Title: STIMULATION OR INHIBITION OF GAMMA DELTA T-CELLS TO PROMOTE OR INHIBIT BONE GROWTH

Abstract: The invention provides a method to promote bone healing, a method to inhibit bone healing; a pharmaceutical composition that promotes healing of a bone injury, disease, or defect; a pharmaceutical composition that inhibits healing of a bone injury; a method to screen for a agent that promotes bone healing; and a method to screen for an agent that inhibits bone healing.
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STIMULATION OR INHIBITION OF GAMMA DELTA T-CELLS TO
PROMOTE OR INHIBIT BONE GROWTH

Government Funding

The invention described herein was developed with the support of the Department of Health and Human Services. The United States Government has certain rights in the invention.

Field of the Invention

The invention relates to the fields of immunology and orthopaedics. Specifically, the invention relates to stimulation or inhibition of gamma delta T-cells (γδ T-cells). More specifically, the invention relates to inhibition or stimulation of gamma delta T-cells to promote or inhibit bone growth respectively.

Background of the Invention

Bone injury is a common occurrence that disables people from working and otherwise carrying on their daily lives. Such injuries commonly result from accidents, such as vehicular accidents, work related injuries, or sports injuries, and present an important health concern. Proper healing of bone injuries allows people to resume working and tending to their daily matters. However, when fracture healing goes awry, the ability of a bone to heal without a scar is lost. This can result in disfigurement, pain, and loss or impairment of bodily function.

Abnormalities in bone healing are associated with many factors. Delayed or non-unions can occur as a result of inadequate alignment or fixation, infection, or poor blood supply. Improper healing can also occur in certain patient populations that are prone to non-healing, such as diabetics, patients on chronic steroid therapy, those that are immunocompromised, or those that are malnourished.

Bone fracture healing involves a series of phases. The initial phase of all fracture healing is localized hematoma formation followed by inflammatory repair. Hematoma formation includes the modulation and induction of cells from the bone, the marrow and the fracture site which causes immunomodulatory
agents to be released into the site of injury. Examples of such immunomodulatory agents include kinins, prostaglandins, and growth factors.

Inflammatory cells, such as neutrophils, lymphocytes, macrophages, and mast cells, usually appear within 48 hours of injury. The macrophage is a major cell type critical in the regulation of transforming growth factor beta 1 and 3, and fibroblast growth factor, which are potent stimulators of mesodermal cell division. These growth factors effect proliferation of cells that actively participate in bone fracture repair, such as chondrocytes, osteoblasts, and periosteal cells. In contrast, other cells of the immune system produce interleukin-6 and interferon gamma which decreases bone formation by influencing bone resorption and exhibiting antiproliferative effects on bone-derived cells respectively. Interleukin-6 and interferon gamma are especially active on osteoblasts. Accordingly, the control of bone formation is highly dependent on an exacting and controlled initial inflammatory response.

Following the inflammatory phase, subsequent stages of repair include formation of granulation tissue and a callus of collagen, cartilage, and immature bone. Intramembranous and endochondral bone formation are both represented. The most clinically applicable inducers responsible for signaling replacement of the early fracture granulation tissue to osseous callus have been the bone morphogenic proteins (BMPs), particularly BMP-2, BMP-3, BMP-4, and BMP-7.

Previous research efforts on understanding and improving fracture healing have been directed primarily at manipulation of cells involved at a secondary level of bone repair, for example, by inducing osteoprogenator cells to become bone forming cells. The osteoinductive properties of many procedures, tissues, and substances have been explored. These include bone grafts, bone marrow implants, demineralized bone matrix, growth factors, recombinant fibroblasts, as well as pulsed electromagnetic fields. Currently, surgical autotransplantation of bone, or surgical augmentation with growth factors, have been the only consistently effective treatment for pathologic bone repair.

To date, no systemic treatment has been administered exogenously to accelerate fracture healing in a reproducible manner. It is known that the primary response that occurs when the bone is injured is immunological. However, methods to manipulate the immune response are poorly understood
due to the molecular complexity of the response. Only when bone pathophysiology is understood at its molecular and immunological level can the healing response be manipulated with the use of small molecules and other therapeutics. Accordingly, there exists a need to further understand the mechanisms that control bone healing, and to develop therapeutics that can be used to control bone healing.

Summary of the Invention

These and other needs are met by the present invention. The invention relates to the discovery that gamma delta T-cells are centrally involved in bone healing. Consequently, the invention provides a method to promote bone healing; a method to inhibit bone healing; a method for treating bone-related diseases and defects; a pharmaceutical composition that promotes healing of a bone injury, disease, or defect; a pharmaceutical composition that inhibits healing of a bone injury, disease, or defect; a pharmaceutical composition that is combined with, or impregnated on or within a bone compatible biodegradable or non-degradable matrix; a method to screen for an agent that promotes bone healing; and a method to screen for an agent that inhibits bone healing.

The invention provides a method to promote bone healing in an organism having a bone injury, disease, or defect. The method involves administering to an organism an agent that at least partially inhibits the activity of gamma delta T-cells, so that bone healing is promoted, wherein the organism has or is suspected of having a bone injury, disease, or defect. The agent can be a cytokine that at least partially inhibits gamma delta T-cell activity. In other embodiments, the agent is a peptide aptamer. In other embodiments, the agent is an antibody. In further embodiments, the agent is a soluble gamma delta T-cell receptor. Preferably the cytokine that inhibits gamma delta T-cell activity is transforming growth factor beta. More preferably the cytokine that inhibits gamma delta T-cell activity is interleukin-10. Preferably the antibody binds to a gamma delta T-cell. More preferably the antibody binds to a gamma delta T-cell receptor. Most preferably the antibody binds to a proinflammatory cytokine secreted by a gamma delta T-cell. Preferably the antibody is a polyclonal antibody. More preferably the antibody is a monoclonal antibody. Even more preferably the antibody is a single-chain antibody. Still even more preferably the
anti-body is a chimeric antibody. Most preferably the antibody is a humanized antibody. Preferably an antibody that binds to a gamma delta T-cell is conjugated to a toxin. Preferably the peptide aptamer binds to a gamma delta T-cell. More preferably the peptide aptamer binds to a gamma delta T-cell receptor. Preferably a peptide aptamer that binds to a gamma delta T-cell is conjugated to a toxin. Preferably the peptide aptamer or antibody causes stimulation of a gene that encodes a protein involved in gamma delta T-cell apoptosis. More preferably the peptide aptamer or antibody causes stimulation of a protein involved in gamma delta T-cell apoptosis. Even more preferably the peptide aptamer or antibody causes stimulation of a gene that encodes a protein involved in direct inhibition of gamma delta T-cell proliferation. Still even more preferably the peptide aptamer or antibody causes stimulation of a protein involved in direct inhibition of gamma delta T-cell proliferation. Most preferably the peptide aptamer or antibody causes stimulation of a transcription factor that is involved in direct inhibition of gamma delta T-cell proliferation. Preferably the organism is an avian. More preferably the organism is a mammal. Most preferably the organism is a human. Preferably the bone injury, disease, or defect heals through bone formation. Preferably the bone injury is a crack in the bone. More preferably the bone injury is a fracture. Preferably the agent is administered to the organism systemically. More preferably the agent is administered to the organism at a site peripheral to the site of bone injury. Most preferably the agent is administered to the organism at the site of the bone injury. Preferably the agent inhibits gamma delta T-cell localization to a site of bone injury, disease, or defect. More preferably the agent inhibits gamma delta T-cell expansion. Even more preferably the agent inhibits expression of a gamma delta T-cell receptor by a gamma delta T-cell. Most preferably the agent inhibits secretion of cytokines by a gamma delta T-cell.

The invention provides a method that involves administering an agent that at least partially inhibits the activity of a gamma delta T-cell related cytokine to the organism to promote healing of a bone injury, disease, or defect. The organism can be an avian organism. Alternatively, the organism is a mammal. In some embodiments, the organism is a human. Preferably the gamma delta T-cell related cytokine is secreted by a gamma delta T-cell. Preferably the bone injury heals through bone formation. Preferably the bone
injury, disease, or defect is a crack in the bone. More preferably the bone injury is a fracture. Preferably the agent is administered to the organism systemically. More preferably the agent is administered to the organism at a site peripheral to the site of bone injury. Most preferably the agent is administered to the organism at the site of the bone injury. Preferably the gamma delta T-cell related cytokine is interleukin 1. More preferably the gamma delta T-cell related cytokine is interleukin 7. Even more preferably the gamma delta T-cell related cytokine is tumor necrosis factor. Still even more preferably the gamma delta T-cell related cytokine is interleukin-2. Yet still even more preferably the gamma delta T-cell related cytokine is interleukin-6. Most preferably the gamma delta T-cell related cytokine is interferon gamma. Preferably the agent that inhibits the activity of a gamma delta T-cell related cytokine is a peptide aptamer. More preferably the agent that inhibits the activity of a gamma delta T-cell related cytokine is an antibody. Preferably the antibody is a polyclonal antibody. More preferably the antibody is a monoclonal antibody. Even more preferably the antibody is a single-chain antibody. Still even more preferably the antibody is a chimeric antibody. Most preferably the antibody is a humanized antibody.

The invention provides a method that involves administering an agent that at least partially stimulates the activity of a gamma delta T-cell to an organism to inhibit healing of a bone injury. Preferably the organism is an avian. More preferably the organism is a mammal. Most preferably the organism is a human. Preferably the agent is a gamma delta T-cell antigen. Preferably the agent stimulates the gamma delta T-cell receptor. Preferably the agent is vitronectin. More preferably the agent is a CD40 ligand. Most preferably the agent is an aminobisphosphonate. Preferably the agent is a peptide aptamer that binds and inactivates a cytokine that inhibits the activity of gamma delta T-cells. More preferably the agent is an antibody that binds and inactivates a cytokine that inhibits the activity of gamma delta T-cells. Preferably the agent is an antibody that binds to Syk. More preferably the agent is an antibody that binds to FcεRIγ. Still more preferably the agent is an antibody that binds to CD3. Most preferably the agent is a M. tuberculosis gamma delta T-cell antigen. Preferably the antibody is a polyclonal antibody. More preferably the antibody is a monoclonal antibody. Even more preferably the antibody is a single-chain antibody. Still even more preferably the antibody...
is a chimeric antibody. Most preferably the antibody is a humanized antibody. Preferably the cytokine that inhibits the activity of gamma delta T-cells is interleukin-10. More preferably the cytokine that inhibits the activity of gamma delta T-cells is transforming growth factor beta.

