PLATFORM TECHNOLOGIES FOR SPONTANEOUSLY OCCURRING DISEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent applications 61/178,391, filed on May 14, 2009, and 61/186,342, filed on June 11, 2009, the disclosures of both provisional applications are herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The endeavor to improve human lives includes the discovery of new biological pathways and mechanisms of action as well as new treatment and diagnostic modalities. The discovery of new drugs, compounds, methods, or the combinations of any of the foregoing, for combating various diseases, such as cancer, is difficult due to regulatory mandates as well as time and cost considerations. A comprehensive study of multiple treatments is very hard to achieve in human clinical trials for the same reasons. These reasons act as real life barriers that impede the efforts of companies, non-profit organizations, and individuals to save human lives and/or improve living conditions of humans who are afflicted with various diseases. What is needed is an improved system for studying various diseases such that a combination of factors can be investigated to determine the most optimal biological and/or physiological response and outcome. Such system can be utilized to translate the information to generate new or improved drugs, compounds and treatment protocols to provide the maximally efficient use of medical and scientific efforts to help individuals with various diseases, such as spontaneously occurring diseases that involve host-induced responses (e.g., diabetes, cancer, autoimmune, neurological, allergic diseases).

[0003] Spontaneously occurring diseases, such as diabetes, have been observed in companion animals, such as dogs and cats (Hoenig M, *Mol. Cell. Endocrinol.* 197: 221-229 (2002)). For example, Davison et al. describes studies performed on autoantibodies to GAD65 and IA-3 in spontaneously occurring diabetes mellitus (Davison LJ et al., *Veterinary Immunology and Immunopathology*, 126: 83-90 (2008)). Hoenig et al. described a qualitative assay for beta cell antibodies in dogs with diabetes in *Veterinary Immunology and Immunopathology*, 32: 195-203 (1992)). Other naturally occurring diseases in dogs have been described in various references, e.g., Tsai et al., *Mamm. Genome*, 18:444-451 (2007).

[0004] In addition to diabetes, other spontaneously occurring diseases have been observed, such as cancer and autoimmune disease. Paoloni et al. describe the integration of the study of dogs with naturally occurring cancer with the study of human cancer biology to identify cancer-associated genes, study environmental risk factors, understand tumor biology and progression and evaluate and develop novel cancer therapeutics. (*Nature*, 8: 147-156 (2008)). The Canine Comparative Oncology and Genomics Consortium (CCOGC) is the result of many collaborative efforts to use the dog as a model of naturally occurring cancer for investigating cancer research in efforts to better both humans and dogs. *Nature Biotechnology* 24(9): 1065-1066 (2006). Examples of cancers that dogs naturally develop include: non-Hodgkin lymphoma, osteosarcoma, melanoma, prostate carcinoma, lung carcinoma, head and neck carcinoma, mammary carcinoma, and soft-tissue carcinoma. *Ibid.* Trials in pet dogs have been reported to help better define the safety and activity of new anticancer agents, assist in the identification of relevant biomarkers associated with the response or exposure to these anticancer drugs, and may allow rational development of combination strategies to improve the success of these new drugs in human clinical trials. *Ibid.* Candolfi et al describe the use of adenoviral-mediated gene transfer into dogs that spontaneously develop glioblastoma mutliforme (GBM) (Candolfi M et al, *Neurosurgery* 60: 167-178 (2007)). Paolini et al. reported that the Comparative Oncology Trials Consortium (COTC) evaluated a targeted AAV-phage vector delivering tumor necrosis factor (RGD-A-TNF) to αV integrins on tumor endothelium. *PLoS ONE* 4(3): e4972 (2009).

[0005] The invention described herein provides platform technologies for studying spontaneously occurring diseases that can be translated into therapeutic treatments and diagnostic methods.

[0006] All references cited herein, including patents, patent applications and publications, are hereby incorporated by reference in their entirety.

BRIEF SUMMARY

[0007] The disclosure provides for platform technologies for investigating biological pathways, the effects (e.g., synergistic effects) of various combination of agents that affect biological and/or physiological pathways, underlying mechanisms of action, biological participants in complex physiological conditions and other parameters that can be useful for development of agents for treatment, diagnosis, or prophylaxis of various physiological

conditions and/or diseases. Such complex physiological conditions can include, but are not limited to, cancer, autoimmune disease, allergies, hypersensitivity, neurological diseases, hereditary genetic disorders, and infectious diseases.

[0008] Accordingly, in one aspect, there is provided a method for identifying a combination of anti-cancer agents with synergistic effects comprising: (1) administering two or more anti-cancer agents to a companion animal with a spontaneously occurring cancer; (2) monitoring the companion animal for a biological and/or physiological effect; and (3), identifying a combination of anti-cancer agents with synergistic effects when the biological and/or physiological effects are synergistic, wherein the anti-cancer agents comprise bisphosphonates and cationic CpG.

[0008a] The disclosure provides for methods for identifying a combination of anti-cancer agents with synergistic effects comprising: (1) administering two or more anti-cancer agents to a companion animal with a spontaneously occurring cancer; (2) monitoring the companion animal for a biological and/or physiological effect; and (3), identifying a combination of anti-cancer agents with synergistic effects when the biological and/or physiological effects are synergistic. In one embodiment, the anti-cancer agent is selected from the group consisting of: bisphosphonates, platinum-based chemotherapeutics, inhibitors of the protein phospholipase D, alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, podophyllotoxins, antibodies, tyrosine kinase inhibitors, hormone treatments, soluble receptors, and antineoplastics. In another embodiment, the agents are clodronate and cationic CpG.

[0009] In another embodiment, the disclosure provides for methods for identifying a treatment modality for treatment in humans comprising testing a combination of compositions in a companion animal with a spontaneously occurring disease and identifying the combination that has a higher probability of success in humans by comparing the results of the testing in the companion animal with a spontaneously occurring disease to the results of the testing in an animal without a spontaneously occurring disease.

[0010] In another embodiment, the disclosure provides for methods of identifying an autoantigen associated an autoimmune disease comprising: (a) determining one more antigens in a companion animal with a spontaneously occurring autoimmune disease; (b) obtaining an antigen profile of the disease in the companion animal; (c) comparing the profile to a control companion animal that does not have the spontaneously occurring disease; and (d) identifying an autoantigen associated with autoimmune disease.

[0011] In another embodiment, the disclosure provides for methods of targeting multiple antigens associated with or suspected of being associated with cancer in a human comprising: (a) administering one or more agents that is suspected of having anti-cancer effects to a companion animal with a spontaneously occurring cancer; (b) monitoring a biological or physiological effect of the agent in the companion animal; (c) identifying one or more

antigens in the companion animal for which the agent had a biological or physiological effect and (d) administering the same agent to the human if the agent has an anti-cancer effect in the companion animal.

[0012] In another embodiment, the disclosure provides for methods of targeting multiple antigens associated with or suspected of being associated with an infectious disease in a human comprising: (a) administering one or more agents that is suspected of having effects against the infectious disease to a companion animal with a spontaneously occurring infectious disease; (b) monitoring a biological or physiological effect of the agent in the companion animal; (c) identifying one or more antigens in the companion animal for which the agent had a biological or physiological effect and (d) administering the same agent to the human if the agent has a beneficial effect in the companion animal. In one embodiment, the infectious disease is selected from the group consisting of influenza, septicemia (e.g., *Klebsiella pneumoniae* septicemia), bacterial infections (e.g., *Staphylococcus aureus*, other Staph infections, E. coli and enterococci), Pseudomonas aeruginosa, Leishmania infantum, Brucellosis, Coccidiosis, and *Salmonella enterica* Serovar Typhimurium.

[0013] In any of the aspects or embodiments, the companion animal is a dog. The dog can be a purebred dog or a mongrel dog. The dog can have a homogeneous genetic background or a heterogeneous genetic background.

[0014] In any of the aspects or embodiments, the companion animal is a cat. The cat can be a purebred or a mongrel. The cat can have a homogeneous genetic background or a heterogeneous genetic background.

[0015] Accordingly, in another embodiment, the disclosure provides a companion animal model system for identifying a treatment modality for treatment in humans comprising a combination of compositions that have a higher probability of success for identifying the treatment modality than a standard model. In one embodiment, the combination of compositions comprises two or more antigens. In another embodiment, the combination of compositions comprises at least 1 antigen and an adjuvant. In another embodiment, the companion animal is a dog or cat. In another embodiment, the companion model system is a canine system and has a heterogeneous genetic background. In another embodiment, the companion animal is a purebred dog. In another embodiment, the companion animal is a mongrel dog.

[0016] In another embodiment, the disclosure provides for methods for identifying an anti-cancer agent comprising: (a) procuring companion animal model system for testing the agent wherein the companion animal model system has a spontaneously occurring cancer; (b) administering the agent to the companion animal model system; (c) monitoring more than one cancer antigen in the companion animal model system for biological and/or physiological effects; and (d) identifying the agent as anti-cancer based on the biological and physiological effects. In one embodiment, the companion animal is a dog or a cat. In another embodiment, the cancer antigen is not a glioblastoma multiforme antigen.

[0017] In another embodiment, the disclosure provides for methods of identifying an autoantigen associated an autoimmune disease comprising: (a) procuring companion animal model system wherein the companion animal model system has a spontaneously occurring autoimmune disease; (b) determining one more antigens to obtain a profile of the disease in the companion animal model system; (c) comparing the profile to a control companion animal model system that does not have the spontaneously occurring disease; and (d) identifying an autoantigen associated with autoimmune disease. In one embodiment, the companion animal is a dog or a cat. In another embodiment, the autoimmune disease is selected from the group consisting of diabetes, dilated cardiomyopathy, and discord lupus. In another embodiment, the autoantigen is not GAD65 or full-length IA-2, juxtamembrane domain (aa 605-682 of IA2). In another embodiment, the autoantigen is not myosin heavy chain, alpha cardiac actin, mitochondrial aconitate hydratase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or brain glycogen phosphorylase (GPBB).

[0018] In another embodiment, the disclosure provides for methods of targeting multiple antigens associated with or suspected of being associated with cancer in a human comprising: (a) procuring companion animal model system for testing the agent wherein the companion animal model system has a spontaneously occurring cancer; (b) administering to the companion animal model system one or more agents that is suspected of having anti-cancer effects; (c) monitoring the effects of the agent on the companion animal model system; and (d) administering the same agent to the human if the agent has an anti-cancer effect in the companion animal model system. In one embodiment, the companion animal is a dog or a cat.

[0019] In another embodiment, the disclosure provides for methods of targeting one or more antigens associated with or suspected of being associated with an infectious disease

comprising: (a) procuring companion animal model system for testing the agent wherein the companion animal model system has a spontaneously occurring infectious disease; and (b) administering to the companion animal model system one or more agents that is suspected of having an effect to combat the infectious disease; (c) monitoring the effects of the agent on the companion animal model system; and (d) administering the same agent to the human if the agent has an effect in the companion animal model system. In one embodiment, the companion animal is a dog or a cat. In another embodiment, the infectious disease is selected from the group consisting of influenza, septicemia (e.g., *Klebsiella pneumoniae* septicemia), bacterial infections (e.g., *Staphylococcus aureus*, other Staph infections, E. coli and enterococci), Pseudomonas aeruginosa, Leishmania infantum, Brucellosis, Coccidiosis, and *Salmonella enterica* Serovar Typhimurium.

[0020] In another embodiment, the disclosure provides for methods of improving timing and/or cost for obtaining regulatory approval on an agent for a disease comprising: (a) identifying companion animal model system for the disease wherein the companion animal model system has a spontaneously occurring version of the disease; (b) administering the agent to the companion animal model system; (c) monitoring the animal for biological and physiological effects; (d) determining the effects of the agent on the disease and (e) documenting the effects of the agents on media that is suitable for submission to a regulatory agency. In one embodiment, the companion animal is a dog or a cat.

BRIEF DESCRIPTION OF THE FIGURES

[0021] Figure 1 depicts results which show that once weekly i.v. administration of 200 ul LC to C57Bl/6 mice with established s.c. MCA-205 (sarcoma) tumors produced significant inhibition of tumor growth.

[0022] Figure 2 depicts results which shows a dog with STS treated with a series of treatments with LC alone experienced significant spontaneous tumor regression beginning after the third LC administration.

[0023] Figure 3 depicts results which show that twenty-four hours after i.v. administration of LC in tumor-bearing mice, $CD11b^{+}/Gr\text{-}1^{+}$ MSC were enumerated in spleen, blood, and tumor tissues and that significant MSC depletion occurred in blood.

[0024] Figure 4 depicts results which show that the antitumor activity of LC was almost completely eliminated in $CD8^{-/-}$ mice, whereas the activity of LC was only partially inhibited in $CD4^{-/-}$ mice. Controls also included mice treated with PBS containing liposomes (lip control).

[0025] Figure 5 depicts results from experiments which tested whether MSC depletion using LC could enhance vaccine responses, using humoral immune responses as the readout.

DETAILED DESCRIPTION OF THE INVENTION

[0026] The invention provides platform technologies for studying various aspects of biological pathways, physiological conditions and/or responses, and underlying mechanisms of action for various diseases, such as spontaneously occurring diseases. Such knowledge can be further used for translational medicine for various purposes, including but not limited to developing treatments, diagnostic methods or kits; identifying new pathways, identifying compounds or agents (and combinations thereof) for therapeutic or prophylactic purposes, and/or identifying new disease targets.

[0027] Generally, companion animals with spontaneously occurring diseases are useful for gathering data on various treatment modalities and combinations. Since companion animals are not kept under laboratory conditions (i.e., with limited exposure to every day environmental factors, and exposed to a controlled set of conditions), the use of such animals is one distinguishing factor from the other studies (e.g., beagle studies) using canines kept under laboratory conditions. Furthermore, the diseases are not being induced by reagents under laboratory conditions, i.e., the diseases develop spontaneously. Thus, the benefit of this platform is that it is more reflective of what happens to humans than laboratory animals which have been induced to develop a particular disease or condition.

[0028] Non-human companion animals, such as dogs, spontaneously develop diseases that mirror human diseases. As such, the use of companion animals that develop spontaneously occurring diseases can provide additional benefits by decreasing the time needed to gather scientific data for regulatory approval, decrease the cost associated with such data gathering and increase the amount of scientific data that can be obtained. The use of animal models with spontaneously occurring diseases permits testing of one or more parameters (such as type of antigen(s), combination of antigens, combination of agents, location of delivery, etc.) that would otherwise not be permitted under FDA regulations. Furthermore, companion

animals are also helped by potential discoveries that could cure or treat their spontaneously occurring diseases.

[0029] Accordingly, in one aspect, the invention provides for a companion animal model system as a platform technology for identifying a treatment modality for treatment in humans comprising a combination of compositions that have a higher probability of success for identifying the treatment modality than a standard model. The companion animal can be any animal that are companions to humans, preferably exposed to the same environmental factors (e.g., air, water) as their humans. In one aspect, the companion animal is an animal whose genome is has been determined either partially or fully. Use of genomic information (e.g., at the nucleic acid level, protein or metabolic level) is useful in these platform technologies. Non-limiting examples of companion animals who share similar environmental factors to their humans and have their genome partially or fully sequences include dogs and cats.

[0030] The use of the platform technologies described herein can provide 20-50 fold reduction in the time and/or cost for translational medicine by exploiting the synergies between multiple platforms as well as between multiple antigens and any combination thereof. As described in greater detail herein, the platform technologies can be applied to different subject matter that traditionally have faced difficulties in human trials due to costs, regulatory constraints, timing, size of trials and other road blocks for advancement of science. This subject matter includes, but is not limited to, vaccines (e.g., tolerizing vaccines), cationic lipid CpG, quorum sensing and autoinducer in infectious diseases, multiplexing pathology from biological samples (e.g., urine, saliva, blood or plasma), diagnostic techniques for rapid diagnosis and marker multiplexing technology.

General Techniques

[0031] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney), ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M.

Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991) and *Short Protocols in Molecular Biology* (Wiley and Sons, 1999). Other useful references include Harrison's *Principles of Internal Medicine* (McGraw Hill; J. Isseleacher et al., eds.), *Dubois' Lupus Erythematosus* (5th ed.; D.J. Wallace and B.H. Hahn, eds.; Williams & Wilkins, 1997), *Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat* (Stephen Ettinger, ed., W.B. Saunders Company; 5th edition (January 15, 2000)); and *Kirk's Current Veterinary Therapy XIV* (Bonagura et al, Saunders; 14 edition (July 10, 2008)).

Definitions

[0032] As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise. For example, "an" antigen includes one or more antigens.

[0033] An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, pets, companion animals, primates, mice and rats. In one embodiment, an individual is a human.

[0034] A "companion animal" is a non-human animal that resides in the same household as their human owners for companionship. Companion animals generally are exposed to the same environmental factors as humans (e.g., water, air, carcinogens, allergens, etc.). Non-limiting examples of a companion animal include dogs and cats. In one aspect, a companion animal is not subjected to laboratory conditions (e.g., with limited exposure to every day environmental factors and exposed to a controlled set of conditions).

[0035] As used herein, "spontaneous occurring" or "spontaneously occurring" (or naturally occurring) diseases are diseases which involve host-induced disease states. Host-induced disease states refer to the host mounting some type of biological or physiological response in certain circumstances. In one embodiment, host-induced disease states do not include virally-induced states wherein the virus is the causative agent for the transformation. In another embodiment, "spontaneously occurring" includes biological and/or physiological conditions or responses brought on by viruses. For example, a mouse or rat can be induced to have cancer by injecting the mouse or rat with certain chemicals. The cancer-ridden mouse or rat would not be considered to have "spontaneous occurring cancer."

[0036] "Synergy" as used to describe biological and/or physiological effects of a combination of agents or treatment modalities refers to one or more effects that are greater

than additive of each agent or treatment modality by itself. For example, if administration of one agent results in a 10% antibody increase and administration of another agent results in a 15% antibody increase, then a synergistic effect would be greater than 25% antibody response. In some embodiments, a synergistic effect is 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% more than additive effect. In other embodiments, a synergistic effect is 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more than additive effect. In other embodiments, a synergistic effect is or 125%, 150%, 200%, 300%, 400%, or 500% more than additive effect. In other embodiments, a synergistic effect can be a 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold increase over additive effect.

[0037] "Synergy" can also be used to describe a decrease in biological and/or physiological effects (e.g., autoimmune response) in addition to an increase in biological and/or physiological effects (e.g., antibody production). For example, if administration of one agent results in a 10% decrease in autoimmune response (e.g., antinuclear antibodies for systemic lupus erythematosus) and administration of another agent results in a 15% decrease, then a synergistic effect would be a decrease of more than 25% autoimmune response. In some embodiments, a synergistic effect is 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% less than additive effect. In other embodiments, a synergistic effect is 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less than additive effect. In other embodiments, a synergistic effect is or 125%, 150%, 200%, 300%, 400%, or 500% less than additive effect. In other embodiments, a synergistic effect can be a 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold decrease over additive effect.

[0038] "Agent" can refer any composition of matter, whether it is naturally occurring or synthetic. Non-limiting examples of an agent include: small molecules, antibodies, naturally occurring protein and fragments thereof (e.g., soluble receptors like Axl, EGF, or VEGF or other involved with the growth factors), recombinant proteins and fragments thereof, fusion molecules (e.g., fusion proteins), synthetic molecules, lipids, nucleic acids, and carbohydrates.

[0039] "Biological and/or physiological effect" refers to the effect of an agent on an individual's biological parameters or physiological parameters. Non-limiting examples of biological parameters include: cytokine profile and/or production, immune response, immune

parameters such as antibody response, Th1 or Th2 or Th17 responses, genomic profile and its changes, antigen profile and its changes, lipid profiles, fatty acid and cholesterol profiles and toxicity profiles. Non-limiting examples of physiological parameters include parameters associated with a system, e.g., cardiovascular system. Such cardiovascular parameters can include, but are not limited to, cardiac health, pulmonary artery occlusion, coronary perfusion pressures; cardiac output, pulmonary, systemic vascular resistances. In other embodiments, the physiologic parameters can include, but are not limited to, blood gas and saturation measurements, oxygen delivery, oxygen utilization, renal capacity, and processing and functional capability of organs (e.g., liver for toxins, pancreas for insulin production, etc.).

[0040] "Disease" refers to an abnormal condition of an individual that can impair bodily functions, and is commonly associated with specific symptoms. It may be caused by external factors, such as invading pathogens, or it may be caused by internal dysfunctions, such as autoimmune diseases. "Disease" also encompasses various states and degrees of each disease. For example, the development of a malignant growth is a disease state of cancer. Metastasis is another disease state of cancer. All the symptoms and/or signs reported to be associated with the development of the disease does not necessarily need to be present in an individual for any given disease.

