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(71) Applicant: **THE UNIVERSITY COURT OF THE UNIVERSITY OF EDINBURGH** [GB/GB]; Old College, South Bridge, Edinburgh EH8 9YL (GB).

(72) Inventors: **KAISER, Peter**; c/o The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian United Kingdom EH25 9RG (GB). **WELCH, Louise**; c/o The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian United Kingdom EH25 9RG (GB).

(74) Agent: **HARRISON GODDARD FOOTE LLP**; Delta House, 50 West Nile Street, Glasgow United Kingdom G1 2NP (GB).

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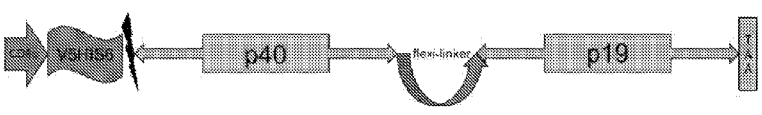
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(54) Title: AVIAN PROTEINS, NUCLEIC ACID AND USES THEREFOR

Fig 4



(57) Abstract: The present invention relates to novel avian cytokine peptides, in particular to avian IL-23α/p19 peptides and to proteins including these peptides such as avian IL-23. The invention also relates to nucleic acids encoding such peptides, and to compositions comprising such peptides or nucleic acids, and to methods of using them.

Avian Proteins, Nucleic Acid and Uses Therefor

The present invention relates to novel avian cytokine peptides, in particular to avian IL-23α/p19 peptides and to proteins including these peptides such as avian IL-23. The invention also relates to nucleic acids encoding such peptides, and to compositions comprising such peptides or nucleic acids, and to methods of using them.

Cytokines are a diverse group of soluble secreted cell signalling molecules which provide critical communication among the cells of the immune system and between immune and non-immune system cells. Many cytokines form homodimeric or heterodimeric complexes which are essential to their function.

Cytokines or cytokine antagonists (e.g. anti-cytokine antibodies) are used as immunomodulators in various therapies. For example, cytokines are used in cancer therapy and in combatting various viral infections. Antibodies to pro-inflammatory cytokines, such as TNF-α, are used to combat inflammatory diseases such as rheumatoid arthritis. Cytokines also have utility as immune stimulators in adjuvants or as adjuvants themselves to improve efficacy of vaccines.

In developed countries, most chickens raised commercially are vaccinated against common avian pathogens. Typical vaccinations are against Newcastle Disease Virus, Infectious Bursal Disease Virus, Marek's Disease Virus, Infectious Bronchitis Virus, Fowlpox Virus, Infectious Laryngotracheitis Virus, *Mycoplasma*, *Salmonella* and Coccidiosis. There is a need for improved vaccines that can be used pre-hatching or immediately at hatching. Immunising newly hatched birds or *in ovo* immunisation does not produce an immune response that is as effective as immunising 2-3 weeks after hatching. Agents that can improve the response of such birds to vaccination have the potential to improve vaccine performance.

A group of heterodimeric cytokines has been identified in mammals which include the p40 protein subunit first identified in respect of IL-12. In mammals, IL-23 is a heterodimer of IL-12β (p40) and IL-23α (p19). IL-23 is produced by dendritic cells

and macrophages. Its production is stimulated by pattern recognition receptor (PRR) agonists, and drives Th17 responses. The receptor of IL-23 is formed by the beta 1 subunit of the IL-12 receptor (IL-12Rβ1) and an IL-23-specific subunit, IL-23R. Both IL-23 and IL-12 activate the transcription activator STAT4, and stimulate the production of IFN-γ. In contrast to IL-12, which acts mainly on naive CD4+ T cells, IL-23 preferentially acts on memory CD4+ T cells.

