ACTIVE IMMUNIZATION FOR TREATING ATOPIC DERMATITIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit and priority of U.S. Patent Application No. 63/527,735, filed on July 19, 2023, the entire content of which is hereby incorporated by reference in its entirety.

BACKGROUND

[0001] Field of the invention

[0002] The invention relates to vaccine technology, immunotherapy, veterinary and medicine. The invention relates to recombinant fusion proteins and immunogenic compositions, methods for engineering and producing the recombinant fusion proteins and their applications for immunotherapy to diseases involving inflammation and/or chronic pruritus associated with inflammatory skin disorders such as in atopic dermatitis (AD) in mammals, including canine, feline, equine. The invention relates to the use of immunogenic fusion proteins and immunogenic compositions in active immunization or vaccination and in methods for treating and/or preventing inflammatory-associated skin disorders and/or AD and its associated symptoms including pruritus and secondary skin lesions.

[0003] Discussion of the related art

[0004] Atopic dermatitis (AD) is a common chronic skin disease in companion animals including dogs, cats, and equines, characterized by inflammatory and pruritic skin lesions. AD is considered as a multifactorial allergic disease triggered by environmental allergens involving allergen specific IgE, that seems to affect genetically predisposed animals [2; 3]. AD is characterized by extensive itching, scratching, hair loss, and secondary infections of the skin, affecting quality of life and well-being of companion animals and owners [4; 5]. AD is a chronic lifelong condition with partial flares and remissions and is often complicated by secondary infections.

[0005] The estimated prevalence of canine AD (CAD) is variable but can affect 10 to 15% of dogs depending on the breed, life conditions, climate, and geographical

regions [5; 6; 7]. CAD tends to develop in dogs between the ages of 6 months to 2 years. Clinically, CAD is characterized by moderate to severe pruritus, often associated with local erythema, erythematous eruptions, scratch-induced alopecia, excoriations, skin thickening [8; 9]. Depending on the breeds, the skin lesions can be localized in the face, pinnae, ear canals, paws, axillae, ventrum, and inguinum. The disease may be perennial or seasonal [10]. Secondary microbial infections in affected skin areas are a common complication. Considering that several millions of dogs are chronically affected by AD, a current challenge for veterinary medicine is the development of effective, easy to administer, and affordable therapies allowing a disease-modifying effect of AD.

[0006] Cats and horses also develop AD atopic syndromes which include skin inflammation, cutaneous lesions, and chronic recurrent pruritus as a predominant sign. Common clinical presentations of allergic dermatitis in cats include local skin lesions in neck and head, including self-induced alopecia, excoriations, erosions, and ulcers [11; 12]. Complications may lead to more complex lesions including plaques, granulomas, and chronic ulcers. Equine atopic dermatitis is often presented with recurrent pruritus in the face, legs, or trunk, which are associated to different skin lesions such as erythema, urticaria, papules and alopecia [2].

[0007] Systemic treatments for inflammatory skin conditions, itching, and atopic dermatitis include a range of medications aimed at reducing inflammation, immune response, and symptom severity. Corticosteroids are commonly used for their anti-inflammatory effects, while immunosuppressants like cyclosporine and methotrexate help manage severe cases by suppressing the immune system. Biologics targeting interleukin 31 pathway, target specific pathways in the immune response and are effective for moderate to severe atopic dermatitis. Antihistamines can alleviate itching, though their effectiveness may vary. Additionally, newer oral treatments like Janus kinase (JAK) inhibitors, such as Upadacitinib, baricitinib or oclacitinib, offer targeted immune modulation with promising results for atopic dermatitis. These treatments are often used in combination with topical therapies to manage symptoms and improve quality of life for patients.

[0008] In veterinary medicine, the current immunotherapies approved for the treatment of atopic dermatitis primarily include monoclonal antibody treatments and Janus kinase (JAK) inhibitors. Lokivetmab (Cytopoint) is a monoclonal antibody that

targets and neutralizes interleukin-31 (IL31), a cytokine involved in the itch signal pathway, providing relief from pruritus in dogs. These treatments offer improvements in managing atopic dermatitis, focusing on alleviating symptoms and improving the quality of life for affected animals.

[0009] Developmental active immunization-based therapies for the treatment of atopic dermatitis in veterinary medicine are focused on stimulating the animal's immune system to recognize and combat allergens or specific immune components contributing to the condition. These therapies often involve vaccines designed to induce an immune response against cytokines or other molecules implicated in the inflammatory process. For example, research is ongoing into vaccines targeting IL31, a key cytokine involved in itch signaling, aiming to reduce pruritus and inflammation over a prolonged period.

[0010] Developmental therapies targeting Substance P for itching or atopic dermatitis are focused on blocking the neuropeptide neurokinin-1 receptor, which is known to play a significant role in transmitting itch signals and promoting inflammation. Substance P interacts with the neurokinin-1 receptor (NK-1R) on skin cells and nerve fibers, contributing to the sensation of itch and the inflammatory response. Therapies in development include NK-1R antagonists, which aim to inhibit the action of Substance P, thereby reducing itch and inflammation. These antagonists can be administered topically or systemically, and some are being evaluated in clinical trials for their efficacy and safety in treating atopic dermatitis. No approved or development treatment based on a vaccine or antibody-based blockade, or neutralization of Substance P has been reported.

[0011] To date, no studies have evaluated therapies which simultaneously target both IL31 and SP to control itching, skin inflammation and neurogenic inflammation and efficiently treat, alleviate and/or prevent AD-associated symptoms and/or secondary skin lesions.

SUMMARY OF THE INVENTION

[0012] The invention relates to immunogenic fusion proteins which comprise at least one IL31 polypeptide (IL31) or immunogenic fragment thereof; and at least one substance P peptide (SP). The immunogenic fusion protein is capable of eliciting a production of neutralizing polyclonal antibodies against IL31 and SP.

[0012a] Disclosed herein is an immunogenic fusion protein comprising: at least one IL31 polypeptide (IL31) or immunogenic fragment thereof, wherein said at least one IL31 polypeptide comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or an immunogenic fragment thereof; and at least one substance P polypeptide (SP), wherein said at least one SP peptide comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9, wherein said immunogenic fusion protein is capable of eliciting a production of neutralizing polyclonal antibodies against IL31 and SP, and preferably, wherein said at least one SP peptide is optionally one, three or five SP peptides.

[0013] The invention also relates to active immunization, peptide vaccines and immunogenic compositions for treating inflammation associated with AD, mediated by neuroimmune factors such as the pro-inflammatory and pruritogenic cytokine IL31 and the neuropeptide substance P (SP) responsible for neurogenic inflammation and pruritus.

[0014] The immunogenic compositions of the invention may derive from canine, feline, equine, or human sequences encoding for IL31 and SP expressed as fusion proteins.

[0015] The immunogenic compositions include at least one immunogenic fusion protein comprising at least one sequence of IL31 or immunogenic fragment thereof, linked by flexible linkers to at least one SP sequence (i.e., IL31-1SP or IL31-3SP). The immunogenic fusion proteins are non-natural, may lack the physiological activity of IL31 and/or SP, may be capable of overcoming immune tolerance to self-antigens and inducing the production of neutralizing antibodies directed against endogenous IL31 and SP. Notably, the immunogenic fusion proteins of the invention may have a synergistic immune effect with a bivalent capability against the inflammatory pathways of the immune system and the peripheral nervous system, thus providing therapeutic and/or prophylactic benefits against inflammatory skin conditions, such as AD, in particular pruritus.

[0016] Advantageously, when administered to mammals, such as dogs, cats, horses or humans, the immunogenic compositions of the invention alleviate skin

inflammatory-associated symptoms such as pruritus and reduce the need for systemic medications, including corticosteroids, cyclosporine, antihistamines, or tyrosine kinase inhibitors. The immunogenic compositions according to the invention are preferably administered as bivalent vaccines targeting IL31 and SP. The immunogenic compositions may include at least one adjuvant to potentiate the therapeutic immune response in immunized mammals.

[0017] The invention relates to the design and production of bivalent non-natural, fusion proteins, which when used for active immunization stimulate the production of neutralizing antibodies to the pro-inflammatory immune mediator IL31 and the neurogenic mediator, substance P.

[0018] The immunogenic fusion protein of any of the preceding embodiments elicits the production of neutralizing antibodies in a mammal and modulates the function of IL31 and SP.

[0019] When administered in a mammal, the immunogenic fusion protein of any of the preceding embodiments yields higher neutralizing antibody titers compared to that of a combination of a non-fused IL31 polypeptide and a non-fused SP peptide administered as single agents.

[0020] The immunogenic fusion protein of any of the preceding embodiments comprises at least one IL31 polypeptide that comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or an immunogenic fragment thereof.

[0021] The immunogenic fusion protein of any of the preceding embodiments comprises at least one SP peptide that comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.

[0022] The immunogenic fusion protein of any of the preceding embodiments comprises one, three or five SP peptides.

[0023] The immunogenic fusion protein of any of the preceding embodiments comprises one IL-31 polypeptide or immunogenic fragment thereof, fused to one, three or five SP peptides.

[0024] The immunogenic fusion protein of any of the preceding embodiments comprises at least one SP peptide fused to at least one IL31 polypeptide or immunogenic fragment thereof by a flexible amino acid linker GSGS.

[0025] The immunogenic fusion protein of the preceding embodiment comprises one IL31 polypeptide fused to one SP peptide.

[0026] The immunogenic fusion protein of the preceding embodiment comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23 or SEQ ID NO: 29.

[0027] The immunogenic fusion protein of any of the preceding embodiments comprises one IL31 polypeptide and three SP peptides linked with flexible peptide linkers.

[0028] The immunogenic fusion protein of the preceding embodiment comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 14, SEQ ID NO: 20, SEQ ID NO: 26 or SEQ ID NO: 32.

[0029] The invention also relates to a recombinant vector comprising at least a nucleotide encoding the immunogenic fusion protein of any of the preceding embodiments.

[0029a] Disclosed herein is an recombinant vector comprising at least a nucleotide encoding the immunogenic fusion protein as defined herein, preferably, wherein said recombinant vector comprises: a) a nucleotide sequence encoding for said immunogenic fusion protein having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 or SEQ ID NO: 31; or b) a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

[0030] The recombinant vector of the preceding embodiment comprises a nucleotide sequence encoding for an immunogenic fusion protein having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 or SEQ ID NO: 31.

[0031] The recombinant vector of the preceding embodiment comprises a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

[0032] The invention relates to a method for producing the immunogenic fusion protein of the any of the preceding embodiments in a procaryotic or eukaryotic expression system.

[0033] Also disclosed herein is a method for producing the immunogenic fusion protein as defined herein, wherein said immunogenic fusion protein is produced in a procaryotic or eukaryotic expression system as inclusion bodies, said immunogenic fusion protein being solubilized in high molar concentrations of urea, preferably, wherein the recombinant vector comprises a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

[0033] The method of the preceding embodiment wherein the immunogenic fusion protein is obtained from inclusion bodies that are soluble in high molar concentrations of urea.

[0034] The method of any of the preceding embodiments, wherein the recombinant vector comprises a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

[0035] Disclosed herein is an immunogenic composition produced according to the method of any of the preceding embodiments.

[0036] The invention also relates to an immunogenic composition comprising at least one immunogenic fusion protein of any of the preceding embodiments.

[0037a] Disclosed herein is an immunogenic composition comprising at least one immunogenic fusion protein as defined herein, preferably, wherein said immunogenic composition further comprises an acceptable carrier and/or an adjuvant selected from the group consisting of oil-in-water adjuvant, polymer and water adjuvant, water-in-oil adjuvant, aluminum hydroxide adjuvant and combinations thereof, and, more preferably, wherein the adjuvant is a complete or incomplete Freund's adjuvant, a MontanideTM, Gel 01 PR, aluminum salts (alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, nonionic block copolymers, derivatized polysaccharides, cytokines, or bacterial derivatives.

[0037] The immunogenic composition of any of the preceding embodiments further includes an acceptable carrier and/or an adjuvant selected from the group consisting of oil-in-water adjuvant, polymer and water adjuvant, water-in-oil adjuvant, aluminum hydroxide adjuvant and combinations thereof.

[0038] The immunogenic composition of the preceding embodiment comprises at least one adjuvant selected from a complete or incomplete Freund's adjuvant, a polymeric adjuvant such as Montanide^{TM} gel, aluminum salts (alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, nonionic block copolymers, derivatized polysaccharides, cytokines, or bacterial derivatives.

[0039] The immunogenic composition of any of the preceding embodiments may be used as a medicament, preferably a vaccine.

[0039a] Disclosed herein is an immunogenic composition as defined herein for use as a medicament, preferably a vaccine, in the treatment and/or the prevention of inflammation in pruritus-related skin disorder, preferably AD, most preferably chronic and/or refractory AD, preferably, wherein AD is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

[0040] The immunogenic composition of the preceding embodiment may be used in the treatment and/or the prevention of inflammation in pruritus-related skin disorder, preferably AD, most preferably chronic and/or refractory AD.

[0041] The immunogenetic composition of the preceding embodiment may be used in the treatment of AD that is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

[0042] The invention also relates to a pharmaceutical composition for treating or preventing AD and/or AD-related symptoms in a subject comprising at least one immunogenic fusion protein of any of the preceding embodiments and a pharmaceutically acceptable carrier.

[0042a] Disclosed herein is a pharmaceutical composition for treating or preventing AD and/or AD-related symptoms in a subject comprising at least one immunogenic fusion protein as defined herein and a pharmaceutically acceptable carrier , preferably, wherein the AD-related symptoms are pruritus and/or secondary skin lesions, and, more preferably, wherein AD is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

[0043] The pharmaceutical composition of the preceding embodiment may be used to reduce AD-related symptoms such a pruritus and/or secondary skin lesions.

[0044] The pharmaceutical composition of the preceding embodiment may be used when AD is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor, and cyclosporine.

[0045] The invention also relates to a method for treating or preventing AD and or AD-related symptoms in a mammal comprising administering an effective amount of the immunogenic composition of any of the preceding embodiments.