[0041] "Antigen," as used herein, refers broadly to any substance that can be recognized by an organism's immune system. In one aspect, antigens can induce the production of antibodies. Antigens are typically proteins or polysaccharides. Antigens include, but are not limited to, parts (coats, capsules, cell walls, flagella, fimbrae, and toxins) of bacteria, viruses, and other microorganisms. Antigens do not necessarily have to elicit an immune response by themselves alone. Antigens encompass immunogens, which do elicit an immune response (e.g., antibody response). Types of antigens include, but are not limited to, exogenous antigens (antigens that have entered the body from the outside, for example by inhalation, ingestion, or injection), endogenous antigens (antigens that have been generated within the cell, for example, as a result of normal cell metabolism, or because of viral or intracellular bacterial infection), autoantigens, tumor antigens and allergic antigens.

[0042] An "autoantigen" is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease. These antigens should, under normal conditions, not be the target of the

immune system, but, due to mainly genetic and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients.

[0043] As used herein, "treatment" is an approach for obtaining beneficial or desired results, preferably including clinical results. For example, in the context of this invention, one desired results would be the halt of the growth of cancer cells. Treatment does not necessarily require that the disease be eradicated or that the individual with the disease be cured.

[0044] "Receiving treatment" includes initial treatment and/or continuing treatment.

[0045] "Therapy" includes both prophylactic therapy (i.e., before disease occurrence) and therapeutic treatment (i.e., after disease occurrence).

[0046] "Beneficial effect" refers to a biological or physiological effect on the individual (e.g., human or companion animal) that improves the well-being of the individual. Non-limiting examples of a beneficial effect include: reduction of cancerous tumors or nodules, reduction in the number of malignant cells, increased antibody production against cancer or pathogens, secretion of cytokines that assist in eliminating cancer cells and/or pathogens, decrease in the amount of immune reaction against self-molecules, reduction in the autoimmune response, palliating symptoms of a disease, palliating undesired pain in an individual, increasing the comfort level of an individual, increasing the robustness of the individual's immune system, and reconstituting an individual's immune system.

[0047] As used herein, "combination" refers to all the possible variations for the combination of any agent, antigen, composition, compound, adjuvant, etc. with each other. This includes the use of more than one of any one agent, antigen, composition, adjuvant and the like within its own group (e.g., multiple agents) or with other groups. For example, "combination" contemplates the use of one agent with one adjuvant or two compositions with several adjuvants.

Compositions of Treatment Modalities

[0048] The invention provides platform technologies that utilize a companion animal model system of spontaneously occurring diseases to investigate aspects of human diseases. Companion animal models that may be used include any animal who resides with humans. In

this manner, the companion animal is exposed to similar environmental factors as their human co-inhabitants. Such environmental factors include, but are not limited to, breathing the same air, drinking the same water, exposure to the same household contents (e.g., carpets, cleaners, etc.) Unlike laboratory animals that are typically used for experiments (e.g., mice and rats), companion animals are exposed to the factors that a human is and, as such, provide a more accurate background for correlation for human diseases and/or physiological conditions. Any treatments that are beneficial for humans can be used to help the companion animal as well, which includes not only treating the disease and/or physiological condition, but also to improve their quality of life.

[0049] In one aspect, the examination of multiple modalities is conducted using a canine model system. In one embodiment, multiple modalities can refer to the use of multiple antigens in the system. The study of a single antigen may not provide sufficient insight into a biological system for generating an efficient immune response. For example, the identification of a single antigen associated with prostate cancer, e.g., prostatic acid phosphatase, may mount an immune response but the identification of other antigens would provide additional, even synergistic, immune response to combat prostate cancer. The study of multiple antigens in human clinical trials is not feasible due to regulatory constraints (e.g., FDA approval), cost, time, and/or other biological barriers. In this regard, the use of a canine model system is useful for examination of multiple antigens since dogs spontaneously develop prostate cancer. One of skill in the art can use the canine model system to examine multiple antigens, for example, cancer antigens, to identify novel antigens that can be used for targets (e.g., antibodies against the antigen, small molecules, etc.). In addition, the use of canine model system can assist to identify new pathways and/or biological niches that the antigen is associated and be utilized as a basis for additional therapies.

[0050] In another aspect of the invention, multiple modalities can refer to the use of one or more antigens plus one or more adjuvants. The term "adjuvant" is well-known in the art. It commonly refers to a pharmacological or immunological agents that can modify the effect of other agents (e.g., drugs or vaccines) while having few if any direct effects when given by themselves. An adjuvant can be an immunological adjuvant, which can modify or augment the effects of a vaccine by stimulating the immune system to respond to the vaccine more vigorously, and thus providing increased immunity to a particular disease. Non-limiting examples of immunological adjuvants include: alum, Freud's complete adjuvant, Freud's

incomplete adjuvant, Ribi adjuvant, aluminum salts, and immunomodulatory polynucleotides (e.g., CpG-containing polynucleotides). An adjuvant can also be a pharmaceutical adjuvant which have few or no pharmacological effects by themselves, but may increase the efficacy or potency of other drugs when given at the same time. A non-limiting example of this is caffeine, which has minimal analgesic effect on its own, but may have an adjuvant effect when given with paracetamol (acetaminophen). Adjuvant can also refer to additional therapy in the cancer therapy context, for example, in chemotherapy. In this context, adjuvant therapy refers to additional treatment, usually given after surgery where all detectable disease has been removed, but where there remains a statistical risk of relapse due to occult disease. In a non-limiting example, radiotherapy or chemotherapy is can be given as adjuvant treatment after surgery for a breast cancer. In some embodiments, one adjuvant is used. In other embodiments, two or more adjuvants are used. In yet other embodiments, 3, 4, 5, 6, 7, 8, 9, or 10 adjuvants are used.

[0051] The use of "reverse adjuvants" is also contemplated within the scope of this invention and is encompassed by the term "adjuvant." Reverse adjuvants can have tolerizing effects when used with a tolerizing vaccine (*see, e.g.*, Ho et al, *J. Immunology* 175: 6226-6234, 2005). One example of a reverse adjuvant is GpG oligonucleotide which has suppressive effects in contrast with CpG oligonucleotides, which tend to have immunostimulatory properties (*see, e.g.*, Ho et al, *J. Immunology* 171: 4920-4926, 2003).

[0052] The combination of various antigens and adjuvants and their effect on various spontaneously occurring diseases, has been difficult to study in human trials for reasons discussed above. Using mice or rats as animal models does not provide as accurate of information as using canine model system of spontaneously occurring diseases since the genetic translation to humans and disease progression does not parallel as closely as dogs to humans. (Tsai et al., *Mamm. Genome*, 18:444-451 (2007). As such, the use of canine model system as a platform technology for examining spontaneously occurring diseases allows for one of skill in the art to identify a treatment modality with a greater probability of success than using a standard mouse model where the diseases are induced.

[0053] Various types of adjuvants can be studied using the canine model system disclosed herein. In one embodiment, adjuvants that act through the toll-like receptor (TLR) agonists can be studied using the platform technology of canine model system of spontaneously occurring diseases. Various TLR include, but are not limited to, TLR 1, 2, 3, 4, 5, 6, 7, 8, and

9. One example is CpG-containing compounds that act through the TLRs. Such adjuvants have been tested in the context of hepatitis B. Other non-limiting examples of adjuvants that can be studied include keyhole limpet hemocyanin (KLH) and MF59.

[0054] In another aspect, the platform technologies described herein allows one of skill in the art to explore the use of multiple modalities against a heterogeneous genetic background. One of skill in the art will appreciate that there are various degrees of heterogeneity and homogeneity in genetic backgrounds. On one end of spectrum, homogeneous genetic backgrounds are commonly seen in cloned animals or animals such as mice that have been inbred for many generations such that their genetic background is the same as the next mouse.

[0055] Further down the spectrum are heterogeneous animals (e.g., with less degree of homogeneity than cloned animals), such as purebred dogs. Although they are purebred, the dogs have slightly different genetic code from each other but yet they retain the same morphological traits that characterize them as being that particular purebred. Dogs are unique among mammalian species in that they can show differences in morphological traits (such as height, weight, shape) and yet within breed, exhibit traits that are inherited within a narrow range. For example, purebred chihuahua dogs are generally +/- 6 inches of each other at the shoulder. Ostrander et al., *Am J Hum Genet* 61:475-480 (1997). Even further down the spectrum are even more heterogeneous dogs which are not purebred but instead are mongrels. In one embodiment, heterogeneous animals do not include non-obese diabetic (NOD) mouse. It is against this backdrop of heterogeneous genetic background that different treatment modalities are explored. The heterogeneous nature of the dogs does not necessarily allow one of skill in the art to predict *a priori* what the biological response will be, and even more so in the case where multiple treatment modalities (e.g., multiple antigens) are utilized. The foregoing is equally applicable with other companion animals, such as cats.

Advantages of Using a Spontaneously Occurring Disease Model

[0056] The use of spontaneously occurring disease model is beneficial in various aspects. In one aspect, the immune system of the disease model is kept relatively intact as compared to animal model of disease where the animal has been induced to have the disease. In the latter case, artificial induction of diseases throws off the balance of the immune system, causes the immune system (including various immune cells such as T cells, B cells,

neutrophils, macrophages, regulatory Tcells, NK cells, NKT cells) and the interactions between immune cells and various branches of the immune system to be perturbed by the artificial induction of the diseases. Accordingly, the invention provides a platform technology that allows for the study of various diseases/disease states as well as complex physiological conditions without the immune dysregulation associated with the artificial induction of the disease. This provides more meaningful findings which facilitates the discovery and/or identification of new pathways, mechanisms of action, biological participants (e.g., cellular receptors or cell types) in these pathways or mechanisms and further understanding to the underpinnings of complex physiological conditions. Such complex physiological conditions can include, but are not limited to, cancer, autoimmune disease, allergies, hypersensitivity, neurological diseases, hereditary genetic disorders, and infectious diseases.

[0057] The invention also encompasses the use of the platform technology for the identification of one or more biomarkers associated with various physiological states and diseases. In some instances, biomarker can refer to the presence or absence of one or more genes or proteins, various isoforms of genes or gene splicing and their product(s), single nucleotide polymorphisms, gene expression profiles, proteomic profiles or metabolomic profiles. In some non-limiting examples, multiplexing biomarkers are used for screening, staging, imaging, diagnosing and/or monitoring the response to various therapies. For example, changes to expression of one or more genes, metabolome and epigenetic changes are contemplated within the scope of invention. In one non-limiting example, methylation patterns on gene chips can be used to study normal vs. abnormal methylation patterns for various diseases/disease states. Another non-limiting example is the use of magnetic arrays that can house multiple biomarkers (e.g., 15-18 biomarkers) and in one embodiment, are detectable at low amounts (e.g., 1 pg/ml). Another non-limiting example is the use of aptamers where hundreds of biomarkers can be assessed simultaneously or nearly simultaneously. One of skill in the art can utilize the screening, staging, imaging, diagnosing and/or monitoring in combination with treatment protocols and the refinement of any therapies that are being contemplated. One of skill in the art, e.g., a physician, can modify the therapy as to most effectively prevent, delay the development of, ameliorate the symptoms of, or treat the disease or physiological condition.

Cancer

[0058] The use of companion animals with spontaneously occurring cancer allows for one of skill in the art to not only seek durable cures for companion animals but also to use the companion animal as a model for studying scientific aspects of cancer (including spontaneously occurring cancers) in humans, which may lead to discoveries for treatments and therapies for various types of cancers for mankind.

[0059] Incidence rates of human and companion animal cancers vary considerably. In some cases, human cancers are not commonly found in pets, and comparative oncology in not practical. In other cases, tumors in companion animals closely resemble their human counterparts and in some cases may occur more frequently, affording the opportunity to study diseases that are rare in human cancer patients.

[0060] Companion animals such as dogs develop various types of spontaneously occurring cancers. Common cancers include, but are not limited to, bone cancer (e.g., osteosarcoma), lymphoma (e.g., non-Hodgkin lymphoma), hemangiosarcoma, other sarcomas, mammary cancer, testicular cancer, mast cell cancer, nasosinal cancer, bladder cancer, head and neck cancer, prostate cancer, melanoma, leukemia, brain cancer, lung carcinoma, and soft-tissue carcinoma. Some breeds develop certain cancers more often than other breeds. For example, hemangiosarcomas, an aggressive cancer that arises from the blood vessels, are seen more in German Shepherds, Golden Retrievers, Boxers, and English Setters than other breeds. In one aspect, one of skill in the art can observe the differences in the cancer progression when different biological procedures that normally would be applied to the companion animal are done. For example, prostate cancer progression can be observed in dogs who have been neutered and compared to prostate cancer progression to dogs whose owners have chosen to not have them neutered.

[0061] In one aspect, the use of purebred dogs allows for the study of a more homogeneous genetic background and comparison with mongrel dogs with heterogeneous genetic background. The use of various genetic backgrounds of companion animals, such as dogs, permits the identification of various antigens or biomarkers that are associated with cancer. The resulting information gleaned from such studies can be translated into diagnostics or therapies for humans by using antigens as targets for drug discovery or immunotherapy and/or by using biomarkers in imaging techniques.

[0062] Companion animals with spontaneously occurring cancer can be used to examine the effects of a combination of anti-cancer agents to identify a combination that produces synergistic effects. The combination of agents can be two or more agents, for example, 3, 4, 5, 6, 7, 8, 9, or 10 agents. The agents can be given at the same time or in two or more administrations. The dosage of each agent can be same or varied, especially when using a group of companion animals with spontaneously occurring cancers where a range of dosage of agents tested can indicate which combination results in most efficacious biological response.

[0063] Various classes of anti-cancer agents can be used. Non-limiting examples include: alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, podophyllotoxin, antibodies (e.g., monoclonal or polyclonal), tyrosine kinase inhibitors (e.g., imatinib mesylate (Gleevec® or Glivec®), hormone treatments, soluble receptors and other antineoplastics.

[0064] Alkylating agents can alkylate many nucleophilic functional groups under conditions present in cells. Cisplatin and carboplatin, and oxaliplatin are alkylating agents. They impair cell function by forming covalent bonds with the amino, carboxyl, sulfhydryl, and phosphate groups in biologically important molecules.

[0065] Anti-metabolites resemble purine ((azathioprine, mercaptopurine)) or pyrimidine and prevent these substances from becoming incorporated in to DNA during the "S" phase of the cell cycle, stopping normal development and division. They also affect RNA synthesis.

[0066] Plant alkaloids and terpenoids are derived from plants and block cell division by preventing microtubule function. Since microtubules are vital for cell division, without them, cell division cannot occur. Some non-limiting examples are vinca alkaloids and taxanes.

[0067] Vinca alkaloids bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules (M phase of the cell cycle). The vinca alkaloids include: vincristine, vinblastine, vinorelbine, and vindesine.

[0068] Podophyllotoxin is a plant-derived compound which has been reported to help with digestion as well as used to produce two other cytostatic drugs, etoposide and teniposide. They prevent the cell from entering the G1 phase (the start of DNA replication) and the replication of DNA (the S phase).

[0069] Taxanes as a group includes paclitaxel and docetaxel. Paclitaxel is a natural product, originally known as Taxol and first derived from the bark of the Pacific Yew tree. Docetaxel is a semi-synthetic analogue of paclitaxel. Taxanes enhance stability of microtubules, preventing the separation of chromosomes during anaphase.

[0070] Topoisomerase inhibitors are also another class of anti-cancer agents that can be used. Topoisomerases are essential enzymes that maintain the topology of DNA. Inhibition of type I or type II topoisomerases interferes with both transcription and replication of DNA by upsetting proper DNA supercoiling. Some type I topoisomerase inhibitors include camptothecins: irinotecan and topotecan. Examples of type II inhibitors include amsacrine, etoposide, etoposide phosphate, and teniposide. These are semisynthetic derivatives of epipodophyllotoxins, alkaloids naturally occurring in the root of American Mayapple (Podophyllum peltatum).

[0071] Antineoplastics include the immunosuppressant dactinomycin, doxorubicin, epirubicin, bleomycin, mechlorethamine, cyclophosphamide, chlorambucil, ifosfamide. The antineoplastic compounds generally work by chemically modifying a cell's DNA.

[0072] Soluble receptors can include the extracellular portion of receptors known to bind to growth factors and especially growth factors that are associated with cancer. Non-limiting examples are: Axl, VEGF, and EGF. The soluble receptors can be recombinant/synthetic or naturally occurring receptors (e.g., purified or concentrated preparation). The extracellular portion of receptors can also be fused to portions to promote half-life and other desirable pharmacokinetics to create fusion proteins.

[0073] In the case of antigens, the invention contemplates the study of one or more antigens associated with cancer. In one embodiment, the platform technology refers to the use of companion animals with spontaneously occurring cancer to study multiple (i.e., two or more) antigens. In other embodiments, at least about 2, 3, 4, 5, 6, 7, 8, 9, or 10 antigens are monitored in the companion animals with spontaneously occurring cancer. In other embodiments, at least about 10 or more antigens are monitored in the companion animals with spontaneously occurring cancer. In one embodiment, the cancer antigen is a not a glioblastoma multiforme antigen.

Osteosarcoma

[0074] Osteosarcoma is a relatively rare form of cancer afflicting a disproportionate percentage of children, with an annual incidence of 900 new patients per year including 400 < 20 years old. Though rare, it is the 6th leading form of cancer in children under the age of 15, about 3% of all childhood cancers. The current standard of care is amputation or limb-salvage orthopedic surgery combined with chemotherapy (high dose methotrexate with leucovorin rescue, intra-arterial cisplatin, adriamycin, ifosfamide, etoposide, and muramyl tri-peptide). Survival rates have improved since the 1960s when the only treatment option was amputation and only 5-20% of diagnosed patients survived more than 2 years, but despite improvements through chemotherapy the survival rate for osteosarcoma remains among the lowest among pediatric cancers. The current 5-year survival rate for non-metastatic osteosarcoma patients is > 70% while for patients with metastases the rate is approximately 30%. Progress toward improved treatment options for this young population is slowed by its rare incidence and the resultant challenges in patient accrual for clinical studies.

[0075] In contrast to human incidence, osteosarcoma is a relatively common cancer in larger breeds of dogs (>60 pounds), particularly in Great Dane, Wolfhound, and Rottweiler. The incidence of osteosarcoma is 3-4% of all canine cancers, afflicting up to 10,000 dogs per year in North America. Human and canine osteosarcoma share common features of anatomical distribution and metastasis. In both species, >75% of cases occur in long bones (distal radius > proximal humerus; distal femur > tibia), predominantly in males (2:1). The high metastatic rate in dogs (90%) is comparable to that in humans (80%), and sites of metastasis have a similar hierarchy of lung>bone>soft tissue. Furthermore, primary osteosarcoma and metastases are histologically indistinguishable between human and canine patients. Like humans, dogs also respond to chemotherapy- treatment with cisplatin, doxorubicin, or carboplatin following amputation produces a mean survival time of 9-11 months, a significant improvement over the median survival of 3-4 months following amputation alone. Given the shared histology, metastatic pattern, and chemotherapy responsiveness, canine osteosarcoma offers an excellent model for testing alternative therapies. With a higher incidence and more rapid progression, clinical trials can be recruited and completed more rapidly in dogs, informing new therapeutic strategies for both human and canine patients.

Soft Tissue Sarcomas

[0076] Soft tissue sarcomas are a diverse group of tumors derived from mesenchymal tissues (e.g. connective tissue, fibrous tissue, muscle). They account for less than 1% of all new cancer cases per year; in 2006 there were approximately 9,500 new cases diagnosed in the United States, more commonly in older patients (>50 years old) though some subtypes (e.g., rhabdomyosarcoma, a sarcoma of the skeletal muscle) are more common in children and adolescents. Soft tissue sarcomas as a class are more common in companion animals, representing 15% of all subcutaneous cancers in dogs and 7 % in cats. With the exception of hemangiosarcomas, this class of tumors is locally aggressive but rarely metastasizes. Nevertheless, the soft tissue sarcomas of both humans and companion animals are only moderately responsive to chemotherapy.

[0077] Because they resemble human tumors of the same origin and are detected relatively late, providing greater tumor bulk for analysis, canine soft tissue sarcomas have served as models for optimizing therapeutic strategies. Protocols aimed at increasing local control, particularly those using adjuvant radiation, often coupled with hypothermia, have guided new treatment protocols for human patients. Interest in localized hyperthermia was stimulated by the observation that heat could increase the efficacy of radiation or chemotherapy. Local and whole body hyperthermia studies tested pharmacological approaches to inducing hyperthermia such as vasoactive drugs. Studies in dogs have also modeled the effect of hyperthermia on the pharmacokinetics of chemotherapeutic drugs and aided the development of biomarkers of hypoxia and prognostic imaging techniques. Soft tissue sarcomas in companion animals have also served as models for testing new chemotherapeutic formulations. For example, the efficacy of slow release cisplatin in a biodegradable polymer was tested in canine soft tissue sarcoma, and efficacy of liposome-encapsulated doxorubicin (Doxil) was tested in vaccine-associated feline sarcoma.