The invention provides a pharmaceutical composition that at least partially promotes healing of a bone injury, disease, or defect in an organism that includes an agent that inhibits gamma delta T-cell activity, and a pharmaceutically acceptable carrier. Preferably the pharmaceutical composition that promotes healing of a bone injury, disease, or defect is combined with, or impregnated on or within a bone compatible matrix. Preferably the bone compatible matrix is non-biodegradable. More preferably the bone compatible matrix is biodegradable. Preferably the organism is an avian. More preferably the organism is a mammal. Most preferably the organism is a human. Preferably the agent is a peptide aptamer. More preferably the agent is an antibody. Even more preferably the agent is soluble gamma delta T-cell receptor. Most preferably the agent is a cytokine that inhibits the activity of gamma delta T-cells. Preferably the peptide aptamer binds to a gamma delta T-cell. More preferably the peptide aptamer binds to a gamma delta T-cell receptor. Most preferably the peptide aptamer binds to a proinflammatory gamma delta T-cell related cytokine. Preferably the antibody binds to a gamma delta T-cell. More preferably the antibody binds to a gamma delta T-cell receptor. Most preferably the antibody binds to a proinflammatory gamma delta T-cell related cytokine. Preferably the cytokine that inhibits the activity of gamma delta T-cells is interleukin-10. More preferably the cytokine that inhibits the activity of gamma delta T-cells is transforming growth factor beta. Preferably the proinflammatory gamma delta T-cell related cytokine is interleukin-1. More preferably the proinflammatory gamma delta T-cell related cytokine is interleukin-2. Still more preferably the proinflammatory gamma delta T-cell related cytokine is interleukin-7. Even still more preferably the proinflammatory gamma delta T-cell related cytokine is interleukin-6. Yet even still more preferably the proinflammatory gamma delta T-cell related cytokine is tumor necrosis factor. Most preferably the proinflammatory gamma delta T-cell related cytokine is interferon gamma.
The invention provides a pharmaceutical composition that at least partially inhibits healing of a bone injury, disease, or defect in an organism that includes an agent that stimulates gamma delta T-cell activity, and a pharmaceutically acceptable carrier. Preferably the pharmaceutical composition that inhibits healing of a bone injury, disease, or defect is combined with, or impregnated on or within a bone compatible matrix. Preferably the bone compatible matrix is non-biodegradable. More preferably the bone compatible matrix is biodegradable. Preferably the organism is an avian. More preferably the organism is a mammal. Most preferably the organism is a human.

Preferably the agent is an antibody that binds CD3. More preferably the agent is a peptide aptamer that binds CD3. Even more preferably the agent is an antibody that binds to Syk. Still even more preferably the agent is an antibody that binds to FcεRIγ. Yet still even more the agent is an antibody that binds to a cytokine that inhibits gamma delta T-cell activity. Even yet still even more preferably the agent is an antibody that binds to a cytokine that inhibits gamma delta T-cell activity. Most preferably the agent is a M. tuberculosis gamma delta T-cell antigen. Preferably the cytokine that inhibits gamma delta T-cell activity is interferon-10. More preferably the cytokine that inhibits gamma delta T-cell activity is transforming growth factor alpha.

The invention provides a method to screen for an agent that modulates gamma delta T-cell activity. The method involves contacting a gamma delta T-cell with a candidate agent, and determining if the candidate agent promotes or inhibits gamma delta T-cell activity. Preferably the agent stimulates gamma delta T-cells. More preferably the agent inhibits gamma delta T-cells.

Preferably the gamma delta T-cell activity is upregulation of a cytokine receptor. More preferably the gamma delta T-cell activity is expression of a gamma delta T-cell receptor. Even more preferably the gamma delta T-cell activity is cytokine production. Still even more preferably the gamma delta T-cell activity is gamma delta T-cell proliferation. Most preferably the gamma delta T-cell activity is apoptosis. Preferably the cytokine is interleukin-1. More preferably the cytokine is interleukin-2. Even more preferably the cytokine is interleukin-6. Still even more preferably the cytokine is interleukin-7. Yet still even more preferably the cytokine is tumor necrosis factor. Most preferably the cytokine is interferon gamma.
This invention is related to U.S. Application Ser. No. 60/518278, filed November 7, 2003, which is herein incorporated in its entirety.

**Brief Description of the Figures**

Figure 1 provides an example of a load-displacement curve. Energy expenditure is defined as the area under the curve, calculated to maximum load (area A) and to failure (area A + B).

Figure 2A-F illustrates Gamma Delta T-cell receptor cellular expression during fracture healing of control (wild type) animals. Representative micrographs for days 1, 5 and 10 after fracturing are shown. Areas of darker staining indicate T-cell receptor expression. Original magnifications: x10 (lower magnification); x40 (higher magnification). Higher magnifications were used to depict the type of cells that were expressing the γδ T-cell receptor (TCR), while lower magnifications give an overview of the tissue stage.

Figure 3 graphically illustrates the percent γδ TCR+ lymphocytes in control (wild type) animals by flow cytometry. Scores are expressed as mean with ± standard deviation. The symbol * indicates P<0.05, by Student’s t-test.

Figure 4A and 4B provide comparative histology scores for control and gamma delta T-cell knockout mice relating to the quality of fracture union and bone marrow maturity as evidenced by colonization of marrow, respectively. Ten micron sections are shown that were representative of the entire callus. The sections were stained with Masson-Trichrome and hematoxylin and eosin stains. Scores are expressed as mean with ± standard deviation. Day 5 knockout animals had higher scores in both categories. The symbol * indicates P<0.05, by Student’s t-test.

Figure 4C and 4D provide representative histological sections for control and gamma delta T-cell knockout mice, respectively, during the reparative phase at Day 5. Arrows point to new osseous elements formed in each group, which are more apparent in knockout animals as compared to controls. Original magnifications X10.

Figure 4E provides an image of a 2% agarose gel containing electrophoretically separated reverse transcription polymerase chain reaction (RT-PCR) products generated from Day 5 RNA obtained from fracture calluses of wild type control (CON) and gamma delta T-cell knockout (KO) mice. The
RT-PCR primers employed were specific for Collagen II (a chondrogenic marker), bone sialoprotein (BSP, an osteogenic marker), and bone morphogenic protein-2 (BMP-2, a matrix marker). Knockout animals exhibited increased expression of all three genes compared with control animals.

Figure 5 provides a bar graph illustrating radiographic scores for a total of 120 control and gamma delta T-cell knockout animals based on quality of union. Scores are expressed as mean with ± standard deviation. The symbol * indicates, P< 0.05, by the Student’s t-test.

Figure 6 provides a bar graph comparing total numbers of peripheral lymphocytes in control and gamma delta T-cell knockout animals, as assessed by flow cytometry. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test. Both control and knockout groups showed a remarkable reduction in the absolute number of total peripheral lymphocytes, as seen by flow cytometry, in the first day following injury.

Figure 7A graphically illustrates an overall decrease in IL-2 expression in gamma delta T-cell knockout knockout animals during the inflammatory phase as compared with control animals. Scores are expressed as means. The symbol * indicates P< 0.05, by Student’s t-test.

Figures 7B and 7C graphically illustrate expression of proinflammatory cytokines interferon-γ and interleukin-6, respectively, in fractured bones during repair, as detected by ELISA. The expression of these cytokines in control and gamma delta T-cell knockout fractured bones is compared. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

Figure 8A and 8B illustrate CD44+ expression in osteoblasts of gamma delta T-cell knockout animals as detected by immunolocalization during the reparative phases. Original magnifications: X40 (Day 5, Figure 8A); X10 (Day 10, Figure 8B).

Figure 9 graphically illustrates the percentage of total lymphocytes expressing the CD44 surface marker as assessed by flow cytometry for control and gamma delta T-cell knockout animals. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

Figure 10 graphically illustrates the percent of γδ TCR+ and γδ TCR+/CD44+ lymphocytes at the systemic level at 1, 5, 10 and 28 days after
fracture, as detected by flow cytometry. The number of days since fracture is plotted on the y-axis. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

Figure 1 illustrates that gamma delta T-cell knockout mice have fewer peripheral CD 44 hi cells during all phases of fracture repair. The number of days since fracture is plotted on the y-axis. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

Figure 12 provides a graphic comparison of gamma delta T-cell knockout and control healing callus rigidity and elastic modulus, which are measures of the material and structural strengths of the healing fractures, as obtained by biomechanical testing at 2, 4, 6, and 8 weeks after fracturing. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

Figure 13 provides a comparison of energy absorption by control and gamma delta T-cell knockout healing calluses. The energy absorption to maximum load and to failure of healing calluses was measured at 2, 4, 6 and 8 weeks after fracture. Scores are expressed as mean with ± standard deviation. The symbol * indicates P< 0.05, by Student’s t-test.

**Detailed Description of the Invention**

It has been discovered that the stability of bone healing at a site of bone fracture is promoted when gamma delta T-cells are not present at the fracture site, as compared to the stability of bone that heals in the presence of gamma delta T-cells. This discovery provides for the treatment of patients having a bone fracture, disease, or defect with gamma delta T-cell inhibitors that will promote bone healing. Alternatively, bone healing can be promoted by administering agents to the injury site that decrease or eliminate the action of cytokines secreted by gamma delta T-cells at the injury site. In addition, the discovery provides for the treatment of an injury site with cytokines produced by gamma delta T-cells, or agents that stimulate the activity of gamma delta T-cells, to inhibit bone healing. Such treatments will stop unwanted bone growth in patients who suffer from certain diseases, such as fibrodysplasia ossificans progressiva or heterotopic ossification.
Gamma delta T-cells are a distinct lineage of T lymphocytes found in mammals and birds. Gamma delta T-cells express a particular antigen receptor (i.e., T-cell receptor or TCR) that includes a gamma chain and a delta chain. The gamma and delta chains are distinguished from the alpha and beta chains that make up the TCR of the more commonly referenced T-cells known as alpha beta T-cells. A gamma delta heterodimer is expressed on the surface of gamma delta T-cells and, like the alpha beta heterodimer of alpha beta T-cells, is associated with the CD3 complex on the cell surface. The gamma and delta chains of the gamma delta T-cell receptor should not be confused with the gamma and delta chains of the CD3 complex.

Gamma delta T-cells have been termed innate lymphocytes. This term refers to the portion of the immune system that relies on mechanisms that are present prior to injury or infection. As such, gamma delta T-cells have the ability to directly recognize products of stressed cells through receptors on their cell surface. These receptors can bind products of stressed cells directly. This contrasts with other types of T-cells that only recognize antigens when the antigens are bound and presented to the T-cells by another type of cell, called an antigen presenting cell.

Gamma delta T-cells are a diverse and heterogeneous population whose function differs according to tissue distribution, and can perform specialized functions related to the repair of tissue damage. Gamma delta T-cells constitute only a small proportion, 1-5% of the lymphocytes that circulate in the blood, but can comprise up to 50% of lymphocytes in epithelial tissue.

Gamma delta T-cells are activated during the initial inflammatory phase of fracture healing to produce cytokines that include interferon gamma (IFN-γ) and interleukin-6 (IL-6). These cytokines have been determined to inhibit bone healing by inducing cell death (apoptosis), and activating cells that inhibit bone formation. After the initial inflammatory phase has passed the immune response stops and maturation of the callus proceeds.