[0046] In the method of the preceding embodiment, the AD-related symptoms may be pruritus and/or secondary skin lesions.

[0047] In the method of any of the preceding embodiments, the administering of the effective amount of the immunogenic composition results in the reduction of a pruritus score. The pruritus score is determined using a pruritus scale as defined in **Fig. 6A** (e.g., scratching, biting, licking, and rubbing against objects or the floor).

[0048] In the method of any of the preceding embodiments, the AD-related symptoms may be refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor, and cyclosporine.

[0049] In the method of any of the preceding embodiments, the immunogenic composition is administered in combination with at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

[0050] In the method of any of the preceding embodiments, the immunogenic composition is administered in combination with at least one receptor antagonist that blocks a nociceptive signaling pathway, preferably a neurokinin-1 receptor (NK1-R) antagonist, most preferably compound CP-96,345.

[0051] In the method of any of the preceding embodiments, the immunogenic composition may be administered orally, subcutaneously, intramuscularly, or transdermally, preferentially subcutaneously.

[0052] The method of any of the preceding embodiments includes the administering of the immunogenic composition as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, over a period of time needed to sustain high neutralizing antibody titers.

[0053] The method of any of the preceding embodiments may be performed in a mammal such as a canine, human, feline or equine.

[0054] A method for treating or preventing AD or AD-associated symptoms in a dog comprises the subcutaneous administering of a bivalent vaccine as a priming dose followed by three booster doses at 2 weeks intervals, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time needed to sustain high neutralizing antibody titers, wherein said bivalent vaccine comprises an

immunogenic fusion protein IL31-SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% % sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

[0055] In the method of any of the preceding embodiments, the pruritus score which is evaluated according to the pruritus scale defined in **Fig. 6A** (e.g., scratching, biting, licking, and rubbing against objects or the floor) is reduced in the treated mammal.

[0056] In the method of the preceding embodiment, the pruritus score ranges from 1 to 3.

[0057] The invention relates to a bivalent vaccine for treating or preventing an inflammation-related skin disorder, preferably AD or AD-associated symptoms including pruritus, erythema and/or associated hair loss in dogs comprising at least one immunogenic fusion protein of any of the preceding embodiments.

[0057a] Disclosed herein is a bivalent vaccine for treating or preventing AD or AD-associated symptoms in a dog, wherein said bivalent dose is administered subcutaneously as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time required to sustain high neutralizing antibody titers, wherein said bivalent vaccine comprises an immunogenic fusion protein IL31-SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

[0057b] Disclosed herein is a bivalent vaccine for treating or preventing an inflammation-related skin disorder, preferably AD or AD-associated symptoms in mammals comprising the immunogenic fusion protein as defined herein.

[0057c] Disclosed herein is a bivalent vaccine for treating or preventing AD and/or pruritus in dogs comprising an immunogenic fusion protein IL31-1SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or

100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

[0058] The bivalent vaccine of the preceding embodiment contains an immunogenic fusion protein IL31-SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

BRIEF DESCRIPTION OF THE DRAWINGS

[0059] **Fig. 1** is a schematic representation showing the pathophysiological contribution of IL31 and Substance P on AD itching and inflammation and an illustration of the mechanism of vaccination-induced neutralization to alleviate chronic inflammation. (Modified from Ständer and Yosipovitch, 2019 [1]). A) Pathway of IL-31 Induced Itch Perception and Role of Substance P. This figure illustrates the pathway by which IL-31 induces itch perception, highlighting the involvement of Substance P and its receptors. **Top Panel**: Diagram showing the epidermis and underlying dermis, indicating the presence of keratinocytes, immune cells, and nerve terminals. Allergen exposure and inflammation in the epidermis lead to the production and release of IL-31 by immune cells. **Middle Panel**: Zoom-in view detailing the interaction between IL-31, Substance P, and their receptors. IL-31 produced by inflammatory immune cells acts on IL-31 receptors located on peripheral sensory nerve terminals. This interaction stimulates the release of Substance P from the sensory nerve terminals. **Bottom Panel**: Pathway of sensory signal transmission from the peripheral nerve terminals to the brain. The released Substance P binds to its receptors on second-order neurons in the dorsal horn of the spinal cord. These neurons then transmit the itch signal to the brain, leading to the perception of itch. B) Schematic representation of the vaccination process to neutralizeIL-31 and substance P in the host. The process begins with the administration of a non-natural recombinant fusion protein to the host (1), which elicits the production of neutralizing antibodies against IL31 and substance P (2). These antibodies then neutralize the inflammatory mediators in the skin (3), leading to a significant reduction in chronic inflammation, decreased itching and scratching behaviors, and an overall improvement in the host's quality of life (4)..

[0060] **Fig. 2**. illustrates the construction, expression, and purification of the recombinant fusion proteins IL31-1SP and IL31-3SP. Panel A shows the genetic constructs with 6xHis tags, TEV sites, and sequences for IL31 and one or three SP fragments, connected by GSGS linkers. Panel B outlines the expression process in E. coli BL21, followed by purification, endotoxin removal, and quality testing. Panel C presents SDS-PAGE analysis confirming the expected molecular weights of the purified proteins. Panel D displays mass spectrometry data, confirming the molecular weights and sequence coverage, validating the correct assembly of IL31 and SP fragments.

[0061] **Fig. 3**. This figure illustrates the immunogenic response in mice vaccinated with IL31-1SP and IL31-3SP. Panel A details the immunization protocol, with Group 1 receiving IL31-1SP and Group 2 receiving IL31-3SP, each followed by three booster doses. Panel B shows significant increases in IgG titers specific to IL31-1SP, IL31-3SP, native IL31, and SP post-vaccination in both groups. Panel C demonstrates that the presence of competitive antigens (non-fused canine IL31 and SP) reduced IgG binding to IL31-3SP in a dose-dependent manner, confirming the specificity of the antibodies generated against IL31 and SP (*$p<0.05$, **$p<0.01$).

[0062] **Fig. 4**. shows the immunogenic response in mice vaccinated with IL31-3SP compared to the combination of non-fused canine IL31 and non-fused canine SP. Panel A outlines the immunization protocol where Group 1 received IL31-3SP and Group 2 received non-fused IL31 and SP, with a priming dose followed by three booster doses at 2-week intervals. Panel B shows significantly higher IgG titers specific to native canine IL31, native canine SP, and IL31-3SP in the IL31-3SP vaccinated group compared to the group receiving non-fused proteins. This indicates the superior immunogenic properties of the IL31-3SP fusion protein over the separate administration of its components, demonstrating enhanced antibody responses against both native IL31 and SP. Statistical significance is indicated by *$p < 0.05$.

[0063] **Fig. 5**. shows the immunogenic response in dogs vaccinated with IL31-1SP. Panel A outlines the immunization protocol, with dogs receiving a priming dose of 5 mg of IL31-1SP followed by three booster doses at 2-week intervals, with blood collection and clinical evaluation points indicated. Panel B displays specific antibody titers to native canine IL31, showing significant increases post-vaccination in all dogs. Panel C illustrates specific antibody titers to native canine SP, also showing significant

increases post-vaccination. This indicates that the IL31-1SP recombinant fusion immunogen effectively induces a robust immune response against both IL31 and SP in vaccinated dogs.

[0064] **Fig. 6**. evaluates the effectiveness of IL31-1SP treatment in client-owned dogs with atopic dermatitis. These results suggest that IL31-1SP effectively reduces itching and the need for corticosteroids in dogs with atopic dermatitis. Evaluation of itch severity and corticosteroid use in atopic dermatitis affected dogs vaccinated with the recombinant fusion immunogen IL31-1SP. **A.** Scoring system for measuring itching severity: A 1-10 scale is used to assess the level of itching in dogs with atopic dermatitis. The scale categorizes the severity of symptoms, secondary symptoms, and recommended treatments. Scores range from 1 (sporadic scratching with no lesions) to 10 (compulsive scratching requiring an E collar). **B.** Scratching scores before and after IL31-1SP treatment: The graph displays the scratching scores of dogs before treatment with IL31-1SP and at 1 month, 1.5 months, and 2 months post-treatment. The data indicate a significant reduction in scratching scores over time, with the most notable decrease observed at 2 months post-treatment (*$p<0.05$). **C.** Number of dogs consuming corticosteroids: The bar graph shows the number of dogs requiring corticosteroids before vaccination and 90 days after vaccination with IL31-1SP. There is a significant reduction in the number of dogs consuming corticosteroids 90 days post-vaccination, indicating an improvement in symptoms and reduced need for corticosteroid treatment.

DETAILED DESCRIPTION

[0065] The invention is directed to a comprehensive approach to AD therapeutics based on the simultaneous control of the synergistic effects of excessive IL31 and SP-mediated neurogenic inflammation in AD. In particular, an embodiment is directed to a self-antigen vaccine based on an immunogenic fusion protein or immunogen comprising IL31 linked with SP by a flexible linker. When administered into a mammal as a vaccine, the immunogen elicits an immune response including the production of antibodies against native IL31 and SP with the potential to neutralize excessive levels of both peptides in AD and/or pruritus. Advantageously, the use of immunogenic fusion proteins IL13-1SP or IL31-3SP in the treatment of AD leads to the control of neurogenic inflammation and pruritus by inducing a synergistic antibody

response against IL31 and SP. In particular, it provides a long-lasting relief of itching, scratching in immunized animals.

[0066] The pathophysiology of AD is complex and not fully understood. It is recognized that canine AD is a multifactorial disease that involves a combination of genetic, environmental, and immunological factors [10]. The immune system plays a critical role in the development and progression of AD with abnormal immune response to environmental allergens being the main triggers. The abnormal immune response is closely associated to defective function of keratinocytes and the skin barrier, as well as a sensitization of nerve fibers that innervate the skin and signal itching [13]. Keratinocytes influence the interaction between dendritic cells and lymphocytes, with thymic stromal lymphopoietin playing a role in connecting skin barrier damage to the modulation of T-helper (Th)2 response, and an imbalanced immune response characterized by elevated Th2, Th17, and CD4+ CD25+ regulatory T cells has been documented [14].

[0067] The biological causes of pruritus and skin inflammation in AD are believed to be mediated by a complex interface between sensitized skin Th2 lymphocytes, keratinocytes, and cutaneous pruritogenic nerve fibers that project to the CNS. However, the neurogenic component of AD is not addressed by any specific current therapeutics. In fact, an antagonist of neurokinin-1 receptor (NK1-R) that blocks the nociceptive signaling pathway, has been shown to exert no significant improvement of AD, except in one study using animal models [59].

[0068] Current treatments of AD in companion animals remain limited, considering the disease is a chronic allergic condition with no definite cure. The main therapeutic objective involves environmental management, allergen avoidance, hygienic measures, topical therapies, and allergen-specific immunotherapy. The pharmacological treatment of canine AD has seen advances in recent years with an increasing complexity. The use of systemic medications, including corticosteroids, antihistamines, cyclosporine or tyrosine kinase inhibitors targeting JAK1-dependent pathways have been shown to control skin inflammation and relieve the symptoms of itching and scratching, although their chronic use may lead to adverse effects [4].

[0069] Passive immunotherapy targeting cytokines such as IL31 have been recently developed for clinical use veterinary medicine given its role as a neuroimmune

mediator. Lokivetmab (Cytopoint, Zoetis) is a canine IL31 monoclonal antibody that has been approved for the treatment of CAD. It provides a long-lasting relief of itching in dogs with AD and allergic dermatitis [49; 50; 51; 52; 53; 54; 55]. In human patients with AD, the humanized monoclonal antibody nemolizumab targeting the IL31 receptor (IL31RA), has been recently approved in Japan [56]. Systemic immunization using IL31-based vaccines has also been shown to ameliorate the symptoms of AD in dogs [57] and insect bite hypersensitivity skin lesions in horses [58], although these vaccines remain in developmental stage and use virus like particle technology. Despite the recent advances in the treatment of CAD, management of the disease in canines remains difficult and costly. Furthermore, the cost of anti-IL31 monoclonal antibodies is significant and requires frequent and long-term dosing, limiting its use to a fraction of affected animals worldwide. Unfortunately, the failure of low-cost conventional therapies results in many patients having diminished quality of life and medical complications.

[0070] IL31 signaling plays a pathogenic role in the development of pruritus and atopic dermatitis-like skin lesions [17]. IL31 belongs to the IL-6 family of cytokines and is predominantly expressed during inflammation and immune-related processes [18]. Several studies have shown that IL31 is expressed by immune cells, including sensitized effector Th2 cells, eosinophils, basophils, mast cells, monocytes, macrophages and dendritic cells, with effector memory Th2 cells representing the major source of IL31 [19; 20; 21; 22; 23]. The immune sensitization of IL31-producing effector Th2 cells in AD is a complex process involving the recognition of allergens by antigen presenting cells from the skin, activation of T cells, production of IgE antibodies by B lymphocyte, and release of pro-inflammatory mediators upon re-exposure to the allergen [24]. Upon release, IL31 binds to a heterodimeric receptor composed of the IL31RA chain, which is expressed on itch-conducting dorsal root ganglia neurons and on their skin nerve terminals, but also in other cell types such as keratinocytes, fibroblasts and other subsets of sensory neurons [25; 26]. In IL31RA+ neurons, IL31 activates the ion channels TRPV1 and TRPA1 which mediate intracellular Ca2+ mobilization [23]. These neurons are believed to signal to the dorsal horn of the spinal cord and then to projection neurons that transport the information to the brain [27]. In addition, IL31 also induces a distinct transcriptional program in sensory neurons, leading to sprouting and branching of nerve terminals in AD skin [28; 29]. The ability of IL31 to increase the density of neuronal networks and activate ion

channels in nerve terminal may explain the increased sensitivity to subtle stimuli inducing itch in AD and the triggering of neurogenic inflammation. Other cellular targets of IL31 include keratinocytes and dermal fibroblasts. IL31 prevents the normal differentiation of keratinocytes and hence contributes to disrupt the skin's barrier function [30]. IL31 also stimulates the production of cytokines, chemokines, and pruritus mediators by keratinocytes and dermal fibroblasts, which intensifies inflammation and tissue remodeling in the skin [31].