Hemangiosarcoma

[0078] Hemangiosarcoma (HSA) is a tumor of the vascular endothelial cells characterized by rapid and extensive metastasis. It is rare in humans, accounting for less than 1% of all tumors, but accounts for 5-7% of all canine malignancies. Assuming a lifetime cancer risk for dogs in the range of 30-50% this cancer may affect 1.5-2.5 million of the estimated 72 million pet dogs in the United States. HSAs originate most often in the spleen, but can also

form in the liver, right atrium of the heart, and skin. They tend to occur in middle aged dogs (> 6 years old), with higher prevalence in Bernese Mountain Dogs, Boxers, Flat Coated Retrievers, German Shepherds, Golden Retrievers, Portugese Water Dogs, and Skye Terriers; according to one survey the incidence of HSA in Golden Retreivers is almost 1 in 5. Canine HSAs appear comparable to angiosarcomas in humans, and because they occur with far greater frequency may prove an important surrogate for clinical testing. Chemotherapy, typically combinations of doxorubicin and cyclophosphamides +/- vincristine, are the most common therapeutic approach for HSA, but median survival times are only 145-180 days.

Mammary Carcinoma

[0079] Breast cancer and canine mammary gland tumors have several epidemiological and physiological similarities. Breast cancer is the leading cause of cancer in North American women, accounting for nearly 30% of all cancer; the lifetime risk of breast cancer in American women is 12%. Mammary gland tumors (MGTs) account for 52% of all tumors in female dogs, and occur in 26% of all unsprayed dogs. There are significant genetic and histological similarities between breast cancer and MGT, but also key differences in gene expression and drug response that complicate efforts to translate therapeutic strategies between species. MGTs are hormone-dependent; 50-60% of these tumors express estrogen receptors or progesterone receptors, and ovariohysterectomy (spaying) reduces the risk of developing MGT to 0.5%. Human breast cancer is also hormone-dependent and often treated with drugs that affect estrogen or progesterone receptors, but the estrogen receptor antagonist tamoxifen does not have demonstrable anti-tumor activity in dogs. There are also similarities and differences on the genetic level. Expression of the oncogene c-erbB-2 correlates with a more aggressive malignant phenotype in human breast cancer. Similarly, c-erbB-2 is overexpressed in 74% of malignant canine mammary tumors, but in 0% of benign tumors. Mutations of the tumor-suppressor gene BRCA1/BRCA2 are associated with increased risk of human breast cancer. The expression and variants of BRCA1 are less documented in canine mammary gland tumors, though recent reports of splicing variants of BRCA1 in some MGTs and upregulation of BRCA2 and RAD51(which interacts with BRCA1 and BRCA2) in metastases of MGTs point to the need for more extensive analysis of gene expression in these canine tumors. As with hormone treatment, the application of chemotherapeutic agents to the treatment of MGT is uncertain. According to some reviews, no chemotherapeutic agents have proven consistently effective in canine MGT, though a few partial responses to

doxorubicin have been documented and cisplatin is sometimes recommended. Despite many similarities it remains unclear whether canine MGT is a relevant therapeutic model for human breast cancer. Additional studies of gene expression may identify common targets for human breast cancer and MGT and guide the application of human chemotherapies for treatment of the canine tumor.

Melanoma

[0080] Skin cancer is the most common of all cancers in the United States. Although melanoma is a relatively uncommon form, accounting for less than 5% of skin cancer cases, it is responsible for 75% of skin cancer deaths. The rate of new cases was relatively stable over the past 8 years, with estimates of 68,720 new cases in 2009 resulting in over 8,650 deaths. According to a World Health Organization report, there are approximately 48,000 melanoma-related deaths worldwide per year. The overall risk of melanoma varies with ethnicity, ranging from 2% for Caucasians to 0.5% for Hispanics and 0.1% in African Americans. Current treatment options include surgical resection and chemotherapy (including single or combination treatments with dacarbazine, carmustine, cisplatin, tamoxifen, vinblastin, temozolomide, and paclitaxel). Melanoma is the fourth most common cancer in dogs, frequently occurring in the oral cavity but also originating in the digits, skin, and eye. Oral melanoma is reportedly most commonly observed in Dachshunds, Golden Retrievers, Poodles, and Scottish Terriers. As with advanced melanomas in humans, melanomas in dogs are generally resistant to chemotherapy and radiation, and aggressive metastasis is the primary cause of treatment failure and death.

[0081] Because canine and human melanomas share common features of physiology and response to treatment, clinical trials in dogs can provide an important translational bridge to new treatment strategies for human melanoma. Immunotherapy approaches have included autologous tumor cell vaccines (unmodified or transfected with immunostimulatory cytokines and/or melanosomal differentiation antigens), allogeneic tumor vaccines transfected with immunostimulatory cytokines (e.g. IL-2, GM-CSF), innate immune stimulants (e.g. L-MTP-PE), and DNA vaccine (e.g., plasmids encoding Fas ligand, IL-2, or GM-CSF). A randomized clinical trial of L-MTP-PE in canine melanoma showed an 80% long-term survival benefit in stage I melanoma, but no benefit in more advanced (stage II and III) melanoma. In a phase I clinical trial, vaccination with GM-CSF-transfected autologous melanoma cells induced localized inflammation and some histological evidence of tumor

disruption. Other vaccine approaches have injected plasmid DNA directly into the melanoma. A phase I clinical trial of 9 dogs with stage II-IV advanced malignant melanoma injected DNA encoding the melanosomal differentiation antigen tyrosinase in attempt to induce cell mediate immunity against tumor cells expressing tyrosinase. This immunotherapy induced an antibody response in 33% of the treated dogs and extended the median survival time to 389 days, significantly longer than the 1-5 months survival conferred by conventional therapies.

Non-Hodgkins Lymphoma

[0082] Non-Hodgkins lymphoma (NHL) is the sixth leading cause of cancer death, with an incidence rate of 3-4% in the United States, resulting in an estimated 66,000 new cases in the US in 2009, and a 5 year survival rate of 50-60% for patients treated with chemotherapy. Over 95% of new cases occur in adults, with an average age of onset of 60 years old. NHL is also relatively common in dogs; its incidence rate is 25/100,000, accounting for 5% of all malignancies and 83% of all hematopoietic malignancies. Approximately 70-80% of canine NHL cases are of B lymphocyte origin, while the rarer T cell lymphomas are associated with a significantly poorer prognosis. The highest prevalence of NHL occurs in German Shepherds, Boxers, Poodles, Basset Hounds, and Saint Bernards. Most canine cases resemble stages III-IV of human NHL, and in the absence of therapy disease progression is relatively rapid, resulting in death within 4-6 weeks after diagnosis. In addition to histological similarities, canine and human NHL share similar chemotherapeutic drug sensitivities, including responsiveness to doxorubicin, cyclophosphamide, and vinca alkaloids. As with human clinical practice, most current treatment protocols for canine NHL employ multiple, alternating combinations of drugs, resulting in reported response rates in the range of 86-91%.

[0083] With an incidence rate of 125/100,000, NHI is the most common cancer in cats, comprising nearly one third of all feline tumors. In contrast to canine NHL, a significant proportion of feline NHL is of T lymphocyte lineage, the result of transformation by a retrovirus, feline leukemia virus (FeLV). As with dogs, feline NHL is very chemoresponsive- sequential combination chemotherapy achieves remission rates of 60-70%. Based on their similarities with human tumors, both canine and feline NHL have served as surrogates for optimizing therapeutic approaches (*see* Examples).

Bladder Cancer

[0084] Bladder cancer is the fourth most common cancer in men and the ninth most in women in the United States. The disparity in incidence, 50,000 men and 16,000 women annually, may be related to the major role of androgen receptors in the development of bladder cancers. The majority of bladder cancers are transitional cell carcinoma (90%), originating in the cells lining the inside of the bladder; the remaining 10% include squamous cell carcinoma, adenocarcinoma, sarcoma, and small cell carcinoma. Transitional cell carcinoma (TCC) is also the most common form of canine urinary bladder cancer, closely resembling invasive human TCC in histology, biologic behavior, and response to therapy. As with other cancers, there is variation in susceptibility related to breed; for example, Scottish Terriers have an 18-fold increased risk to develop TCC.

[0085] Current treatment options for bladder cancer patients include surgery, radiation, and chemotherapy. Canine TCC is responsive to these approaches as well and has been a useful model for development and optimization of novel therapeutics. Canine TCC shows modest response to platinum and anthracycline-based protocols, with objective response rates of ~30% and MSTs of 4-8 months. Treatment with the cyclooxyenase inhibitor piroxicam results in an objective response rate of 18% which can be further improved by the addition of cisplatin, but at the cost of unacceptable nephrotoxicity. Canine TCC has proved a useful model for preclinical investigation of photodynamic therapy.

[0086] The invention also encompasses the use of the platform technology for the identification of one or more biomarkers associated with cancer and in some cases, a gene expression profile, proteomic profile or metabolomic profile of cancers. In one aspect of the invention, the platform technology can be used for multiplexing biomarkers. In one non-limiting example, methylation patterns on gene chips can be used to study normal vs. abnormal methylation patterns for various cancers. Another non-limiting example is the use of magnetic arrays that can house multiple biomarkers (e.g., 15-18 biomarkers) and in one embodiment, be detectable at low amounts (e.g., 1 pg/ml). Another non-limiting example is the use of aptamers where hundreds of biomarkers can be assessed simultaneously or nearly simultaneously.

[0087] In some cases of cancer, paraneoplastic syndrome is observed. In one aspect, paraneoplastic syndrome is a disease or symptom that is the consequence of the presence of

cancer in the body, but is not due to the local presence of cancer cells. These phenomena can be mediated by humoral factors (by hormones or cytokines) excreted by tumor cells or by an immune response against the tumor. Sometimes the symptoms of paraneoplastic syndromes show even before the diagnosis of a malignancy. Paraneoplastic syndromes can be divided into 4 main categories: endocrine, neurological, mucocutaneous and hematological paraneoplastic syndromes. In another aspect, paraneoplastic syndromes can be a group of rare disorders that are triggered by an abnormal immune system response to a cancerous tumor or a "neoplasm." Without being bound by theory, in one aspect, paraneoplastic syndromes can happen when cancer-fighting antibodies or white blood cells (e.g., T cells) mistakenly attack normal cells in the nervous system. Accordingly, in one embodiment, the immune system is left intact so that paraneoplastic syndrome can be more effectively studied.

[0088] In another aspect of the invention, the use of companion animals with spontaneously occurring cancer allows for the study of cancer is a form that has not been induced to progress to a more severe state. In one embodiment, the cancer being studied is pre-metastatic. The use of anti-cancer drugs may cause inflammation which may cause the cancer to progress from pre-metastatic cancer to a metastatic cancer. By using an animal model of spontaneously occurring diseases, such as cancer, the cancer that is examined is not further induced to progress into a form that it would otherwise not have progressed absent the chemotherapeutic and/or radiation intervention.

[0089] In this manner, the immune system of the animal is kept in as close of the natural state as possible. This makes for more accurate studying of the biological or physiological state of the immune system and thus, allows for the generation of more meaningful scientific data. This scientific data can then be used for identification of anti-cancer therapeutic agents.

Autoimmune and Neurodegenerative Diseases and/or Disorders

[0090] Another class of spontaneously occurring diseases which are observed in companion animals and can be leveraged for use in translational medicine is the class of autoimmune diseases. Another class of spontaneously occurring disease which re observed in companion animals and can be leveraged for use in humans is neudegenerative and neurological diseases and/or disorders, as detailed below. Autoimmune diseases include, but are not limited to, diabetes (e.g., juvenile diabetes, pemphigus vulgaris, myasthenia gravis, autoimmune hemolytic anemia, rheumatoid arthritis, polyarthritis, polymyositis, systemic lupus

erythematosus (SLE), discoid lupus erythematosus, cardiomyopathy (e.g., dilated cardiomyopathy), narcolepsy, and thrombocytopenia.

[0091] The platform technologies described herein can be used to identify one or more novel autoantigens that are associated with various autoimmune diseases. In one aspect of the invention, multiple antigens and/or autoimmune biomarkers are evaluated using companion animals with spontaneously occurring autoimmune disease. These autoantigens and/or autoimmune biomarkers can be targets for therapies and other treatment modalities for addressing autoimmune disease in humans.

The Canine and Feline Major Histocompatibility Complex

[0092] The human major histocompatibility complex (MHC), termed the human leukocyte antigen (HLA) complex, contains >200 loci, including >40 coding for immune function molecules, in a 3.6 Mb stretch of DNA. HLA class I molecules (A, B, C) bind endogenous peptides and present them to CD8 T cells for surveillance of intracellular pathogens and other disruptions of normal cellular function. HLA class II molecules (DR, DP, DQ) bind exogenous peptides processed in specialized cells (e.g., macrophages, dendritic cells) and present them to CD4 T cells for surveillance of extracellular pathogens. Many HLA genes have a high level of allelic polymorphism, allowing the human population to bind a wide range of peptides from potential pathogens. HLA molecules also bind peptides derived from self proteins, and T cell reactivity to these HLA-self peptide combinations is usually eliminated during early development, resulting in tolerance to self. When tolerance breaks down, activated T cells and autoantibodies attack self proteins and the tissues expressing them, causing autoimmune disease. More diseases are associated with the HLA than any other genomic region, and specific autoimmune diseases are associated with specific HLA alleles. The etiology of autoimmune disease is unknown, but HLA genes are generally the highest genetic risk factor. Susceptibility and resistance to a wide range of autoimmune diseases correlate with specific HLA class I and II alleles, and these associations differ among autoimmune diseases. Study of HLA alleles is aiding the understanding of autoimmune disease and the development of therapeutic strategies.

[0093] In canines, the equivalent to the HLA family of genes is termed the dog leukocyte antigen (DLA) region. Analyzing DLA genetics in pedigreed dog breeds provides defined subpopulations that, like certain human ethnicities and isolated genetic populations, show

strong correlations with specific autoimmune disorders. Mapping of the canine genome has lagged behind human and mouse genomes, but has received increasing scrutiny in the past decade. Analysis of 711,521 bp in the canine classical and extended MHC class II regions revealed 45 loci, including 29 predicted to be functionally expressed. In 2005, typing 360 dogs representing 25 AKC registered dog breeds identified broad DLA class II allelic diversity across breeds, with 31/61 published DLA-DRB1 alleles, 11/18 published DLA-DQA1 alleles, and 31/47 published DLA-DQB1 alleles identified among the 25 breeds tested. In contrast to the allelic diversity between breeds, within an individual breed the allelic diversity in DLA class II genes is severely limited. Some DLA alleles are shared by many breeds, whereas others are unique to a single breed or small related set of breeds. For example, seventeen of the 31 DRB1 alleles identified were found in only a single breed, and only 7 alleles were shared by = 7 breeds, including DLA-DRB1*00101 (16 breeds) and DLA-DRB1*01501 (19 breeds). DLA-DQA1*00101 and DLA-DQA1*00601 alleles were also shared by many breeds. Similarly, DLA-DQB1*00201 and DLA-DQB1*02301 were found in many breeds, shared by 17 and 18 breeds respectively. In individual pedigreed dogs, homozygosity at HLA alleles was common- 40% of dogs tested were homozygous at DLA-DRB1, 52% were homozygous at DLA-DQA1, and 44% were homozygous at DLA-DQB1. North American and European purebred dogs had similar frequencies of HLA alleles, consistent with founder effects, but the North American breeds may have lost some DLA class II diversity when established in North America. Sequencing HLA genes in other dog populations have revealed further diversity, including alleles shared with gray wolves. As genetic studies have become more refined, increasing instances of specific DLA alleles associated with autoimmune diseases have been documented, as discussed *infra.*

[0094] In one aspect of the invention, the autoantigens do not include one or more of the following diabetes antigens: GAD65, full-length IA-2, juxtamembrane domain (aa 605-682 of IA2). In another aspect of the invention, the autoantigens do not include one or more of the following dilated cardiomyopathy autoantigens: myosin heavy chain, alpha cardiac actin, mitochondrial aconitate hydratase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and brain glycogen phosphorylase (GPBB).

Neurological and Neuromuscular Disorders

Inflammatory Myopathy (IM)

[0095] Inflammatory myopathies are a group of muscle diseases characterized by chronic muscle inflammation, sometimes termed myositis, and muscle weakness. The three main types of inflammatory myopathy are polymyositis, dermatomyositis, and inclusion body myositis. Polymyositis affects skeletal muscles and rarely develops before age 18, with the majority of cases in patients between 31-61 years old. Progressive muscle weakness can cause difficulty walking, climbing stairs, swallowing, speaking, and reaching overhead objects. Dermatomysotis is a skin rash that precedes or accompanies progressive muscle weakness. Unlike polymyositis, it can accompany tumors of the breast, lung, or bowel. Some cases of dermatomyositis include calcium deposits under the skin or in the muscle, termed calcinosis. Inclusion body myositis resembles polymyositis but has an earlier age of onset, first appearing in children ages 2-15 years. Symptoms include proximal muscle weakness and inflammation, edema, muscle and abdominal pain, fever, contractures (shortened muscles or tendons around joints) and difficulty swallowing and breathing. Inclusion body myositis is more common in males, unlike polymyositis and dermatomyositis. Diagnosis of these conditions is based on symptoms and medical history, confirmed by elevated levels of certain muscle enzymes (e.g. creatine kinase) and autoantibodies, electromyography, ultrasound, MRI, and biopsy. The etiology of IMs is unknown, but HLA associations and recently discovered autoantibodies point to an autoimmune origin. Sporadic inclusion body myositis has been linked to HLA-DR3 (specifically to DRB1*0301) and other components of the ancestral haplotype HLA-A1, B8, DR3. Recent evidence suggests that detection of autoantibodies against certain proteins in about half of idiopathic IM cases correlates with patient subsets and clinical outcomes. For example, 23% of patients with juvenile dermatomyositis have detectable anti-p140 autoantibodies. Autoantibodies against aminoacyl-transfer RNA synthetases, anti-signal recognition particle, and Mi-2 are detected in other subsets of IM patients. Polymyositis and dermatomyostitis are treated first with high dose prednisone or other corticosteroids; patients unresponsive to prednisone are administered common immunosuppressant drugs such as azathioprine and methotrexate to reduce inflammation. Other treatments can include intravenous immunoglobulin, cyclosporine A, cyclophosphamide, and tacrolimus. There is no standard regimen for treating

inclusion body myositis as it is generally unresponsive to corticosteroids and immunosuppressive drugs.

[0096] Dogs also develop inflammatory myopathies, and investigation into their pathology and treatment are guiding therapeutic strategies in humans. Masticatory muscle myositis (MMM), an inflammatory disease affecting the muscles controlling chewing, is the most common inflammatory myopathy in dogs. This disease primarily afflicts large breed dogs, including German Shepherds and Cavalier King Charles Spaniels. A similar disease affects the eye muscles of some Golden Retrievers. Corticosteroids such as prednisone are the primary treatment for MMM, with decreasing doses for up to 4-6 months. Cases of polymyositis are also treated with corticosteroids as an anti-inflammatory and immunosuppressive strategy, with escalation to Cytoxan and Imuran for refractory cases. MMM is characterized by 2M fibers in muscles of the jaw, a type of fiber resembling proteins found on the surface of bacteria but nowhere else in the body. A study of 53 dogs with MMM, 32 with polymyositis, and 4 dogs with both suggest that both inflammatory myopathies are CD8+-mediated autoimmune diseases that initiate muscle fiber destruction, leading to the production of autoantibodies against myosin. Other studies of canine MMM identified autoantibodies against a novel member of the myosin binding protein-C family, named masticatory myosin binding protein-C, that is expressed only within masticatory muscle fibers and that is also expressed in human muscle. The discovery of muscle-specific autoantigens in canine inflammatory myopathies may guide the search for equivalent targets in human myopathies.

Myasthenia Gravis (MG)

[0097] Myasthenia gravis is relatively rare, with an estimated prevalence of 200-400 cases per million, approximately 36,000 – 60,000 cases in the U.S. (14, 15). MG is caused by defect on the muscle side of the neuromuscular junction (NMJ) resulting in suboptimal signaling and muscle weakness. In normal muscles, nerve impulses release acetylcholine which migrates across the NMJ and binds to acetylcholine receptors (AChR) on muscle, opening the ion channel formed by AChR subunits to cause sodium ion flux, membrane depolarization, and muscle contraction. Very rare cases of congenital MG are caused by functional mutations in one of the AChR subunits. Acquired MG is an autoimmune disorder of unknown etiology characterized by an immune response to the proteins on the muscle side of the NMJ. In 80-90% of cases patients develop antibodies against AChR that reduce the

density of functional receptors at the NMJ and cause complement-mediated damage to the postsynaptic membrane; 10-20% of autoimmune MG patients are seronegative for anti-AChR antibody and instead have antibodies against other NMJ components such as muscle specific kinase (MuSK) or ryanodine receptor (RyR). MG may be limited to ocular muscles, with symptoms including drooping eyelids (ptosis) and double vision (diplopia), or may extend to the limbs, diaphragm, oropharyngeal and other muscle groups with attendant difficulties in walking, swallowing, and breathing that can require assisted ventilation. MG is commonly treated with neostigmine or pyridostigmine, inhibitors of acetycholinesterase that permit prolonged presence of acetylcholine in the NMJ where it can bind to the limited AChRs. In some cases, immunosuppressive drugs such as prednisone, cyclosporine, mycophenolate mofetil, or azathioprine are added to acetylcholinesterase inhibitors to control the autoimmune response. Thymectomy, the surgical removal of the thymus, reduces symptoms in the 10-15% of MG patients with thymoma and may benefit other MG patients as well, although the benefit may not occur until 2-5 years post surgery.