The present discovery was made through use of gamma delta knockout mice, which are mice that lack gamma delta T-cells. It was determined that bone healing in these gamma delta knockout mice was superior to bone healing in mice that contained gamma delta T-cells. The gamma delta knockout mice demonstrated quantitative and temporal alterations in the expression of type II
collagen (Col 2), bone sialoprotein (BSP), and bone morphogenic protein 2 (BMP-2), as well as CD44 adhesion molecule in the fracture callus of a bone injury. Histological, immunolocalization, and biomechanical analysis corroborated these findings.

The increased strength noted in a callus of smaller size that formed in gamma delta knockout animals can be explained in a three fold manner. First, since T-cells represent a population of osteoprogenator cells, their number is reduced in knock-out animals, and subsequently less cells are available to respond to the injury site initially. This results in the formation of a smaller callus size. Second, the environmental milieu that pervades at an injury site in the absence of gamma delta T-cells provides an increased proliferative and adhesive capacity within undifferentiated cells that are present at the injury site. Third, the accelerated expression of osseous, chondral, and morphogenic elements confers a stability seen during the early reparative phase that is more reminiscent of the later remodeling phase.

Accordingly, manipulations that modulate immune responsiveness can lead to improvement in fracture healing. Modulation of cytokine production and release by immune cells leading to the restoration of cellular homeostasis provide an approach for decreasing the susceptibility of patients to nonunion.

The results reported herein leave no doubt that a specific interaction exists between the immune, hematopoietic, and musculoskeletal systems as a result of skeletal injury.

I. **Method to promote bone healing in a mammal**

The invention provides a method to promote healing of a bone injury, fracture, bone disease, or defect, in an organism, such as a mammal or avian. In one aspect, the invention involves administering an agent that inhibits the activity of gamma delta T-cells to an organism, such as a human, having a bone injury, disease, or defect.

**Soluble T-cell receptors**

An example of an agent that can be used to reduce or eliminate expansion or activation of gamma delta T-cells is a soluble gamma delta T-cell receptor. Soluble gamma delta T-cell receptors are thought to bind gamma delta T-cell specific ligands, and thereby out-compete binding of the ligands by receptors
expressed on the surface of gamma delta T-cells. This results in reduction or elimination of expansion or activation of gamma delta T-cells. Soluble gamma delta T-cell receptors and methods to prepare such soluble receptors have been described in United States Patent Application, Publication Number US 2003/0175212 A1.

A soluble T cell receptor is a T cell receptor consisting of the chains of a full-length (e.g., membrane bound) receptor, except that, minimally, the transmembrane region of the receptor chains are deleted or mutated so that the receptor, when expressed by a cell, will not associate with the membrane. Most typically, a soluble receptor will consist of only the extracellular domains of the chains of the wild-type receptor (i.e., lacks the transmembrane and cytoplasmic domains).

A soluble gamma delta T-cell receptor can include a single gamma chain and a single delta chain linked by a disulfide bond. A soluble gamma delta T-cell receptor can be a multimer of soluble gamma delta T-cell receptors that include gamma chains and delta chains that are linked by disulfide bonds. The soluble gamma delta T-cell receptor can include any combination of Vgamma and Vdelta chains. For example, a soluble gamma delta T-cell receptor can include a human Vgamma chain chosen from, but not limited to: Vgamma8 or Vgamma9. A soluble gamma delta T-cell receptor can include, but is not limited to, a human Vdelta1 chain.

Numerous human gamma and delta chains are known in the art and have been assigned accession numbers. Examples of human gamma chains include, but are not limited to: Vgamma8 (WHO Designation hGV1; GenBank Accession No. M13434; this receptor chain has also been referred to as Vgamma1 in humans); Vgamma9 (WHO Designation hGV2; GenBank Accession No. X72500; this receptor chain has also been referred to as Vgamma2 in humans). Examples of human delta chains include, but are not limited to: Vdelta2 (WHO Designation hDV102; GenBank Accession No. X72501); Vdelta3 (WHO Designation hDV103; GenBank Accession No. X13954); and Vdelta4 (WHO Designation hADV6; GenBank Accession No. M21624). World Health Organization (WHO) Designations are also given for clarity. More complete lists of human Vgamma and Vdelta chains have been described (Arden et al.,
Soluble gamma delta T-cell receptors can be produced by any suitable method known to those of skill in the art, and are most typically produced recombinantly. A recombinant nucleic acid molecule useful for producing a soluble gamma delta T-cell receptor typically includes a recombinant vector and a nucleic acid sequence encoding one or more segments (e.g., chains) of a gamma delta T-cell receptor as described herein. A recombinant vector is an engineered (i.e., artificially produced) nucleic acid molecule that is used as a tool for manipulating a nucleic acid sequence of choice and/or for introducing such a nucleic acid sequence into a host cell. The recombinant vector is therefore suitable for use in cloning, sequencing, and/or otherwise manipulating the nucleic acid sequence of choice, such as by expressing and/or delivering the nucleic acid sequence of choice into a host cell to form a recombinant cell. Such a vector typically contains heterologous nucleic acid sequences, that is, nucleic acid sequences that are not naturally found adjacent to a nucleic acid sequence to be cloned or delivered, although the vector can also contain regulatory nucleic acid sequences (e.g., promoters or untranslated regions) which are naturally found adjacent to nucleic acid sequences which encode a protein of interest (e.g., the T-cell receptor chains), or which are useful for expression of the nucleic acid molecules. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a plasmid.

A recombinant nucleic acid molecule may include at least one nucleic acid molecule operatively linked to one or more transcription control sequences. As used herein, the phrase "recombinant molecule" or "recombinant nucleic acid molecule" primarily refers to a nucleic acid molecule or nucleic acid sequence operatively linked to a transcription control sequence, but can be used interchangeably with the phrase "nucleic acid molecule," when such nucleic acid molecule is a recombinant molecule as discussed herein. The phrase "operatively linked" refers to linking a nucleic acid molecule to a transcription control sequence in a manner such that the molecule is able to be expressed when transfected (i.e., transformed, transduced, transfected, or conjugated) into a host cell. Transcription control sequences are sequences which control the initiation, elongation, or termination of transcription. Particularly important
transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in a host cell or organism into which the recombinant nucleic acid molecule is introduced.

One or more recombinant molecules can be used to produce an encoded product (e.g., a soluble gamma delta T-cell receptor). For example, an encoded product can be produced by expressing a nucleic acid molecule as described herein under conditions effective to produce the protein. A host cell can be transfected with one or more recombinant molecules to form a recombinant cell. Suitable host cells to transfact include, but are not limited to, any bacterial, yeast, fungal, insect, plant or animal cell that can be transfected. Host cells can be either untransfected cells, or cells that are already transfected with at least one other recombinant nucleic acid molecule.

Resultant proteins may either remain within the recombinant cell, be secreted into the culture medium, be secreted into a space between two cellular membranes, or be retained on the outer surface of a cell membrane. The protein may be obtained by collecting the whole culture medium containing the protein and need not imply additional steps of separation or purification. Proteins can be purified using a variety of standard purification techniques, such as, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing, and differential solubilization. Proteins can be obtained in substantially pure form.

Substantially pure refers to a purity that allows for the effective use of the soluble gamma delta T-cell receptor in a composition and method for the promotion of bone growth.

Recombinant nucleic acid constructs containing the relevant gamma and delta genes (e.g., nucleic acid sequences encoding the desired portions of the gamma and delta chains of a gamma delta T-cell receptor) can be produced by PCR of T-cell receptor cDNAs derived from a source of gamma delta T-cells (e.g., hybridomas, clones, transgenic cells) that express the desired receptor. The PCR amplification of the desired gamma and delta genes can be designed so that the transmembrane and cytoplasmic domains of the chains will be omitted (i.e.,
creating a soluble receptor). Preferably, portions of the genes that form the interchain disulfide bond are retained, so that the gamma delta heterodimer formation is preserved. In addition, if desired, sequence encoding a selectable marker for purification or labeling of the product or the constructs can be added to the constructs. Amplified gamma and delta cDNA pairs are then cloned, sequence-verified, and transferred into a suitable vector, such as a baculoviral vector containing dual baculovirus promoters (pAcUW51, Pharmingen Corp., San Diego, Calif).

The soluble gamma delta T-cell receptor constructs are then co-transfected into a suitable host cell (e.g., in the case of a baculoviral vector, into suitable insect host cells) which will express and secrete the recombinant receptors into the supernatant. Culture supernatants containing soluble gamma delta T-cell receptors can then be purified using various affinity columns, such as anti-C delta (GL3) sepharose affinity columns.

Antibodies against gamma delta T-cell receptors

Antibodies can be used as agents to down-regulate the expression of T-cell receptors on gamma delta T-cells and thereby decrease the activity of the gamma delta T-cells. An antibody that binds a gamma delta T-cell receptor can be administered to an organism, such as a human, having a bone injury, disease, or defect to down-regulate expression of the gamma delta T-cell receptor and thereby promote bone healing. Antibodies that specifically bind to gamma delta T-cells are known in the art and have been used to down-regulate gamma delta T-cell receptor expression in mammals and avians (Cihak et al., Scand. J. Immunol., 38:123 (1993); Rose et al., Infect. Immun., 64:4854 (1996)). For example, the monoclonal antibody GL3 specifically binds the murine gamma delta T-cell receptor (Goodman and Lefrancois, J. Exp. Med., 170:1569 (1989)). The GL3 antibody has been shown to down-regulate expression of the gamma delta T-cell receptor following intraperitoneal injection into mice (Kaufmann et al., Proc. Natl. Acad. Sci. USA, 90:9620 (1993)).

Antibodies that specifically bind gamma delta T-cells can also be conjugated to a toxin and used to deplete gamma delta T-cells in a human or animal. For example, an antibody that binds the gamma delta T-cell receptor, UC7-13D5, was conjugated to daunomycin and used to deplete gamma delta T-cells in mice (Seo et al., J. Immunol., 163:242 (1999)).

The preparation of polyclonal antibodies is well-known to those skilled in the art (Green et al., Production of Polyclonal Antisera, in Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan et al., Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in Current Protocols in Immunology, section 2.4.1 (1992), and Harlow et al., Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. 1988)). For example, an antigen is injected into an animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animal is bled periodically. Polyclonal antibodies specific for the antigen may then be purified from such antisera by, for example, affinity chromatography using the polypeptide or peptide fragment coupled to a suitable solid support.