[0071] Neurogenic inflammation in AD is mediated by the release of neuropeptides, including SP, from primary afferent sensory C-fiber nerve terminals innervating the skin [1; 32]. Upon release, SP locally interacts with surrounding endothelial cells, keratinocytes, macrophages, and mast cells inducing inflammatory phenotypes [33]. Abundant SP-positive nerve fibers have been shown in lesioned skin from patients with AD but not in healthy controls [34; 35]. SP-positive nerve fibers close to dermo-epidermal junction as well as the SP receptor NK1R, were overexpressed in pruritic skin [36]. In addition, SP-positive fibers spatially associate with mast cells in AD and may induce degranulation in mast cells, increasing the release of histamine and serotonin, proteolytic enzymes as well as cytokines and trophic factors [37].

[0072] Neurogenic inflammation is involved in AD and is mediated by the release of neuropeptides such as Substance P (SP) from sensory nerve terminals of the skin [15; 16]. Thus, increased expression of IL31 by immune cells and keratinocytes, together with increased release of SP from nerve terminals may synergistically mediate AD symptoms, including pruritus, skin inflammation, and remodeling of the affected epithelium. While IL31 induces itch sensation and inflammation in the skin, substance P acts as a neuropeptide involved in local neurogenic inflammation and indirectly transmitting itch signals through the induction of mast cell degranulation which comprises pruritogenic histamine. Evidence indicates that the binding of IL31 to its receptor on sensory nerve fibers triggers the release of SP, which further enhances itch perception and promotes inflammatory processes in atopic dermatitis. Likely, this interaction between IL31 and Substance P contributes to the persistent itching and chronic inflammation characteristic of the disease. In addition, evidence indicates significant changes in nerve innervation pattern underlying chronic itching and inflammation associated with AD [38]. Repeated scratching and rubbing of the affected

skin can lead to an overgrowth or hyperplasia of nerve fibers, particularly those that transmit itch signals to the brain. Evidence indicates that increased neuropeptide NGF and histamine levels in the AD skin stimulate growth and proliferation of nerve fibers, including upregulation of ion channels and SP [39]. Over time, these nerve fibers become more abundant and sensitive, resulting in a heightened perception of itchiness contributing to the vicious cycle of scratching and further skin damage. Therefore, substance P is currently considered as one of the key pruritogenic inflammatory factors in AD [40; 41]. As a mediator of itch signaling, SP acts on NK1R which are expressed in skin cells and in the central nervous system. Antagonists of SP-NK1R signaling have been shown to disrupt itch behavior to some extend [1; 42]. SP and neurogenic inflammation have been proposed as a key mediator by which stress worsen skin inflammation [16]. In addition, SP is known to stimulate degranulation of skin mast cells through the receptor Mas-related G protein-X2 (MRGPRX2), which may also play a pathogenic role in AD [43; 44].

[0073] In humans, topical application or intradermal administration of SP to the skin is sufficient to elicit flare and pruritus [33; 45; 46; 47]. In mice, cutaneous injection of SP resulted in dose-dependent increases in scratching at the injection site and skin scratching resulted in an increased number of SP-immunoreactive cutaneous nerve fibers [36], further suggesting a link between sensitized skin nerve terminals and neurogenic inflammation mediated by SP. However, various reports have found little or no therapeutic effect of pharmacological blockade of the NK1R in AD [1], suggesting that alternative receptors such as expressed in mast cells may be main signaling pathways of SP in AD [44; 48]. Thus, IL31 and SP delineate a complex and interactive pathological axis in AD. While IL31 acts on various cells in the skin, including keratinocytes, immune cells, and sensory nerves to induce itching, it also induces long term changes in skin nerve terminals that become sensitized. In turn, SP released by nerve terminals can activate mast cells, keratinocytes, endothelial and immune cells to induce local inflammation and further infiltration of immune cells.

[0074] Synergistic effects of IL31 and SP on AD itching and inflammation model **(Fig. 1)**. Following allergen exposure, immune cells in the skin release IL31, which leads to pruritogenic signal transmission to the CNS via sensory c-fibers. Additionally, IL31 increases the expression of other itch-related molecules, such as TRPV1 ion channels and neuropeptides, which contribute to increasing the density of

sensory nerve terminals in AD skin. Activated C-fibers release Substance P (SP) at nerve endings, causing neurogenic inflammation and vasodilation. Histamine and cytokines released by immune cells in response to SP amplify the primary AD trigger, perpetuating skin inflammation. Neurogenic and neuroendocrine mechanisms triggered by psychosocial stress can further promote immune cell activation. Thus, IL31 and SP act together to enhance the signaling and perception of itch, resulting in scratching behavior and exacerbating skin inflammation in AD. The neutralization of excessive levels of IL31 and SP by therapeutic antibodies elicited by the IL31-1SP or IL31-3SP vaccine can ameliorate AD signs and recurrence, reduce scratching, and restore natural skin barriers to allergens. (**Fig. 1).**

[0075] Some embodiments of the current invention are discussed in detail below. In describing embodiments, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. A person skilled in the relevant art will recognize that other equivalent components can be use and other methods developed without departing from the broad concepts of the current invention. All references cited anywhere in this specification, including the Background and Detailed Description sections, are incorporated by reference as if each had been individually incorporated.

[0076] Definitions are included herein for the purpose of understanding the present subject matter and the appended claims. The abbreviations used herein have their conventional meanings within the chemical and biological arts.

[0077] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. *See, e.g.,* Singleton et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY 2nd ed., J. Wiley & Sons (New York, NY 1993); Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, Cold Springs Harbor Press (Cold Springs Harbor, NY 1989). Any methods, devices and materials similar or equivalent to those described herein can be used in the practice of this invention. The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

[0078] The present description identifies certain nucleotide and amino acid sequences (polynucleotides and polypeptides) as part of the invention. It is to be

understood that the specifically identified sequences adequately describe other sequences that contain less than 100% sequence identity but to the identified sequences that provide the same function. For example, a nucleotide sequence may have 90% sequence identity or 95% sequence identity to a polynucleotide specifically disclosed herein and still encode for an entirely equivalent or functionally equivalent polypeptide. Similarly, a polypeptide may contain less than 100% sequence identity to a polypeptide specifically identified herein and provide the same function. For example, a polypeptide may have 90% sequence identity, 95% sequence identity, or 99% sequence identity to a polypeptide specifically disclosed herein and still retain the same or sufficiently similar activity or functionality as the specifically identified polypeptide.

[0079] As used throughout, the term "gene" refers to a nucleotide sequence or a part thereof having a functional role in protein coding or transcription, or regulation of other gene expression. The gene may be composed of all nucleotides encoding a functional protein or a part of the nucleotide encoding or expressing the protein. The nucleotide sequence may include a gene mutation in exon, intron, initiation or termination region, promoter sequence, other regulatory sequence, or a unique sequence adjacent to the gene.

[0080] As used throughout, the term “neurogenic mediator or factor” refers to a neuropeptide that directly or indirectly promotes inflammation, including but not limiting to SP. In the context of the invention, the release of a neurogenic factor into the skin contributes to the development of inflammation leading to atopic dermatitis and pruritus.

[0081] As used throughout, the term “immune factor or mediator” refers to a polypeptide, particularly cytokines e.g., IL31, whose biological activity affects the immune system and inflammation. In the context of the invention, the immune factor has a pro-inflammatory and pruritogenic activity such as IL31.

[0082] As used throughout, the term “immunogenic fragment or immunogen” refers to an amino acid sequence that has the ability to induce a humoral and/or cell-mediated immune response. For example, an immunogenic fragment derived from IL31 is capable of eliciting the production of antibodies against IL31.

[0083] As used throughout, the term “neurogenic inflammation” refers to the physiological process by which mediators are released directly from the sensory nerves

to initiate an inflammatory reaction. This results in production of local inflammatory responses including erythema, swelling, temperature increase, tenderness, and pain. Fine unmyelinated afferent somatic C-fibers, which respond to low intensity mechanical and chemical stimulations, are largely responsible for the release of inflammatory mediators. When stimulated, these nerve fibers in the cutaneous nerves rapidly release active neuropeptides such as SP into the microenvironment, triggering a series of inflammatory responses.

[0084] As used throughout, the term “antibody or immunoglobulin” refers to a protein produced by the B-cells of the immune system that can identify, bind and neutralize an antigen. In the context of the invention, an antibody is produced by the immune system and binds an endogenous protein or polypeptide, such as a neurogenic mediator or a cytokine. The antibody may have neutralizing properties and may be capable of suppressing or reducing the biological activity of the downstream pathway mediated by the neurogenic mediator or cytokine such as blocking its binding to its specific receptor.

[0085] As used throughout, the term “self-antigen” refers to any molecule or chemical group of an organism which acts as an antigen in inducing antibody production in another organism but to which the healthy immune system of the parent organism is tolerant. Immunization/vaccine against self-antigens requires a specific design of the immunogens and formulations that allows the vaccination to break the self-tolerance in a specific organism. Due to several central and peripheral tolerance mechanisms, it is extremely challenging to induce long lasting immune response to self-antigens. In the context of the invention, the self-antigens are immune mediators such as endogenous IL31 and neurogenic mediators such as SP.

[0086] As used throughout, the term “active immunization” refers to immunization that stimulates the immune system to produce antibodies against self-antigens. Active immunization can be induced through vaccination. In the context of the invention, a bivalent vaccine or immunogenic composition comprises at least one immunogenic fusion protein comprising at least 2 self-antigens or endogenous peptides such as IL31 and SP. When injected to a mammal, the vaccine induces the production of polyclonal antibodies against different epitopes without causing any illness or uncontrolled side effects. Such antibodies may have neutralizing or therapeutic properties that will capture the self-antigens, thereby modulating their respective

function(s). Active immunization is often long-lasting and may be reactivated by repeated injection of boosters. In contrast, passive immunization occurs when antibodies directed against specific antigen are administered to a subject.

[0087] As used throughout, the term “adjuvant” refers to a substance that increases the intensity of the immune response after co-administration with an immunogen. An adjuvant may act as an immunopotentiator useful for enabling immunogenic composition or vaccine to induce potent and persistent immune responses, while reducing the dose and number of boosters. Adjuvant may also increase the stability of the immunogenic composition or vaccine.

[0088] As used throughout, the term “recombinant protein or recombinant polypeptide” may be used interchangeably and refer to a protein or polypeptide encoded by recombinant nucleotide that has been cloned in an expression vector that supports expression of the gene and translation of messenger RNA. *Escherichia coli* (bacteria) is one of the organisms of choice for the production of recombinant proteins. Its use as a cell factory is well-established and it has become the most popular expression platform. High-level expression of many recombinant proteins in *Escherichia coli* leads to the formation of highly aggregated protein commonly referred to as inclusion bodies. Inclusion bodies are normally formed in the cytoplasm. Bacterial inclusion bodies are mesoscale protein aggregates commonly observed in recombinant bacteria, primarily formed by recombinant protein. Other expression system may include but are not limited to insect cells and yeast cells.

[0089] As used throughout, the term “fusion protein or fusion polypeptide” refers to a hybrid protein or polypeptide having an amino acid sequence comprising at least two partial or complete sequences derived from, obtained from, or isolated from different polypeptides that are not naturally adjoined. The terms “fusion” and “chimeric” may be used interchangeably throughout. A fusion protein or fusion polypeptide is the functional product of a fusion gene or fusion nucleotide sequence. Fusion gene can further be modified by mutation, deletion, insertion or substitution of heterologous sequences, or by any means available using recombinant DNA technology.

[0090] As used throughout, the term “fragment” refers to a peptide or polypeptide of chain-type polymer formed by at least 6 amino acid residues which are

linked to each other via peptide bonds. It may also include the complete amino acid sequence of the native polypeptide or protein. It may include amino acid sequences that are conservative variations. The terms fragment, peptide and polypeptide are used interchangeably. In the context of the invention, an immunogenic fragment is a peptide or polypeptide capable of eliciting an immune response cellular and/or humoral, including the production of specific antibodies directed to that immunogenic fragment or to the protein having the immunogenic fragment. A person having ordinary skill in the art may introduce substitutions to obtain immunogenic fragments having higher immunogenicity. For example, one aspect disclosed in the present application provides fragments corresponding to amino acid sequences (e.g., SEQ ID NOS: 1-9), as well as analogues, homologs, isomers, derivatives, amidated variations, and conservative variations thereof, as long as the immunogenicity of the fragment remains. The IL31 polypeptide may comprise at least one of an immunogenic fragment derived from the canine IL31 polypeptide defined as SEQ ID NO: 1, the equine IL31 polypeptide defined as SEQ ID NO:2, the human IL31 polypeptide defined as SEQ ID NO:3, or the feline IL31 polypeptide defined as SEQ ID NO:4. The SP peptide may comprise one of the canine SP peptide defined as SEQ ID NO: 5, the equine SP peptide defined as SEQ ID NO: 6, the mouse SP peptide defined as SEQ ID NO: 7, the human SP peptide defined as SEQ ID NO: 8, or the feline SP peptide defined as SEQ ID NO: 9. Minor modifications to primary amino acid sequences disclosed in the present application may result in fragments which have substantially equivalent or enhanced immunogenicity, as compared to the specific fragments described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous.

[0091] All peptides, polypeptides or fragments may be synthesized using L-amino acids, but D forms of all the peptides may be synthetically produced. In addition, C-terminal derivatives, such as C-terminal methyl esters and C-terminal amidates, may be produced to increase the immunogenicity of the peptide according to one embodiment disclosed in the present application.