[0098] MG is likely the most common canine neuromuscular disorder. As with the human version, canine MG symptoms include weakness in facial and extraocular muscles and weakness in the limbs that worsens with exercise. Other symptoms can include difficulty swallowing, an enlarged esophagus (megaesophagus), loss of tone and difficulty transporting food to the stomach, and regurgitation that can lead to aspiration pneumonia. As with human myasthenics, there are several diagnostic tests available to confirm MG in dogs. Diagnosis is often based on detection of serum antibodies against AChR in the serum; this test is available through the Comparative Neuromuscular Laboratory at the University of California, San Diego. Other diagnostics tests include decreased AChR levels in muscle biopsy, electromyography, X-ray to check for megaesophagus, and temporary improvement in clinical symptoms following administration of the short acting cholinesterase inhibitor edrophonium chloride (the Tensilon test). Over 90% of dogs diagnosed with MG have detectable anti-AChR serum titers, comparable to the frequency of human MG patients who are seropositive for AChR antibodies. In these human patients, and in animal models of MG induced by immunization with purified AChR and adjuvant, a high percentage of these AChR antibodies bind to a conformational epitope formed by amino acid residues 61-76 of the AChR alpha subunit, an area termed the major immunogenic region (MIR). Similarly, in canine MG 68 % of anti-AChR antibodies bind to the MIR. Other similarities include weakness in a limited set of muscles in some canines, termed focal MG, resembling the

restriction to extraocular muscles in some human MG patients. As with human MG, a subset of canine acquired MG cases include a thymoma, a tumor of the cranial mediastinum of the thymus. Thymectomy is the common treatment for human MG patients with or without thymoma, but it is not common practice for the treatment of myasthenic dogs and cats.

[0099] The average age of onset of acquired MG in dogs is 5 years. A comparison of incidence in pure bred and mixed breed dogs in 1,154 cases of canine MG recorded between 1991-1995 documented elevated risk of acquired (spontaneous autoimmune) MG for Akitas, German Shorthaired Pointers, Chihuahuas, Scottish Terriers and others in the Terrier group; Rotweillers, Doberman Pinschers, Dalmatians, and Jack Russell Terriers had a lower relative risk of acquired MG. Other sources cite an elevated risk of acquired MG in larger breeds, especially German Shepherd, Golden Retriever, and Labrador Retriever, whereas congenital MG was more common in Jack Russell Terrier, Springer Spaniel, and Smooth-Haired Fox Terrier. Two separate studies report a mortality rate of 17%. As with humans, the common treatment for canine MG is pyridostigmine (Mestinon), an acetylcholinesterase inhibitor that prolongs the presence of acetylcholine in the neuromuscular junction. Corticosteroids such as prednisone are administered if anticholinesterase therapy is not effective. Stronger immunosuppressants, such as azathioprine, are used only if corticosteroids are contraindicated due to diabetes mellitus, high blood pressure, concurrent infection, or if the case of MG is refractory to standard treatment. In many cases, therapeutic intervention may be unnecessary. Unlike human patients, nearly 90% of myasthenic dogs have a spontaneously remission within 18 months of disease onset, even without therapeutic treatment. In a study of 53 dogs with muscle weakness and positive AChR antibody titers, spontaneous clinical and immunological remission occurred in 47 of 53 dogs (88.7%) at an average time of 6.4 months; neoplasia was noted in all 6 of the dogs that did not spontaneously remit. During spontaneous remission, AChR titers either decline or fluctuate. However, vaccination against infectious agents can induce a recurrence of MG in dogs that were in spontaneous remission. The role of regulatory T cells in maintaining or re-establishing tolerance has recently become an area of intense research, and it may be instructive to monitor the balance between effector and regulatory T cells specific for AChR during MG onset and spontaneous remission in dogs. Such studies may indicate whether vaccination causes general effector T cell increases, overriding a remission driven by increasing regulatory T cells. If this proves the case, it may be prudent to avoid broad immunosuppression that may also decrease regulatory T cells and instead focus future therapeutic strategies on increasing the proportion of regulatory T cells.

Narcolepsy

[0100] Narcolepsy is a chronic neurological disorder caused by a dysregulation of sleep-wake cycles resulting in excessive daytime sleepiness and inappropriate, often sudden onset of sleep. Along with these irregular sleep episodes, narocleptics may also exhibit related symptoms including cataplexy, a sudden loss of voluntary muscle tone sometimes induced by strong emotions, vivid hallucinations during the onset or cessation of sleep, and brief episode of total paralysis at the start or end of the sleep cycle. The length of total sleep during a 24 hour period is similar in narcoleptic and normal sleep, but the number of sleep periods and ratio of non-REM to REM sleep are significantly different. A typical sleep cycle is 100-110 minutes, beginning with non-REM sleep and transitioning to REM sleep after 80-100 minutes. In contrast, narcoleptic patients may enter REM sleep within minutes of falling asleep and have a larger number of shorter sleep cycles distributed more sporadically through the day. Narcolepsy prevalence varies among populations, affecting 1 in 2,000 individuals in the U.S., 1 in 500,000 in Israel, and 1 in 600 in Japan. Most cases of narcolepsy first manifest between the ages of 10-25.

[0101] Without being bound by theory, narcolepsy is caused by reduced levels of hypocretin, a hormone that promotes wakefulness. These lower levels are due to a decrease in the neurons which secrete hypocretin in the brain. However, except in rare cases the hypocretin gene is not mutated in narcolepsy patients. Narcolepsy can occur in multiple family members, but these instances account for fewer than 10% of cases, and studies of twins indicate a strong influence of nongenetic factors, suggesting an environmental trigger. The first documented genetic association with narcolepsy was mapped to the human histocompatibility haplotype HLA-DR2, and was subsequently localized to the DQB1*0602 allele. More than 90% of narcoleptics with cataplexy have the DQB1*0602 allele, significant increase over the 25% frequency in Caucasian controls. Narcolepsy is also strongly associated with DQB1*0602 in Asians and African Americans, an unusually strict HLA allelic association. Based on this HLA association, it has been suggested that narcolepsy is an autoimmune reaction to an environmental trigger. Attempts to confirm an autoimmune pathology in narcolepsy have been challenging and controversial. Support for the autoimmune hypothesis comes from induction of narcolepsy-like symptoms in mice injected with antibodies from narcoleptic humans. However, a radioligand binding assay screening for autoantibodies against hypocretin, hcrt-1, and hcrt-2 detected comparably low frequencies in

the serum of narcoleptics with cataplexy (5%) and healthy controls (3%). In contrast, a recent study suggested that Tribbles homolog 2 (Trib2), an enriched transcript in hypocretin-producing neurons and an autoantigen in autoimmune uveitis, may be the elusive narcolepsy autoantigen. An ELISA assay detected high autoantibody titers to Trib2 in sera and CSF of narcolepsy patients, and this serum bound to >86% of hypocretin neurons in mouse hypothalamus. Further evidence of an autoimmune etiology comes from a recent genome wide association study of 807 HLA-DQB*0602 positive Caucasian narcoleptics and 1074 matched controls. The genetic analysis identified 3 markers in high linkage disequilibrium in an 18 Kb segment of the TCRA locus, a region of the T cell receptor gene encoding the joining segment, and another marker in the V segment of the T cell receptor beta (TCRB) locus. This study asserts an autoimmune origin of most human narcolepsy cases. Spontaneous narcolepsy was first described in dogs in the 1970s. However, in most dogs this is an autosomal recessive trait not associated with the canine histocompatibility complex, DLA. Nevertheless, the canine form of narcolepsy has been crucial to the understanding of the human condition. In 1999, studies of narcoleptic Doberman Pinscher and Labrador Retriever laboratory colonies established the linkage between narcolepsy and dysregulation of the hypocretin/orexin receptor (Hcrtr-2) gene. Despite differences in genetic origin, the naturally occurring canine model has been useful for the optimization of treatments for human narcolepsy patients.

Neuronal Ceroid Lipofucsinoses/Batten Disease

[0102] Neuronal ceroid lipofucsinoses (NCLs or CLNs) are a group of autosomal recessive neurodegenerative lysosomal storage disorders affecting children. As a group, NCLs are characterized by intracellular accumulation of lysosomal storage bodies resembling lipofucsin in neurons and other cells, leading to cellular degeneration, including retinal and brain atrophy. They are the most common progressive neurodegenerative diseases in childhood, with an incidence of one in 12,500 live births and approximately 440,000 carriers in the U.S. Subtypes are classified based on age of onset and responsible gene: Haltia-Santavuouri disease (infantile NCL, CLN1); Jansky-Bielschowsky disease (late infantile NCL, CLN2); Batten disease (juvenile NCL, CLN3); Kufs disease (adult NCL, CLN4); and two late infantile variant forms, CLN5 and CLN6. However, some physicians classify all NCLs as Batten disease. CLN1 encodes the lysosomal enzyme palmitoyl protein thioesterase (PPT1) a lysosomal protein thiolesterase and CLN2 encodes a lysosomal tripeptidyl protein

peptidase (TPP1). CLN 8 is associated with epilepsy and progressive mental retardation. CLN3 encodes a protein of unknown function which resides in the lysosomal membrane and co-localizes with synaptic vesicle proteins. Nearly three quarters of Batten disease patients carried a 1.02 kb deletion in CLN3 genes on both chromosomes, with missense mutations, nonsense mutations, deletions, insertions, and other defects in CLN3 accounting for the rest. The defective CLN3 leads to seizures, mental impairment, progressive loss of sight, speech, and motor skills, and is often fatal by the late teens or twenties. Batten disease patients have an autoimmune response to glutamic acid decarboxylase (GAD65). A survey of patient sera revealed anti-GAD65 autoantibodies in all 20 individuals tested. Glutamic acid decarboxylase is an enzyme responsible for converting the excitatory neurotransmitter glutamate to the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), and therefore anti-GAD autoantibodies could cause excess excitatory neurotransmitters, leading to seizures. Autoantibodies to GAD are also detected in other degenerative CNS diseases, including stiff-person syndrome, and cerebellar ataxia. These autoantibodies inhibit the activity of GAD, whereas autoantibodies to GAD detected in insulin dependent diabetes mellitus (IDDM, type 1 diabetes) are not inhibitory. The potential link between an autosomal disorder and an autoimmune response is intriguing and further study is needed in both patients and animal models to understand the cause and develop therapeutic interventions.

[0103] Hereditary NCLs have been reported in mice and several domestic animal species, including cattle, sheep, cat, and specific dog breeds. Dog breeds with reported occurrences of NCLs include English Setters, Tibetan Terriers, American Bulldogs, Dachshunds, Polish Lowland Sheepdogs, Border Collies, Dalmatians, Miniature Schnauzers, Australian Shepherds, Australian Cattle Dogs, and Golden Retrievers. NCLs in dogs are characterized by progressive degeneration in the CNS and accumulation of fluorescent material in nerve cells. Genomic sequences and transcripts of canine CLN2 (PPT1), CLN5, CLN6, and CLN8 are conserved relative to their human counterparts. NCLs in English setters are associated with a single point mutation in CLN8. The progressive neurodegeneration causes intractable seizures and death at approximately two years of age. A late onset form of NCL can occur in Tibetan Terriers and Polish Owczarek Nizinny (PON) dogs. A form of NCL discovered in Dacshunds is caused by a mutation in CLN2 (TPP1), resulting in a retinal degeneration that resembles the late infantile NCL in humans. The first documented case of NCL in Border Collies was recorded in 1980, and the responsible mutation is located in CLN5. Diagnostic

DNA tests are now available for American Bulldogs, Dachshunds, English Setters, and Tibetan Terriers.

[0104] Although there are mouse models of most NCLs, their limited size, lifespan, and relatively primitive nervous system are detractions for the testing of therapeutic approaches. The characterization of canine NCLs should provide a better understanding of disease pathology, including the role of autoantibodies, and a better opportunity to test experimental therapies to halt disease progression and correct genetic defects.

Skin Disorders

Pemphigus

[0105] Pemphigus is a group of rare autoimmune skin diseases characterized by chronic, often painful blistering. Pemphigus is caused by autoantibodies against desmoglein, the molecular "glue" that attaches adjacent epidermal cells via attachment sites termed desmosones. Autoantibodies binding desmoglein disrupt this connection, causing blisters that slough off leaving open sores. Several categories of pemphigus are classified based on the target autoantigen and the location of blisters and sores. Pemphigus vulgaris (common pemphigus) is caused by antibodies against desmoglein 3, resulting in a loss of cohesion between keratinocytes and the basal layer of the epidermis; severity is proportional to levels of desmoglein 3. Sores often originate in the mouth, impeding eating. Although it may occur at any age, pemphigus vulgaris usually begins in patients between ages 40-60, and is more common in Ashkenazi Jews. Genotyping of North American Caucasian non-Jewish and Ashkenazi Jewish pemphigus vulgaris patients revealed a strong HLA association to DRB1*0402 and DQB1*0503. Pemphigus foliaceus, the least severe form of pemphigus, is caused by autoantibodies against desmoglein 1. Because desmoglein 1 is expressed only on the top dry layer of skin, sores are superficial and generally less painful than pemphigus vulgaris. Another difference from pemphigus vulgaris is that sores do not form in the mouth; rather, they usually begin on the scalp and may spread to the chest, back, and face. Genomic comparison of 31 Caucasian pemphigus foliaceus patients and 84 healthy controls showed increased susceptibility associated with HLA-DR alleles DRB1*0102, DRB1*0402, DRB1*0406, and DRB1*1404. Paraneoplastic pemphigus is the least common and most severe form of pemphigous. This rare form accompanies some forms of cancer, including certain of lymphomas and leukemias. Painful sores occur on the mouth, lips, and esophagus,

and may also cause constrictive bronchiolitis in the lungs. Pemphigus is most generally treated with oral corticosteroids, especially prednisone or prednisolone. Effective management often requires high doses of these anti-inflammatory drugs. Immunosuppressive drugs are frequently added to the treatment regimen, including mycophenolate mofetil (CellCept), aziathioprine (Imuran), cyclophosphamide (Cytoxan), and methotrexate. Intravenous gamma globulin can be useful in severe cases, especially paraneoplastic pemphigus.

[0106] It is estimated that < 2% of dogs in the US have some form of autoimmune skin disease, though this may be an underestimate. Pemphigus vulgaris is the most common form, manifesting as lesions in the mouth and mucocutaneous junctions, the borders of haired skin and mucosal tissues (e.g. eyelids, lips, nostrils, anus, and genitals). These blisters are thin and easily ruptured. Human pemphigus vulgaris patients have autoantibodies against desmoglein 3 and desmoglein 1. Similarly antibodies recognizing desmoglein 3 were detected in 60% of sera from dogs with pemphigus vulgaris. These antibodies caused dissociation when incubated with sheets of normal human keratinocytes, confirming their role in pathogenesis. Pemphigus vegetans is characterized by thick, irregular, open lesions around the groin and between the legs and trunk. Pemphigus foliaceus is rare, generally confined to the face, ears, feet, and groin. Blisters are temporary, presenting with redness, crusting, and hair loss. As in human pemphigus foliaceus, dogs with this skin disorder have pathogenic IgG4 autoantibodies. These antibodies are difficult to detect by *in vitro* binding assays, but can be demonstrated bound to keratinocytes. Pemphigus erythematosis resembles foliaceus and is frequently limited to the nose. Autoantibody characterization in canine pemphigus is at an early stage. Further characterization of the autoantigens involved in these disorders may advance our understanding the pathology of human pemphigus autoimmunity.

Endocrine and Gastrointestinal Disorders

Thyroiditis

[0107] Thyroiditis is an inflammation of the thyroid gland. Hashimoto's thyroiditis is the most common form, characterized by destruction of follicles in the thyroid gland mediated by antibodies against thyroid peroxidase and/or thyroglobuin. This autoimmune disease is the most common cause of primary hypothyroidism in North America, with an average incidence of 1-1.5 case per 1,000 people. Grave's hyperthyroid disease is mediated by autoantibodies

against thyrotropin receptor, stimulating thyroid function and causing hypersecretion of thyroid hormones. In European countries, an atrophic form of autoimmune thyroiditis, termed Ord's thyroiditis, is more common than Hashimoto's thyroiditis. Onset of thyroiditis usually occurs between 45-65 years of age. As with many autoimmune diseases prevalence is higher in women, but the estimated ratio of 10:1 - 20:1 occurrence in women:men is unusually high amongst autoimmune disorders. There is also evidence of geographical and seasonal correlates with the disease, a feature seen in other autoimmune diseases as well. Many of the symptoms of autoimmune thyroiditis, such as fatigue, weight gain, depression, and constipation, also occur in other conditions and can lead to misdiagnosis. Advanced cases are treated with hormone-replacement therapy such as synthetic T4 hormone levothyroxine.

[0108] Hyperthyroidism is the most common endocrine disease in dogs. The majority of cases are autoimmune, resembling Hashimoto's thyroiditis in man, and as in human autoimmune diseases there is an association with expression of certain histocompatibility alleles. Genotyping of 173 hypothyroid dogs in a range of breeds showed a significant association with DLA-DQA1*00101, a rare DLA class II haplotype.

[0109] Similarly, analysis of 27 Doberman Pinschers affected by hypothyroid disease revealed an increase in a rare DLA haplotype in affected dogs compared to unaffected dogs; this haplotype is only found in Doberman Pinschers and Labradors. Larger dogs are at higher risk whereas toy and miniature breeds are rarely affected. In additions to Doberman Pinschers, breeds with reported susceptibility to thyroiditis include Golden Retrievers, Borzois, Giant Schnauzers, Akitas, Irish Setters, Old English Sheepdogs, Shetland Sheep Dogs, Skye Terriers, Beagles, Great Danes, and English Cocker Spaniels.

Type 1 Diabetes

[0110] Diabetes is a metabolic disorder affecting an estimated 23.6 million people in the U.S., roughly 7.8% of the population. Type 1 diabetes is an autoimmune disorder resulting from the destruction of insulin producing beta cells in the pancreas, resulting in a dysregulation of glucose metabolism. The onset of symptoms is relatively rapid, though the underlying destruction of beta cells may progress for a longer period of time before the effects are detectable. Symptoms of type I diabetes may include increased thirst and urination, continual hunger, blurred vision, weight loss, and fatigue. If untreated, patients may lapse into a diabetic coma, also known as diabetic ketoacidosis, which can be fatal. Type

2 diabetes is far more common, accounting for 90-95% of diabetes cases. It is not autoimmune, occurs at a later average age of onset, and is associated with obesity, a family history of diabetes, physical inactivity, and certain ethnic backgrounds. In type 2 diabetes, insulin is produced but for unknown reasons the body fails to use it properly. As in type 1 diabetes, the result is a buildup of glucose in the blood and inefficient energy metabolism and storage. Some clinicians and investigators also recognize a category termed "latent autoimmune diabetes in adults" (LADA). These cases generally begin after the age of 30 and may be a slower developing form of type 1 diabetes as patients have antibodies against the insulin producing beta cells and eventually the beta cells are destroyed. LADA may account for as many as 10% of type 2 diabetes cases.

[0111] Unlike many other autoimmune diseases, type 1 diabetes occurs equally among males and females. It occurs more frequently in Caucasian than non-Caucasian populations, and is rare in most African, American Indian, and Asian populations. Certain northern European countries, such as Finland and Sweden, have high rates of type 1 diabetes. It can develop at any age, but onset most often occurs during childhood. Although the etiology is unknown, type 1 diabetes clusters in families, with an overall genetic risk ratio of approximately 15. Concordance of type 1 diabetes amongst monozygotic and dizygotic twins is also evidence of a strong genetic component in susceptibility. Allelic variation in the HLA region accounts for 40-50% of the family clustering in type 1 diabetes. Numerous studies have demonstrated that specific alleles of HLA region genes DRB1, DQA1, and DQB1 are strongly associated with type 1 diabetes. Detailed analysis of 607 Caucasian families and 38 Asian families revealed several susceptible and protective DR-DQ halplotyes and a marked hierarchy in type 1 diabetes risk based on these haplotypes. The haplotype DRB1*0301-DQA1*0501-DQB1*0201 conferred the highest susceptibility with an odds ratio of 3.64, whereas the most protective haplotypes had associated odds ratios of 0.02. In addition to HLA, genome wide association studies (GWAS) have identified several other genes that contribute to susceptibility in Caucasians, including INS, CTLA4, PTPN22, and IL2RA/CD25. In GWAS comparisons of Caucasian and Asian type 1 diabetics, the disease association of CTLA4 is concentrated in the subset of diabetics with autoimmune thyroid disease in both ethnic populations, the association with IL2RA/CD25 is similar in both populations, and the association with PTPN22 is stronger in Asian patients. As with other human autoimmune disorders, susceptibility is strongly linked to alleles in the HLA region and to a lesser extent to a series of additional genes, some linked to inflammatory pathways.