Mammalian IL-23 is an important part of the inflammatory response against infection. It promotes up-regulation of the matrix metalloprotease MMP9, increases angiogenesis and reduces CD8+ T cell infiltration. Recently, IL-23 has been implicated in the development of cancerous tumours. In conjunction with IL-6 and TGF-β1, IL-23 stimulates naive CD4+ T cells to differentiate into Th17 cells, which are distinct from the classical Th1 and Th2 cells. Th17 cells produce IL-17A, a pro-inflammatory cytokine that enhances T cell priming and stimulates the production of pro-inflammatory molecules such as IL-1β, IL-6, TNF-α, NOS-2 and chemokines resulting in inflammation. They also produce IL-17F, IL-21 and IL-22. Knockout mice deficient in either p40 or p19, or in either subunit of the IL-23 receptor (IL-23R and IL12R-β1), develop less severe symptoms of multiple sclerosis and inflammatory bowel disease highlighting the importance of IL-23 in the inflammatory pathway.

IL-23 is mainly produced by cells of the innate immune system such as dendritic cells and tissue-resident macrophages (Basso *et al.*, 2009). The full differentiation and maintenance of Th17 cells is dependent on IL-23 (Korn*et al.*, 2009; McGeachy *et al.*, 2009). However, after identification of the differentiation factors required for Th17 cells, it became clear that IL-23 was not involved in the initial differentiation stage (Korn *et al.*, 2009) but is fundamental to maintain Th17 cells *in vitro* and *in vivo* (Chen *et al.*, 2011). Naive T cells do not express the IL-23R. The transcription factor RORγt controls the expression of the IL-23R on newly primed T cells, expanding their responsiveness to IL-23 by expression of the IL-23R on activated/memory T cells (Aggarwal *et al.*, 2003; Kastelein *et al.*, 2007; Kolls and Khader, 2010), allowing Th17 cells to become responsive to IL-23 and IL-23 to serve as a survival factor for committed Th17 cells (Veldhoen *et al.*, 2006). Th17 cells are preferentially induced in response to infections with extracellular bacteria and fungi, but are also induced during classical Th1 and Th2 responses.

The chicken cytokine repertoire has been established based on the currently available versions of the chicken genome. All of the cytokines annotated as such in the chicken genome have been cloned and sequenced. However, there are obvious gaps in the chicken's repertoire compared to those of mammals (Kaiser *et al.*, 2005). These include obvious gaps in the IL-1 family, the IL-17 family, the TNF super family and the chemokines (reviewed in Kaiser, 2010).

Adaptive immune responses are driven by cytokines produced by CD4+ T helper (Th) cells. The Th1-Th2 paradigm has long been used to explain adaptive immune responses. Briefly, cytokines produced by Th1 cells (e.g. IFN-γ) control infections with intracellular pathogens and cytokines produced by Th2 cells (e.g. Il-4, IL-5 and IL-13) control infections with extracellular pathogens. These cytokines and responses are present in chickens (Degen *et al.*, 2005; Powell *et al.*, 2009), with the exception that IL-5 is switched off during Th2 responses in the bird.

However, it became apparent that this simple paradigm did not explain all adaptive immune responses in mammals. It is now apparent that there are many more CD4+ Th cell subsets, including Th17, Th9, follicular Th cells, and natural and inducible regulatory T cells (Tregs). Of these, only the existence of natural Tregs has been demonstrated in the chicken (Shanmugasundaram and Selvaraj, 2011).

It is currently unclear how many of the mammalian immunomodulatory systems will ultimately be found in birds. Certainly, given the evolutionary distance between birds and mammals, it seems unlikely that all components of mammalian immune systems will be fully replicated in birds. Furthermore, despite the publication of the chicken genome in 2004, the p19 subunit of IL-23 (and many other cytokines) has been neither identified in chickens, nor in any other avian species.

The chicken genome sequence is not complete; there are thousands of contig gaps even on the best assembled chromosomes, and at least six of the micro-chromosomes as yet have no sequence assigned to them, although there is a large amount of unmapped sequence that may belong on these. It is currently not known

how much of the avian genome remains to be sequenced, or why certain regions of the genome seem to be so difficult to sequence.