[0092] The present invention is directed to the fusion of endogenous peptides IL31 and SP (i.e., self-antigens) as peptide-based vaccines for active immunization in mammals suffering from inflammatory skin conditions, pruritus (itching) and dermatitis. The recombinant fusion proteins are obtained from inclusion bodies and are soluble in high molar concentrations of urea (4-8M). The sequence and biophysical

properties support the fact that the recombinant fusion immunogens exhibit non-native conformations, which are not occurring naturally. These features allow the recombinant fusion protein to be highly immunogenic, overpassing the natural immune tolerance to self-antigens such as native IL31 and SP, while having the unexpected ability to elicit a therapeutic immune response, including antibodies that cross-react with IL31 and SP.

[0093] In contrast to traditional vaccines targeting pathogens, the bivalent vaccines against self-antigens such as IL-31 and SP of the present invention requires a specific design where sequences of the peptides and/or polypeptides are appropriately connected by flexible peptide linkers comprising Glycine (Gly) and Serine (Ser) residues in short sequences such as GSGS within a recombinant fusion protein. As a result, the recombinant fusion protein adopts a non-native conformation allowing recognition as non-self and triggering an immune response that is not subject to central and peripheral tolerance. The bivalent immunogenicity of the recombinant fusion protein induces high anti-IL31 and anti-SP antibodies titers which are not observed when non-fused components IL-31 and SP are administered as single agents.

[0094] As used herein, “high neutralizing antibody titers” refers to neutralizing antibody titers that are therapeutically more significant compared to the administration of IL31 and SP as single agents, either individually or in combination. High neutralizing antibody titers may also refer to titers that are sustained throughout a therapeutically more effective window compared to administration of IL31 and SP as single agents, either individually or in combination, thus providing longer term immune memory.

[0095] **Immunogenic fusion proteins targeting IL31 and SP**

[0096] In the context of the invention, the terms “fusion protein”, and “recombinant fusion protein” are used interchangeably since the fusion protein is the product of a recombinant DNA and is generated by a recombinant expression system.

[0097] In some embodiments, an immunogenic fusion protein includes immunogenic fragments derived from IL31 and SP and elicits the production of immunoglobulins that are capable of neutralizing endogenous IL31 and/or SP.

[0098] In some embodiments, the immunogenic fusion protein comprises a IL-31 polypeptide and one or more SP peptides, for example three or five SP peptides,

each connected by a flexible amino acid linker, potentially enhancing the immunogenicity of the recombinant fusion protein. The linker is of a length such that the polypeptides are linked without substantial interference. In some embodiments, a linker may be chosen to maintain structural flexibility while ensuring non-native conformations of the recombinant fusion protein. In other embodiments, a linker may also provide additional beneficial properties to the protein, such as increased protein expression in expression systems, improved biophysical properties such as stability and solubility, improved protein purification and detection and/or increased enzymatic activity. Exemplary linkers may have the formula Ser(Gly4Ser)n or (Gly-Ser)n residues with some Glu or Lys residues dispersed throughout to increase solubility, where n can be an integer from 1 to 20, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In another aspect of the invention, the at least one SP peptide is fused to the at least one IL31 polypeptide or immunogenic fragment thereof by a flexible amino acid linker, preferably GSGS.

[0099] Antibodies possess significant advantages as therapeutic agents because of their higher specificity and reduced off-target effects. By binding to their respective self-antigens, polyclonal antibodies may inhibit or reduce the biological activity of the neurogenic SP and immune IL31 mediators and/or their downstream signaling pathways such as blocking the binding to their specific receptor or suppressing the cellular response(s) the mediators induce. In some embodiments, systemic active immunization of a mammal (e.g., dogs, horse, human or cat) affected by AD and pruritus with at least one immunogenic fusion protein of the embodiments such as IL31-1SP or IL31-3SP results in unexpectedly high titers of antibodies that cross-react with endogenous IL31 and SP.

[00100] In some embodiments, the recombinant fusion protein exhibits an inherent immunogenicity due to its design and is capable of overcoming immune tolerance against the self-antigens IL31 and/or SP when administered to a mammal. In some aspects of the embodiments, the immunogenic fusion protein may be recognized non-self, and triggering an immune response that is not subject to immune tolerance with the production of neutralizing antibodies against IL-31 and/or SP. The fusion of multiple sequences derived from IL31 and SP results in a recombinant protein that exhibits enhanced immunogenic properties. Notably, the fusion protein induces higher

antibody titers against endogenous IL31 and SP when compared to that of the equivalent amounts of non-fused IL31 and non-fused SP administered as single agents.

[00101] In some embodiments, the recombinant fusion protein comprises engineered sequences derived from at least one IL31 polypeptide and at least one SP peptide which exhibits enhanced immunogenic properties. Support for the chimeric approach in conceiving a non-natural recombinant fusion protein comes from its unique immunogenic profile against native IL-31 and SP, as compared to the individual IL-31 and SP peptides administered individually or in combination as single agents. In one aspect of the embodiments, the immunogenic fusion protein may comprise multiple copies of SP peptide, preferably one, three or five SP peptides. Increasing the number of SP sequences within the fusion protein enhances SP exposure and immunogenicity. Without being bound by theory, it is believed that several features contribute to the improved immunogenicity. 1) Structure-function relationship: substance P acts as a neurogenic mediator involved in inflammation and itch sensation. Amplifying the number of SP peptides within the fusion protein may increase the number of potential conformational epitopes presented and recognized by immune cells, such as B cells, thereby enhancing a humoral response. 2) Multivalent immune recognition: the presence of multiple SP copies in a recombinant fusion protein increases the local antigen concentration and provides multivalent antigenic stimuli. Multivalent antigens have the potential to engage multiple B cell receptors simultaneously, leading to enhanced immune activation and antibody production. This strategy capitalizes on the concept of avidity, where increased binding interactions can result in stronger immune responses compared to monovalent antigens. 3) Moreover, extending the length of peptides into longer polypeptides may overcome immune tolerance given that the recombinant fusion protein are recognized as non-self, and potentially triggering immune cell responses. After vaccination, long peptides may be processed by antigen-presenting cells (APCs) for presentation and immune cell activation, thus alleviating the potential immune tolerance and enhancing the vaccine potency. In some embodiments, the length and conformation of the immunogenic fusion protein are optimized to facilitate its internalization and processing by APCs, enhancing the overall immune response to endogenous IL31 and/or SP.

[00102] In preferred embodiments, the immunogenic fusion protein induces an immune response specific to IL31 and SP epitopes, thus reducing the likelihood of off-target effects.

[00103] In some embodiments, the immunogenic fusion protein IL31-1SP comprises one IL31 polypeptide or immunogenic fragment thereof fused to one SP peptide, each separated by a flexible peptide linker, for example GSGS. In other embodiments, the immunogenic fusion protein IL31-3SP comprises one IL31 polypeptide or immunogenic fragment thereof fused to 3 SP peptides, each separated by a flexible linker. Similar methodologies can be implemented to prepare immunogenic fusion proteins having more than one IL31-derived immunogenic fragment with one or more than one SP peptide.

[00104] In some embodiments, the mammal is a canine, and the immunogenic fusion protein comprises at least one IL31 polypeptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 1 and at least one SP peptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 5. In one aspect of the embodiments, the at least one IL31 polypeptide is fused to the at least one SP peptide with a flexible linker, for example GSGS. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to one SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 11. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to three SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

[00105] In some embodiments, the mammal is an equine, and the immunogenic fusion protein comprises at least one IL31 polypeptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 2 and

at least one SP peptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 6. In one aspect of the embodiments, the at least one IL31 polypeptide is fused to the at least one SP peptide with a flexible peptide linker, for example GSGS. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to one SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 17. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to three SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 20.

[00106] In some embodiments, the mammal is a human, and the immunogenic fusion protein comprises at least one IL31 polypeptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 3 and at least one SP peptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 8. In one aspect of the embodiments, the at least one IL31 polypeptide is fused to the at least one SP peptide with a flexible peptide linker, for example GSGS. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to one SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 23. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to three SP peptide. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 26.

[00107] In some embodiments, the mammal is a feline, and the immunogenic fusion protein comprises at least one IL31 polypeptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 and at least one SP peptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 9. In one aspect of the embodiments, the at least one IL31 polypeptide is fused to the at least one SP peptide with a flexible peptide linker, for example GSGS. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to one SP peptide connected by a flexible peptide linker, for example GSGS. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 29. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to three SP peptides, each separated by a flexible linker, preferably GSGS. In a preferred embodiment, the immunogenic fusion protein comprises an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 32.

[00108] In some embodiments, the mammal is a mouse, and the immunogenic fusion protein comprises at least one IL31 polypeptide and at least one SP peptide having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 7. In one aspect of the embodiments, the at least one IL31 polypeptide is fused to the at least one SP peptide with a flexible linker. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to one SP peptide connected by a flexible peptide linker, for example GSGS. In another aspect of the embodiments, the immunogenic fusion protein comprising one IL31 polypeptide fused to three SP peptides, each separated by a flexible linker, preferably GSGS.

[00109] In some embodiments, the nucleotide sequence encoding for the immunogenic fusion protein has at least 90%, at least 95 %, at least 99% sequence, or

100% nucleotide sequence identity to one of SEQ ID NO:10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 or SEQ ID NO: 31.

[00110] Therapeutic uses

[00111] The immunogenic fusion proteins of the invention are immunogens suitable for the treatment of diseases where inflammatory skin lesions and pruritus are predominant such as in AD.

[00112] In some embodiments, the immunogenic composition comprising an immunogenic fusion protein IL31-1SP and/or IL31-3SP significantly enhances the humoral immune response against endogenous IL31 and SP when administered in a mammal, as evidenced by the higher production of cross-reactive, high-affinity antibodies against endogenous IL31 and SP compared to that of a combination of non-fused IL31 and non-fused SP administered as single agents. In some aspects of the embodiments, the immunogenic composition combining at least one immunogenic fusion protein IL31-1SP and/or IL31-3SP and at least one appropriate adjuvant shows synergistic effects that further enhance the immune response, resulting in the production of antibodies that cross-react with native IL31 and SP, which mediate pathological effects in treated mammals. The results presented herein support the synergistic effects of combining IL31 and SP in a recombinant fusion protein to reduce inflammation and pruritus.

[00113] In the context of active immunization, the immunogenic composition may be formulated as a bivalent vaccine.

[00114] Advantageously, systemic immunization of a mammal with the immunogenic fusion protein of the invention, such as IL31-1SP or IL31-3SP provides long-term immunologic memory with persistent high antibody titers in the mammal’s immune system.

[00115] Systemic immunization with a combination of non-fused IL31 and non-fused SP administered as single peptide fails to yield significant antibody titers, as compared to the systemic immunization with the immunogenic composition comprising at least one recombinant fusion protein. The systemic immunotherapy using the immunogenic composition comprising at least one immunogenic fusion protein directed

to the neurogenic SP and immune IL31 mediators improves clinical symptoms associated with pruritus and secondary skin lesions while reducing the use and/or dose of conventional anti-inflammatory drugs such as corticoids, antihistamines, tyrosine kinase inhibitors. This is particularly relevant since chronic use of anti-inflammatory drugs are associated with the severe side effects.

[00116] The concomitant blockade of the IL31 and SP mediators results in a synergistic effect, thereby decreasing the dosage of immunogenic composition required to obtain an efficacious therapeutic benefit while preventing adverse effects. Surprising and unexpected results (i.e., neutralizing antibodies titers) show more than the additive effect that would be expected from immunization with native canine IL31 or native canine SP as single agents.

[00117] Notably, the capacity of the IL31-1SP and IL31-3SP immunogens to produce cross reactive and therapeutic antibodies that bind native IL31 and SP was unexpected, given that the immunogenic fusion proteins are non-natural, non-physiological proteins which differ from native IL31 and SP. The immunogenic fusion proteins may harbor distinct structural epitopes as those predicted for IL31 and SP. Since the fusion proteins are molecularly heterogenous, they may adopt conformations displaying a plurality of conformational epitopes. When used as a vaccine, the IL31-1SP or IL31-3PS immunogens elicit complex immunological responses including the production of antibodies to endogenous IL31 and SP, accounting for the therapeutic response.

[00118] Advantageously, systemic active immunization with the recombinant fusion protein IL31-1SP or IL31-3SP produced from inclusion bodies, is effective in treating AD in mammals affected by inflammatory and pruritic skin lesions.

[00119] Surprisingly, systemic immunizations with the bivalent fusion proteins as disclosed herein are effective in alleviating symptoms associated with pruritus including excessive scratching, itching and/or reducing or preventing secondary skins lesions. Such active immunization results, unexpectedly, in enhanced treatment with long-lasting therapeutic effects (e.g., immune memory) and few adverse effects. Moreover, the systemic immunizations with the bivalent fusion proteins as disclosed herein allow for a reduced intake of corticoid (medications) conventionally used to manage pruritus symptoms.

[00120] In some embodiments, the method includes administering the immunogenic fusion protein as an immunogenic composition or a vaccine e.g., IL31-1SP or IL31-3SP in combination with at least one therapeutic agent to enhance the therapeutic benefits in a mammal affected or susceptible of developing symptoms associated with diseases mediated by IL31 and SP overexpression. Non-limiting examples of therapeutic agents are anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor, and cyclosporine or receptor antagonist that blocks a nociceptive signaling pathway, preferably a neurokinin-1 receptor (NK1-R) antagonist, most preferably compound CP-96,345.

[00121] In some embodiments, administering in a mammal in need an effective amount of the immunogenic fusion protein e.g., IL31-1SP or IL31-3SP treats and/or prevents inflammatory skin conditions and itching mediated by IL31 and SP. In other embodiments, administering an effective amount of the immunogenic fusion protein IL31-1SP or IL31-3SP in a mammal in need treats and/or prevents allergies mediated by IL31 and SP.

[00122] In some embodiments, the immunogenic composition is used in the treatment and/or prevention of inflammation and pruritus-related skin disorders, preferably AD, most preferably chronic and/or refractory AD. AD is refractory when AD-associated symptoms cannot be efficiently treated with the current standard of care centered on anti-inflammatory compound such as corticosteroid, antihistamine, tyrosine kinase inhibitor, and cyclosporine.