The preparation of monoclonal antibodies is also routine (Kohler & Milstein, Nature, 256:495 (1975); and Harlow et al., Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. 1988)). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. The hybridomas can be grown in vitro for antibody production. Production in vitro provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies.
Antibodies can also be prepared through use of phage display techniques. In one example, an organism is immunized with an antigen and then lymphocytes are isolated from the spleen of the immunized organism. Total RNA is isolated from the splenocytes and mRNA contained within the total RNA is reverse transcribed into complementary deoxyribonucleic acid (cDNA). The cDNA encoding the variable regions of the light and heavy chains of the immunoglobulin is amplified by polymerase chain reaction (PCR). To generate a single chain fragment variable (scFV) antibody, the light and heavy chain amplification products may be linked by splice overlap extension PCR to generate a complete sequence and ligated into a suitable vector. E. coli are then transformed with the vector encoding the scFV, and are infected with helper phage, to produce phage particles that display the antibody on their surface. Alternatively, to generate a complete antigen binding fragment (Fab), the heavy chain amplification product can be fused with a nucleic acid sequence encoding a phage coat protein, and the light chain amplification product can be cloned into a suitable vector. E. coli expressing the heavy chain fused to a phage coat protein are transformed with the vector encoding the light chain amplification product. The disulphide linkage between the light and heavy chains are established in the periplasm of E. coli. The result of this procedure is to produce an antibody library with up to $10^9$ clones. The size of the library can be increased to $10^{18}$ phages by later addition of the immune responses of additional immunized organisms that may be from the same or different hosts. Antibodies that recognize a specific antigen can be selected through panning. Briefly, an entire antibody library can be exposed to an immobilized antigen against which antibodies are desired. Phage that do not express an antibody that binds to the antigen are washed away. Phage that express the desired antibodies are immobilized on the antigen. These phage are then eluted and again amplified in E. coli. This process can be repeated to enrich the population of phage that express antibodies that specifically bind to the antigen. After phage are isolated that express an antibody that binds to an antigen, a vector containing the coding sequences for the antibody can be isolated from the phage particles and the coding sequences can be recloned into a suitable vector to produce an antibody in soluble form. Phage display methods to isolate antigens and antibodies are known in the art and have been described (Gram et al., Proc. Natl. Acad. Sci.,


One approach that can be used to make mouse-human chimeric antibodies is join the original variable region of the murine mAb, to constant regions of a human immunoglobulin. Chimeric antibodies and methods for their production are known in the art (Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Taniguchi et al., European patent Application 171496 (published Feb. 19, 1985); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Robinson et al., International Patent Publication #PCT/US86/02269 (published May 7, 1987); Liu et al., Proc. Natl. Acad. Sci. USA, 84:3439 (1987); Sun et al., Proc. Natl. Acad. Sci. USA, 84:214 (1987); Better et al., Science, 240:1041 (1988)). Generally, DNA segments encoding the H and L chain antigen-binding regions of the murine mAb can be cloned from the mAb-producing hybridoma cells, which can then be joined to DNA segments encoding C_H and C_L regions of a human immunoglobulin, respectively, to produce murine-human chimeric immunoglobulin-encoding genes. Humanized antibodies can also be made by

Antibody fragments that bind to gamma delta T-cells can also be administered to a human or animal to promote healing of a bone fracture.

5 Antibody fragments of the invention can be prepared by proteolytic hydrolysis of the antibody, or by expression in E. coli of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulphydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described (U.S. patents No. 4,036,945; 4,331,647; and 6,342,221, and references contained therein; Porter, *Biochem. J.*, 73:119 (1959); Edelman et al., *Methods in Enzymology*, Vol. 1, page 422 (Academic Press 1967); and Coligan et al. at sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise, an association of VH and VL chains. This association may be noncovalent (Inbar et al., *Proc. Nat'l Acad. Sci. USA*, 69:2659 (1972)). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (Sandhu, *Crit. Rev. Biotech.*, 12:437 (1992)). Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are
Another form of an antibody fragment is a peptide that forms a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (Larrick et al., Methods: A Companion to Methods in Enzymology, Vol. 2, page 106 (1991)).

An antibody of the invention may be coupled to a toxin. Such antibodies may be administered to an organism, such as a human, to promote healing of a bone injury by depleting gamma delta T-cells. For example, an antibody that binds to a gamma delta T-cell receptor may be coupled to a tetanus toxin and administered to a human or animal having a bone injury, disease, or defect. The toxin-coupled antibody will bind to the receptor on the gamma delta T-cell and will kill the cell.

Peptide aptamers to the gamma delta T-cell receptor can also be used to down-regulate expression of a gamma delta T-cell receptor, or to deplete gamma delta T-cells in a human or animal. Peptide aptamers are peptides that bind to a gamma delta T-cell receptor with affinities that are often comparable to those for monoclonal antibody-antigen complexes. In one example, aptamers can be isolated according to mRNA display through use of a DNA library that contains a promoter, a start codon, a nucleic acid sequence coding for random peptides, and a nucleic acid sequence that codes for a histidine tag. This library is transcribed using a suitable polymerase, such as T7 RNA polymerase, after which a puromycin-containing poly A linker is ligated onto the 3' end of the newly formed mRNAs. When these mRNAs are translated in vitro, the nascent peptides form covalent bonds to the puromycin of the linker to form an mRNA-peptide fusion molecule. The mRNA-peptide fusion molecules are then purified through use of Ni-NTA agarose and oligo-dT-cellulose. The mRNA portion of the fusion molecule is then reverse transcribed. The double-stranded DNA/RNA-peptide fusion molecules are then incubated with gamma delta T-
cells, or gamma delta T-cell receptors, and unbound fusion molecules are washed away. The bound fusion molecules are eluted from the immobilized gamma delta T-cells, or T-cell receptors, and are then amplified by PCR. This process may be repeated to select for aptamers having high affinity for gamma delta T-cell receptors. The sequence of the nucleic acid coding for the aptamers can then be determined and cloned into a suitable vector. Methods for the preparation of peptide aptamers have been described (Wilson et al., Proc. Natl. Acad. Sci., 98:3750 (2001)). These peptide aptamers can be conjugated to a toxin and used to deplete gamma delta T-cells.

Numerous toxins may be used to deplete gamma delta T-cells. Examples of toxins include, ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, pokeweed antiviral protein, a radioactive isotope, cisplatin, Bacillus anthracis protective antigen, and the like.

Toxins may be coupled to antibodies or peptide aptamers through routine methods. For example, an antibody may be conjugated to daunomycin according to the following method. Daunomycin (40 mg/ml) can be oxidized with 0.1 M sodium periodate (in PBS) at 20°C for 20 min in a dark room (final 1-ml volume). Immediately, 100 μl of 1 M glycerol can be added to the mixture to stop the reaction, and incubation proceeded at 20°C for 30 min. Supernatant obtained after centrifugation of the mixture can be used as an oxidized daunomycin solution. Antibody or peptide aptamer dissolved in 0.15 M potassium carbonate buffer (pH 9.5) is mixed with the oxidized daunomycin solution and incubated at 20°C for 2 h. After centrifugation, the supernatant (2 ml) containing daunomycin-conjugated antibody or peptide aptamer can be reduced by the addition of 0.6 mg of sodium borohydride and incubation at 4°C for 2 hours. The solution can be subjected to gel filtration (Bio-Gel P-100, BioRad, Hercules, CA) to separate daunomycin-bound antibody or peptide aptamer from free daunomycin.

Antagonists against cytokines produced by gamma delta T-cells

Antagonists to certain Th1 cytokines can also be administered to a human or animal having a bone fracture to promote healing of the bone fracture. According to the invention, certain Th1 cytokines that are released by γδ cells reside can play an antagonistic role to osteoprogenitor cells that may later lead to pathologic bone repair. Thus, agents that block or antagonize these types of
cytokines may be used to promote healing of bone fractures and other injuries. Examples of cytokines whose function can be locked or antagonized to promote bone healing include interleukin-1, interleukin-2, interleukin-6, interleukin-8, interleukin-12, tumor necrosis factor alpha, and interferon-γ.

The invention therefore contemplates antibodies that bind to cytokines secreted by gamma delta T-cells. These antibodies can be administered to a human or animal to promote bone healing by reducing or eliminating the bone healing inhibition caused by gamma delta T-cells. These antibodies may be administered systemically, or administered directly to the site of injury. These antibodies can be formulated for administration to bone tissues. Examples of such cytokines include interleukin-1, interleukin-2, interleukin-6, interleukin-8, interleukin-12, tumor necrosis factor alpha, and interferon gamma. Antibodies can be prepared to these cytokines according to methods known in the art and described above. Alternatively, antibodies are commercially available that bind to cytokines produced by gamma delta T-cells. For example, antibodies that bind to human interleukin-2, interleukin-6, and interferon gamma are commercially available (Anogen Inc., Mississauga, Ontario, Canada; Assay Designs Inc., Ann Arbor, MI; BD Biosciences Parmingen, San Diego, CA; Biodesign International, Saco, Maine; CalTag, Burlingame, CA). These antibodies can be obtained in a purified form, or isolated from hybridomas according to standard techniques. In addition, nucleic acid sequences can be isolated from hybridomas that encode these antibodies. These nucleic acid sequences can be used to create additional antibodies recombinantly; such as humanized antibodies, single-chain antibodies, antibody fragments, and the like.

Cytokines that inhibit gamma delta T-cells

Cytokines that inhibit the activity of gamma delta T-cells can be administered to a human or animal having a bone fracture to promote healing of the bone fracture. For example, interleukin-10 or transforming growth factor beta can be administered to a human or animal to inhibit gamma delta T-cells and thereby promote healing of a bone fracture (Rojas et al., Infect. Immun., 67:6461 (1999)).
II. Method to inhibit bone healing

The invention provides a method to inhibit bone healing in an organism, such as a human, having a bone fracture, disease, or defect comprising contacting the organism with an effective amount of a cytokine secreted by a gamma delta T-cell, or an agent that stimulates gamma delta T-cells. Such a method may be used to inhibit bone formation in patients with diseases where unwanted bone growth is debilitating.

Gamma delta T-cell activity can be stimulated with numerous agents. For example, an anti-CD3 antibody, vitronectin, CD 40 ligand, proteins along the gamma delta T-cell signaling pathway, such as Syk or FcεRIγ, (Meissner et al., J. Immunol., 170:356 (2003)), aminobisphonates (Das et al., Blood, 98:1616 (2001)), or a mycobacterial antigen can be used to stimulate gamma delta T-cells (Rojas et al., Infect. Immun., 67:6461 (1999)). A mycobacterial antigen is a product of a mycobacterium that activates a gamma delta T-cell. A mycobacterial antigen can be obtained from M. tuberculosis. Examples of such antigens include cell membrane components, proteins, peptides, oligosaccharides, and the like that activate gamma delta T-cells.

T-cell function is modulated by numerous proinflammatory cytokines which include, interleukin-1, interleukin-6, interleukin-7, interleukin-8, interleukin-12, and tumor necrosis factor alpha. Accordingly, these cytokines can be administered to a human or animal alone, or in combination, to stimulate gamma delta T-cell activity and inhibit bone fracture healing.