The present invention is based upon the identification of the IL-23 p19 subunit in a portion of the chicken genome; a portion of the IL-23 p19 subunit cDNA sequence was pieced together from shotgun sequences that were not included in the current genome build (presumably because their sequence scores did not pass the automatic thresholds for inclusion in the assembly), and this was used as a template to generate the rest of the cDNA sequence by 5' and 3' RACE. This discovery provides a novel avian cytokine with potential for use in immunomodulation in birds. In particular, IL-23 and protein variants derived therefrom may be particularly useful to enhance the effectiveness of avian vaccines, e.g. to be used as an adjuvant.

Statements of the Invention

According to a first aspect of the invention there is provided a protein comprising an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 1 or a functional fragment thereof. SEQ ID NO 1 represents the amino acid sequence of the chicken p19 subunit of IL-23.

The sequence depicted in SEQ ID NO 1 represents a polypeptide having a molecular weight of approximately 19 kD, and will be referred to generally as chicken p19 or IL23α. It was identified from chickens and thus represents chicken p19. Other avian forms of p19 are highly likely to exist (e.g. duck or turkey p19), and will have sequences that are similar to chicken p19. Such other avian forms of p19 are also included in the present invention.

As used herein, the term "protein" can be used interchangeably with "peptide" or "polypeptide", and means at least two covalently attached amino acids linked by a peptidyl bond. The term protein encompasses purified natural products, or products which may be produced partially or wholly using recombinant or synthetic techniques. The term protein may refer to an aggregate of a protein, such as a dimer or other multimer, a fusion protein, a protein variant, or derivative thereof. The term also includes modified proteins, for example, a protein modified by glycosylation,

acetylation, phosphorylation, pegylation, ubiquitination, and so forth. A protein may comprise amino acids not encoded by a nucleic acid codon.

The protein of the present invention can be a multi-subunit protein wherein at least one subunit comprises the said sequence. Alternatively, it can be a single subunit protein.

An exemplary multi-subunit protein comprises both the p19 and p40 subunits. Preferably the p19 and p40 subunits are linked together. When linked by disulphide bonds this heterodimer forms an avian interleukin-23 (IL-23). Of course, p19 and p40 subunits can be linked by other means (e.g. by a linker peptide to form a fusion protein) to form a modified IL-23 which retains IL-23 functionality. Avian IL-23, in both wild-type and modified forms, is a preferred embodiment of the present invention.

It should be noted that avian p19 could potentially form multi-subunit proteins with other proteins, which may also be useful entities. Such other entities that contain avian p19 and which provide useful effects also form embodiments of the present invention.

Accordingly, in a preferred embodiment, the invention also provides a protein comprising a subunit comprising an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 1 or a functional fragment thereof (i.e. p19) and a subunit having a sequence showing at least 80% similarity with the sequence as depicted in SEQ ID NO 3 or a functional fragment thereof (i.e. the amino acid sequence of the chicken p40 subunit of IL-23, which is shared with IL-12).

The proteins of the invention are in principle avian cytokines that can be used for various purposes. The cytokine proteins according to the invention, especially avian IL-23, more particularly chicken IL-23, may be used as an adjuvant in avian vaccines to enhance an immune response. Avian IL-23 may be especially useful in improving the ability of an avian to generate a memory response to an antigen. This would be very useful in providing long term immunity to pathogens in avian species.

It will be obvious that proteins having minor modifications in the sequence are equally useful, and the invention also provides for a protein comprising a polypeptide sequence which has at least 80%, or preferably at least 90%, more preferably 95%, more preferably at least 99%, even most preferably 100% similarity to the sequence in SEQ ID NO 1 or a functional fragment thereof, and also in respect of SEQ ID NO 3 or a functional fragment thereof.