[00123] The methods for treating AD and pruritic skin lesions include administering to a mammal at least one immunogenic fusion protein designed and produced as bivalent fusion proteins, that stimulate a humoral immunological response and the production of specific antibodies that will simultaneously bind and neutralize the activity of neurogenic and immune mediators upregulated in the affected mammal.

[00124] In some embodiments, a method for treating AD and/or (or, in other embodiments, preventing) symptoms associated with pruritus includes the administering of an effective amount of a bivalent vaccine or an immunogenic composition comprising at least one immunogenic fusion protein e.g., IL31-1SP or IL31-3SP to alleviate excessive scratching, itching and/or reducing or preventing

secondary skins lesions. In some embodiments, vaccination will be administered up to 4 boosters or more to alleviate AD-associated symptoms, particularly pruritus.

[00125] In an embodiment, the immunogenic composition or the bivalent vaccine is used in an active immunization protocol. Active immunization with the IL31-1SP or IL31-3SP immunogen is safe and has a therapeutic effect in dogs affected by chronic atopic dermatitis that usually required systemic corticoid medications. After the administration of the bivalent vaccine comprising the immunogen and at least one adjuvant (priming and 3 boosters), the immunized dogs showed a significant improvement of the skin lesions as assessed by clinical observation.

[00126] In an embodiment, the administration of the immunogenic composition reduces AD-related symptoms such as pruritus and reduced the need of corticosteroid medications. (**Fig. 6**) Vaccination with the immunogenic fusion protein, including IL31-1SP significantly reduced the frequency of scratching in the treated dogs within one month after completing the immunization cycle. (**Fig. 5**) Such therapeutic effect is long-lasting, over several months. In addition, there is a significant reduction in the number of dogs consuming corticosteroids long after post-vaccination, indicating an improvement in symptoms and reduced need for corticosteroid treatment. In some aspects of the embodiments, three months after immunization, IL31-1SP immunized dogs may be off corticosteroid, further confirming the potent effect of the bivalent vaccine in efficiently reducing skin inflammation and pruritis-related symptoms..

[00127] Using the pruritus scale as defined in **Fig. 6A**, an effective treatment may be defined as achieving a pruritus score ranging from 1 to 3, wherein the dog experiences occasional or mild itching that does not significantly impact its overall quality of life. Evaluation of itch severity and corticosteroid use in atopic dermatitis affected dogs before and after treatment (i.e., immunization protocol) with the recombinant fusion immunogen described herein, preferably, IL31-1SP or IL31-3SP may be performed using the scale as defined in **Fig. 6A**. A. The scoring system provides a comprehensive evaluation of symptoms such as itching severity and appropriate treatments. A 1-10 scale is used to assess the level of itching in dogs with atopic dermatitis. The scale categorizes the severity of symptoms, secondary symptoms, and recommended treatments. Scores range from 1 (sporadic scratching with no lesions) to 10 (compulsive scratching requiring an E collar).

[00128] As shown in **Fig. 6**, IL31-1SP treatment is efficacious in reducing itching severity and corticosteroid use in dogs with atopic dermatitis. Dogs treated with IL31-1SP showed a significant reduction in scratching scores over a two-month period, with fewer dogs requiring corticosteroids 90 days post-vaccination. These results suggest that IL31-1SP is a promising therapeutic option for managing atopic dermatitis in dogs.

[00129] Notably, the active immunization utilizing the immunogenic fusion protein yields promising results, reducing the pruritus score within few months after treatment completion. Additionally, the active immunization can result in a substantial reduction in corticosteroid consumption, thus underscoring the efficacy and safety profile of the immunogenic fusion protein of the invention. The immunogenic fusion protein provides therapeutic benefits and has an impact on pruritus management.

[00130] The mechanisms underlying skin inflammation and pruritus in AD include increased expression of IL31 and SP which synergize leading to pruritus and neurogenic inflammation [1; 29; 31; 32; 33; 34; 35; 36]. In the present study, evidence shows that vaccination with IL31-1SP immunogens in both mice and dogs induces an immune response characterized by the production of neutralizing antibodies that recognize endogenous IL31 and SP. Thus, the concomitant immunological neutralization of both IL31 and SP, likely explains the potent and long-lasting therapeutic effects of IL31-1SP or IL31-3SP immunization in canine AD.

[00131] Various authors have reported the safety and efficacy of systemic immunization with IL31 or as its main epitope sequences linked to virus-like particles, both in canines with CAD and in horses suffering from dermatitis secondary to hypersensitivity to insect bites [57; 58]. These vaccines generate (therapeutic) neutralizing antibodies to IL31 in the host, which results in a decrease in pruritus and inflammatory lesions secondary to scratching. These treatments are still experimental and have not been tested in controlled clinical trials and in chronic treatment regimens. However, the proposed mechanisms of action appear to be the neutralization of endogenous IL31, which is upregulated in atopic dermatitis.

[00132] The therapeutic effects of the IL31-1SP vaccine in AD dogs suggest that the neutralization of IL31 by specific antibodies is enough to alleviate the main symptoms of canine AD, regardless of how the immune system is stimulated. However,

the fact that the IL31-1SP vaccine also generates antibodies against SP, makes it a different immunization strategy. The antibodies neutralizing SP likely potentiate the effects of anti-IL31 antibodies, downregulating the synergism of both mediators inducing skin inflammation and pruritus. Neutralizing antibodies to SP likely decrease the reported effects of SP mediating neurogenic inflammation, including increased skin vascular permeability, immune cell infiltration, mast cell degranulation and in AD. Given that SP activates mast cell MRGPRX2 receptors which mediates the release of inflammatory mediators from mast cells, including the pruritogenic monoamine histamine [43; 44], the neutralization of SP through systemic immunization may become a significant therapeutic mechanism in several pathological conditions involving neurogenic inflammation.

[00133] The safety and efficacy of systemic immunization with the IL31-1SP immunogen in mice and dogs with AD have been established. The recombinant immunogen is able to elicit an immune response in the mammal, including the production of neutralizing antibodies to endogenous IL31 and SP, thereby resulting in the reduction of skin inflammation and pruritus-related symptoms.

[00134] In some embodiments, the immunogenic composition or bivalent vaccine is administered as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time required to sustain high neutralizing antibody titers.

[00135] In some embodiments, treating or preventing an inflammation-related skin disorder, preferably AD or AD-associated symptoms such as pruritus in a mammal in need such as a dog involves the subcutaneous administering of as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time required to sustain high neutralizing antibody titers. The bivalent vaccine comprises an immunogenic fusion protein IL31-SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

[00136] The vaccine can be administered to a mammal in need via various suitable routes. For example, the vaccine can be administered together or separately, and/or simultaneously and/or sequentially, orally, subcutaneously, intramuscularly or transdermally. In some embodiments, the invention provides a pharmaceutical composition for treating AD comprising an effective amount of a bivalent or multivalent vaccine, and a pharmaceutically acceptable carrier.

[00137] In an embodiment, the medicament which comprises at least one immunogenic fusion protein is capable of eliciting high therapeutic antibody titers against endogenous IL31 and SP.

[00138] In an embodiment, the immunogenic fusion protein is used in the preparation of a medicament for the treating or preventing AD or/and pruritis in a mammal.

[00139] In the method of treatment according to an embodiment, the immunogenic composition including the immunogenic fusion protein as an active ingredient may be administered by intravenous, intra-arterial, intraperitoneal, intramuscular, intrasternal, percutaneous, topical, intraocular or subcutaneous route.

[00140] In an embodiment, the immunogenic composition is capable of inducing therapeutic antibodies, resulting in the i*n vivo* inhibition of the biological activity of IL31 and SP that act in concert to mediate AD, in the preparation of a medicament.

[00141] **Methods for producing the immunogenic fusion protein**

[00142] In some embodiments, the immunogenic fusion protein is produced in a genetically modified microorganism as inclusion bodies. In preferred embodiments, the immunogenic fusion proteins harbor multiple epitopes directed to IL31 and/or SP.

[00143] In some embodiments, a method for producing an immunogenic fusion protein involves i) transfecting *E. coli* cells with an expression vector comprising the nucleotide sequence encoding the immunogenic fusion protein as described herein; ii) isolating the inclusion bodies containing the produced immunogenic fusion; iii) purification and solubilization of the immunogenic fusion protein in a solubilizing agent. In some aspects of the embodiments, the solubilizing agent used to maintain the solubility of the immunogenic fusion protein is urea 4M-8M.

[00144] The sequence and biophysical properties support the fact that the immunogenic fusion protein has an artificial structure, which is non-naturally occurring, thus being a product of human ingenuity. These features allow the immunogenic fusion protein to be highly immunogenic, overcoming the natural immune tolerance to self-antigens IL-31 and SP, and having the unexpected ability to elicit a therapeutic immune response, including the production of high affinity antibodies that cross-react with IL31 and SP.

[00145] In some embodiments, the method for producing a immunogenic composition or a bivalent vaccine comprising the immunogenic fusion protein IL31-1SP or IL31-3SP, involves the steps of cloning, expressing, purifying, and formulating the fusion protein with suitable adjuvants. Selecting the appropriate adjuvant may enhance the versatility of the therapeutic formulation. For example, a particular adjuvant may be more effective depending on the species being treated (e.g., horse, human, cat, dog) and/or the route of administration.

[00146] Surprisingly, the immunogenic fusion proteins produced as inclusion bodies elicit a humoral immune response with the production of therapeutic antibodies directed against the endogenous IL31 and/or SP which synergize under pathological conditions.

[00147] Inclusion bodies are readily obtainable (as a source of immunogenic species that) and induce strong immune reactions. The immunogenic composition or bivalent vaccine comprising at least one immunogenic fusion protein is easy to administer and lacks the common challenges associated with the preparation and production of monoclonal antibodies, such as high-cost production.

[00148] In some embodiments, the self-antigens IL31 and SP are expressed in bacterial cells as a fusion protein. The homogeneity of the composition produced guarantees precise dosing by the simple design of the immunogenic fusion proteins and further isolation and purification from the inclusion bodies.

[00149] In some embodiments, the recombinant expression vector has a nucleotide sequence having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 18, SEQ ID NO: 21,, SEQ ID NO: 24, SEQ ID NO: 27, SEQ ID NO: 30, or SEQ ID NO: 33.

[00150] In some embodiments, the recombinant expression vector comprises a nucleotide sequence encoding for an immunogenic fusion protein having at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28, or SEQ ID NO: 31.

[00151] In some embodiments, the recombinant expression vector having the nucleotide sequence encoding for at least one immunogenic fusion protein may be inserted or recombined in the host cell genome.

[00152] In other embodiments, the nucleotide sequence encoding for at least one immunogenic fusion protein refers to any nucleotide including a nucleotide sequence competent to replicate spontaneously as an episome. Such a vector may include a linear nucleotide, a plasmid, a phagemid, a cosmid, an RNA vector, a viral vector, etc.

[00153] In some embodiment, the vector may be genetically engineered to incorporate the nucleotide sequence encoding IL31 in an orientation either N-terminal and/or C-terminal to a nucleotide sequence encoding SP, and in the correct reading frame so that the immunogenic fusion protein may be expressed. A flexible linker encoding for a flexible linker rich in Gly and Ser, such as GSGS for example connects the sequences. For example, the expression vector may include SEQ ID NO: 10 encoding for the canine polypeptide IL31-1SP, or SEQ ID NO: 13 encoding for the canine polypeptide IL31-3SP. It is understood that the expression vector may comprise any combination of at least one immunogenic fragment derived from IL31 and at least one immunogenic fragment derived from SP.

[00154] Expression vectors may also be selected from those readily available for use in prokaryotic or eukaryotic expression systems.

[00155] Standard recombinant nucleotide methods may be used to express a genetically engineered immunogenic fusion protein. In some embodiments, the nucleotide sequence encoding the immunogenic fusion protein may be cloned into an expression vector e.g., with appropriate signal and processing sequences and regulatory sequences for transcription and translation. In other embodiments, the immunogenic fusion protein may be synthesized using automated organic synthetic methods.

[00156] In order to obtain high level expression of the immunogenic fusion protein, a cDNA encoding the immunogenic fusion protein may be subcloned into an expression vector that includes a strong promoter for directing transcription, a transcription/translation terminator, and in the case of a nucleotide encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art. Bacterial expression systems for expression of the recombinant fusion protein are available in, e.g., *E. coli*, *Bacillus sp*., and *Salmonella*. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. The eukaryotic expression vector may be preferably an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[00157] In some embodiment, the nucleotide sequence encoding the immunogenic fusion protein may be present in a vector in which the nucleotide sequence is operably linked to regulatory sequences capable of providing for the expression of the nucleotide sequence by a suitable host cell.

[00158] Within an expression vector, the term "operably linked" is intended to mean that the nucleotide sequence of the immunogenic fusion protein is linked to the regulatory sequence(s) in a manner which allows for transcription of the nucleotide sequence. The term "regulatory sequence" is intended to include promoters, enhancers, and other expression control elements. Such operable linkage with the expression vector can be achieved by conventional gene recombination techniques known in the art, while site-directed DNA cleavage and linkage are carried out by using conventional enzymes known in the art.

[00159] The expression vectors may contain a signal sequence or a leader sequence for membrane targeting or secretion, as well as regulatory sequences such as a promoter, an operator, an initiation codon, a termination codon, a polyadenylation signal, an enhancer and the like. The promoter may be a constitutive or an inducible promoter. Further, the expression vector may include one or more selectable marker genes for selecting the host cell containing the expression vector and may further include a polynucleotide sequence that enables the vector to replicate in the host cell in question.

[00160] The expression vector constructed according to an embodiment may be the vector where the polynucleotide encoding the recombinant fusion protein is inserted within the multiple cloning sites (MCS) of a pT7 vector.

[00161] The recombinant fusion protein may be introduced into an appropriate host cell, e.g., a bacterial cell, a yeast cell, an insect cell, or a tissue culture cell. The recombinant protein may also be introduced into embryonic stem cells in order to generate a transgenic organism. Large numbers of suitable vectors and promoters are known to those skilled in the art and are commercially available for generating the recombinant protein.