Type 1 diabetes is preferably diagnosed based on measurement of blood glucose levels following 8 hours of fasting, where a level of 126 mg/dL is considered indicative. Although there is no cure, the disease can be managed by injections of insulin.

[0112] Diabetes is relatively common in dogs; for example, the estimated prevalence in the UK is 0.32%, and other studies report prevalence ranging from 0.005% to 1.5%. As in man, clinical symptoms of canine diabetes include excess thirst (polydipsia), urination (polyuria), weight loss, and high levels of glucose in the blood and urine. The onset of canine diabetes typically occurs between the ages of 5 and 12, with an average onset at 9 years, an older age of onset than the equivalent age for type I diabetes in humans. The classification system developed for human diabetes is not readily applied to canine diabetes. Some have characterized cases as either insulin dependent or non-insulin dependent, but nearly all diabetic dogs require insulin therapy. An alternative system classifies cases as either primary insulin deficient diabetes (IDD) or primary insulin resistance diabetes (IRD). In IDD, there is immune-mediated progressive loss of pancreatic beta cells. IRD is usually caused by antagonism of insulin function by other hormones, and may be secondary to other endocrine disorders. Pancreatitis, an inflammation of the pancreas, has been reported in 28-40% of diabetic dogs, but in another study, only 8 of 253 diabetic dogs had clinical and biochemical signs of pancreatitis. Separate studies over the past several decades attest to the heterogeneous pathology of canine diabetes, with some detecting similarities with human type 1 diabetes and insulitis in 6 out of 18 cases, while others report less evidence of pancreatic beta cell destruction than in humans and rodents. Autoantibodies to insulin, canine GAD65, and/or canine islet antigen-2 have been identified in some newly diagnosed diabetic dogs. Lymphocyte infiltration of pancreatic islets is only seen in a subset of dogs with adult-onset diabetes, and is not observed in dogs with juvenile-onset diabetes. Therefore, canine diabetes may be comparable to the latent autoimmune diabetes in adults (LADA) characteristic of the adult form of type 1 diabetes in man, which manifests a slow progressive destruction of beta cells. There is no evidence for the canine equivalent of human type 2 diabetes.

[0113] According to a database of >6,000 diabetic dogs from 24 veterinary schools in North America, susceptible breeds include Miniature Schnauzer, Bichon Frise, Miniature Poodle, Samoyed, and Cairn Terrier. Similarly, in a UK study Samoyed, Tibetan terrier and Cairn Terrier were found predisposed to diabetes. In contrast, Boxer and German Shepherd breeds

are less susceptible. Diabetes is more prevalent in female than male dogs, with a bias of 53-70% according to separate studies.

[0114] As with human autoimmune diseases including type 1 diabetes, canine diabetes originates from a complex interaction of susceptibility alleles and environmental triggers. As with human diabetics, canine diabetes has a seasonal pattern, with twice as many cases diagnosed between the winter months of November-January as between the summer months of July-September, perhaps reflecting common environmental triggers. Several genes are linked to diabetes susceptibility, with the strongest association found in alleles of the canine major histocompatibility complex, DLA. The first reported association was with the haplotype DLA DRB1*009, DQA1*001, DQB1*008. Subsequent DLA typing of 530 diabetic dogs and > 1,000 controls found associations between diabetes and 3 DLA haplotypes, with the strongest association seen with DLA DRB1*009, DQA1*001, DQB1*008. Haplotype DLA DRB1*009, DQA1*001, DQB1*008 is common in diabetes-susceptible breeds (Samoyed, Cairn Terrier, Tibetan Terrier), but rare in diabetes-resistant breeds (Boxer, German Shepherd, Golden Retriever). There is also evidence that DLA-DQA1*001 is associated with hypothyroidism in dogs. In contrast, one DLA-DQ haplotype, DQA1*004/DQB1*013, is significantly underrepresented in an analysis of 460 diabetic dogs, potentially indicative of a resistance alleles. As noted above, a series of genetic studies have identified several loci associated with type I diabetes in humans, including several in the Human leukocyte antigen (HLA) region, the insulin variable number tandem repeat, PTPN22, CTLA4, IL-4, and IL-13. Some of these loci were also identified in GWAS analyses of diabetic dogs. A study of 483 cases of canine diabetes and 869 controls identified 37 SNP allele associations- 13 were protective and 24 increased susceptibility. Genes associated with increased susceptibility included IFN-gamma (IFN-γ), IL-10, IL-2beta (IL-2β), IL-6, insulin, PTPN22, IL-4, and TNF-alpha (TNF-α). Most of the cytokines associated with increased risk of developing canine DM were from the Th2 subset with IL-4, IL-6, and IL-10 being predominant. Several other genes were protective, including IL-4, PTPN22, IL-6, insulin, IGF2, TNF-alpha (TNF-α). However, individual SNPs were variable between breeds, and in a few cases a SNP that was protective in some breeds was associated with increase risk in others. This disparity may reflect the relatively small sample size of individual breeds. It is also possible that canine diabetes has a different etiology in different breeds.

[0115] Historically, dogs have played a significant role in understanding diabetes pathology and in testing therapeutic strategies. Experiments in 1889 revealed that removal of the pancreas from healthy dogs led to polyuria and polydipsia, leading to the conclusion that the pancreas secretes an "anti-diabetogenic factor," subsequently identified as insulin, enabling the body to utilize glucose. In 1921 a diabetic dog was the first recipient of insulin therapy. Although the spontaneous NOD mouse model has been the focus for testing experimental drug strategies, the canine diabetes model may offer opportunities for preclinical testing of drugs and delivery systems in a larger animal model.

Inflammatory Bowel Disease (IBD)

[0116] Inflammatory bowel disease is a category of chronic inflammatory gastrointestinal tract disorders, including Crohn's disease and ulcerative colitis. Ulcerative colitis is a recurring inflammation of the mucosal layer of the colon, invariably involving the rectum and sometimes extending to other portions of the colon. Crohn's disease can affect any part of the gastrointestinal tract, with the majority of cases initiating in the terminal ileum. Whereas the inflammation in ulcerative colitis is restricted to the mucosal lining of the gut, Crohn's disease affects the entire bowel wall, which can lead to fibrosis, obstruction, and fistulas. Reported incidence rates in North America range from 2.2-14.3 cases per 100,000 person years for ulcerative colitis, and 3.1-14.6 cases per 100,000 person years for Crohn's disease. Based on a survey of 9 million insurance claims, the prevalence of ulcerative colitis in adults is 238 per 100,000 population, and the prevalence of Crohn's disease is 201 per 100,000. The incidence of both these major inflammatory bowel diseases is lower in Asia, Japan, and South America, and in Europe as well as in the U.S. the incidence decreases in more southern latitudes. Spondyloarthrpathies, a group of related diseases (e.g. ankylosing spondylitis, reactive and psoriatic spondyloarthritis, undifferentiated spondyloarthritis) are frequent extraintestinal manifestations of inflammatory bowel disease, with reported prevalence of 45.7% and 9.9% in cases of Crohn's disease and ulcerative colitis respectively. A recent genome wide association study of DNA samples from 1,052 ulcerative colitis patients and 2,571 controls, all of European ancestry, linked susceptibility to a region spanning BTNL2 to HLA-DQB1 and to the IL23R locus. Other genome studies show some overlap in the genes associated with both major inflammatory bowel diseases. Crohn's disease, bur not ulcerative colitis, is associated with genetic variations in NOD2 and ATG16L1, two genes that can affect the intracellular processing of bacteria. Both Crohn's disease and ulcerative colitis are

associated with variations in genes encoding IL-23R, and the IL12B, STAT3, and NKX2-3 gene regions.

[0117] IBD is a common digestive disorder in both cats and dogs. Canine and feline IBD share more characteristics with human IBD than with Johne's disease in cattle, with little evidence of bacteria in tissue and response to drugs such as corticosteroids and sulfasalazine. Incidence is similar in males and females, and onset peaks in middle aged dogs. Breeds that are at an increased risk for this disease include Boxers, German Shepherds, Soft Coated Wheaten Terriers, Rottweilers, French Bulldog, Doberman Pinscher, Mastiff, Alaskan Malamute, and Shar-peis. As with humans, the onset of IBD in dogs is hypothesized to originate from an abnormal intestinal response to commensal gut microflora. Toll-like receptors (TLR) may be central to the initial inflammatory response, as TLR-2, -4, and -9 are upregulated in dogs with IBD, paralleling the activation of TLR-4 noted in human IBD cases. Alterations in microflora may be critical as well, as IBD dogs have different small intestinal bacteria than healthy dogs. Similar shifts have been noted in the intestinal microflora of human IBD patients. Despite similarities in pathology and involvement of the innate immune system, there are some differences in the adaptive immune response to IBD in dogs and humans. In human IBD the Th1 lymphocyte subset is predominant, whereas in canine IBD there is a mixed activation of Th1 and Th2 lymphocytes. Corticosteroids (e.g. prednisone) are generally administered as the first course of treatment for cats diagnosed with IBD. Corticosteroids are also used for dogs when dietary management and sulfasalazine do not provide relief. Sulfasalazine, 5-A SA, mesalamine, and related compounds are the preferred treatment option for dogs with IBD primarily confined to the large intestine, but these drugs can affect tear production. Sulfasalazine and related compounds contain salicylates which can be very toxic to cats, and therefore corticosteroids are the primary therapeutic for cats. Metronidazole, an antibiotic and anti-inflammatory agent, can also be used alone or in combination with either corticosteroids or sulfasalazine. If corticosteroids fail, the immunosuppressive drugs azathioprine and cyclophosphamide can be used. Although parallels between IBD in humans and dogs are incompletely understood, further research may provide opportunities to test experimental therapeutics for the benefit of both species.

Addison's Disease

[0118] Damage to the adrenal glands causing an insufficient production of the hormones cortisol and aldosterone is termed primary adrenal insufficiency, also known as Addison's

disease. It affects 1-4 in every 100,000 people. Secondary adrenal insufficiency, a far more common condition than Addison's disease, results from failure by the pituitary gland to produce enough adrenocorticotropin (ACTH) to stimulate the adrenal glands to produce cortisol. Up to 80% of Addison's disease cases are caused by autoimmune destruction of the adrenal cortex, leading to adrenal insufficiency including deficiencies of mineralcorticoids (aldesterone) and glucocorticoids (cortisol) when >90% of the cortex is destroyed. Addison's disease is rare in Western European populations. As with other autoimmune diseases, it is a polygenic disorder, including a strong association with a specific major histocompatibility allele, in this case HLA DRB1*04 and DQ; other associations include specific alleles of CTL-4, Cyp27B1, VDR, and MIC-A and MIC-B loci.

[0119] Canine hypoadrenocorticism resembles the human condition and occurs in several breeds at frequencies ranging from 1.5-9%. The Portuguese Water Dog is one of the significantly affected breeds; analysis of 11,384 Portuguese Water Dogs between 1985-1996 indicated an incidence of 1.5%. Hypoadrenocorticism in this breed resembles human Addison's disease both in pathology and in genetic associations with susceptibility. Two disease-associated loci were identified on chromosome regions analogous to human HLA allele DRB1*04 and DRB1*0301 and to human locus CTLA-4. Nova Scotia Duck Tolling Retrievers are also at elevated risk for Addison's Disease, and genotyping of affected and unaffected dogs showed 7 different haplotypes with an elevated incidence of haplotype DLA-DRB1*01502/DQA*00601/DQB1*02301 in diseased dogs. Dogs with this adrenal gland disorder were also more likely homozygous in the susceptibility haplotype, and homozygous dogs had an earlier disease onset.

Bone and Joint Disorders

Rheumatoid Arthritis (RA)

[0120] Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease primarily attacking the synovial joints. The disorder is characterized by excess synovial fluid and overgrowth of synovial cells accompanied by articular cartilage destruction and joint stiffness. RA is the most prevalent autoimmune disease. Approximately 1% of the world's population is afflicted, with a threefold higher incidence in women than in men. Onset occurs most frequently between the ages of 40-50. A genetic predisposition to RA is associated with several alleles of the HLA-DRB1 locus, particularly the HLA-DRB1*04 subtypes:

DRB1*0401, *0404, *0405, and *0408 in Caucasians and subtypes DRB1*0101, *0102, and *1001 in other ethnic groups. All RA-associated HLA-DRB1 alleles encode related amino acid sequences in positions 70-74, the third hypervariable region: QKRAA (*0401). QRRAA (*0404, *0405, *0408, *0101, *0102), or RRRAA (*1001). This "shared epitope" occurs in at least one HLA-DRB1 locus of 80-90% of Caucasian RA patients.

[0121] Canine arthritis is relatively common, with reported incidence as high as 65% in dogs over 6 years of age. Up to 90% of these cases are osteoarthritis, with rheumatoid arthritis accounting for the rest. RA most commonly occurs in toy or small breeds, generally between the ages of 5-6. As with human cases, there is a strong link between susceptibility and certain genes in the major histocompatibility complex. In one genomic study, DNA samples from 61 dogs with clinically diagnosed small-joint polyarthritis and from 425 controls were compared. Several DLA-DRB1 alleles were associated with increases risk for RA, including DLA-DRB1*002, DRB1*009, and DRB1*018. A conserved amino acid motif, QRRAA/RKRAA found in the third hypervariable region of most HLA-DRB1 alleles of human RA patients was also noted in DLA-DRB1 alleles associated with canine RA. Corticosteroid treatments result in clinical remission in about 50% of cases. In more severe cases, treatments with Cytoxan or Imuran are administered to induce remission.

Circulatory Disorders

Autoimmune Hemolytic Anemia (AIHA) aka Immune-Mediated Hemolytic Anemia (IMHA)

[0122] There are many types of hemolytic anemia, defined as anemia caused by the lysis of red blood cells. Some forms are inherited and result from defects in erythrocyte structure, including sickle cell anemia, Thallasemia, and hereditary spherocytosis. In contrast, acquired hemolytic anemias are not inherited and can arise from exposure to toxic chemicals and drugs, antiviral agents (e.g. ribavirin), physical damage, infections, and immune disorders. Autoimmune hemolytic anemia (AIHA) accounts for over half of all hemolytic anemias. In AIHA the autoantibodies fix complement and lyse red blood cells, lowering the hematocrit and causing anemia and weakness. Evidence of AIHA includes elevated serum bilirubin, lactic dehydrogenase, and reduced plasma haptoglobin due to red cell destruction, and elevated levels of circulating reticulocytes and erythroid hyperplasia in the bone marrow in compensation for the cell loss. In some cases, AIHA is associated with another underlying disease, such as systemic lupus erythematosus (SLE) or chronic lymphocytic leukemia

(CLL); approximately 11% of CLL patients with advanced disease develop AIHA. Treatment regimens are predicated on whether the autoimmune attack is mediated by IgG or IgM antibodies. In the case of IgG-associated AIHA, cortisone and other immunosuppressive drugs are recommended. IgM autoantibodies are less responsive to cortisone, and the form of AIHA mediated by this isotype is sometimes referred to as cold agglutinin disease because binding to red cells occurs at lower temperatures. When the body temperature drops from 37 degrees Celsius to 28-31 degrees Celsius, as can occur in the extremities in winter months, IgM antibodies in this form of AIHA can bind to the polysaccharide region of glycoproteins (typically the I, i, and Pr antigens) on the surface of red blood cells. In such cases, avoidance of cold temperatures is recommended and folic acid supplements are administered to boost red blood cell production.

[0123] Autoimmune hemolytic anemia is the most common canine immune-mediated disease, but is uncommon in cats. Clinical signs include weakness, lethargy, anorexia, increased heart rate and respiration, pale mucous membranes, and in more severe cases fever and jaundice (icterus), a yellow discoloration of the gums, eyes, and skin, due to a buildup of bilirubin, a breakdown product of hemoglobin. The target membrane antigens in canine AIHA include the anion exchange molecule (band 3), the cytoskeletal molecule spectrin, and a series of membrane glycoproteins (glycophorins). As with human AIHA, the standard diagnostic is detection of antibody bound to erythrocytes based on the Coombs' test. Cases occur in clusters and onset can be seasonal- in one study, 40% of cases were diagnosed between May and June, suggesting a possible viral etiology. The median age of onset is 6.4 years, and females are more commonly affected. The acute form of AIHA has a breed association with Cocker Spaniels. Like other autoimmune disorders, susceptibility to AIHA is associated with specific alleles encoded in the canine histocompatibility complex, DLA. Genotyping of 108 dogs with Coombs' positive IMHA identified two DLA haplotypes increased on dogs with IMHA: DLA DRB1*00601, DQA1*005011, DQB1*00301 and DLA DRB1*015, DQA1*00601, DQB1*00301. Most affected dogs are maintained on corticosteroids for the rest of their lives, most often prednisone. In some cases, Cytoxan (cyclophosphamide), cyclosporan A, or Imuran (azathioprine) may be added to the therapeutic regimen, though some studies suggest that these supplemental drugs have no added value. A range of other drugs, including danazol, aziothioprine, cyclophosphamide, or cyclosporine A, are sometimes co-administered with glucocorticoids to reduce the steroid dose and side effects.

Immune-Mediated Thrombocytopenia (IMT)

[0124] Thrombocytopenia is a drop in the platelet count; in immune-mediated thrombocytopenia (IMT), this is the result of antibody and complement-mediated destruction of platelets within the reticuloendothelial system (spleen, bone marrow, and liver).

[0125] IMT is relatively common in dogs, but uncommon in cats. Symptoms include hemorrhages of skin and mucous membranes, bruising, excessive bleeding following trauma, surgery, or estrus, and blood in the urine or stool. Approximately 70% of all thrombocytopenia cases in dogs are apparently of autoimmune origin. The target membrane antigens in canine ITP are the platelet membrane glycoproteins GPIIb and GP111a. Cases of IMT may occur in isolation or may occur in combination with immune-mediated hemolytic anemia or systemic lupus erythematosus. Most cases occur in middle aged dogs, and female are afflicted more commonly than males. Diagnosis is hampered by the lack of definitive tests for canine IMT. Like other canine autoimmune disorders, it is usually treated with high doses of immunosuppressive corticosteroids, especially prednisone. Unresponsive cases are also treated with cyclophosphamide and vincristine, the latter drug enhancing thrombopoiesis as well as suppressing phagocytosis of antibody-coated platelets by macrophages.

Immune-Mediated Neutropenia (IMN)

[0126] Immune-mediated neutropenia (IMN), also known as autoimmune neutropenia, resembles the more common immune thrombocytopenic purpura, a neutropenia deficiency in children. Like other autoimmune diseases, the etiology is unknown, though some studies suggest an association with parvovirus B19 infection. The autoimmune response is mediated by antibodies (generally IgG) binding to cell surface antigens on neutrophils. The neutrophil glycosylated isoforms of Fc-gamma-IIIb or FcγIIIb (CD16b), a glycoprotein tethered to the membrane through a glycosylphosphatidlyinositol anchor, is a common target. Antibodies are also often directed against the human neutrophil antigen (HNA), especially HNA-1. In some clinical cases a small number of mature neutrophils are detectable, suggesting that immune attack occurs in the peripheral circulation rather than the bone marrow. Serum levels of granulocyte colony-stimulating factor (G-CSF) are normal, but levels of ICAM-1, TNF-a, and IL-1b are inversely correlated with the neutrophil count, suggesting a low degree of inflammation. IMN is often associated with systemic immune-mediated disorders, including systemic lupus erythematosus (SLE), rheumatoid arthritis, and Felty's syndrome. Over half of

SLE patients also have neutropenia, and many more have detectable antibodies bound to neutrophils.

[0127] IMN is relatively uncommon in both dogs and cats, accounting for <1% of all cases of neutropenia in dogs. It was initially documented in 1983 and subsequently few reports have appeared in the literature. Affected animals may present with anorexia, pyrexia, and lethargy, but a definitive diagnosis requires demonstration of anti-neutrophil antibodies, and such tests are not readily available. Immunosuppressive doses of corticosteroids such as prednisone generally produce a rapid rebound in circulating neutrophil counts within 48-72 hours. Approximately 25% of dogs and humans with IMN also have thrombocytopenia.

Multi-Systemic Disorders

Systemic Lupus Erythematosus (SLE)

[0128] Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by antinuclear antibodies (ANA), circulating immune complexes, and activated complement. Other hallmarks include decreased CR1 expression, defective Fc receptor function, and deficiencies in early complement components (e.g. C4A). SLE is a multi-organ disorder, causing widespread vascular lesions and also potentially affecting joints, skin, kidney, brain, lung, heart, serosa, and the gastrointestinal tract. The reported annual incidence of SLE in the U.S. varies from 6 to 35 new cases per 100,000 population in low-risk to high-risk groups. In Northern Europe, the rate is lower, approximately 40 per 100,000. Individuals of non-European descent may have a higher frequency and greater severity of SLE, ranging as high as 159 per 100,000 individuals of Afro-Caribbean descent. The incidence of SLE in the U.S. increased over the two decades from 1995 to 1974 from 1.0 to 7.6; it is not clear whether this increased frequency is due to improved diagnostic accuracy, changing demographics, environmental changes, or a combination of these and other factors.