III. Pharmaceutical compositions

The invention provides pharmaceutical compositions that can be used to promote healing of a bone injury, such as a bone fracture. A composition of the invention can include an agent that inhibits gamma delta T-cells and a pharmaceutically acceptable carrier, such that administration of the pharmaceutical composition to a human or animal in need thereof promotes bone healing. A composition of the invention can include an agent that inhibits reduces or eliminates that effects of a cytokine produced by a gamma delta T-cell and a pharmaceutically acceptable carrier, such that administration of the pharmaceutical composition to a human or animal in need thereof promotes bone healing. Examples of agents that can be formulated as pharmaceutical
compositions of the invention include soluble gamma delta T-cell receptors, antibodies that bind to gamma delta T-cell receptors, antibodies that bind to cytokines produced by gamma delta T-cells, and the like.

The invention also provides pharmaceutical compositions that can be used to inhibit healing of a bone fracture. A composition of the invention can include an agent that stimulates gamma delta T-cells and a pharmaceutically acceptable carrier, such that administration of the pharmaceutical composition to a human or animal in need thereof inhibits bone healing. A composition of the invention can include one or more cytokines that are produced by a gamma delta T-cell, and a pharmaceutically acceptable carrier, such that administration of the pharmaceutical composition to a human or animal in need thereof inhibits bone healing. Examples of agents that can be formulated as pharmaceutical compositions of the invention include anti-CD3 antibodies, mycobacterial antigens, interleukin-1, interleukin-6, interleukin-8, interleukin-12, tumor necrosis factor alpha, and the like.

Pharmaceutically acceptable carriers include pharmaceutically acceptable excipients and/or delivery vehicles for administering a given agent (i.e., a soluble gamma delta T-cell receptor, cytokine, or antibody) to a human or animal. A pharmaceutically acceptable carrier can be used to deliver an agent useful to promote or inhibit healing of a bone fracture in a human or animal to a suitable in vivo or ex vivo site. Pharmaceutically acceptable carriers are capable of maintaining the agent in a form that, upon administration of the agent to a human or animal and/or at a target cell (if the procedure is ex vivo), the agent is capable of interacting with its target (i.e., a ligand for the gamma delta T-cell, a cytokine, or a gamma delta T-cell receptor). Such an interaction will reduce or prevent the activity of an endogenous gamma delta T-cell, a gamma delta T-cell ligand, or a cytokine produced by a gamma delta T-cell.

Pharmaceutically acceptable carriers include pharmaceutically acceptable excipients and/or delivery vehicles for administering a given agent (i.e., a cytokine, antibody, or mycobacterial antigen) to a human or animal. A pharmaceutically acceptable carrier can be used to deliver an agent useful to inhibit healing of a bone fracture in a human or animal to a suitable in vivo or ex vivo site. Pharmaceutically acceptable carriers are capable of maintaining the agent in a form that, upon administration of the agent to a human or animal
and/or at a target cell (if the procedure is ex vivo), the agent is capable of interacting with its target (i.e., a gamma delta T-cell receptor, a cytokine, or a gamma delta T-cell). Such an interaction will increase the activity of an endogenous gamma delta T-cell, or will stimulate other cell types that are activated by gamma delta T-cells.

Suitable excipients of the present invention include excipients or formularies that transport or help transport, but do not specifically target an agent to a cell. Examples of pharmaceutically acceptable excipients include, but are not limited to, water, phosphate buffered saline, Ringer's solution, dextrose solution, serum-containing solutions, Hank's solution, other aqueous physiologically balanced solutions, oils, esters, glycols and dry-powder inhalers. Aqueous carriers can contain suitable auxiliary substances required to approximate the physiological conditions of the recipient, for example, by enhancing chemical stability and isotonicity.

Suitable auxiliary substances include, for example, sodium acetate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, and other substances used to produce phosphate buffer, Tris buffer, and bicarbonate buffer. Auxiliary substances can also include preservatives, such as thimerosal, m-cresol, o-cresol, formalin and benzol alcohol. Compositions of the present invention can be sterilized by conventional methods and/or lyophilized.

One type of pharmaceutically acceptable carrier includes a controlled release formulation that is capable of slowly releasing an agent into a human or animal. A controlled release formulation can comprise an agent and other components of the pharmaceutical composition in a controlled release vehicle.

Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. A pharmaceutical composition can also be formulated for delivery by an inhaler device.

A pharmaceutical composition can include components in addition to an agent that promotes or inhibits bone fracture healing. Such components include, but are not limited to, cytokine antagonists (e.g., anti-cytokine antibodies, soluble cytokine receptors), cytokine receptor antagonists (e.g., anti-cytokine receptor antibodies), cytokines, anticholinergics, immunomodulating drugs,
leukotriene synthesis inhibitors, leukotriene receptor antagonists,
glucocorticosteroids, steroid chemical derivatives, anti-cyclooxygenase agents,
anti-cholinergic agents, beta-adrenergic agonists, methylxanthines, anti-
histamines, cromones, zyleuton, surfactants, anti-thromboxane reagents, anti-
serotonin reagents, ketotiphen, cytoxin, cyclosporin, methotrexate, antibiotics,
heparin, low molecular weight heparin, and mixtures thereof.

Acceptable protocols to administer an agent, such as a soluble gamma
delta T-cell receptor or antibody, including the route of administration and the
effective amount of the agent to be administered to a human or animal, can be
determined and accomplished by those skilled in the art. An agent can be
administered in vivo or ex vivo. Suitable in vivo routes of administration can
include, but are not limited to, intravenous administration, intraperitoneal
administration, intramuscular administration, intraarterial administration,
subcutaneous administration, transdermal delivery, intratracheal administration,
intraventricular administration, inhalation, intranasal, oral, bronchial, rectal,
topical, vaginal, urethral, pulmonary administration, impregnation of a catheter,
and direct injection into a tissue. Some preferred routes of administration
include, intravenous, intraperitoneal, subcutaneous, intradermal, intramuscular,
transdermal, inhaled, intranasal, rectal, vaginal, urethral, topical, oral, and
intraarticular. Combinations of routes of delivery can be used and in some
instances, may enhance the therapeutic effects of the pharmaceutical
composition. In some embodiments, the agents and therapeutic compositions of
the invention can be formulated for administration to bone.

Ex vivo refers to performing part of the administration step outside of a
human or animal, such as by removing cells from the human or animal,
contacting the cells in vitro with an antibody that binds to a gamma delta T cell
receptor, and returning the cells, or a subset thereof to the human or animal.

A suitable dose of a pharmaceutical composition to administer to a
human or animal to promote bone fracture healing is a dose that reduces or
eliminates the inhibition of bone fracture healing by endogenous gamma delta T-
cells when the composition is administered one or more times over a suitable
time period.

A suitable dose of a pharmaceutical composition to administer to a
human or animal to inhibit bone fracture healing is a dose that increases
inhibition of bone fracture healing by endogenous gamma delta T-cells when the
composition is administered one or more times over a suitable time period.

Agents and pharmaceutical compositions that promote or inhibit bone
healing can also be incorporated into, associated with, or impregnated within, a
bone-compatible matrix. A matrix can be a porous composite, solid or semi-
solid substance. The matrix can have pores or spaces that are sufficiently large
to allow cells to populate the matrix. The term matrix can include matrix-
forming materials, such as materials which can form matrices within a defect site
in cartilage or bone.

The matrix can be placed near to bone, or in contact with bone or
surrounding tissue. The matrix may become impregnated with an agent by
soaking the matrix in a solution containing the agent, or by preparing the matrix
in combination with an agent or pharmaceutical composition. Matrices of the
invention include are all those in which an agent or pharmaceutical composition
of the invention is adsorbed, absorbed, or otherwise maintained in contact with
the matrix.

Numerous types of bone-compatible matrices may be used with the
agents and pharmaceutical compositions of the invention. Such matrices have
all the features commonly associated with being "biocompatible", in that they
are in a form that does not produce an adverse, allergic or other unwanted
reaction when administered to an animal, such as a human, and they also suitable
for placing in contact with bone tissue. This latter requirement takes into
consideration factors such as the capacity of the matrix to provide a structure for
the developing bone and, preferably, its capacity to resorbed into the body after
the bone has been repaired.

The choice of matrix material will differ according to the particular
circumstances and the site of the bone that is to be treated. Matrices such as
those described in U.S. Pat. No. 5,270,300 may be employed. Physical and
chemical characteristics, such as biocompatibility, biodegradability, strength,
rigidity, interface properties and even cosmetic appearance may be considered in
choosing a matrix, as is well known to those of skill in the art. Matrices may
deliver an agent or pharmaceutical composition and also provide a surface for
new bone growth. For example, a matrix may act as an in situ scaffolding
through which cells may migrate.
Another aspect of the present invention is its use in connection with orthopaedic implants and interfaces and artificial joints. These include the implants themselves and functional parts of an implant, such as surgical screws, pins, and the like.

Non-biodegradable matrices may also be used, such as sintered hydroxyapatite, bioglass, aluminates, other bioceramic materials and metal materials, particularly titanium. A suitable ceramic delivery system is that described in U.S. Pat. No. 4,596,574. Bioceramics may be altered in composition, such as in calcium-aluminate-phosphate; and they may be processed to modify particular physical and chemical characteristics, such as pore size, particle size, particle shape, and biodegradability. Polymeric matrices may also be employed, including acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Pat. Nos. 4,526,909, and 4,563,489. Additional examples of polymeric matrices include acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Pat. Nos. 4,526,909, and 4,563,489, respectively. Examples of useful polymers are those of orthoesters, anhydrides, propylene-cofumarates, or a polymer of one or more \( \alpha \)-hydroxy carboxylic acid monomers, (e.g. \( \alpha \)-hydroxy acetic acid (glycolic acid) and/or \( \alpha \)-hydroxy propionic acid (lactic acid)).

Biodegradable matrices may also be used. A biodegradable matrix is generally defined as one that is capable of being resorbed into the body. Potential biodegradable matrices for use in connection with the agents and pharmaceutical compositions of the invention include biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyanhydrides, matrices of purified proteins, semi-purified extracellular matrix compositions, and the like. Matrices may also be prepared from tendon or dermal collagen, as may be obtained from a variety of commercial sources (Sigma and Collagen Corporation). Collagen matrices may also be prepared as described in U.S. Pat. Nos. 4,394,370 and 4,975,527, or obtained commercially (UltraFiber \textregistered, Norian Corp., Mountain View, Calif.).

Additional matrices may be prepared from combinations of materials, such as polylactic acid and hydroxyapatite, or collagen and tricalciumphosphate. Although sufficient sequestration and subsequent delivery of an agent or pharmaceutical composition is in no way a limitation of the present invention,
should it be desired, a porous matrix and agent or pharmaceutical composition combination may also be administered to the bone tissue site in combination with an autologous blood clot. The basis for this is that blood clots have previously been employed to increase sequestration of osteogenic proteins for use in bone treatment (U.S. Pat. No. 5,171,579).

Matrix-forming materials may require addition of a polymerizing agent to form a matrix, such as adding thrombin to a solution containing fibrinogen to form a fibrin matrix. Other matrix materials include collagen, combinations of collagen and fibrin, agarose, and gelatin. Calcium phosphate may be used alone or in combination with other matrix materials in treating defects in bones. (U.S. Patent No. 5,270,300).