[00162] Known methods may be used to construct vectors including the polynucleotide sequence according to one embodiment disclosed in the present application and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic recombination.

[00163] Another aspect of the embodiment provides a transformant transformed with the recombinant expression vector.

[00164] As used herein, “introducing” of a protein, a peptide, an organic compound into a cell may be used interchangeably with the expression of “carrying,” “penetrating,” “transporting,” “delivering,” “permeating” or “passing.”

[00165] It is understood that the host cell refers to a eukaryotic or prokaryotic cell into which one or more DNAs or vectors are introduced and also to the progeny or potential progeny thereof. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[00166] The host cells may be preferably bacterial cells, and as the bacterial cells, there are, in principle, no limitations. They may be eubacteria (gram-positive or gram-negative) or archaebacteria, as long as they allow genetic manipulation for insertion of a gene of interest, preferably for site-specific integration, and they may be cultured on a manufacturing scale. Preferably, the host cells may have the property to allow cultivation to high cell densities.

[00167] Examples of bacterial host cells that may be used in the preparation of the recombinant fusion protein are *E. coli*, *Bacillus subtilis*, *Pseudomonas fluorescens* as well as various *Corynebacterium* and *Lactococcus lactis* strains. Preferably, the host cells are *Escherichia coli* cells.

[00168] In an embodiment, the host cell may include an RNA polymerase capable of binding to a promoter regulating the gene of interest. The RNA polymerase may be endogenous or exogenous to the host cell.

[00169] In an embodiment, host cells with a foreign strong RNA polymerase may be used. For example, *Escherichia coli* strains engineered to carry a foreign RNA polymerase (e.g., like in the case of using a T7 promoter a T7-like RNA polymerase in the so-called "T7 strains") integrated in their genome may be used. Examples of T7 strains, e.g., BL21(DE3), HMS174(DE3), and their derivatives or relatives (see Novagen, pET System manual, 11th edition), may be widely used and commercially available. Preferably, BL21-CodonPlus (DE3)-RIL or BL21-CodonPlus (DE3)-RIPL may be used. These strains are DE3 lysogens containing the T7 RNA polymerase gene under control of the lacUV5 promoter. Induction with IPTG allows production of T7 RNA polymerase which then directs the expression of the gene of interest under the control of the T7 promoter.

[00170] The host cell strains, *E. coli* BL21(DE3) or HMS174(DE3), which have received their genome based T7 RNA polymerase via the phage DE3, are lysogenic. It is preferred that the T7 RNA polymerase contained in the host cell has been integrated by a method which avoids, or preferably excludes, the insertion of residual phage sequences in the host cell genome since lysogenic strains have the disadvantage to potentially exhibit lytic properties, leading to undesirable phage release and cell lysis.

[00171] The method for preparing the recombinant fusion protein includes preparing the recombinant expression vector; preparing the transformant using the recombinant expression vector; culturing the transformant; and recovering the recombinant fusion protein expressed by culturing.

[00172] Culturing may employ the addition of a feed medium, for fed-batch mode, semi-continuous mode, or continuous mode, and the bacterial expression host cells may include a DNA construct, integrated in their genome, carrying the DNA

sequence encoding the protein of interest under the control of a promoter that enables expression of said protein.

[00173] There are no limitations in the type of the culture medium. The culture medium may be semi-defined, i.e., containing complex media compounds (e.g. yeast extract, soy peptone, casamino acids), or it may be chemically defined, without any complex compounds. Preferably, a defined medium may be used. The defined media (also called minimal or synthetic media) are exclusively composed of chemically defined substances, i.e., carbon sources such as glucose or glycerol, salts, vitamins, and, in view of a possible strain auxotrophy, specific amino acids or other substances such as thiamine. Most preferably, glucose may be used as a carbon source. Usually, the carbon source of the feed medium serves as the growth-limiting component which controls the specific growth rate.

[00174] Host cells may be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or the use of cell lysing agents. There are a number of general methods known in the art for purifying recombinant (and non-recombinant) proteins. The methods may include, e.g., ion-exchange chromatography, size-exclusion chromatography, affinity chromatography, selective precipitation, dialysis, and hydrophobic interaction chromatography. These methods may be adapted to devise a purification strategy for the cell permeable recombinant fusion protein. If the cell permeable recombinant fusion protein includes a purification handle, such as an epitope tag or a metal chelating sequence, affinity chromatography may be used to easily purify the protein.

[00175] The amount of the protein produced may be evaluated by detecting the advanced macromolecule transduction domain directly (e.g., using Western analysis) or indirectly (e.g., by assaying materials derived from the cells for specific DNA binding activity, such as by electrophoretic mobility shift assay). Proteins may be detected prior to purification, during any stage of purification, or after purification. In some implementations, purification or complete purification may not be necessary.

[00176] Pharmaceutical composition and Formulation

[00177] In some embodiment, a pharmaceutical composition includes at least one immunogenic fusion protein as an active ingredient. The pharmaceutical composition may further include at least one pharmaceutically acceptable carrier. The

pharmaceutical composition may be used for treating or preventing AD and/or AD-related symptoms such as pruritus or secondary skin lesions in a mammal, preferably in a dog. The pharmaceutical composition may be efficacious in treating AD refractory to anti-inflammatory compounds such as corticosteroid, antihistamine, tyrosine kinase inhibitor, and cyclosporine.

[00178] In some embodiments, the pharmaceutical composition may be for injectable (e.g. subcutaneous, intraperitoneal, intramuscular) and may include the active ingredient in an amount of 0.001 mg/kg to 1000 mg/kg, preferably 0.01 mg/kg to 100 mg/kg, more preferably 0.1 mg/kg to 20 mg/kg for human, more preferably 0.001 mg/kg to 10 mg/kg for dogs, more preferably 0.001 mg/kg to 5 mg/kg for cats, and more preferably 0.001 mg/kg to 20 mg/kg for horses.

[00179] For example, dosages normally fall within the range of about 0.001 to about 1000 mg/kg of body weight. In the treatment of adult humans, the range of about 0.1 to about 50 mg/kg/day, in single or divided dose, is especially preferred. However, it will be understood that the concentration of the recombinant fusion protein actually administered will be determined by a physician or veterinarian, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the age, weight, and response of the individual subject, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances, dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effects, provided that such larger doses are first divided into several smaller doses for administration throughout the day.

[00180] The pharmaceutical composition according to an embodiment may be prepared by using pharmaceutically suitable and physiologically acceptable additives, in addition to the active ingredient, and the additives may include excipients, disintegrants, sweeteners, binders, coating agents, blowing agents, lubricants, glidants, flavoring agents, etc.

[00181] For formulation of the immunogenic composition or bivalent vaccine into a liquid preparation, a pharmaceutically acceptable carrier which is sterile and biocompatible may be used, such as saline, sterile water, a Ringer's solution, buffered

saline, an albumin infusion solution, a dextrose solution, a maltodextrin solution, glycerol, and ethanol, and these materials may be used alone or in any combination thereof. If necessary, other common additives, such as antioxidants, buffers, bacteriostatic agents, etc., may be added. Further, diluents, dispersants, surfactants, binders, and lubricants may be additionally added to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions. Furthermore, the composition may be preferably formulated, depending upon diseases and ingredients, using any appropriate method known in the art.

[00182] In an embodiment, the effective amount is the amount of immunogenic fusion protein as the active ingredient, or a pharmaceutical composition disclosed herein, when administered to a mammal for the treatment or prevention of AD or/and pruritis, is sufficient to effect such treatment or prevention of the disease. Any improvement in the mammal is considered sufficient to achieve treatment. An effective amount of an active ingredient or a pharmaceutical composition disclosed herein, used for the treatment of AD and/or pruritus may vary depending upon the manner of administration, the age, body weight, and general health of the mammal. Ultimately, the prescribers will decide the appropriate amount and dosage regimen.

[00183] In an embodiment, the immunogenic composition or bivalent vaccine includes an effective amount between 0.5 μg and 10000 μg of the immunogenic fusion protein.

[00184] In some embodiment, the pharmaceutical composition comprising an immunogenic fusion protein e.g., IL31-1SP or IL31-3SP is for veterinary use and as such is formulated with a veterinary adjuvant suitable for administration in an animal affected from conditions associated with IL31 and SP. The optimal adjuvant will merely depend on the animal species (e.g., dog, horse, cat) and the route of immunization (e.g., by injection or mucosal).

[00185] In some embodiments, the adjuvant includes an oil-in-water adjuvant, a polymer and water adjuvant, a water-in-oil adjuvant, an aluminum hydroxide adjuvant or combinations thereof.

EXAMPLES

[00186] Specific embodiments will now be demonstrated by reference to the following examples. It should be understood that these examples are disclosed solely by way of illustrating the invention and should not be taken in any way to limit the scope of the present invention.

[00187] **Material and Methods**

[00188] **Expression and purification of recombinant IL31-1SP and IL31-3SP immunogenic proteins from inclusion bodies.**

[00189] The IL31-1SP and IL31-3SP fusion proteins as defined in SEQ ID NO: 11 and SEQ ID NO: 14, respectively were expressed by transforming the plasmid pT7 with IL31-1SP genes into competent One Shot™ BL21 Star™ (DE3) Chemically Competent E. coli (Thermo Fisher C601003), following the manufacturer's instructions.

[00190] To produce the immunogenic fusion proteins IL31-1SP and IL31-3SP, the transformed E. coli cells were grown at 37°C in Lauria-Bertani Broth supplemented with 100µg/mL Ampicillin (Sigma A0166) overnight at 37°C and 220rpm. Then, one liter of Terrific Broth was inoculated with 10mL of pre-culture and incubated at 37°C and 220rpm until reaching an optical density at 600nm of 2. Induction was performed by adding 1mM IPTG (Euromedex EU0008-B) and incubating for 4 hours at 37°C and 220 rpm. After 4 hours, the cells were harvested by centrifugation at 10,000xg and 4°C for 10 minutes and then resuspended in a buffer containing 30mM Tris (pH 8), 150mM NaCl, and 0.5mg/mL lysozyme. The cell suspension was sonicated using a 1:20 minutes cycle (20 seconds ON and 1 minute OFF at 50%) and centrifuged at 20,000xg and 4°C for 40 minutes. The resulting pellet was resuspended in Washing Buffer I (WBI) composed of 50mM Tris (pH 8), 50mM NaCl, 0.5% Triton X-100, 1.5mM β-Mercaptoethanol, and 1.6M Urea. The resuspended pellet was then centrifuged at 20,000xg and 4°C for 20 minutes. This washing step was repeated three times. Subsequently, the pellet was resuspended in Washing Buffer II (WBII) containing 30mM Tris (pH 8) and 150mM NaCl, followed by centrifugation at 20,000xg and 4°C for 20 minutes. This step was repeated twice. Finally, the sample was resuspended in Final Buffer (FB) consisting of 20mM Tris (pH 8), 500mM NaCl, 30mM Imidazole, and 8M Urea.

[00191] Purification by Ni2+ affinity chromatography was performed at room temperature. The protein in the Final Buffer was applied to a pre-equilibrated 5mL

HisTrap chelating HP column charged with Ni+2 (GE17-5248-02, Cytiva) at a maximum flow rate of 2mL/min. The column was then washed with 5 column volumes (CV) of the Final Buffer. The protein was eluted in three steps of 10mL each using an elution buffer composed of 20mM Tris (pH 8), 500mM NaCl, 500mM Imidazole, and 8M Urea.

[00192] All fractions obtained were analyzed by SDS-PAGE, and imidazole was removed by dialysis.

[00193] To deplete endotoxins, Endotoxin Removal Beads from Miltenyi Biotec (130-093-657) were used according to the manufacturer's instructions. The sample was subjected to 5 successive passages through the magnetic beads to lower the endotoxin levels below 10 UE/mg of protein.

[00194] The level of endotoxins was measured using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher A39553) following the manufacturer's instructions.

[00195] Protein quantification was performed using the Bradford protein assay, and a standard curve was generated using the Final Buffer (FB). The amount and purity of the protein were confirmed by mass densitometry using Acrylamide gels and 1D electrophoresis. The identity of the sample was confirmed by mass spectrometry.

[00196] Mass Spectromery (MS) analysis.

[00197] **Bottom-up LC-MS analysis**. For bottom-up LC-MS analysis, the respective purified recombinant fusion proteins IL31-1SP and IL31-3SP were alkylated with 50 mM iodoacetamide (IAM), then digested using Sequencing Grade Modified Trypsin in a pH 7 carbonate buffer for 16 hours at 37ºC, subsequently the samples were concentrated and desalted by ZipTip C18. Finally, 3 µL of each sample at a concentration of 1µg/L were injected into the Thermo Fisher Scientific Q-Exactive Plus (Q-Orbitrap) LC-MS system. LC Gradient used: An HPLC gradient was used with the following conditions: Time (min): 0 - 110, Flow (µL/min): 0.2 constant % B: 0 min: 1%, 15 min: 1%,75 min: 35%, 90 min: 99%, 100 min: 99%, 102 min: 1%, 110 min: 1%. Both the guard column and the column are from Thermo Fisher Scientific. IL31-1SP and IL31-3SP were identified using Patternlab V software using the theoretical sequence of the protein of interest.

[00198] **Direct injection MS1**. For MS1 analysis, the protein was precipitated in a phosphate buffer, centrifuged and washed. The final precipitate was dissolved in a solution of 1% acetic acid. The protein was directly injected and analyzed by MS1 on an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer. Software from Agilent: ExDviewer Version 4.6.12.

[00199] **Mice immunization**. To evaluate the safety of the product, three B6 mice were immunized with 5mg/kg of protein every 15 days, administered subcutaneously. The immunization protocol comprised a priming injection and 3 subsequent boosters. Each dose was prepared using Tris 20mM pH 8, NaCl 500mM, Urea 4M, and 3% adjuvant Montanide GEL 01 PR (Seppic). Submandibular blood samples were collected before the start and 7 days after each injection. The blood samples were incubated at room temperature for 2 hours and then centrifuged at 3000xg for 10 minutes at 4°C to separate the serum.