[0129] Estimates of the prevalence of SLE in the U.S. also vary, ranging from 250,000 to 500,000 total cases, but estimated as high as 1-2 million based on a telephone survey commissioned by the Lupus Foundation of America. Regional differences in prevalence may reflect the impact of environmental and/or ethnic variations. For example, a survey of women in the greater Birmingham, Alabama metropolitan area reported a prevalence of 500 per 100,000. SLE disproportionately affects women of child bearing age; 60% of SLE patients

experience onset between puberty and the fourth decade of life, and within the age range the ratio of females to males is 9:1; in younger and older patients, the ratio is 3:1.

[0130] The etiology of SLE is unknown, but based on family histories, genetic analysis, and geographic distribution its initiation appears influenced by genetic predisposition, sex hormones, and environmental trigger(s). Occurrence of SLE in monozygotic twins is evidence of a hereditary component, but the moderate concordance rate of 25-60% suggests that other factors are also responsible for the disorder. Like other autoimmune disorders, the strongest genetic association is with genes encoded in the human major histocompatibility complex, HLA. SLE patients have a statistically increased percentage of HLA-DR2 and HLA-DR3 alleles, and there is also an increased frequency of the extended haplotype HLA-A1, B8, DR3. Other genes cited as risk variants associated with SLE include IRF5, PTPN22, STAT4, ITGAM. BLK, TNFSF4, and BANK1.

[0131] Diagnosis of SLE presents several challenges. Symptoms and affected organ involvement are variable: 80% of SLE cases affect skin and joint; 90% affect the musculoskeletal system; 80% affect skin, often including a characteristic butterfly shaped rash across the bridge of the nose and cheeks; 50% include alopecia; 50% have inflammatory serositis of the pleura, pericardium, or peritoneum; 10% have hemolytic anemia; 50% have neuropsychiatric complications, including seizures in 25% of cases. Detection of ANA in the serum can indicate SLE, but 5-10% of patients are seronegative. Furthermore, 25-40% of normal, healthy adult females may be transiently ANA positive without developing SLE or other connective tissue disorders. Therefore, proper diagnosis requires a panel of supporting tests that slow definitive identification and treatment. As symptoms of SLE vary, so do the treatments. Disease-modifying anti-rheumatic drugs (DMARDS) reduce the frequency of flares, including methotrexae and azathioprine. Hydroxychloroquine, an FDA-approved antimalarial drug, is also administered. For severe glomerulonephritis, patients are prescribed cyclophosphamide. Despite the broad and serious organ involvement, the prognosis has improved in recent decades, and the ten year survival of diagnosed SLE patients now exceeds 80-90%.

[0132] As with the human autoimmune disorder, canine SLE targets multiple organs and shows a genetic predisposition. Nova Scotia duck tolling retrievers are predisposed to SLE-related diseases, including immune-mediated rheumatic disease (IMRD) and steroid-responsive meningitis-arthritis (SRMA). IMRD symptoms resemble those in human SLE,

including persistent lameness, stiffness after resting, and joint pain. The majority of IMRD-afflicted dogs have antinuclear autoantibody (ANA)-reactivity. Comparative sequencing of dogs with IMRD (51 cases), SRMA (49 cases), and healthy controls (78 cases) revealed that homozygosity for the DLA risk haplotype DRB1*00601/DQA1*005011/DQB1*02001 increased the risk for IMRD (OR = 4.9; ANA-positive IMRD, OR = 7.2) relative to other genotypes. The risk haplotypes contains the five amino acid epitope RARAA previously identified as a shared epitope for human HLA-DRB1 alleles rheumatoid arthritis.

[0133] Discoid lupus is a subset of SLE characterized by a scarring skin disease and patients usually lack ANA or any other autoantibodies. Symptoms usually remain localized, spreading to systemic illness in only about 10% of cases. The canine equivalent, discoid lupus erthematosis, is considered a benign form of systemic lupus. It is primarily a facial dermatitis, most common in the Collie, German Shepherd, Shetland Sheepdog, German Shorthair Pointer, Siberian Huskie, Akita, Chow Chow, Brittany Spaniel, and Sheltie. Discoid lupus erthematosis also shows gender disequilibrium, with 60% of cases in females.

[0134] Additional autoimmune and neurodegenerative diseases are taught in various publications, for example, Lewis R, et al., "Autoimmune Diseases In Domestic Animals," Annals of the New York Academy of Sciences, Volume 124 Issue Autoimmunity-Experimental and Clinical Aspects: Part I, Pages 178 – 200 (Published Online: 16 Dec 2006).

Infectious Disease

[0135] Infectious diseases are another class of spontaneously occurring disease which is observed in companion animals. The use of companion animals, such as dogs, with spontaneously occurring infectious disease as an animal model for studying various infectious diseases is beneficial for several reasons. In one aspect, the creation of additional antibiotic resistance strains of infectious agents is minimized and/or avoided. In another aspect, the creation of mutant infectious agents with undesirable characteristics, for example, "supershedder" strains of *Salmonella enterica* Serovar Typhimurium, is minimized and/or avoided.

[0136] Spontaneously occurring infectious diseases observed in companion animals include, but are not limited to, influenza, septicemia (e.g., Klebsiella pneumoniae septicemia), bacterial infections (e.g., Staphylococcus aureus, other Staph infections, E. coli and enterococci), Pseudomonas aeruginosa, Leishmania infantum, Brucellosis, and Coccidiosis.

[0137] One or more aspects of infectious diseases, such as antigens, pathogens and parts thereof, can be examined at the same time in companion animals with spontaneously occurring infectious diseases to provide valuable information for the design of therapeutics or diagnostics (e.g., imaging techniques, etc.) to combat infectious diseases.

Other Diseases/Disease States

[0138] The invention also provides platform technologies for studying any of the hereditary genetic disease that occur in canines. See, for example, Online Mendelian Inheritance in Animals at <www.omia.angis.org.au> which catalogs genes, inherited disorders and traits in various animals, including dogs. Canines experience approximately 450 hereditary diseases (Ostrander et al., *Am J Hum Genet* 61:475-480 (1997). Using the canine model system of the invention, various modalities (e.g., multiple antigens) can be studied in approximately 220 of these 450 canine hereditary diseases that parallel the same disease or disease states in humans. Examples of such hereditary disease include, but are not limited to, nephropathy, kidney disease, narcolepsy, retinal degeneration, hemophilia, and muscular dystrophy.

[0139] In another aspect, the invention provides for a companion animal model system, such a canine or feline, of spontaneous allergies, hypersensitivity (including delayed type hypersensitivity), and asthma as a platform technology for examination of one or more aspects of allergy, hypersensitivity or asthma. In one embodiment, cats spontaneously develop idiopathic asthma. As such, the feline model of spontaneously developed asthma is useful for investigating underlying biological mechanisms (e.g., immune cell involvement, airflow obstruction) of asthma development, progression and recurrence. Understanding the biological underpinning of asthma can be used for development treatments and other agents that can ameliorate the symptoms of asthma.

[0140] In another aspect of the invention, the platform technologies provided herein are applied to tolerizing vaccine and other toleragens. For example, food allergies are common in the daily setting of schools, homes and workplaces. Extreme hypersensitivity to nuts, such as peanuts, may be investigated using the platform technologies described herein. Tolerization to various food products (nuts, eggs, dairy, etc) can be investigated on a single platform or combination of the platforms (e.g., multiple food allergy antigens) in the companion animal model system.

[0141] Similarly, the invention provides for a companion animal model system, such as canine or feline, for investigating neurological disease and potential therapeutic or diagnostic agents. For example, the pathogenesis of various neurological diseases or conditions is observed in a companion animal model system in which spontaneously occurring neurological disease occurs. One non-limiting example is beta-amyloid accumulation in canine brains (e.g., beagle). Beta-amyloid accumulation is involved in the development and/or progression of Alzheimer's disease. Other neurological diseases or conditions for which the platform technologies of this invention are contemplated include, but are not limited to, Parkinson's disease, Amyotrophic lateral sclerosis, cognitive impairment, aneurysms, degenerative myelopathy, myasthenia gravis, tremors, and seizures.

Machine Readable Storage Media

[0142] The data generated by using the companion animal model system can be stored on machine readable media. Such data can include information about the biological responses, physiological parameters of responses to agents that are administered, antigen(s) which are identified, structure of agents that are administered, structure (including sequences, both nucleic acid and amino acid) of antigens or immunogens. This information can be stored on machine readable media and be further utilized to generated novel agents that have similar structure to known agents that have elicited a desirable immune response in the canine model system. In this manner, novel agents that have desirable biological effects are identified for potential use in treatment of humans.

[0143] In another aspect of the invention, the machine readable storage media can be used for educational purposes, for example, instruction materials or manuals. In another aspect, the invention contemplates promoting collaborations between individuals, such as scientists, philanthropists, and veterinarians. Such collaboration can be fostered by dissemination of the data generated by use of companion animal model system of spontaneously occurring diseases. This dissemination can be accomplished by distribution of this data on tangible media, for example, a machine readable storage media.

[0144] Accordingly, the invention thus further provides a machine-readable storage medium including a data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, displays a graphical three-dimensional representation of any of the molecule or molecular complexes of this invention

that have been described above. In a preferred embodiment, the machine-readable data storage medium includes a data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, displays a graphical three-dimensional representation of a molecule or molecular complex.

[0145] For example, a system for reading a data storage medium may include a computer including a central processing unit ("CPU"), a working memory which may be, e.g., RAM (random access memory) or "core" memory, mass storage memory (such as one or more disk drives or CD-ROM drives), one or more display devices (e.g., cathode-ray tube ("CRT") displays, light emitting diode ("LED") displays, liquid crystal displays ("LCDs"), electroluminescent displays, vacuum fluorescent displays, field emission displays ("FEDs"), plasma displays, projection panels, etc.), one or more user input devices (e.g., keyboards, microphones, mice, touch screens, etc.), one or more input lines, and one or more output lines, all of which are interconnected by a conventional bidirectional system bus. The system may be a stand-alone computer, or may be networked (e.g., through local area networks, wide area networks, intranets, extranets, or the internet) to other systems (e.g., computers, hosts, servers, etc.). The system may also include additional computer controlled devices such as consumer electronics and appliances. This allows for collaborative efforts to be pooled for better results.

[0146] Input hardware may be coupled to the computer by input lines and may be implemented in a variety of ways. Machine-readable data of this invention may be inputted via the use of a modem or modems connected by a telephone line or dedicated data line. Alternatively or additionally, the input hardware may include CD-ROM drives or disk drives. In conjunction with a display terminal, a keyboard may also be used as an input device.

[0147] Output hardware may be coupled to the computer by output lines and may similarly be implemented by conventional devices. By way of example, the output hardware may include a display device for displaying a graphical representation of an active site of this invention using a program such as QUANTA. Output hardware might also include a printer, so that hard copy output may be produced, or a disk drive, to store system output for later use.

[0148] In operation, a CPU coordinates the use of the various input and output devices, coordinates data accesses from mass storage devices, accesses to and from working memory,

and determines the sequence of data processing steps. A number of programs may be used to process the machine-readable data of this invention. Such programs are discussed in reference to the computational methods of drug discovery as described herein. References to components of the hardware system are included as appropriate throughout the following description of the data storage medium.

[0149] Machine-readable storage devices useful in the present invention include, but are not limited to, magnetic devices, electrical devices, optical devices, and combinations thereof. Examples of such data storage devices include, but are not limited to, hard disk devices, CD devices, digital video disk devices, floppy disk devices, removable hard disk devices, magneto-optic disk devices, magnetic tape devices, flash memory devices, bubble memory devices, holographic storage devices, and any other mass storage peripheral device. It should be understood that these storage devices include necessary hardware (e.g., drives, controllers, power supplies, etc.) as well as any necessary media (e.g., disks, flash cards, etc.) to enable the storage of data.

[0150] The following examples are provided for illustrative purposes only and are not meant to limit the scope of the invention in any manner.

EXAMPLES

Example 1 Preparation of Delivery Vehicles For Use in the Animal Model of Spontaneously Occurring Diseases

[0151] Delivery vehicles that selectively seek out a cancer cell instead of a normal cell is prepared by using molecular or physical property or biomarker properties that allows for selective targeting. In this example, the delivery vehicle is a liposome, liposome-like particle or nanoparticle. The liposomes can be charged (e.g, cationic) or non-charged. These liposomes, liposome-like particles or nanoparticles are made both with and without receptor or ligands or biomarkers.

[0152] Liposomes, liposome-like particles or nanoparticles are also made which carries one or more oncolytic viruses (for example, any of the oncolytic viruses discussed in "Viral Therapy of Cancer," Harrington, Vile and Pandha, co-editors, Wiley Publishing, 2008). Other liposomes, liposome-like particles or nanoparticles are made which carry prodrugs and RNAi targets, with or without immune cells or chemotactic agents or immune modulators.

[0153] The prodrugs are incorporated into the liposomes, liposome-like particles or nanoparticles and used for testing in the animals with spontaneously occurring diseases. Non-limiting examples of products are cancer therapeutics and other drugs for cancer. Similarly, RNAi targets are incorporated into the liposomes, liposome-like particles or nanoparticles. For RNAi targets, proto-oncogenes and oncogenes, the RNAi(s) serves to turn off, block, or reduce the activation of proto-oncogenes and oncogenes. For tumor suppressors, the RNAi(s) serves as an agonist to turn on or increase the activity of the tumor suppressors.

[0154] The liposomes, liposome-like particles or nanoparticles of this example are also packaged with immune modulators, which include cytokines, chemokines, exosomes (small particles secreted by various immune cells, such as mastocytes, T and B lymphocytes, dendritic cells, platelets) or immune factors that promote differentiation/maturation/clonal expansion of immune cells (e.g., CTLA-4). Immune modulators that are incorporated into the liposomes, liposome-like particles or nanoparticles of this example can also target the immunosuppressor cells (e.g., T regulatory cells or MDSCs) to potentiate cancer immunotherapy.

[0155] Liposomes, liposome-like particles or nanoparticles of this example are also packaged with factors that affect epigenetics, for example, methylation, prenylation, acetylation and de-acetylation (e.g., histone acetyltransferases (HATs) and histone deaceytlases (HDACs)), chromatin modifications, X-inactivation, and imprinting.

[0156] Liposomes, liposome-like particles or nanoparticles of this example are engineered with various molecular properties that are helpful to make these delivery vehicles more effective. Cancer antigens and other types of biomarkers (metabolic markers) are examples of molecular properties. See Example 3 for more details on cancer antigens. Another molecular property is ligand binding. Pre-metastatic niches are targeted by using the appropriate ligand for binding. Similarly, metastatic niches are also targeted. Some markers are used for certain types of cancer. For example, the Axl receptor is used to target pancreatic cancer since it is expressed >50% in metastatic pancreatic cancer. *Cancer Biol Ther.* 8(7):618-26 (2009). An example of a metabolic marker is formerly N-linked glycopeptides that change in abundance upon cAMP treatment in glioblastomas. *Proteomics* 9(3):535-49 (2009).

[0157] Liposomes, liposome-like particles or nanoparticles of this example are also engineered by using various physical properties. Tumor tissues have a different physical gradient than non-tumor tissue. Pressure gradient of tumor versus non-tumor tissues are measured by standard techniques known to those of skill in the art. The liposomes, liposome-like particles or nanoparticles as described above are made for preferential targeting to tumors and tumor-bearing regions of the body by following the pressure gradient of the tumors.

Example 2 Timing and Dosing of Delivery of Agent(s)

[0158] In this example, a cohort of a homogeneous or heterogeneous canine population is used as own control. The dosing of one or more agents under investigation is about one week in between doses. The order of delivery between cancer therapy and immune modulator is varied and the biological responses are measured and/or monitored. In one group of canines, cancer therapy is administered first and then the immune modulator(s). In another group of canines, the immune modulator(s) is administered first and then cancer therapy.

[0159] In another group of animals, the order of delivery of immune modulators with or without chemotaxis agent is switched and biological responses are then measured.

Example 3 Canine and Cancer Antigen/Biomarkers

[0160] Multiple cancers antigens and/or biomarkers are used for translational studies in various combinations with each other. For osteosarcoma, cancer antigens and/or biomarkers that are examined include but are not limited to: the antigen that is bound by monoclonal antibodies TP-1 and TP-3 (which detect an antigen expressed on the cells of human osteosarcoma), *erbB-2* (human epidermal growth factor receptor 2/neu) proto-oncogene, vimentin, osteopontin, PCNA, p53, MMP-2 and MMP-9.

[0161] For lymphoma (e.g., non-Hodgkin lymphoma), antigens and/or biomarkers that are examined include but are not limited to: CD3 antigen (*J Vet Diagn Invest* 5:616-620, 1993), T200 (homologue of the lymphocyte differentiation antigen)(*Can J Vet Res.* 51(1): 89–94, 1987), and the antigen that is bound by canine lymphoma monoclonal antibody 231 (*Cancer Therapy,* Vol 7, 59-62, 2009).

[0162] For hemangiosarcoma, antigens and/or biomarkers that are examined include but are not limited to: c-kit, CD34, CD133, CD45 (*Exp Hematol.*, 34(7):870-8, 2006), factor VIII-related antigen, ICAM-1, avß3 integrin (*Research in Veterinary Science*, 81(1): 76-86, 2006), VEGF receptors 1 and 2, CD31, CD146, and avß3 integrin (*Neoplasia*, 6(2): 106–116, 2004).

[0163] For mammary cancer, antigens and/or biomarkers that are examined include but are not limited to: Receptor-binding cancer antigen expressed on SiSo cells (RCAS1) (*Journal of Veterinary Medical Science*, 6 (6): 651-658, 2004), Sialyl Lewis X and T/Tn (*Vet Pathol* 46:222-226, (2009).

[0164] For testicular cancer, antigens and/or biomarkers that are examined include but are not limited to: proliferating cell nuclear antigen (PCNA)(*Journal of Comparative Pathology*, 113(4): 301-313, 1995), GATA-4 (transcription factor expressed in Sertoli cells and less commonly in Leydig (interstitial) cells) (*Veterinary Pathology*, doi:10.1354/vp.08-VP-0287-R-BC, 2009), inhibin-alpha and vimentin (*J. Vet. Sci.*, 10(1), 1-7, 2009).

[0165] For mast cell cancer, antigens and/or biomarkers that are examined include but are not limited to: CD117 (*BMC Vet Res.* 3:19, 2007), chromosome nucleolar organizer regions stained with silver (AgNORs), and anti-proliferating cell nuclear antigen (PCNA)(*Veterinary Pathology*, Vol 31, Issue 6, 637-647, 1994).

[0166] For bladder cancer, antigens and/or biomarkers that are examined include but are not limited to: V-TBA, or urinary tumor bladder antigen (*Am J Vet Res.* 64(8):1017-20, 2003) and basic fibroblast growth factor (bFGF).

[0167] For prostate cancer, antigens and/or biomarkers that are examined include but are not limited to: prostatic phosphatic acid antigen, prostate specific antigen (PSA), prostate specific membrane antigen (PMSA), and downregulation of epithelial Na, K-ATPase expression (*Cancer Cell Int.* 3:8, 2003).

[0168] For melanoma, antigens and/or biomarkers that are examined include but are not limited to: canine melanoma antigen recognized by the murine monoclonal antibody IBF9 (*Am J Vet Res.* 58(1): 46-52, 1997), S100, human melanosome specific antigens (HMSA) 1 and 5, neurone specific enolase (NSE), vimentin and IBF-9 (http://www.vetscite.org/publish/articles/000038/index.html).

[0169] For leukemia, antigens and/or biomarkers that are examined include but are not limited to: rearrangement of TCR Vß genes (e.g., detection of seven distinct canine TCR Vß genes)(*Veterinary Immunology and Immunopathology*, Vol. 69, Issues 2-4, Pages 113-119, 1999)

[0170] For lung carcinoma, antigens and/or biomarkers that are examined include but are not limited to: proliferating cell nuclear antigen (PCNA) and Ki-67 (MIB1) proteins (*Journal of Comparative Pathology*, 120(4): 321-332, 1999)

Example 4 Cancer Associated with Chronic Inflammation

[0171] Dogs and cat with spontaneously occurring chronic inflammation are used to study the various disease states and disease progression of chronic inflammation. This information is translated to helping humans with chronic inflammation as well as helping the dogs and cats with alleviating the symptoms of chronic inflammation. Without being bound by theory, breaking the loop that feeds back to generate more chronic inflammation can help to lessen, and in some cases, prevent or delay the development of cancer. Features of chronic inflammation that are examined are the role and effect of IL-17 and myeloid-derived suppressor cells (MDSCs).

[0172] In one experiment, companion animals such as dogs and cats with spontaneously occurring inflammation are tested with Cox-2 inhibitor used to determine if a decrease of cancer associated with extended inflammation is seen. In another experiment, chemokine gradient and other gradients needed for inflammation to target liposomes and nanoparticles to a tissue with chronic inflammation are tested in these animals. (*Journal of Experimental Medicine*, Vol 181, 1179-1186, 1995). Other types of immune cells that are beneficial for surveillance, such as CIK cells, NKG2D and NKT cells, and innate immune cells (e.g., gamma delta T cells) are monitored as well.