IV. A method to screen for agents that promote or inhibit bone fracture healing or bone formation

The invention provides a method to identify agents that promote bone fracture healing or bone formation. Generally, the method involves contacting a gamma delta T-cell with a candidate agent, and determining if the candidate agent inhibits gamma delta T-cell activity.

The invention provides a method to identify agents that inhibit bone fracture healing. Generally, the method involves contacting a gamma delta T-cell with a candidate agent, and determining if the candidate agent stimulates gamma delta T-cell activity.

Gamma delta T-cell activities include cytokine production, gamma delta T-cell receptor expression, gamma delta T-cell expansion, as well as other activities generally associated with gamma delta T-cells.

Changes in the expression or activity of gamma delta T-cell receptors, and T-cells expressing such receptors, can be measured using any technique known to those of skill in the art for evaluating the presence and expression of a cell surface molecule, and/or the activity of a T-lymphocyte and particularly, a gamma delta T-lymphocyte. Such techniques include, but are not limited to, detecting the expression of specific receptors using protein or nucleic acid detection methods, measurement of changes in the numbers of cells, measurement of changes in T-lymphocyte biological function. For example, characteristics of T-cell receptor expression and T-cell activation can be
determined by a method including, but not limited to: measuring receptor expression (e.g., by flow cytometry, immunoassay, RNA assays); measuring cytokine production by the T-cell (e.g., by immunoassay or biological assay); measuring intracellular and/or extracellular calcium mobilization (e.g., by calcium mobilization assays); measuring T-cell proliferation (e.g., by proliferation assays such as radioisotope incorporation); measuring upregulation of cytokine receptors on the T-cell surface, including IL-2R (e.g., by flow cytometry, immunofluorescence assays, immunoblots, RNA assays); measuring upregulation of other receptors associated with T-cell activation on the T-cell surface (e.g., by flow cytometry, immunofluorescence assays, immunoblots, RNA assays); measuring reorganization of the cytoskeleton (e.g., by immunofluorescence assays, immunoprecipitation, immunoblots); measuring upregulation of expression and activity of signal transduction proteins associated with T-cell activation (e.g., by kinase assays, phosphorylation assays, immunoblots, RNA assays); and, measuring specific effects or functions of the T-cell (e.g., by proliferation assays). Methods for performing each of these measurements are routine in the art.

The invention will be further described by reference to the following detailed examples, which are given for illustration of the invention, and are not intended to be limiting thereof.

**EXAMPLE 1: Materials and Methods**

This Example illustrates some of the procedures that were used in the development of the invention.

*Animals and methods* Mice homozygous for the Tcrδ<sup>tm1mem</sup> delta chain targeted mutation and C57BL/6 background wild type controls were obtained from The Jackson Laboratories (Bar Harbor, ME). The Animal Care and Use Committee of the National Institutes of Health approved the animal experiments described. Animals were individually housed in a pathogen free environment of the animal care facilities of the National Institutes of Health. Maintenance and experimental procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health 1996).

*Animal model:* Mice homozygous for the Tcrδ<sup>tm1mem</sup> delta chain targeted mutation had a 4 kb deletion encompassing most of the delta chain constant
region sequences. This mutation results in a deficiency of gamma delta T-cell receptor expression in all adult lymphoid and epithelial organs. However, mice homozygous for the Tcrδtm1monm delta chain mutation have normal development of the alpha beta T-cell lineage. Itohara et al. 1993 Cell 72(3):337-48.

**Experimental groups:** A target number of sixty adult male γδ knockout mice and sixty control mice (23-28 g) were assigned to six experimental groups as follows:

1) Basic histology (n=8 controls and 10 knockouts); 2) Immunohistochemistry (n=4 controls and 5 knockouts); 3) Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) (n=8 in each group); 4) Enzyme-linked immunosorbent assays (ELISA) (n=8 in each group); 5) Flow cytometry (n=9 pre-operative animals each group, n=14 control and 12 knockout animals); and 6) Biomechanical and geometric properties (n=9 controls and 8 knockouts).

**Fracture Procedure:** With the animals under general anesthesia using isoflurane, fractures were created in the right mid tibia by three-point bending using a specially designed fracture apparatus (see Bonnarens F & Einhorn TA 1984 Production of a standard closed fracture in laboratory animal bone. J Orthop Res 2(1):97-101). Fractures were then instrumented with intramedullary pins. Post-operative radiographs confirmed the fracture and correct pin placement. Animals were not included for analysis in any experimental group if the animals had comminuted fractures, displaced pins, or signs or symptoms of infection at any time point. Unrestricted weight bearing was allowed immediately post-operative. The four phases of fracture healing was evaluated at the time points of 1, 5, 10, and 28 days in groups 1 through 5. For biomechanical testing and basic histomorphometrics tissue was processed at 2, 4, 6, and 8 weeks. All animals received radiographs at each of their respective days of analysis. Each radiograph was scored as 0, 1, or 2 based on quality of union with the presence, partial presence, or absence of the fracture line, respectively. Knockout animals were compared to controls with regard to radiographic evidence of healing.

**Histology:** Calluses were excised, fixed in phosphate-buffered 4% paraformaldehyde (pH 7.2) for 3 days at room temperature, and decalcified in 20% ethylenediaminetetraacetic acid at room temperature for 7 days. The tissue samples were then dehydrated through a graded ethanol series and embedded in
paraffin. Ten micron sections representative for the entire callus were obtained, and stained with Masson-Trichrome and hematoxylin and eosin stains.

Histological scoring was conducted in a blind fashion using a modified method of Bos et al, 1983 J Bone Joint Surg Am 65(1):89-96. Scores of 0 through 5 were assigned to two categories for quality of union and colonization of bone marrow, based on the maturity of the callus and bone marrow reconstitution, respectively. (Table 1)

<table>
<thead>
<tr>
<th>QUALITY OF UNION</th>
<th>COCOLORATION OF BONE MARROW</th>
</tr>
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<tbody>
<tr>
<td><strong>Histological Description</strong></td>
<td><strong>Score</strong></td>
</tr>
<tr>
<td>No Evidence</td>
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<td>Osseochondral</td>
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<td>Bony Union</td>
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</tr>
<tr>
<td>Remodeled</td>
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</tbody>
</table>

**Immunohistochemistry:** Calluses were excised, fixed, decalcified, and paraffin embedded as above. Three serial sections from both knockout and controls at each time point were used for immunostaining for the CD44 adhesion molecule, up-regulated by both lymphocytes and bone cells at sites of injury. See Kelleher et al. 1995 J Leukoc Biol 58(5):539-46; Yamazaki et al. 1999 J Bone Joint Surg Br 81(3):508-15. Controls alone were immunostained for the γδ TCR. The avidin-biotin-peroxidase complex method of Hsu et al (1981 J Histochem Cytochem 29(4):577-80) was used with a commercial kit from Zymed (San Francisco, CA). The sections were dewaxed, rehydrated, and treated with 3% hydrogen peroxide in 100% methanol for 20 minutes to block the endogenous peroxidase activity. In order to optimize antigen retrieval, sections were irradiated at 750 W in a microwave oven in BD Retrieval Solution. (BD Pharmigen, San Diego, CA) Primary monoclonal antibodies for CD44, (BD Bioscience), and γδ TCR (Caltag, Burlingame, CA)
were diluted at 1:25 for optimal staining in TRIS-buffered saline and incubated on sections overnight at 4°C. Hematoxylin was used as a counterstain. The specificity of the reactions was controlled using isotype antibodies in parallel sections. All control slides were negative and showed no background staining.

The sequential changes in the expression of CD44 and γδ TCR immunoreactivities in regard to percentage and types of cells stained at the four phases of fracture healing were scored using the method reported by Fujii et al. 1999 Histochem Cell Biol 112(2):131-8.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

(Messenger ribonucleic acid (m-RNA) expression of bone specific (bone sialoprotein), cartilage specific (collagen II), and bone matrix proteins (bone morphogenic protein-2 (BMP-2)) were assessed to represent the early predominant message of each stage of repair. Calluses were harvested with 3 mm of bone proximal and distal to the fracture site, snap-frozen in liquid nitrogen and stored at -80°C. Two specimens each from control and knockout were pooled for each time point. Total RNA was isolated using the single step method of Chomczynski & Sacchi, 1987 Anal Biochem 162(1):156-9. Frozen tissue samples were crushed under liquid nitrogen, and homogenized in Trizol (Gibco BRL, Gaithersburg, MD). A soluble fraction was obtained by centrifugation (12,000 U/min for 5 minutes) and then purified with isopropanol and repeated ethanol precipitations. The quantity and purity of RNA obtained was determined by spectrophotometry at 260 and 280 nm. Semi-quantitative PCR was performed for 30 cycles on single-strand complementary DNA prepared from RNA (1 μg) using a SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen Life Technologies Carlsbad, CA). The gene specific nucleotide sequences for primers employed (IDT Coralville, IA) and the expected sizes of the PCR product are listed in Table 2.

Table 2

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<tr>
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</table>

Amplification was performed in a thermocycler (Minicycler, MJ Research) under the following conditions: denaturation at 94°C for 30 seconds; primer annealing at 53°C for 45 seconds; and extension at 72°C for 60 seconds. To control for the integrity of the various RNA preparations, the expression of glyceraldehyde phosphate dehydrogenase (GAPDH) was also assessed. The PCR products were electrophoresed onto a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. Photographs of the stained gels were analyzed with a Foto/Analyst Image Analysis System (Fotodyne, Hartland, WI).

**Enzyme-linked immunosorbent assays (ELISA):** For ELISA, calluses were harvested and homogenized in the same manner as outlined above. Two specimens each from control and knockout were pooled for each time point. The homogenates were centrifuged at 15,000 rpm for 15 minutes at 4°C to remove the debris. Aliquots (100 μl) taken from the soluble fraction were analyzed for the presence of cytokines, INF-γ, IL-2, and IL-6, by double antibody sandwich method with commercially available ELISA kits (Quantikine M; R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. All samples and standards were assayed in triplicate. The plates were read on a
Perkin Elmer Victor V microplate reader at 405 nm. All data related the total protein concentration according to the proportional luminance. Cytokine concentrations (pg/ml) were presented as the mean ± standard error.

Flow Cytometry: In order to analyze the systemic response distant from the site of injury, flow cytometry analysis of total lymphocytes, subset γδ T-cells, and cells expressing the CD44 marker were performed on peripheral blood samples. At euthanasia peripheral blood mononuclear cells were obtained by intracardiac puncture with the animals under anesthesia. Lymphocytes were separated using the Ficoll technique. Cell suspensions containing 1 x 10^5 cells were incubated for 30 minutes at room temperature with either conjugated anti-CD44-phycocerythrin (PE) or anti-γδ TCR-trichrome diluted 1:100 in PBS with 1% bovine serum albumin and 0.1% sodium azide. Cells were washed to remove antibody, pelleted, and resuspended in 400 μl of PBS and 1% paraformaldehyde. Non-specific mouse IgG was used as a negative control.