[00200] **Synergistic immunogenic effect of IL31-3SP as compared to non-fused IL31 and SP indivually administered as antigens.** In other experiment, 2 groups of three B6 mice were immunized with 1 mg/kg of recombinant fusion protein IL31-3SP every 15 days, for a total immunization protocol comprising a priming and 3 boosters, administered subcutaneously. Each dose was prepared using the IL31-3SP antigen, Tris 20mM pH 8, NaCl 500mM, Urea 4M, and 3% of adjuvant Montanide GEL 01 PR (Seppic). For comparison, other group of 3 B6 mice were immunized with the non-fused recombinant canine IL31 (CYT 604, PROSPEC) and synthetic substance P (RP10178 GENSCRIPT) as antigens, in a solution prepared with Tris 20mM pH 8, NaCl 500mM, and 3% of adjuvant Montanide GEL 01 PR (Seppic). Submandibular blood samples were collected before the immunization and 7 days after the third booster. The blood samples were incubated at room temperature for 2 hours and then centrifuged at 3000xg for 10 minutes at 4°C to separate the serum.

[00201] **Antibody titers in serum samples by ELISA**

[00202] The ELISA assays for measuring antibody titers were performed as follows: The plates were coated with either 2,5 µg/mL of IL31-1SP, IL31-3SP, 2,5 µg/mL of IL31 (CYT 604, PROSPEC) or 5µg/mL of Substance P (RP10178, Genscript) in Carbonate buffer 0.05M pH 9.6 and incubated overnight at 37°C. The plate was then blocked with 1% Gelatin (G9382, Sigma) in PBS. Then, the wells were

incubated with the indicated sera dilutions in PBS-Tween 20 0.1%, Gelatin 0.5% and incubated 1h at 37°C. Then, the wells were washed 5 times with PBS-Tween 20 0.1% and incubated 1h at 37°C with the secondary anti-mouse and anti-canine IgG antibodies diluted (1/5000) in PBS-Tween 20 0.1%, Gelatin 0.5%.

[00203] Competitive ELISA

[00204] For competitive ELISA, the plates were coated with 2.5 µg/mL of IL31-3SP in 10 mM Na2CO3 pH 8.0 buffer for 24 hours. After the incubation, the plates were blocked with 3% gelatin in 50 mM PBS for 1 h at 37°C. After blocking the plates were incubated with mice sera for 1h at 37°C in a dilution of 1/32000, which were previously incubated during one hour at 37°C with one of the different competitor antigens as follows: 5 ug/mL of recombinant IL31-3SP (canine sequences), 5 ug/mL of recombinant canine IL31 (CYT 604, PROSPEC) and 20 ul/mL of canine SP (RP10178 Genscript). Then, the plates were washed with 1.5% gelatin, 0.1% Tween, 50 mM PBS buffer, and incubated with the secondary antibody (goat anti-mice IgGs). Next, the plate was washed six times with 1.5% gelatin, 0.1% tween in PBS 50 mM and developed with TMB substrate and the reaction was stopped with 1M H2SO4. The Multiskan FC (Thermo scientific) was used to measure the absorbance of OD450. The results were analyzed using the two-tailed Paired T-test statistical test (P value < 0.05).

[00205] Immunization of dogs with atopic dermatitis with recombinant fusion IL31-1SP protein.

[00206] A total of 5 client-owned dogs were selected for a proof-of-concept clinical trial, following an open label, non-placebo-controlled design. These 5 dogs had been living with treatment-refractory atopic dermatitis. All selected dogs were currently receiving some form of medication for dermatitis treatment, and no medication was discontinued prior to initiating the immunization cycle, which had an add-on protocol to the ongoing medication regimen.

[00207] Formulation and administration of IL31-1SP vaccine in dogs with atopic dermatitis.

[00208] Each dose of the vaccine for dogs was prepared using IL31-1SP as antigen (5 mg per dose), solubilized in Tris 20mM pH 8, NaCl 500mM, Urea 4M, and

20% Montanide Gel 01 PR (SEPPIC), in a total volume of 1 ml. The components were mixed by vortexing for 5 minutes.

[00209] A total of 5 dogs received the active immunotherapy with IL31-1SP vaccine. Each dog was administered a total of four doses, with each dose given every 15 days. Before each injection and 15 days after the final dose, a venous blood sample was taken to analyze the antibody titer.

[00210] Dog's itching score.

[00211] To assess the progression of the dermatological condition and its evolution during the immunization protocol with IL31-1SP immunogen, a team of 3 trained veterinarians used a scratching and itching scale ranging from 1 to 10, as shown in **Fig.6.** The scale is designed to measure the severity of pruritus in dogs. Pruritus may include scratching, biting, licking, and rubbing against objects or the floor [60]. At the time of diagnosis, the score was applied to each of the recruited dogs, and it was repeated 15 days after each booster.

[00212] Ethic concerns

[00213] All procedures using laboratory animals were performed under the national and international guidelines and were approved by the Institutional Animal Committee for animal experimentation (CEUA Approved protocol: #012-16 to Dr. Martina Crispo). This study was carried out in strict accordance with the Institute Pasteur de Montevideo Committee's requirements and under the current ethical regulations of the Uruguayan Law N° 18.611 for animal experimentation that follow the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All procedures using client-owned dogs living with atopic dermatitis were performed under the national and international guidelines and were approved by the Institutional Animal Committee for animal experimentation (CEUA Approved protocol: #008-20 to Dr. Luis Barbeito). This study was carried out in strict accordance with the Institute Pasteur de Montevideo Committee's requirements and under the current ethical regulations of the Uruguayan Law N° 18.611 for animal experimentation that follow the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All procedures were supervised during the entire procedure by three experts in veterinary medicine. Dog owners received a detailed description of the protocol and signed an informed consent form before the screening process.

Owners incurred in no costs for participation. After the screening of potential candidates, a total of 5 dogs were recruited in the study. Systemic subcutaneous immunization using IL31-1SP immunogen was started on day 0 and boost injections were repeated on days 15, 30, and 45 as described in **Fig. 3**. Blood was collected before priming and each booster. Also, blood was collected 2 weeks and 12 weeks after completion of the immunization.

[00214] **Sequences**

[00215] ***Table 1.***

Species	**IL31** SEQ ID NO:	**Substance P** SEQ ID NO:
CANIS LUPUS FAMILIARIS	1 NP_001159386.1 IL31 PRECURSOR	5
EQUUS CABALLUS	2 XP_023503836.1 IL31 PARTIAL	6
HOMO SAPIENS	3 NP_001014358.1 IL31 PRECURSOR	8
FELIZ CATUS	4 XP_044897990.1	9
MUS MUSCULUS		7

[00216] ***Plasmid pT7***

[00217] In an embodiment, plasmid pT7 was used as an expression vector to clone the recombinant DNA. T7 modified plasmid was described in Correa et al 2014. The gene synthesis of the genes and their cloning in the pT7 vector was carried out by

Genscript (Project U407GHG110). All sequences were optimized for their expression in E. coli and inserted into T7 plasmid between BamHI/BamHI restriction sites.

[00218] In an embodiment, nucleotide sequences of certain recombinant pT7 plasmids are disclosed in Table 2

[00219] ***Table 2. Sequences of the IL31-1SP and IL31-3SP immunogenic fusion proteins***

Species	**IL31-1SP** SEQ ID NO:			**IL31-3SP** SEQ ID NO:		
	DNA	aa	T7Plasmid	DNA	aa	T7Plasmid
CANIS LUPUS FAMILIARIS	10	11	12	13	14	15
EQUUS CABALLUS	16	17	18	19	20	21
HOMO SAPIENS	22	23	24	25	26	27
FELIZ CATUS	28	29	30	31	32	33

[00220] As examples, the amino acid sequences of certain immunogenic fusion proteins are SEQ ID NOS: 11, 17, 23, 29, 14, 20, 26, 32-5. IL31-1SP contains one IL31-derived immunogenic fragment fused to one SP peptide with specific amino acid linkers. IL31-3SP contains one IL31-derived immunogenic fragment fused to 3 SP peptides, each separated by the amino acid linkers. Similar methodologies can be implemented to prepare recombinant fusion polypeptides having more than one IL31-derived immunogenic fragments with one or more than one SP peptide. Similar methodologies can be implemented to prepare recombinant proteins having the IL31- and SP-derived immunogenic fragments with the respective feline, equine or human sequences (See table 2)

[00221] Results

[00222] Example 1. Construction and recombinant production, purification and analysis of recombinant fusion IL3-1SP and IL31-3SP immunogenic proteins.

[00223] Two plasmid constructs, pT7-IL31-1SP and pT7-IL31-3SP, were successfully designed and generated for expressing the recombinant fusion immunogen IL31-1SP and IL31-3SP with different signal peptides separated by 4 amino acid linkers. A flexible GSGS linker separates the coding sequences of IL31 and substance P in both constructs. Both constructs included a 6xHis tag for purification and a TEV protease cleavage site, ensuring efficient downstream processing (**Fig. 2A**). The IL31-1SP and IL31-3SP fusion proteins were expressed in E. coli BL21(DE3) cells. Induction with IPTG resulted in high levels of expression, as evidenced by the inclusion bodies observed in the cell lysates. The inclusion bodies were solubilized and subjected to Ni-NTA affinity chromatography for purification. The purified proteins were further processed to remove endotoxins, ensuring their suitability for in vivo applications (**Fig. 2B**). The purified proteins were not soluble in saline buffers but were soluble in 4-8M urea, further suggesting their aberrant folding and non-natural sequence and conformation.

[00224] **Fig. 2C** shows the SDS-PAGE analysis of IL31-1SP and IL31-3SP after isolation and purification, with distinct bands observed at approximately 22 kDa and 25.2 kDa, respectively. Lanes 1 and 2 shows IL31-1SP and IL31-3SP respectively, both purified by IMAC chromatography. The asterisks indicate the protein of interest, and the arrows indicate the theoretical molecular weights for both IL31-1SP (22 kDa) and IL31-3SP (25.2 kDa). (**Fig. 2C**). The purity of the proteins was assessed to be high, with minimal contaminating bands, indicating efficient purification. Further analysis with Mass Spectrometry provided detailed characterization of the purified proteins (**Fig. 2D**). MS1 analysis confirmed the expected molecular weight of IL31-1SP and IL31-3SP (22011 Da and 25249 Da), respectively. The spectra show that both proteins matched to the respective theoretical protein molecular weights as indicated, and also identified poorly represented species with posttranslational carbamoyl modification sites (arrows). (**Fig. 2D**). Top-down MS2 analysis of IL31-1SP and IL31-3SP. The MS analysis of peptides after trypsin digestion. The analysis confirmed the predicted amino acid sequences of both immunogens and the conservation of both IL-31 and SP

sequences. The sequence coverage for IL31-1SP is 49%, and for IL31-3SP is 83%, indicating the extent of protein sequence identified by mass spectrometry.

[00225] The results demonstrate the successful construction, expression, and purification of IL31-1SP and IL31-3SP fusion proteins having the expected molecular weights and high purity. The endotoxin removal steps ensured that the purified proteins were suitable for subsequent in vivo studies. Overall, these findings validate the methodology employed for producing IL31-1SP fusion proteins and lay the groundwork for their application in immunization studies aimed at investigating their potential therapeutic benefits.

[00226] Example 2. Immunization with recombinant fusion IL31-1SP and IL31-3SP immunogen elicited antibodies against native IL31 and SP in mice.

[00227] Immunogenicity of recombinant fusion IL31-1SP and IL31-3SP immunogenic proteins in mice. **Fig. 3A** describes the mice Immunization Protocol: Mice were divided into two groups for immunization. Group 1 was immunized with the recombinant fusion immunogenic protein IL31-1SP, and Group 2 was immunized with IL31-3SP. Both groups received a priming dose followed by three booster doses (1 mg/kg) at two-week intervals. Blood samples were collected before the initial vaccination (week 0) and after the final booster (week 7) to evaluate the immune response. **Fig. 3B**. IgG titers specific to IL31, IL31-1SP, IL31-3SP, and Substance P: Group 1 (Vaccinated with IL31-1SP): The left panel shows the IgG titers specific to IL31-1SP before and after vaccination. The middle panel indicates the IgG titers specific to native canine IL31, and the right panel shows IgG titers specific to native canine Substance P (SP). A significant increase in IgG titers post-vaccination was observed for IL31-1SP and native IL31, indicating a strong immune response. IgG titers specific to native SP also increased but to a lesser extent. Group 2 (Vaccinated with IL31-3SP): The left panel shows the IgG titers specific to IL31-3SP before and after vaccination. The middle panel indicates the IgG titers specific to native canine IL31, and the right panel shows IgG titers specific to native canine SP. Post-vaccination, there was a significant increase in IgG titers for IL31-3SP and native IL31, and an increase in IgG titers specific to native SP, indicating an effective immune response. **Fig. 3C.** Competitive ELISA for IgG binding to IL31-3SP. The graph shows the binding of IgG to IL31-3SP in the presence of competitive antigens. Mice sera were tested for binding to IL31-3SP in the presence of varying concentrations of competitive

antigens (0.5 µg of IL31-3SP, 0.5 µg of non-fused canine IL31, and 2 µg of non-fused canine SP). The absorbance at 450 nm indicates the level of IgG binding. The results show that IL31-3SP binding was significantly higher than the sham control ($p<0.05$, $^{**}p<0.01$), and the competitive antigens reduced IgG binding to IL31-3SP in a dose-dependent manner.