The term "similarity" refers to a degree of similarity between proteins in view of differences in amino acids, but which different amino acids are functionally similar in view of almost equal size, lipophilicity, acidity, etc. A percentage similarity can be calculated by optimal alignment of the sequences using a similarity-scoring matrix such as the Blosum62 matrix described in Henikoff S. and Henikoff J.G., P.N.A.S. USA 1992, 89: 10915-10919. Calculation of the percentage similarity and optimal alignment of two sequences using the Blosum62 similarity matrix and the algorithm of Needleman and Wunsch (J. Mol. Biol. 1970, 48: 443-453) can be performed using the GAP program of the Genetics Computer Group (GCG, Madison, WI, USA) using the default parameters of the program.

Polymorphic forms of p19 and homologues from other avian species are included in the present invention. Variants of the proteins that also form part of the present invention are natural or synthetic variants that may contain variations in the amino acid sequence due to deletions, substitutions, insertions, inversions or additions of one or more amino acids in said sequence or due to an alteration to a moiety chemically linked to a protein. For example, a protein variant may be an altered carbohydrate or PEG structure attached to a protein. The proteins of the invention may include at least one such protein modification.

Substitutional variants of proteins are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. The proteins of the present invention can contain conservative or non-conservative substitutions.

The term “conservative substitution”, relates to the substitution of one or more amino acid substitutions for amino acid residues having similar biochemical properties. Typically, conservative substitutions have little or no impact on the activity of a resulting protein. For example, a conservative substitution in a cytokine may be an amino acid substitution that does not substantially affect the ability of the cytokine to bind to its receptor or otherwise perform its usual biological function. Screening of variants of the proteins of the present invention can be used to identify which amino acid residues can tolerate an amino acid substitution. In one example, the relevant biological activity of a modified protein is not altered by more than 25%, preferably not more than 20%, especially not more than 10%, when one or more conservative amino acid substitutions are effected.

One or more conservative substitutions can be included in a protein of the present invention. In one example, 10 or fewer conservative substitutions are included in the protein. A protein of the invention may therefore include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative substitutions. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. Alternatively, a polypeptide can be produced to contain one or more conservative substitutions by using peptide synthesis methods, for example as known in the art.

Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val. In one embodiment, the substitutions are among Ala, Val Leu and Ile; among Ser and Thr; among Asp and Glu; among Asn and Gln; among Lys and Arg; and/or among Phe and Tyr. Further information about conservative substitutions can be found in, among other locations, Ben-Bassat *et al.*, (J. Bacteriol. 169:751-7, 1987), O'Regan *et al.*, (Gene 77:237-51, 1989), Sahin-Toth et al., (Protein Sci. 3:240-7, 1994), Hochuli *et al.*, (Bio/Technology 6:1321-5, 1988), WO 00/67796 (Curd et al.) and in standard textbooks of genetics and molecular biology.

Other variants can be, for example, functional variants such salts, amides, esters, and specifically C-terminal esters, and N-acyl derivatives. Also included are peptides which are modified *in vivo* or *in vitro*, for example by glycosylation, amidation, carboxylation or phosphorylation.

Proteins according to the present invention can be modified by a variety of chemical techniques to produce derivatives having essentially the same activity as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable caution or esterified, for example to form a C1-C6 alkyl ester, or converted to an amide, for example of formula $CONR^1R^2$ wherein R^1 and R^2 are each independently H or C1-C6 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6-membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C1-C6 alkyl or dialkyl amino or further converted to an amide. Hydroxyl groups of the peptide side chains may be converted to alkoxy or ester groups, for example C1-C6 alkoxy or C1-C6 alkyl ester, using well-recognized techniques. Phenyl and phenolic rings of the peptide side chains may be substituted with one or more halogen atoms, such as F, Cl, Br or I, or with C1-C6 alkyl, C1-C6 alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide side chains can be extended to homologous C2-C4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides of this disclosure to select and provide conformational constraints to the structure that result in enhanced stability.