[0173] In another experiment, dogs with spontaneously occuring inflammatory myopathies are used for translational model for human myositis (*Veterinary Immunology and Immunopathology*, 113 (1-2): 200-214, 2006).

Example 5 Neurodegenerative Diseases

[0174] An animal, such as a dog, with naturally occurring neurodegenerative disease is used to study various types of similar neurodegenerative disease in humans. A dog with canine degenerative myelopathy is used to study the various diseases states and/or progression of amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease). Various agents that are candidates for halting progression or improving the state of the neurological state can be administered to dogs with canine degenerative myelopathy and monitored for physiological effects to obtain information that can translate to how a human body with ALS would react to the same agents.

[0175] In another experiment for obtaining translational information for neurodegenerative diseases, canines with spontaneously accumulating human type ß-amyloid are used as a translational model for Alzheimer's disease (*J. Neuroscience*, 28(14): 3555-3566, 2008).

[0176] In other experiments, dogs with epilepsy or Parkinson's Disease are used as a translational model for human epilepsy or Parkinson's Disease to investigate biological pathways and therapeutic agents.

Example 6 Myeloid Suppressor Cell Depletion to Augment Tumor Vaccine Responses in a Canine Model of Non-Hodgkin Lymphoma

[0177] This example contains references to publications by use of numbers which correspond to the list of publications at the end of the example. The overall goal of this example is to develop more effective therapeutic cancer vaccines by utilizing MSC depletion to augment immune responses to existing cancer vaccines. The success rate of current tumor vaccines remains low despite a tremendous amount of effort directed to vaccine design. The relative ineffectiveness of current cancer vaccines may stem in part from the immunosuppressive properties of myeloid suppressor cells (MSC), which serve to potently suppress not only antitumor immunity, but may also suppress immune responses to vaccines in general. Preliminary studies indicate that elimination of MSC using liposomal clodronate (LC) can trigger spontaneous T cell-mediated antitumor immunity. Moreover, preliminary studies also indicate that MSC depletion can increase immune responses to vaccines in animals without tumors. Therefore, this example details how MSC depletion affects the generation of antitumor immunity following tumor vaccination, using both mouse and dog tumor models. Next, using a spontaneous canine model of Non-Hodgkin Lymphoma (NHL),

the question of whether the combined MSC depletion/tumor vaccination approach is more effective in reducing minimal residual tumor burden than tumor vaccination alone is examined.

[0178] Aim: To determine using mouse tumor models the optimal timing of MSC depletion for augmenting immune responses to tumor vaccination. The non-binding hypothesis is that depletion of MSC shortly after vaccination will significantly enhance T cell responses to vaccination and trigger significantly enhanced antitumor activity.

Background and Rationale for Cancer vaccines and NHL

[0179] Non-Hodgkin lymphoma (NHL) is an important tumor of humans that has been considered a prime target for vaccine immunotherapy because the tumor cells each express a unique tumor antigen (i.e., the idiotypic surface immunoglobulin molecule). Most forms of NHL are relatively refractory to treatment with chemotherapy and affected patients typically have short survival times. Therefore, a number of tumor vaccine approaches for NHL have been devised (1-5). Most NHL vaccines have utilized the idiotypic antigen receptor as the target antigen for immunization. Numerous vaccine studies have been conducted in NHL patients and three NHL studies have advanced to the point of completing phase III clinical trials (5, 6). Unfortunately, despite encouraging preliminary results, each of the phase III trials completed thus far has failed to meet the original study endpoints (5). The reasons for the vaccine trial failures are not clear, but may be related to vaccine design, insufficient vaccine potency, or patient inclusion criteria.

[0180] Despite a lack of major clinical successes, significant progress has been made in the design and implementation of cancer vaccines over the past two decades. However, there have still been few human cancer vaccines that have advanced beyond phase I trials. Thus, it is apparent that incremental improvements in vaccine design may not be sufficient to overcome the considerable hurdles that cancer vaccines face. Therefore, the focus of research in cancer immunotherapy has now begun to shift towards a better understanding of the role of the tumor microenvironment in regulating tumor immunity. One new strategy to emerge from this refocusing is the idea that modifying or circumventing immune regulatory and inhibitory mechanisms could be used to improve the effectiveness of existing tumor vaccines.

[0181] ***Myeloid suppressor cells (MSC) inhibit antitumor immunity.*** A number of recent studies have begun to more fully define the key role that immature myeloid cells play in

suppressing tumor immunity (7-11). This poorly defined population of myeloid cells are referred to collectively as myeloid suppressor cells (MSC). Recently, the MSC population in animals with cancer has been shown to consist of a mixture of immature monocytes and neutrophils(12). Despite their differing lineage, both monocytic and neutrophilic MSC have been shown to suppress T cell and NK cell function, albeit by different mechanisms. Suppression of T cells and NK cells is mediated by a number of mechanims, including production of reactive nitrogen species, reactive oxygen species, and surface expression of TGF-β, and arginase production. In many cases, inhibition by MSC requires direct or very close contact with T cells. The end result is that T cells and NK cells in the vicinity of MSC are rendered functionally incapable of cytotoxicity, proliferation, and cytokine production. The generation of MSC from the bone marrow is regulated by cytokines and growth factors produced by tumor cells themselves, or produced in response to tumor-associated inflammation. Following release from the bone marrow, MSC distribute to the spleen, bone marrow, draining lymph nodes, and tumor tissues.

[0182] Myeloid suppressor cells are not only generated in response to cancer, but are also elicited by a variety of inflammatory stimuli. For example, expanded numbers of MSC are present in individuals with sepsis, chronic infections (viral, fungal), and chronic inflammatory diseases (12). Thus, it appears that MSC likely have evolved to serve as negative modulators of both acute and chronic inflammation (7, 13). Viewed therefore as regulators of inflammation, without being bound by theory, MSC may also serve to dampen immune responses to vaccines, especially vaccines that elicit significant inflammation. Such a response would be particularly pronounced in individuals with cancer, since they would already possess greatly expanded numbers of MSC(14). In fact, evidence for just such an MSC response to tumor vaccination has been reported in melanoma patients vaccinated with a GM-CSF transduced melanoma vaccine (15, 16). If MSC do in fact inhibit tumor vaccine responses, then eliminating MSC or blocking their effects may help boost effective T cell immune responses to vaccination in patients with cancer. Experimental evidence in favor of this idea comes from studies of all-trans retinoic acid (ATRA) induced differentiation of MSC, which drives the cells to a mature into macrophages or neutrophils and reverses their immunosuppressive properties. When tumor-bearing animals or humans were treated with ATRA, spontaneous anti-tumor immunity was improved and vaccine responses were significantly enhanced (17-19). Similar enhancement of tumor vaccine responses was also reported when ROS production by MSC was inhibited using nitroaspirin (20).

[0183] One question is whether MSC depletion can restore tumor immunity and improve the effectiveness of tumor vaccines. Based on the preceding information, without being bound by theory, elimination of MSC may improve tumor vaccine responses. At present, the only two realistic options for eliminating MSC *in vivo* are to use antibody-mediated depletion or to use liposomal clodronate. Antibody mediated depletion of MSC has shown some effectiveness *in vitro* and *in vivo*, but is not currently considered feasible because a cell surface marker specific for MSC has not been identified (21). However, non-specific depletion of $CD11b^+/Gr\text{-}1^+$ cells with antibodies results in widespread depletion of macrophages, monocytes, and neutrophils and increases the risk of immunosuppression. Liposomal clodronate (LC) has been used extensively in the past to deplete macrophages and monocytes in mice for a variety of immunological investigations (22-26). When the bisphosphonate drug clodronate is encapsulated within neutral liposomes, the liposomes are taken up efficiently by phagocytic myeloid cells (macrophages, monocytes, MSC), followed by intracellular release of clodronate and rapid induction of macrophage apoptosis through competition for ATP binding (27, 28). Because LC does not deplete neutrophils, the risks of significant immunosuppression are considerably reduced.

[0184] More recently, LC treatment has also demonstrated antitumor activity in rodent tumor models, though in these studies the antitumor effects of LC treatment have been attributed to the effects of depletion of tumor-associated macrophages (TAM) and inhibition of tumor angiogenesis (29-31). The use of LC as a macrophage depleting agent in mouse models and in dogs with autoimmune disease has been investigated (32, 33). In addition to depletion of macrophages, systemic (intravenous) administration of LC also induced significant MSC depletion, which was associated with significant anti-tumor activity in mice and in dogs (34).

[0185] However, the query was whether LC treatment might be mediating antitumor activity through induction of systemic immune effects, rather than by local depletion of TAM in tumor tissues. Indeed, the antitumor activity elicited by LC treatment was due to spontaneous, systemic activation of antitumor immunity, rather than by depletion of TAM or inhibition of tumor angiogenesis. Thus, without being bound by theory, MSC depletion using LC could, if administered in the proper sequence relative to vaccination, also significantly augment the effectiveness of tumor vaccines. In fact, experiments combining LC treatment and vaccination against model antigens suggest that just such an effect occurs. Therefore,

without being bound by theory, MSC depletion using LC improves the effectiveness of NHL tumor vaccines. This example investigates this hypothesis first in mouse tumor models and to then conduct proof-of-principle experiments in a canine model of NHL.

[0186] Results: Past studies on the antitumor activity elicited by systemic administration of LC have focused in part on determining how to optimize LC delivery to generate maximal antitumor activity, on assessing the spectrum of tumor types that are susceptible to LC-induced antitumor activity, and on defining the mechanism(s) by which LC generates antitumor activity. Intravenous administration of LC elicits significant inhibition of growth of established tumors in mouse models. For example, once weekly i.v. administration of 200 ul LC to C57Bl/6 mice with established s.c. MCA-205 (sarcoma) tumors produced significant inhibition of tumor growth (Figure 1). Importantly, administration of control PBS containing liposomes (L-PBS) did not elicit antitumor activity. Similar antitumor activity was also generated in BALB/c mice with CT-26 (colon carcinoma) tumors. Significant antitumor activity was also observed in mice with B16 (melanoma) and 4T1 (breast carcinoma) tumors. Thus, LC administration inhibits tumor growth in a tumor type and mouse strain independent fashion.

[0187] Studies in dogs have also demonstrated that LC has antitumor activity. For example, twice monthly i.v. administration of LC to dogs with soft tissue sarcoma (STS) or malignant histiocytosis (MH) elicits tumor regression in approximately 50% of treated patients. As shown in Figure 2, a dog with STS treated with a series of treatments with LC alone experienced significant spontaneous tumor regression beginning after the third LC administration. Treatment responses have also been observed in dogs with MH treated with LC (34). Importantly, treatment with LC was well-tolerated by dogs, even those with advanced cancer, and the only notable side-effect has been transient fever, which has interestingly only been observed in dogs with MH. Thus, LC is also an effective and well-tolerated antitumor agent in dogs with cancer.

[0188] Studies to elucidate the immunological mechanisms by which LC treatment may induce spontaneous antitumor activity have been done. Since LC is known from prior studies to deplete phagocytic cells, whether LC treatment could deplete myeloid suppressor cells (MSC), particularly monocytic MSC(35) was investigated. Twenty-four hours after i.v. administration of LC in tumor-bearing mice, $CD11b^{+}/Gr\text{-}1^{+}$ MSC were enumerated in spleen, blood, and tumor tissues (Figure 3). Significant MSC depletion occurred in blood (Figure 3),

spleen, and tumor tissues, and that most of the depleted cells were monocytic. In addition, there was significant depletion of TAM and inhibition of tumor angiogenesis in LC-treated mice. Thus, systemic administration of LC elicited significant depletion of multiple different populations of phagocytic myeloid cells, including MSC, in animals with cancer.

[0189] Based on the fact that i.v. administration of LC resulted in systemic depletion of MSC, the next step was to investigate whether the antitumor effects of LC treatment were mediated by local effects (i.e., depletion of TAM) or by systemic immunological effects. To address this question, tumor-bearing mice lacking T cells ($RAG2^{-/-}$ mice) were treated with LC and compared MCA tumor growth rates with wild type C57Bl/6 mice treated with LC. The antitumor effect of LC treatment was almost completely abrogated in $RAG2^{-/-}$ mice, which suggested that the antitumor activity of LC was largely mediated by T cells. Therefore, to determine which T cell subset mediated the antitumor activity of LC, the tumor experiment in $CD8^{-/-}$ mice and $CD4^{-/-}$ mice was repeated. The antitumor activity of LC was almost completely eliminated in $CD8^{-/-}$ mice (Figure 4), whereas the activity of LC was only partially inhibited in $CD4^{-/-}$ mice. Controls also included mice treated with PBS containing liposomes (lip control). Therefore, the antitumor activity elicited by i.v. administration of LC was mediated by systemic activation of CD8 T cell anti-tumor immunity, rather than by local effects on TAM or tumor angiogenesis. These results are important because they suggest that MSC depletion and activation of systemic immunity is likely the primary mechanism by which LC generates antitumor activity.

[0190] The preceding experiments, in which LC-mediated depletion of MSC was able to generate spontaneous CD8 T cell-mediated antitumor activity, also suggested that MSC depletion might be capable of enhancing vaccine responses. To address this question, mice were vaccinated s.c. using a CLDC-adjuvanted vaccine (36) containing ovalbumin as a model antigen and asked whether MSC depletion using LC could enhance vaccine responses, using humoral immune responses as the readout (Figure 5). Mice were vaccinated once with the ova/CLDC vaccine alone, or with ova/CLDC plus LC treatment 3 days prior to immunization (LC, then Vacc), or ova/CLDC plus LC treatment 3 days after immunization (Vacc, then LC). Blood was collected and IgG responses to ova determined by ELISA. The mice vaccinated with ova/CLDC and treated with LC 3 days after immunization developed significantly higher antibody responses than mice vaccinated with ova/CLDC alone or ova/CLDC plus LC 3 days before immunization. Thus, these data suggest that in fact MSC depletion can

enhance immune responses to vaccination when administered in the proper sequence relative to the vaccine. Moreover, it should also be noted that this experiment was conducted in non-tumor bearing mice, while the vaccine enhancing effect would be expected to be more pronounced in tumor-bearing animals with much larger populations of MSC.

Experimental Design

Aim: Determine how the timing of MSC depletion affects vaccine-induced T cell responses.

[0191] Though LC is effective in depleting MSC, administration of LC also results in depletion of other relevant myeloid cells, including macrophages and DC. Thus, it is possible that LC administration could inhibit or augment vaccine responses, depending on which cells were depleted and when they were depleted relative to vaccination. Therefore, mouse immunization models are used to determine the effect of timing of systemic LC administration on cellular and humoral immune responses to vaccination with CLDC adjuvanted vaccines. Initial experiments use a model antigen, since the readouts for these experiments are very robust and reproducible. Once the optimal timing of administration is identified, the relevance of these findings in two mouse cancer models are confirmed. The B16 melanoma model was selected because CD8 T cell responses can be tracked using tetramers, while the A20 lymphoma model was selected because of the close similarity with the dog NHL model. In addition, A20 cell lines that have been transfected with the HA antigen are used, which allow more accurate assessment of CD4 T cell responses.

Experimental Approach:

[0192] Aim: to determine the optimal timing of MSC depletion to increase T cell and antibody responses to immunization with a nominal antigen or with a tumor antigen. These experiments are designed to 1) determine whether combined MSC depletion and vaccination enhances immune responses in normal and tumor-bearing mice; 2) identify the optimal timing of MSC depletion relative to vaccination to maximize immune responses; and 3) to assess the effects of combined MSC depletion and immunization on antitumor immunity in two mouse tumor models.

Table 1. Timing of depletion

Group	Vaccinate	MSC deplete
1	-	-
2	+	-
3	-	+
4	+	day -7
5	+	day -3
6	+	day -1
7	+	concurrent
8	+	day +1
9	+	day +3

[0193] *Determine optimal timing of MSC depletion relative to vaccination to elicit maximal T cell responses*. These experiments are designed to determine the optimal timing of MSC depletion using LC treatment for generating maximal T cell responses. In the first experiments, normal C57Bl/6 mice (n = 5 per group) re be vaccinated with Ova, using a potent cationic liposome-nucleic acid (CLDC) adjuvant developed as in the referenced publication (36). The 10 experimental groups of animals to be evaluated are described in Table 1. Mice are vaccinated s.c. with 5 ug Ova in CLDC adjuvant. Depletion of MSC is accomplished using a single injection of 200 ul liposomal clodronate (LC) administered i.v. Mice are euthanized 7 days after vaccination and lymphoid tissues and serum collected. Read-outs include assessment of CD8 responses by flow cytometry (K^b-ova tetramers), CD4 responses (cytokine release and proliferation assays), and humoral responses (serum antibodies to Ova are quantitated by ELISA).

[0194] *Statistical analysis of data*. Immune responses in treated mice are compared to untreated control mice, using non-parametric ANOVA (Kruskal-Wallis), followed by Dunn's multiple means comparison test. Similar analyses are also done for data below. Statistical analysis is done using commercial software (Prism5, GaphPad, San Diego, CA) and significance is defined as $p < 0.05$.

[0195] Treatment with LC either 1 day or 3 days after immunization generates optimal immune responses, which is reflected by increased numbers of Ova-specific CD8 T cells, greater IFN-γ production, and higher antibody titers. These assays are routinely done in the laboratory. The results allow clear identification of the optimal timing of MSC depletion to enhance vaccine responses. If readouts are not clear after a single immunization, then the experiment is repeated using a boost immunization administered 2 weeks after the first immunization to increase the numbers of antigen specific T cells.

[0196] *Assess the effects of MSC depletion on immune responses and antitumor activity following vaccination against tumor antigens*. These experiments are designed to determine whether MSC depletion can augment T cell responses against tumor antigens in mice with established tumors, using two different tumor models. In the first model, C57Bl/6 mice with B16 melanomas are used, because a well-defined tumor antigen (trp2) has been identified in this model which allows accurate quantitation of CD8 T cell responses using tetramer reagents. Using the optimal MSC depletion schedule determined above, mice (n = 5 per group) with established cutaneous B16 tumors are vaccinated s.c. with 5 ug trp2 peptide in

CLDC adjuvant, then boosted 7 days later. Treatment groups include unvaccinated control mice, vaccinated only mice, LC only treated mice, and mice treated with vaccination plus LC treatment. Numbers of trp2-specific CD8 T cells in blood, spleen, and LNs is assessed 5 days after the boost, using flow cytometry and Kb-trp2 tetramers. These experiments are repeated in another group of mice to assess the effects of combined MSC depletion/vaccination on tumor growth responses. In these studies, tumor growth rates are assessed by means of 3 times/week measurement of tumor diameter. In addition, the overall survival times of treated and control mice are evaluated.

[0197] In a second tumor model, immune responses to vaccination in BALB/c mice with A20-HA lymphomas are evaluated. In this model, the tumor has been engineered to express the influenza HA antigen to facilitate measurement of T cell responses. Two different vaccines are evaluated: 1) paraformaldehyde fixed A20-HA cells (1×10^6 inactivated A20 cells per vaccine, admixed with CLDC adjuvant) or 2) HA antigen vaccine, 5 ug rHA in CLDC adjuvant. Mice (n = 5 per group) with established cutaneous A20 tumors are vaccinated s.c. with either autologous A20-HA tumor cells in CLDC adjuvant, or with rHA in CLDC adjuvant, then boosted 7 days later. Treatment groups include unvaccinated control mice, vaccinated only mice, LC only treated mice, and mice treated with vaccination plus LC treatment. Immune responses to be assessed include measurement of cytokine responses to vaccination (cytokine release following *in vitro* restimulation of spleen or LN cells with fixed tumor cells or with HA antigen), proliferative responses (proliferation of spleen or LN cells following *in vitro* restimulation for 96 hours with fixed A20 tumor cells or with rHA antigen) and assessment of *in vivo* CTL activity (*in vivo* killing of adoptively transferred, CFSE-labeled A20 tumor cells; described previously (36). The experiments are repeated in another group of mice to assess the effects of combined MSC depletion/vaccination on tumor responses. In these studies, tumor growth rates of cutaneously implanted A20 are assessed by means of 3 times per week measurement of tumor diameter. In addition, the overall survival times of treated and control mice is determined.

[0198] Combined MSC depletion/vaccination protocol induces a significant increase in the number of trp2-specific CD8 T cells in the B16 tumor model, compared to vaccination alone or MSC depletion alone. If an additive effect of the two treatments is not observed, the experiment is repeated using twice weekly LC administration, in case the numbers of MSC in tumor-bearing mice are still sufficient to inhibit vaccine responses. If the magnitude of trp2-

specific CD8 T cell responses is too low to measure directly *ex vivo*, then the cells for 4-5 days are cultured *in vitro* in the presence of IL-2 and specific peptide to expand numbers of T cells before the tetramer assay is done. The increase in numbers of trp2 specific T cells correlates with a significant reduction in tumor growth rate and increased overall survival times in mice receiving combined MSC depletion/vaccination therapy. The role of CD8 T cells in the antitumor immune response is confirmed using $CD8^{-/-}$ mice, or by antibody mediated depletion of CD8 T cells following immunization.