The flow cytometry (Becton Dickinson) was set at a wavelength appropriate for the fluorochromes of interest and with compensation for multiple stains. Gates were set to exclude debris. Total lymphocyte populations were defined using scatter characteristics and gating in the forward scatter/side scatter (FSC/SSC) dot plots. Results were reported as percent of cells positive for each respective fluorochrome with gating at the 98th percentile of isotype stained cells. Data from up to 100,000 cells were acquired and analyzed using CELLQuest software.

Biomechanical and Geometric Analysis: Bilateral fractured and unfractured tibias were harvested and tested fresh, with the intramedullary pin extracted. Prior to biomechanical testing, the maximum vertical (Dv) and horizontal (Dh) diameter of the gross callus, and the mid-diaphysis of the unfractured tibia were measured by sliding caliper (0.05-mm resolution). The transverse diameters of the marrow space were measured from anteroposterior radiographs using the diameter of the nail as a reference. This allowed geometric calculations of the quantity of callus, expressed as the callus area (Xa), and the area moment of inertia (I) for the direction of load, assuming the cross section to be elliptical with a centrally located hole. The following formulas (Eshbach et al. Handbook of Engineering Fundamentals p. 450 (Wiley, New York, ed. 3, 1975)) were used:
1.) $X_s$ (mm$^2$/g) = $\pi(D_c/2)(D_h/2)$ (The average callus area was adjusted for the weight (gram) of each animal.)

2.) $I$ (mm$^4$) = $(\pi/64)D_hD_c^3$

The biomechanical properties of the healing fractures were analyzed by a destructive three-point bending procedure on a materials testing machine (Instron Model). The callus was positioned on two grooved roller supports of a holding apparatus, spanning a total distance of 6.5 mm with the repair site centered. The machine was set at a maximum local value of 50 N, a chart speed of 10 sec/cm, and a crosshead speed of 2 mm/minute. A load displacement curve was produced as loading was continued until the specimens exhibited evidence of failure (F). (Figure 1) From this curve the following structural or extrinsic parameters were obtained: maximum load ($P_{max}$) (N), deflection at maximum load ($D_{max}$)(mm) and maximum stiffness (N/mm), defined as the slope ($K = \Delta P/\Delta D$). With the slope ($K$) and the test span ($L$=6.5 mm) the rigidity was calculated as follows (see Dumbleton et al. in Clinical Biomechanics, pp.359-400 (Churchill Livingston, New York, 1981)):

3.) $R$ (N/mm$^2$) = $(K)L^3/48$

In addition to structural parameters, the intrinsic or material properties, elastic modulus ($E$) and stress ($\sigma$), were calculated using standard engineering formula derived from simple beam theory (id.). Both material properties, as a function of applied load and moment of inertia, are dictated by callus geometry and therefore reflect properties at the periosteal surface.

4.) $E$ (N/mm$^2$) = $(L^3)(K)/48(I)$

5.) $\sigma$ (N/mm$^2$) = $(ML)(L)(C)/4(I)$ where C is $\frac{1}{2}D_h$

Another material property calculated was the healing bone’s ability to absorb energy during loading. This is defined as the area under the curve, either to maximum load or failure, and is a function of both load and deflection. (Figure 1)

Statistics: All data are reported as mean and standard error (error bars: ±SD). Data points were distributed around the mean in a normal fashion and did not require transformation. Two samples were compared using student’s t-test. Statistical analysis on biomechanical data was tested for normal distribution and
homogeneity of variances (F-test) using the GLM procedure of SAS. Differences were judged as statistically significant when the p value was less than 0.05.

**EXAMPLE 2: γδ T-cells Inhibit Bone Healing**

This Example shows that γδ T-cells, as part of an innate immunological response to injury, are recruited to the local site of fracture repair and illustrates the consequence thereof. As described herein, inhibition of γδ T-cell receptor activity lead to an up regulation of CD 44 adhesion molecules in matrix producing osteoblasts, and a decrease in osteoprogenitor antiproliferative cytokines, IL-6 and interferon gamma. Inhibition of γδ T-cell receptor activity improved stability at the repair site and was correlated with overall superior biomechanical strength.

**γδ T-cells respond to fracture injury:** The presence of γδ T-cells at the local fracture repair site of control animals was first defined by immunolocalization to permit more meaningful conclusions to be made about γδ knockout animals. Representative micrographs for the four phases of fracture healing are shown in Figure 2. Higher magnifications were used to depict the type of cells that were expressing the γδ T-cell receptor (TCR), while lower magnifications give an overview of the tissue stage. At the time of fracture, γδ TCR protein was localized to lymphocytes within the hematoma. In the ensuing inflammatory phase, when the hematoma was replaced with an accumulation of monocytes, macrophages, and immature mesenchymal cells, the γδ TCR was found distributed on both inflammatory and mesenchymal cells.

At Day 5, as fracture callus began to form, more γδ TCR+ cells are found with the morphological appearance of fibroblastic mesenchymal stem cells. During both the chondrogenic and osteogenic phases, the γδ TCR was detected on osteoblasts lining trabecular bone. On Day 28, as the bony callus is remodeled, the γδ TCR was not found, with absence of staining noted specifically with mature osteocytes within lacunae. Moreover, staining was not observed on proliferating, hypertrophic, or mature chondrocytes at any time point. A summary of the immunohistochemical analyses for the relative levels
of cell marker expression for the γδ TCR and CD44 seen at the different phases is presented in Table 3.

When γδ T-cells respond to the local injury site their presence is also noted peripherally by flow cytometry (Figure 3). Peripheral blood γδ TCR expression on lymphocytes was determined at baseline and during the four phases of fracture repair. The percentage of cells expressing the γδ TCR receptors decreased immediately after injury, but increased significantly (p<0.05) above baseline at Day 10. This maximum percentage of positive γδ TCR+ cells noted with flow cytometry agreed with the maximum level of immunohistochemical staining for the same day. As the level of γδ cells increase at the local injury site, their total number was increased peripherally as well.

**Knockout animals exhibit early differences in cartilage and bone formation:** Although typical histological features were seen in a sequential fashion in both groups, knockout animals have better quality of union with more osseous and chondral elements and mature bone marrow at Day 5 (Figure 4A and B). This is evidenced as well at the molecular level by increased m-RNA expression of chondrogenic Collagen II, osteogenic bone sialoprotein (BSP), and matrix protein BMP-2 (Figure 4C). All differences are lost as the callus matured. Further studies (data not shown) confirmed previous reports that characteristic messenger expression of the three cell and matrix specific markers were observed during all phases of fracture healing and correlated with the histological appearance. Hirakawa et al. 1994 J Bone Miner Res 9(10):1551-57; Jingsushi et al. 1992 J Bone Miner Res 7(9):1045-55. By radiographic evaluation, knockout animals did not demonstrate improved healing over controls until Day 28 (Figure 5).

**Knockout animals produce less inflammatory cytokines at the regenerative site:** Both control and knockout groups showed a remarkable reduction in the absolute number of total peripheral lymphocytes, as seen by flow cytometry, in the first day following injury (Figure 6). This result is similar to the T-cell lymphopenia seen in other models of injury. Menges et al. 1999 Crit Care Med 27(4):733-40. Compared to controls, total peripheral lymphocytes in knockout animals remain depressed until remodeling begins.
With the absence of γδ T-cells, there was a significant decrease (p<0.05) in the production of IL-2 at the inflammatory phase (Day 1) and interferon-γ and IL-6 at the reparative phase (Days 5 and 10) in fractured bones as noted by ELISA (Figure 7A,B, and C).

**Knockout animals up-regulate CD 44+ adhesion molecules on osteoblasts:**

Several cell types expressed CD44 throughout the healing process as detected by immuno-localization (Table 2). Similar results have been reported previously by Yamazaki et al. (1999 J Bone Joint Surg Br 81(3):508-15).

However, important spatial and temporal differences were noted between γδ knockout and control animals. On days 1 and 5, only knockout animals demonstrated CD44 expression within the hematoma and on osteocytes, respectively. Moreover, during the reparative phases, only knockout animals had CD44+ adhesion molecules present on osteoblasts (Figure 8). Such expression in osteoblasts (matrix-producing cells) is previously unreported.

By flow cytometry, cells gated for total lymphocytes in both knockout and control animals (Figure 9) and γδ TCR+ lymphocytes in controls alone (Figure 10) were analyzed for CD44 expression. A significant decrease was noted on Day 5 between CD44 expressing peripheral lymphocytes of knockout animals compared with controls. Overall, however, by Days 10 and 28 this difference was lost. The pattern of CD44 expression in total lymphocytes exhibited a biphasic distribution with a population of CD44hi cells noted. Figure 11 compares the distribution of CD44hi cells between controls and knockouts. As shown in Figure 10, lymphocytes expressing both the γδ TCR and CD44 surface markers follow the same pattern as total γδ T-cells, but with a decreased percentage of positive cells from day 1 through day 28. Because stress disturbs normal cell trafficking, it is postulated that such decreases represent depletion of circulating lymphocytes expressing surface adhesion molecules and suggest a homing mechanism that redistributes cells from blood to sequestration at sites of inflammation. Hamzaoui et al. 1994 J Rheumatol 21(12):2301-6; Aguilar et al. 1998 T cells. J Trauma 45(1):14-18; Matsushima et al. 2004 Shock 22(1):11-15. Biphasic lymphocytes expressing CD44 of knockout animals, overall, demonstrate an increased “adhesiveness” when compared to controls.
Biomechanical properties are superior in the absence of γδ T-cells:

Both structural (rigidity) and material (elastic modulus) strength variables were different between γδ knockout and control mice (Figure 12). The average maximum rigidity of the healing fractures in knockout increased to 241, 57, and 17% of the maximum rigidity sustained by controls at 4, 6, and 8 weeks, respectively, while elastic modulus increased to 785, 55, and 82%, respectively, compared with the corresponding values for controls. For all weeks, γδ knockout animals reached statistically significant higher values of maximum load (p=0.0002), stiffness (p=0.05), and elastic modulus (p=0.05) before failure compared with controls. Gamma delta knockout animals had comparatively higher values of maximum stress than controls, but were not statistically significant (p=0.09). Comparison of γδ knockout animals at 8 weeks showed essentially no significant differences between controls with respect to maximum load (p=0.27), rigidity (p=0.46), and elastic modulus (p=0.45).

This overall increase in strength was also reflected in the largest amount of energy to failure for all weeks when γδ knockout animals where compared with controls (p=0.03). In the 4-week knockout group, energy absorbed to failure was 47% greater than that of controls. However, at 6 and 8 weeks the gap narrowed to 10 and 6% respectively, so that at 8 weeks there was no statistically significant difference between the ability of γδ knockout callus to absorb energy than that of control callus (p=0.76) (Figure 13).

