, wherein the RANKL polypeptide comprises amino acids x to y of SEQ ID NO:13, wherein x is any one of amino acids 69-162 of SEQ ID NO:13, and y is any one of amino acids 313-317 of SEQ ID NO:13.

59. The method according to claim 52, wherein the RANKL polypeptide is at least 90% identical to amino acids 1-317 of SEQ ID NO:13, and binds to a RANK polypeptide as shown in SEQ ID NO:6.

60. A method of producing the monoclonal antibody as defined in any one of claims 36, 39, 44, and 47, said method comprising culturing a cloned hybridoma cell that produces said antibody.

61. The method of claim 60 wherein the hybridoma cell is transferred into the peritoneal cavity of a rodent.

62. A cloned hybridoma cell that produces the monoclonal antibody as defined in any one of claims 36, 39, 44, and 47.
63. A composition comprising the antibody as defined in any one of claims 33 to 49 and a pharmaceutically acceptable carrier.

64. Use of the RANKL polypeptide as defined in any one of claims 1 to 17 for the preparation of an antibody that binds specifically to the polypeptide.

65. Use of the fusion polypeptide as defined in any one of claims 18 to 21 for the preparation of an antibody that binds specifically to the first polypeptide.

66. A method to screen for an inhibitor of RANK activation, the method comprising the step of providing the RANKL polypeptide as defined in any one of claims 1 to 21 under conditions where the RANKL polypeptide binds to RANK, in the presence or absence of a test compound; wherein a reduction in binding between the RANKL polypeptide and RANK in the presence of the test compound compared to its absence indicates the test compound is an inhibitor of RANK activation.

67. The method according to claim 66 wherein the reduction in binding between the RANKL polypeptide and RANK is observed as a reduction in RANK activity.

68. Use of the antibody as defined in claim 49 for inhibiting interaction between receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 and RANK polypeptide as shown in SEQ ID NO:6.

69. Use of the antibody as defined in claim 49 in the manufacture of a medicament for inhibiting interaction between receptor activator of NF-kappa B ligand (RANKL) polypeptide as shown in SEQ ID NO:13 and RANK polypeptide as shown in SEQ ID NO:6.
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70. A kit for detecting a receptor activator of NF-kappa B (RANK) or a RANK ligand (RANKL) polypeptide, or for detecting RANK activity, the kit comprising:

(a) instructions for the detecting; and

(b) the RANKL polypeptide as defined in any one of claims 1 to 21 or the antibody as defined in any one of claims 33 to 49.

71. A kit comprising:

(a) the soluble RANKL polypeptide as defined in any one of claims 11 to 14 and 17; and

(b) instructions for using said RANKL polypeptide to monitor receptor activator of NF-kappa B (RANK) related activity.
Figure 1

Day 6, viable/T cell recovery (×10^3/ml)
Figure 3

Control + CD40L + RANKL + ΔH RANKL T cells only

[^3]H-Thymidine Incorporation

cpm

Number of APC per well

20000 15000 10000 5000 0

10^0 10^1 10^2 10^3 10^4 10^5
Figure 5
Figure 5 (cont.)