[00228] Mice were immunized via subcutaneous injection with the respective antigens as shown in **Fig. 3A**. After completion of the immunization protocol both antigens IL31- elicited a strong immune response against the vaccinal antigens IL31-1SP or IL31-3SP in all mice with 100% seroconversion and titers > 1:10000 and 1:50000, respectively. The vaccination was safe and did not generate side effects. Surprisingly, both antigens also elicited antibodies against native species of IL31 and SP as shown in **Fig. 3B**. Mice vaccinated with IL31-1SP showed titers > 1: 10000 and >1: 50 for IL31 and SP, respectively. In comparison, mice vaccinated with IL31-3SP showed titers > 1:10000 and >1:2000 for IL31 and SP, respectively. **Fig. 3C** shows a competitive ELISA study where the binding of IgG elicited by mice immunization with IL31-3SP to the vaccinal antigen can be significantly competed by native recombinant IL31 or native SP. The graph shows the binding of IgG to IL31-3SP in the presence of competitive antigens.

[00229] In sum, **Fig. 3** demonstrates that both IL31-1SP and IL31-3SP induce strong antigen-specific IgG responses in mice. The IL31-3SP construct elicited a higher immune response compared to IL31-1SP, as evidenced by the higher IgG titers against the fusion protein, native canine IL31, and native canine Substance P. Competitive ELISA confirms the specificity of the immune response generated by the recombinant fusion immunogen IL31-3SP against native canine IL31 and SP, with significant competition observed with both non-fused canine IL31 and Substance P.

[00230] Example 3: Comparative immunogenicity of the recombinant fusion immunogen IL31-3SP versus the immunogenicity of the individual non-fused native peptides.

[00231] To determine whether the immunogenicity of IL31-1SP peptides is dependent of the intrinsic non-natural properties of the recombinant fusion proteins, one experiment was performed in mice to compare the immunogenicity of IL31-3SP

with the immunogenicity of a similar vaccine formulation containing equivalent amounts of individual non-fused canine IL31 and SP (**Fig. 4A**).

[00232] The comparative determination of IgG titers by ELISA in both groups of mice showed that the recombinant fusion IL31-3SP antigen elicited robust immune response with titers of antibodies cross reactive with canine IL31 and SP ranging in 1:64000 and 1:2000, respectively (**Fig. 4B**). In contrast, immunization of mice using a mixture of recombinant canine IL31 and SP individually administered together with the same adjuvant, resulted in an almost null immunogenicity. For example, the non-fused peptides induce seroconversion in only 1 of 3 mice for IL31 and in no mice for SP (**Fig. 4B**). These results indicate the low immunogenicity of the non-fused peptides and its unique ability to elicit antibodies that cross-react with native IL31 and SP.

[00233] These results indicate that compared to IL31 and SP individually used as immunogens in a vaccine formulation, the recombinant bivalent fusion immunogenic protein IL31-3SP not only enhances the immune response in terms of magnitude and duration, but also unexpectedly optimizes the quality of the response, producing higher-affinity antibodies that cross-react with native IL31 and SP, which is crucial for vaccine efficacy.

[00234] Comparative immunogenicity of the recombinant fusion immunogen IL31-3SP versus Non-Fused Native Proteins. A. Mice immunization protocol: Two groups of mice were immunized to compare the immune responses elicited by the recombinant fusion immunogen IL31-3SP and a combination of non-fused native canine IL31 and canine Substance P (SP). Group 1 was immunized with IL31-3SP, and Group 2 was immunized with non-fused IL31 and non-fused SP. Both groups received a priming dose followed by three booster doses (1 mg/kg) at two-week intervals. Blood samples were collected before the initial vaccination (week 0) and after the final booster (week 7) for analysis. B. IgG titers specific to recombinant antigen IL31-3SP, and native canine IL31, and native canine SP: IgG Titers Specific to native IL31: The top panel shows a significant increase in IgG titers specific to native IL31 post-vaccination in Group 1 (IL31-3SP) compared to Group 2 (non-fused IL31 + non-fused SP), indicating a stronger immune response induced by the IL31-3SP fusion protein ($*p<0.05$). IgG titers specific to native SP: The middle panel illustrates the IgG titers specific to native SP, with a notable increase post-vaccination in Group 1 (IL31-3SP), demonstrating the cross-immune response elicited by the fusion protein ($*p<0.05$), in

comparison with the immunization with the non-fused native proteins. IgG titers specific to IL31-3SP: The bottom panel displays the IgG titers specific to IL31-3SP, showing a significant immune response in Group 1 (IL31-3SP) compared to Group 2 (non-fused IL31 + non-fused SP) post-vaccination (*$p<0.05$).

[00235] The recombinant bivalent fusion immunogen IL31-3SP exhibits unexpectedly enhanced immunogenic properties compared to the individual peptides administered as non-fused recombinant proteins. This surprising enhancement is likely due to several factors that facilitate a robust and specific immune response when injected into a host animal. Key characteristics of this immunogen include a) Non-natural protein, multiple conformational epitopes and bivalent fusion structure: The fusion of two peptides into an engineered single recombinant molecule which contains linkers and molecular tags unexpectedly enhances the immunogenicity of the protein, including the production of antibodies that cross-react with the native, physiological species of IL31 and SP in the host. b) Evidence is provided for the IL31-3SP protein being capable of breaking the immune tolerance to self-antigens, and more easily captured and processed by APCs due to an optimized size and conformation that favors its internalization and processing within lysosomes. This unexpected advantage increases the humoral immune response. c) In addition, the recombinant bivalent fusion protein IL31-3SP can prolong antigen persistence in the host's immune system, providing continuous stimulation of the immune system and facilitating long-term immunological memory formation. This unexpected persistence is crucial for sustained immune response.

[00236] Example 4: Immunogenicity of the recombinant fusion immunogen IL31-1SP in dogs with atopic dermatitis. IL31-Client-owned dogs living with AD were immunized using IL31-1SP immunogen as described in **Fig. 5A**. After the primer immunization, 3 boosters every two weeks were administered using 5mg of antigen in 4M urea and 20% Montanide (SEPPIC) adjuvant. Blood samples were taken before and after every immunization. Dog immunization protocol: Dogs were immunized with the IL31-1SP fusion protein. Each dog received a priming dose of 5 mg followed by three booster doses at two-week intervals (weeks 2, 4, and 6). Blood samples were collected before the initial vaccination (week 0), after the final booster (week 8), and at a follow-up time point (week 20) for clinical evaluation and to assess the immune response. Two weeks after the last booster antibodies were detected for both native IL31 and SP with

average titers of 1:30000 and 1:600, respectively (**Figs. 5B-C**) The most significant peak in the antibody titers occurred two weeks after the last booster.

[00237] The graph in **Fig. 5B** displays the antibody titers against canine IL31 in individual dogs before vaccination (pre-vaccination) and after the vaccination protocol (post-vaccination) It shows a significant increase in specific IgG titers against canine IL31 was observed in all dogs post-vaccination, indicating a strong immune response elicited by the recombinant IL31-1SP fusion protein. In comparison, the antibody titers against canine SP also increased post-vaccination, demonstrating the cross-immune response induced by the IL31-1SP fusion protein. Such increase in specific IgG titers post-vaccination highlights the potential of IL31-1SP to generate a targeted and effective immune response, against self-antigens such as IL31 and SP, further validating its efficacy as an immunogen in mammals. The graph of **Fig. 5 C.** shows the antibody titers against canine Substance P (SP) in individual dogs before vaccination (pre-vaccination) and after the vaccination protocol (post-vaccination). An increase in specific IgG titers against canine SP was observed post-vaccination, demonstrating the cross-immune response induced by the IL31-1SP fusion protein.

[00238] In sum, **Fig. 5** illustrates the immunogenicity of the IL31-1SP fusion protein in client-owned dogs living with atopic dermatitis. The vaccination protocol successfully induced a robust antibody response specific to both canine IL31 and SP. The increase in specific IgG titers post-vaccination highlights the potential of IL31-1SP to generate a targeted and effective immune response, further validating its efficacy as an immunogen in mamals.

[00239] Example 5: Vaccination with recombinant fusion IL31-1SP antigen improved itch severity and decreased corticosteroid use in dogs with atopic dermatitis.

[00240] To assess the theraputic potential of vaccination with IL31-1SP in dogs with AD, a 1-10 scale was used to quantify the level of itching. The scale categorizes the severity of symptoms and secondary symptoms (**Fig. 6A**). The scratching scores before and after IL31-1SP immunization indicated a significant reduction in scratching scores over time in immunized dogs, with the most notable decrease observed at 2 months post-treatment ($*p<0.05$) (**Fig. 6B**). Also, there was a significant reduction in the number of dogs consuming corticosteroids 90 days post-

vaccination, indicating an improvement in symptoms and reduced need for corticosteroid treatment (**Fig. 6C**).

[00241] These results demonstrate the effectiveness of IL31-1SP treatment in reducing itching severity and corticosteroid use in dogs with atopic dermatitis. The scoring system provides a comprehensive evaluation of symptoms and appropriate treatments. Dogs treated with IL31-1SP showed a significant reduction in scratching scores over a two-month period, with fewer dogs requiring corticosteroids 90 days post-vaccination. IL31-1SP is a promising therapeutic option for managing atopic dermatitis in dogs.

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WE CLAIM

1. An immunogenic fusion protein comprising:

at least one IL31 polypeptide (IL31) or immunogenic fragment thereof, wherein said at least one IL31 polypeptide comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or an immunogenic fragment thereof; and

at least one substance P polypeptide (SP), wherein said at least one SP peptide comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9,

wherein said immunogenic fusion protein is capable of eliciting a production of neutralizing polyclonal antibodies against IL31 and SP, and

preferably, wherein said at least one SP peptide is optionally one, three or five SP peptides.

2. The immunogenic fusion protein of claim 1, comprising one IL31 polypeptide fused to one SP peptide, optionally linked with peptide linkers,

preferably, wherein said at least one SP peptide and at least one IL31 polypeptide or immunogenic fragment thereof are fused by a flexible peptide linker GSGS, and

preferably, wherein said immunogenic fusion protein comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23 or SEQ ID NO: 29.

3. The immunogenic fusion protein of claim 1, comprising:

a) one IL31 polypeptide and three SP peptides, optionally linked with peptide linkers; preferably, wherein immunogenic fusion protein comprises one IL31 polypeptide and three SP peptides, optionally linked with peptide linkers, wherein said immunogenic fusion protein comprises an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 14, SEQ ID NO: 20, SEQ ID NO: 26 or SEQ ID NO: 32; or

b) one IL31 polypeptide and five SP peptides, optionally linked with peptide linkers.

4. A recombinant vector comprising at least a nucleotide encoding the immunogenic fusion protein of any of claims 1-3,

preferably, wherein said recombinant vector comprises:

a) a nucleotide sequence encoding for said immunogenic fusion protein having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO:10, SEQ ID NO: 13, SEQ ID NO: 16, SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 or SEQ ID NO: 31; or
b) a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

5. A method for producing the immunogenic fusion protein of any of claims 1-4, wherein said immunogenic fusion protein is produced in a procaryotic or eukaryotic expression system as inclusion bodies, said immunogenic fusion protein being solubilized in high molar concentrations of urea,
preferably, wherein the recombinant vector comprises a nucleotide sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to one of SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 24, SEQ ID NO: 30, SEQ ID NO: 15, SEQ ID NO: 21, SEQ ID NO: 27 or SEQ ID NO: 33.

6. An immunogenic composition produced according to the method of claim 5.

7. An immunogenic composition comprising at least one immunogenic fusion protein of any one of claims 1-3, preferably, wherein said immunogenic composition further comprises an acceptable carrier and/or an adjuvant selected from the group consisting of oil-in-water adjuvant, polymer and water adjuvant, water-in-oil adjuvant, aluminum hydroxide adjuvant and combinations thereof, and,

more preferably, wherein the adjuvant is a complete or incomplete Freund's adjuvant, a Montanide^{TM}, Gel 01 PR, aluminum salts (alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, nonionic block copolymers, derivatized polysaccharides, cytokines, or bacterial derivatives.

8. The immunogenic composition of claim 7 for use as a medicament, preferably a vaccine, in the treatment and/or the prevention of inflammation in pruritus-related skin disorder, preferably AD, most preferably chronic and/or refractory AD, preferably, wherein AD is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

9. A pharmaceutical composition for treating or preventing AD and/or AD-related symptoms in a subject comprising at least one immunogenic fusion protein of any of claims 1-3 and a pharmaceutically acceptable carrier , preferably, wherein the AD-related symptoms are pruritus and/or secondary skin lesions, and, more preferably, wherein AD is refractory to at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine.

10. The immunogenic composition of claim 7, wherein the immunogenic composition is used in combination with at least one anti-inflammatory compound selected from the group consisting of a corticosteroid, an antihistamine, a tyrosine kinase inhibitor and cyclosporine,

preferably, wherein the immunogenic composition is used in combination with at least one receptor antagonist that blocks a nociceptive signaling pathway, preferably a neurokinin-1 receptor (NK1-R) antagonist, most preferably compound CP-96,345.

11. The immunogenic composition of any of claims 7, 8 or 10, wherein said immunogenic composition is administered orally, subcutaneously, intramuscularly, or transdermally, preferentially subcutaneously,

preferably, wherein said immunogenic composition is administered as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time required to sustain high neutralizing antibody titers.

12. The immunogenic composition of any of claims 7, 8, or 10-11, wherein the mammal is a canine, human, feline or equine.

13. A bivalent vaccine for treating or preventing AD or AD-associated symptoms in a dog, wherein said bivalent dose is administered subcutaneously as a priming dose followed by three booster doses administered approximately 2 weeks apart, and optionally followed by one or more booster doses administered 3 to 6 months apart, or over a period of time required to sustain high neutralizing antibody titers,

wherein said bivalent vaccine comprises an immunogenic fusion protein IL31-SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or

100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.

14. A bivalent vaccine for treating or preventing an inflammation-related skin disorder, preferably AD or AD-associated symptoms in mammals comprising the immunogenic fusion protein of any of claims 1-3.

15. A bivalent vaccine for treating or preventing AD and/or pruritus in dogs comprising an immunogenic fusion protein IL31-1SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 11 or an immunogenic fusion protein IL31-3SP comprising an amino acid sequence having at least 90%, at least 95%, at least 99%, or 100% sequence identity to SEQ ID NO: 14.