Tibia fractures in mice are usually considered healed when strength has reached values of contralateral intact bone, thus acting as an internal control. At 4 weeks, the fractured tibias of knockout animals had obtained 85 and 42% of the rigidity and elastic modulus of the corresponding values of intact bones, whereas the rigidity and elastic modulus of control animals had only reached 28 and 6% of intact bone. Structurally, with respect to stiffness and rigidity, γδ knockout callus more closely approximated values of intact bone for all weeks (p=0.07), than controls that demonstrated statistically greater differences between fractured and intact bone (p=<0.0001). Data obtained for both the healing right tibia and the contralateral intact bone are shown in Tables 4, 5, and 6.
Table 4: Biomechanical Structural Strength Variables in γδ Knock-Out and Control Mice

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<tr>
<th>EXPERIMENTAL GROUP</th>
<th>Week s</th>
<th>n</th>
<th>Max Load Mean (N)</th>
<th>Max Load SD (N)</th>
<th>Stiffness Mean (N/mm)</th>
<th>Stiffness SD (N/mm)</th>
<th>Rigidity Mean (N/mm²)</th>
<th>Rigidity SD (N/mm²)</th>
<th>Deflection Max Load Mean (mm)</th>
<th>Deflection Max Load SD (mm)</th>
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ANOVA (knock-out fracture versus intact) P=0.004 P=0.07 P=0.07 P=0.17

ANOVA (control versus knock-out fracture) P=0.0002 P=0.05 P=0.07 P=0.47
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<th>ELASTIC MODULUS (N/mm²)</th>
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<td>ANOVA (control fracture versus intact)</td>
<td></td>
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<td>10.953</td>
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Table 6: Energy Absorption in γδ Knock-Out and Control Mice

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<th>n</th>
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<th>AREA A+B TO FAILURE (N/mm)</th>
<th>ANOVA (control fracture versus intact)</th>
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</table>

| Knock-Out Fracture   | 2    | 3  | 1.12 .516                | 2.58 .771                 |                                       |
|                      | 4    | 3  | 8.78 3.402               | 15.85 6.787               |                                       |
|                      | 6    | 3  | 4.96 2.402               | 10.00 3.347               |                                       |
|                      | 8    | 2  | 10.51 3.840              | 15.11 6.293               |                                       |

| Knock-Out Intact     | 2    | 2  | 4.69 2.082               | 11.77 1.478               |                                       |
|                      | 4    | 3  | 6.29 2.625               | 11.16 3.040               |                                       |
|                      | 6    | 3  | 4.88 1.060               | 10.53 5.537               |                                       |
|                      | 8    | 2  | 3.54 0.559               | N/A N/A                   |                                       |

ANOVA (knock-out fracture versus intact)  P=0.01  P=0.07

ANOVA (control versus knock-out fracture)  P=0.01  P=0.03

This work demonstrates, for the first time, the expression of γδ T-cells responding to skeletal damage, and illustrates that γδ cells and/or their attendant cytokines can play a detrimental role in fracture healing. When γδ T-cells are not present, overall superior biomechanical strength and stability, as a final outcome, is seen in knockout animals compared to that of controls. Histology, immuno-localization and molecular analysis corroborates these findings with quantitative increases in osseous and chondral elements, temporal augmentation
in the messenger expression of Collagen II, BSP, and BMP-2, increased adhesiveness in lymphocytes responding to injury, as well as CD44 expression in matrix producing osteoblast, providing an explanation for the significant biomechanical stability noted.
References


All patents and publications referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced patent or publication is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such cited patents or publications.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality (for example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.
The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.
WHAT IS CLAIMED:

1. A pharmaceutical composition that promotes healing of a bone injury, bone disease, or bone defect in an organism comprising an agent that inhibits gamma delta T-cells and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the composition is formulated for administration to bone.

3. The pharmaceutical composition according to claim 1, wherein the pharmaceutical composition further comprises a biodegradable or non-biodegradable matrix.

4. The pharmaceutical composition according to claim 1, wherein the organism is a mammal or an avian organism.

5. The pharmaceutical composition according to claim 1, wherein the mammal is a human.

6. The pharmaceutical composition according to claim 1, wherein the bone injury is a bone fracture.

7. The pharmaceutical composition according to claim 1, wherein the agent is a soluble gamma delta T-cell receptor, an antibody, a cytokine, or a peptide aptamer.

8. The pharmaceutical composition according to claim 7, wherein the peptide aptamer binds to a gamma delta T-cell receptor, or a gamma delta T-cell related proinflammatory cytokine.

9. The pharmaceutical composition according to claim 7, wherein the cytokine is interleukin-10 or transforming growth factor beta.
10. The pharmaceutical composition according to claim 7, wherein the antibody binds to gamma delta T-cell receptor, or a gamma delta T-cell related proinflammatory cytokine.

11. The pharmaceutical composition according to claim 10, wherein the gamma delta T-cell related proinflammatory cytokine is interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor, or interferon gamma.

12. The pharmaceutical composition according to claim 1, wherein the agent that inhibits gamma delta T-cells inhibits gamma delta T-cell growth, expression of gamma delta T-cell receptor, or recognition of stressed cells.

13. A pharmaceutical composition that inhibits healing of a bone injury in an organism comprising an agent that inhibits gamma delta T-cells and a pharmaceutically acceptable carrier.

14. The pharmaceutical composition of claim 13, wherein the composition is formulated for administration to bone.

15. The pharmaceutical composition according to claim 13, wherein the pharmaceutical composition is associated with a biodegradable or non-biodegradable matrix.

16. The pharmaceutical composition according to claim 13, wherein the organism is a mammal or an avian.

17. The pharmaceutical composition according to claim 16, wherein the mammal is a human.

18. The pharmaceutical composition according to claim 13, wherein the bone injury is a bone fracture.
19. The pharmaceutical composition according to claim 13, wherein the agent is an anti-CD3 antibody, an \textit{M. tuberculosis} antigen, an aminobisphosphonate antigen, or an antibody that binds to a cytokine that inhibits gamma delta T-cells.

20. The pharmaceutical composition according to claim 19, wherein the cytokine is interleukin-10 or transforming growth factor beta.

21. A method to promote bone healing comprising contacting an organism having a bone injury, bone disease, or bone defect with an effective amount of an agent that inhibits gamma delta T-cell activity.

22. The method according to claim 21, wherein the organism is a mammal or an avian organism.

23. The method according to claim 21, wherein the mammal is a human.

24. The method according to claim 21, wherein the bone injury is a bone fracture.

25. The method according to claim 21, wherein the gamma delta T-cell activity is T-cell expansion, proliferation, apoptosis, secretion of a cytokine, or expression of a gamma delta T-cell receptor.

26. The method according to claim 21, wherein the agent is a soluble gamma delta T-cell receptor.

27. The method according to claim 21, wherein the agent is a cytokine.

28. The method according to claim 27, wherein the cytokine is interleukin-10 or transforming growth factor beta.

29. The method according to claim 21, wherein the organism is contacted at a site of the bone injury, disease, or defect.
30. The method according to claim 21, wherein the agent is an antibody that binds to the gamma delta T-cell.

31. The method according to claim 30, wherein the antibody binds to a gamma delta T-cell receptor on the gamma delta T-cell.

32. The method according to claim 30, wherein the antibody is conjugated to a toxin.

33. The method according to claim 32, wherein the toxin is ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, pokeweed antiviral protein, a radioactive isotope, cisplatin, or Bacillus anthracis protective antigen.

34. The method according to claim 21, wherein the agent is a peptide aptamer that binds to the gamma delta T-cell.

35. The method according to claim 34, wherein the peptide aptamer binds to a gamma delta T-cell receptor.

36. The method according to claim 34, wherein the peptide aptamer is conjugated to a toxin.

37. The method according to claim 36, wherein the toxin is ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, pokeweed antiviral protein, a radioactive isotope, cisplatin, or Bacillus anthracis protective antigen.

38. A method to promote bone healing comprising contacting an organism having a bone injury, bone disease, or bone defect with an effective amount of an agent that at least partially inhibits a gamma delta T-cell related proinflammatory cytokine.
39. The method according to claim 38, wherein the organism is a mammal or an avian.

40. The method according to claim 39, wherein the mammal is a human.

41. The method according to claim 38, wherein the bone injury is a bone fracture.

42. The method according to claim 38, wherein the gamma delta T-cell related proinflammatory cytokine is interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor, or interferon gamma.

43. The method according to claim 39, wherein the mammal is contacted at a site of the bone injury, disease, or defect.

44. The method according to claim 38, wherein the agent is an antibody that binds to the gamma delta T-cell related proinflammatory cytokine.

45. The method according to claim 38, wherein the agent is a peptide aptamer that binds to the gamma delta T-cell related proinflammatory cytokine.

46. A method to promote bone healing comprising contacting a mammal having a bone injury with an effective amount of a daunomycin-conjugated antibody that depletes gamma delta T-cells in the mammal.

47. A method to inhibit bone healing comprising contacting an organism having a bone injury, bone disease, or bone defect with an effective amount of an agent that stimulates gamma delta T-cells.

48. The method according to claim 47, wherein the organism is a mammal or an avian.

49. The method according to claim 48, wherein the mammal is a human.
50. The method according to claim 47, wherein the bone injury is a bone fracture.

51. The method according to claim 47, wherein the agent is an anti-CD3 antibody, an anti-CD 40 ligand antibody, or an anti-FCe RIy antibody.

52. The method according to claim 47, wherein the agent is a *M. tuberculosis* antigen or an aminobisphosphonate antigen.

53. The method according to claim 47, wherein the agent is an antibody or peptide aptamer that binds to a cytokine that inhibits gamma delta T-cells.

54. The method according to claim 53, wherein the cytokine is interleukin-10 or transforming growth factor beta.

55. A method to screen for an agent that alters gamma delta T-cell activity comprising:

   (a) contacting gamma delta T-cells with a candidate agent, and

   (b) determining if the candidate agent at least partially promotes or inhibits gamma delta T-cell activity.

56. The method according to claim 55, wherein the gamma delta T-cell activity is expression of a gamma delta T-cell receptor; cytokine production; intracellular calcium mobilization; extracellular calcium mobilization; T-cell proliferation; T-cell apoptosis, upregulation of a cytokine receptor; cytoskeletal reorganization; upregulation of signal transduction proteins; expression of signal transduction proteins; or activity of signal transduction proteins, or any combination thereof.
57. The method according to claim 56, wherein the cytokine is interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor, interferon gamma, or any combination thereof.
FIG. 1

\[ K = \frac{\Delta P}{\Delta D} \]

LOAD (N)

\[ P_{\text{max}} \]

\[ \Delta D \]

\[ \Delta P \]

\[ D_{\text{max}} (\text{mm}) \]

DEFLECTION

F
FIG. 12

FIG. 13
SEQUENCE LISTING

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