[0199] In the A20-HA model, T cell cytokine release and CTL activity is increased in mice that receive the combination MSC depletion/vaccination therapy. By utilizing both whole tumor cell and HA vaccination and immune assays, interpretable data is generated. Tumor growth rates are significantly slowed and survival improved in mice vaccinated with autologous tumor cells plus MSC depletion. Vaccination with the autologous tumor vaccine are most likely to be more effective than vaccination with the HA antigen alone due to the greater complexity and number of potential antigens on fixed tumor cells.

[0200] Aim: To determine whether tumor vaccination combined with MSC depletion significantly reduces residual tumor burden in a canine model of Non-Hodgkin lymphoma.

[0201] **Rationale.** Experiments in mouse tumor models are useful for optimizing the timing of vaccine and LC administration to maximize cellular immunity, and also for assessing antitumor activity. However, the limitations of mouse tumor models in predicting outcomes in human cancer studies are well-known. Therefore, the best available spontaneous NHL tumor model, dogs with B cell lymphoma, is used. This model has been used in the past to assess the effectiveness of an autologous lymphoma vaccine prepared using CM-CSF transfected tumor cells. The approach of vaccinating dogs with whole, fixed autologous tumor cells may not entirely analogous to human NHL vaccines, which usually consist of recombinant idiotypic Ig molecules however, constructing such a vaccine is highly difficult in the dog tumor model. Immunizing with paraformaldehyde fixed tumor cells, which preserve the surface Ig molecules, generates relevant vaccine responses. Using a conservative study design with 3 treatment groups of dogs, the determination of whether LC treatment can significantly augment NHL tumor vaccine responses is done. Moreover, use of changes in minimal residual disease burden (MRD) following vaccination as the primary endpoint for the study (rather than DFI or OST) allows study endpoints to be achieved much more quickly

and with greater potential accuracy. This type of data is also highly relevant to the evaluation of MSC depletion therapy with LC as a strategy for use with human NHL vaccines.

[0202] *Trial design.* These studies are designed as a proof-of-principal study in dogs with B cell lymphoma, the canine equivalent of NHL in humans. The primary goal of this study is to determine whether vaccination plus MSC depletion generates a greater reduction in residual tumor burden (circulating tumor DNA detectable by qRT-PCR in the bloodstream (37) than vaccination alone or MSC depletion alone. Based in part on studies in mice, group sizes of 8 dogs each should allow the determination of a significant treatment difference, based on a 30% reduction in MRD in vaccinated/MSC depleted dogs compared to dogs that are vaccinated alone or treated with LC alone, with a power of 80% (PS Power and Sample Size calculation software). Therefore, 24 dogs with histologically confirmed B cell lymphoma are enrolled in a randomized clinical trial. Each dog is treated with conventional chemotherapy (doxorubicin plus Lasparaginase) for 10 weeks to achieve complete macroscopically visible tumor remission, at which time dogs are randomized to treatment group 1 (vaccine alone); treatment group 2 (LC treatment alone); or treatment group 3 (vaccine plus LC treatment). Group 1 and 3 dogs are vaccinated once every 2 weeks for 5 total immunizations, using autologous lymphoma cells (1×10^7 paraformaldehyde-fixed cells per vaccination, administered s.c. in 2 ml CLDC adjuvant). Group 2 dogs receive a series of 5 infusions of LC (0.5 ml/kg) once every 2 weeks. Group 3 dogs are vaccinated and treated with LC, using the optimal timing of LC administration relative to vaccination determined in one of the aims above.

[0203] Blood is collected prior to treatment, and on weeks 2, 4, 6, 8, and 10 of treatment for determination of MRD and for immunological assays. Lymph node size is determined at each recheck visit. A CBC is performed at each recheck to assess numbers of monocytes and neutrophils. At the completion of the study, dogs continue to be followed by telephone follow-up to determine the time of first tumor recurrence (disease-free interval; DFI).

[0204] *Preparation of tumor vaccine and LC for MSC depletion.* Autologous tumor vaccines are prepared using lymphoma cells collected from lymph node biopsies obtained from each patient prior to administration of chemotherapy. Single cell suspensions of tumor cells are prepared using gentle enzymatic dissociation. The tumor cells are then fixed overnight in a 1% solution of paraformaldehyde in PBS, which is designed to lightly fix and kill tumor cells, while still preserving surface antigens. Aliquots of fixed tumor cells are

stored frozen until used to produce the vaccine. The vaccine is prepared using 1 x 10^7 tumor cells admixed with 2 ml CLDC adjuvant, using a technique similar to that reported previously to prepare an allogeneic tumor vaccine for dogs with hemangiosarcoma (38). The vaccine is administered intradermally in 2 different sites over the lateral thorax. Vaccination is repeated for a total of 5 immunizations at 2-week intervals. Depletion of MSC is accomplished by i.v. administration of LC, which is prepared as described for treatment of dogs with malignant histiocytosis (34). The LC is administered once every 2 weeks by slow i.v. infusion over 60 minutes, at a dose of 0.5 ml/kg. This dose of LC has been well-tolerated by dogs previously, with transient fever being the most frequent adverse effect in approximately 30% of treated dogs with MH.

[0205] *Assessment of vaccine responses.* Vaccine responses is assessed using PBMCs collected prior to treatment and on weeks 2, 4, 6, 8, and 10 of treatment. The PBMCs are thawed and then incubated with PFA-fixed autologous lymphoma cells at 3 different ratios (1:1, 1:10, 1:100) for 96 hours, and proliferation assessed using BrDU incorporation and flow cytometry. In addition, supernatants from the cultures are collected and assayed for determination of IFN-γ concentrations, using a commercial canine IFN-γ ELISA (R & D Systems). A neoantigen (KLH) is incorporated into the vaccine in order to facilitate assessment of vaccine responses, as reported previously (39). Immune responses to KLH are assessed by proliferation and IFN-γ release, using PBMC incubated with 50 ug/ml KLH *in vitro* for 96 hours. In addition, antibody responses to KLH are assessed using a KLH ELISA (39).

[0206] *Assessment of molecular remission following chemotherapy and vaccination.* Tumor samples are collected at the beginning of the study to design tumor BCR-specific primer sets for amplification of tumor BCR (40, 41). Blood samples for PCR determination of numbers of circulating lymphoma cells (MRD) are collected at the completion of chemotherapy (immediately prior to first vaccine) and at 2-week intervals during the treatment phase of the study. PBMC are separated and frozen in 3 different aliquots, to be used for MRD calculation and assessment of immune function. Circulating tumor cells are quantitated using quantitative real time PCR (qRT-PCR) and a previously described protocol for quantitation of MRD burden in dogs with B cell lymphoma (37). In that study, which utilized PCR primers designed specifically for an individual patients idiotype Ig, the PCR technique was reported to be sensitive enough to detect circulating tumor cells in each of 7 dogs, even following

complete visible tumor remission that was induced using conventional chemotherapy. Moreover, in all 7 studied dogs, the circulating tumor burden increased after the cessation of chemotherapy and the assay was predictive for time to macroscopic tumor recurrence. Thus, the qRT-PCR approach achieves accurate quantitation of the tumor response to vaccination and MSC depletion (i.e., molecular remission). In addition, the between group comparisons should be sufficiently robust to address the primary question of the study (i.e., is combined vaccination/MSC depletion treatment more effective than either alone) without having to include an additional group of dogs with lymphoma treated only with chemotherapy.

[0207] Without being bound by theory, the combined treatment with the autologous tumor vaccine and LC yields greater reduction, even significantly greater reduction, in tumor MRD, compared to dogs receiving the tumor vaccine alone or LC treatment alone. Vaccination alone or LC treatment alone also significantly reduces MRD compared to pre-treatment values, but that the combined vaccine/LC treatment generates synergistic antitumor activity. While MRD reduction is the primary endpoint of the study, the immune assays (proliferation, cytokine production, target cell killing) correlate with MRD assays.

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Example 7 Clinical Trial of Monocyte/Macrophage Activator L-MTP-PE

[0208] Activated monocytes and macrophages eliminate chemotherapy-resistant cancer cells *in vitro*, and therefore agents that activate these effector cells of innate immunity may complement chemotherapy. The minimal peptidoglycan motif muramyl dipeptide (MDP), composed of N-acetylmuramic acid linked to an L-alanine D-isoglutamine dipeptide, is a common membrane component of Gram-negative and Gram-positive bacteria. An important component of complete Freund's adjuvant, MDP activates monocytes and macrophages through the innate immune receptor NALP3. Muramyl tripeptide phosphatidylethanolamine (MTP-PE) is a synthetic conjugation of alanine and dipalmitoylphosphatidylethanolamine to MDP, creating a lipophilic molecule with greater potency, improving cellular uptake and boosting tumoricidal activity. The lipophilic MTP-PE is also more readily incorporated into

liposomes for rapid uptake by phagocytic cells. Pharmacokinetics studies in dogs confirmed rapid clearance and a 10-fold reduction in toxicity. Based on promising preclinical studies, clinical trials were conducted in several canine and feline cancers. Following surgical resection, L-MTP-PE was administered at a dose of 2 mg/m2 twice weekly for 8 weeks alone or in combination with chemotherapy (doxorubicin and cyclophosphamide, or cisplatin). When administered immediately after surgery, L-MTP-PE treatment conferred a median survival time of 222 days, significantly longer ($p < 0.002$) than dogs treated with placebo liposomes (77 days). Non-metastatic dogs treated with L-MTP-PE after cisplatin had a median survival time of 14.4 months, again significantly longer ($p < 0.01$) than dogs treated with cisplatin and placebo (9.8 months); treatment with L-MT-PE concurrent with cisplatin also improved median survival, but the 1.6 month difference was not significant. Longer disease free survival was also noted in treatment of early-stage melanoma, but had no effect in feline or canine mammary tumors following mastectomy.

[0209] Building on the success of these studies in companion animals, a series of exploratory phase I studies were conducted in approximately 150 patients with various advanced cancers (breast, colorectal, lung, melanoma, renal cell carcinoma, stomach and salivary gland cancers as well as sarcoma). These studies determined the L-MTP-PE maximum tolerated dose and optimal biological dose, indicating similar dosing to the canine studies. From 1993-1997, a phase III clinical study assessed the efficacy of L-MTP-PE (mifamurtide) and/or ifosfamide added to the standard regimen of doxorubicin, cisplatin, and high-dose methotrexate in newly diagnosed patients with high-grade osteosarcoma. The trial included 678 patients with non-metastatic resectable osteosarcoma, 332 receiving L-MTP-PE, and 115, and 115 patients with metastatic or unresectable osteosarcoma, with 39 receiving L-MTP-PE. Addition of ifosfamide and three chemotherapeutic drugs did not significantly improve drug free survival (DFS) or overall survival (OS) relative to the standard of care, but addition of L-MTP-PE significantly improved both (DFS p=0.030; OS p=0.039). IDM Pharma Inc submitted an NDA for L-MTP-PE in 2006, but received a nonapprovable letter in 2007 requesting additional data. In March 2009, L-MT-PE (mifamurtide, MEPACT®) was granted a centralized marketing authorization by the European Commission, permitting marketing of the drug in the European Union.

[0210] The development pathway of L-MTP-PE exemplifies the way in which studies in human and canine osteosarcoma can proceed in parallel, providing a two-way flow of

information that can lead to optimization of drugs for the treatment of osteosarcoma in both species.

Example 8 Optimization of Electrochemotherapy (ECT)

[0211] Some drugs, including cancer chemotherapeutics bleomycin and cisplatin, are highly lipophobic and therefore have poor cellular uptake. Bleomycin is so lipophobic it cannot enter target cells through simple diffusion, requiring relatively slow and inefficient uptake through specific protein receptors resulting in < 0.1% internalized in cultured cells. The high systemic doses required due to poor uptake have caused considerable toxicity to normal tissue, impeding the adoption of bleomycin as an anti-cancer agent despite its therapeutic potential. Short electric pulses that temporarily alter target cell permeability offered a solution to this problem. These pulses appear to induce pores in the cell membrane, improving cellular entry of drugs and plasmids. Electropulsation of cells *in vitro* increased the cytotoxicity of bleomycin several thousand-fold, and increased the cytotoxicity of cisplatin by seventy-fold. The first *in vivo* study of this technique was conducted in 1997 in cats with recurring soft tissue sarcoma after adjuvant radiation therapy. A small cohort of cats received bleomycin followed by square pulses, with prolonged survival in 12 cats relative to 11 untreated controls.

[0212] In a subsequent phase I/II study, canine and feline soft tissue sarcoma patients were treated with intralesional bleomycin coupled with biphasic electric pulses, resulting in an overall response rate of 80%, including 40% with long term remissions. This study revealed that canine hemagiopericytomas were particularly responsive to electrochemotherapy (ECT), but also underscored the need for development of customized electrodes adapted to connective tissue. A series of phase II studies were subsequently initiated with the optimized electrodes. Cats with soft tissue sarcoma receiving intraoperative or postoperative bleomycin with electrotherapy had improved average time of recurrence of 12 and 19 months respectively, compared to an average of 4 months with surgery alone. A similar study with canine soft tissue sarcoma patients yielded a median time to recurrence of 730 days and a 95% response rate in dogs treated with bleomycin and electric pulses, with the greatest sensitivity by hemangiopericytomas. A review of over 370 biopsy specimens from ECT trials in a variety of tumors showed a strong correlation between overall survival and necrosis ($p < 0.0001$) and high rates of apoptosis ($p < 0.0001$).

[0213] The period and frequency of electric pulses were also optimized through multiple trials in companion animals, demonstrating that decreasing the period of pulses from 1 second to 100 milliseconds and increasing the repetition frequency from 1 Hz to 5000 Hz could deliver the necessary 400 V/cm electric field to the tumor with less patient discomfort. Although the first *in vivo* studies were initiated just over a decade ago, ECT is already approved for human use and reimbursed in several EU countries. Clinical studies of ECT in veterinary patients began shortly after the first human oncology trials, and the approach is used extensively in several European countries and Brazil for cats, dogs, and horses with a wide variety of cutaneous and subcutaneous tumors. Optimization of the technique has progressed in parallel in human and veterinary clinical trials, exemplifying how similarities between tumors in humans and companion animals and communication between oncologists working in both fields can accelerate development of new therapeutic modalities.

Example 9 Treatment of Hemangiosarcomas

[0214] Liposome encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) proved successful in randomized clinical studies of canine osteosarcoma (above), and therefore this therapeutic strategy was extended to hemangiosarcoma. Thirty-two dogs with HSA and no evident metastases were treated with splenectomy and doxorubicin + cyclophosphamide along with L-MTP-PE or placebo. Dogs that received L-MTP-PE had significantly improved disease-free survival ($p=0.037$) and overall survival ($p=0.029$), with better responses by dogs in clinical stage I than in clinical stage II. Bioassay showed significant elevation of serum tumor necrosis factor and interleukin-6, important immune cytokines. These studies suggest a novel therapeutic approach for this unmet medical need in dogs. Furthermore, studies of canine HSA may inform anti-metastatic strategies for treatment of companion animals and humans.

Example 10 Plasmid DNA Stimulation of Innate and Adaptive Immunity

[0215] T cells activated by bacterial superantigens develop strong cytolytic activity and mediate tumor regression when adoptively transferred. Twenty-six dogs with spontaneous malignant melanoma were treated with plasmid DNA encoding the bacterial superantigen staphylococcal enterotoxin B and either GM-CSF or IL-2 to test the effect of DNA vaccination on tumor regression. The overall response rate (complete and partial remission) for all dogs was 46%, and was highest in smaller tumors. Histological examination revealed

CD4+ and CD8+ T cell infiltrates in the tumors, and demonstrated that tumor regression was correlated with high levels of circulating cytotoxic T lymphocytes. In this study, the plasmid DNA was complexed with cationic lipids to compact the plasmid for greater stability. Subsequent studies revealed that the combination of cationic lipid and bacterial DNA effectively stimulated innate immunity and provoked a strong cytokine response even in the absence of encoded genes.

Example 11 Development of Antiangiogenic Thrombospondin-1 Peptide Mimetics

[0216] This example shows how spontaneous tumors in companion animals can play a key role in bridging therapeutic development from mouse models to human clinical trials. As tumors grow they must induce localized angiogenesis to develop an adequate blood supply supporting further growth. Therefore, blocking angiogenesis is a goal of many cancer therapy efforts. Thrombospondin-1 (TSP-1) is a pleiotropic natural angiogenesis inhibitor, blocking many aspects of endothelial cell activation. Modified nonapeptides based on the angiogenic domain of TSP-1, ABT-526 and ABT-510, share this antagonist activity in a more practical size for drug development. Initial efficacy studies in syngeneic and xenograft mouse models showed that ABT-526 and ABT-510 both slow tumor growth. However, inhibition of angiogenesis is unlikely to rapidly destroy tumors, so establishing the dose for human clinical trial based on a rapidly progressing mouse cancer model was not considered optimal. To better define safety and efficacy, the two TSP-1 peptide mimetics were tested in an open-label nonclinical trial of spontaneous canine tumors. A prospective open-label trial was conducted on 242 dogs with a variety of cancers including NHL, soft tissue sarcoma, mammary adenocarcinoma, head and neck carcinoma, and many other primary and metastatic tumors (115). Pharmacokinetic studies were conducted in a laboratory colony of beagle dogs, providing a bridge between mouse and outbred companion animal studies and establishing initial dose parameters. No dose-limiting toxicities were observed in any dogs in the study. Objective regression (> 50% reduction of tumor size) of measureable lesions were noted in 19 of 180 evaluable dogs and significant disease stabilization occurred in 23 dogs. Most of these responses occurred after 60 days of treatment with the TSP-1 mimetic, confirming the selection of spontaneous tumors in dogs as the appropriate model to optimize dosing and confirm efficacy. This study indicated that NHL was one of the more responsive classes of tumor and that ABT-526 was more active than ABT-510. Based on these results, a controlled double-blinded trial of ABT-526 was conducted on 94 pet dogs with naturally

occurring first-relapse NHL. This study was designed to provide additional definition of optimal biological dose and schedule, identify predictive biomarkers of activity, and to test efficacy in combination with chemotherapy. Dogs received lomustine (CeeNu®, Bristol Myers Squibb) and placebo or ABT-526. In this controlled clinical trial ABT-526 did not increase the number of cases responding to chemotherapy, but modestly enhanced the duration of response. ABT-510 testing was advanced into a series of human phase I and phase II clinical trials. A phase I safety, pharmacokinetic and pharmacodynamic study of ABT-510 in 39 human patients with a range of advanced cancers demonstrated a favorable toxicity profile and caused a decrease in basic fibroblast growth factor, a marker of angiogenesis, and stable disease in 6 patients for at least 6 months.

Example 12 Reduction of Doxil Adverse Effects

[0217] Doxorubicin is an anthracycline antibiotic that intercalates into DNA blocking replication, and is used in the treatment of a wide range of cancers including hematological malignancies such as NHL and in soft tissue sarcomas. Doxil, a peglylated liposome containing doxorubicin, has prolonged circulation and enhanced anti-tumor efficacy with less cardiotoxicity. However, unlike free doxorubicin, Doxil induces a painful skin reaction called palmar-plantar erythrodysesthesia (PPES), sometimes called hand-foot disease. Like humans, dogs are also susceptible to development of PPES following prolonged Doxil therapy. Anecdotal evidence suggested that oral vitamin B6 (pyridoxine) could alleviate or eliminate PPES. To test this, a randomized double-blind study of daily Doxil chemotherapy in combination with oral pyridoxine or placebo was conducted in 41 dogs with NHL (118). No difference was observed in remission rates between treatment groups, but the relative risk of developing PPES was 4.2 times greater in the placebo group. Although pyridoxine did not completely prevent or reverse PPES, it delayed and lessened the symptoms. This exploratory trial in dogs provided the rationale for more extensive testing of this strategy in human patients.

CLAIMS

What is claimed is:

1. A method for identifying a combination of anti-cancer agents with synergistic effects comprising: (1) administering two or more anti-cancer agents to a companion animal with a spontaneously occurring cancer; (2) monitoring the companion animal for a biological and/or physiological effect; and (3), identifying a combination of anti-cancer agents with synergistic effects when the biological and/or physiological effects are synergistic, wherein the anti-cancer agents comprise bisphosphonates and cationic CpG.

2. The method of claim 1 wherein the agents are clodronate and cationic CpG.

3. The method of any one of claims 1 or 2 wherein the companion animal is a dog.

4. The method of claim 3 wherein the companion animal is a purebred dog.

5. The method of claim 3 wherein the companion animal is a mongrel dog.

6. The method of claim 3 wherein the dog has a homogeneous genetic background.

7. The method of claim 3 wherein the dog has a heterogeneous genetic background.

8. The method of any one of claims 1 or 2 wherein the companion animal is a cat.