Proteins comprising only a functional fragment of the p19 sub-unit also form part of the present invention. A functional fragment is a fragment that at least represents the part or parts of the polypeptide subunit or subunits, which are essential for the protein to be able to serve as a cytokine, and can fulfil this function, for example,

when used alone or in a multi-subunit form. Thus, such functional fragments may be polypeptides that are functional *per se*, or the fragments may be functional when linked to other polypeptides, e.g. to obtain chimeric proteins. Such functional fragments are understood to fall within the scope of the present invention. Whether a fragment is functional can be determined using the bioassays herein described.

Fragments can *inter alia* be produced by enzymatic cleavage of precursor molecules, using restriction endonucleases for the DNA and proteases for the polypeptides. Other methods include chemical synthesis of the fragments or the expression of peptide fragments by DNA fragments.

In one particularly preferred embodiment, the present invention includes fragments of the above mentioned proteins in which a signal peptide has been removed or deactivated. The vast majority of genes have a signal peptide that is cleaved to make the mature protein, particularly genes that go to the surface of a cell or are exported; p19 and p40 are no different and include an N-terminal signal peptide. Particularly when expressing a recombinant heterodimer from a single clone, but also in other situations, it is wise to remove the genes' own signal peptide sequences to avoid any expression problems. Removal of the signal peptide can be achieved by cleaving a protein once is has been translated, or it can be achieved by expressing the protein from a nucleic acid in which the sequence encoding the signal peptide has been removed. Deactivation of a signal sequence can be achieved by changing (e.g. by substitution or by partial deletion) the sequence of the signal peptide such that it is no longer functional. For example, embodiments of the present invention include a protein comprising a sequence as set out in SEQ ID NO 1 in which the 19 N-terminal amino acids have been deleted and/or to such an extent that the signal peptide no longer functions. Such a sequence is shown in SEQ ID NO 10. Similarly, where the protein of the present invention further comprises a sub-unit comprising the sequence of SEQ ID NO 3, the 15 N-terminal amino acids can be deleted or altered to such an extent that the signal peptide no longer functions. Such a sequence is shown in SEQ ID NO 11.

Thus, in one embodiment the present application provides a protein comprising an amino acid sequence showing at least 80% similarity with the amino acid sequence

as depicted amino in SEQ ID NO 10. Such a protein may optionally comprise a sub-unit showing an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 11.

In an especially preferred embodiment the invention provides the chicken IL-23, consisting of a p19 subunit having the amino acid sequence of SEQ ID NO 1, or a variant or derivative thereof (e.g. SEQ ID NO 10), and a p40 subunit having the amino acid sequence depicted in SEQ ID NO 3, or a variant or derivative thereof (e.g. SEQ ID NO 11), linked together by any suitable means, for example, by a disulfide bond or linker peptide.

A suitable linker peptide has the sequence GSGSSRGGSGSGGSGGGGSKL (SEQ ID NO 9), but many other sequences would be suitable. Suitable linkers are well known in the art, and are typically rich in glycine for flexibility, and may comprise serine and/or threonine for solubility. Further information can be found, *inter alia*, at George RA and Heringa J. *An analysis of protein domain linkers: their classification and role in protein folding.*Protein Eng, 2002 Nov; 15(11) 871-9.

The linkage of the p19 and p40 subunits can, of course, be established in various ways to provide a multi-subunit IL-23 protein according to the present invention. Numerous means of linking two or more subunits together would be apparent to the skilled person.

The bioactivity of proteins according to the invention can be measured *in vitro* using a suitable bioassay. Suitable bioassays are described below in detail. Suitable assays include measuring the stimulation of chicken BM-DC with purified IL-23 (or variants/derivatives thereof) and measuring the increase in expression of IL-12 p70 and IFN-γ and/or the stimulation of splenocytes with IL-23 (or variants/derivatives thereof) and measuring the increase in expression of IL-17A, IL-17F, IL-21 and/or IL-22. The levels of expression of these markers can be determined by measuring the protein levels directly (e.g. by ELISA) or by measuring the amount of mRNA (e.g. by quantitative RT-PCR).

Such assays can readily be used to compare the bioactivity of any variant or derivative of avian IL-23 proteins with the bioactivity of wild type IL-23.

Preferably proteins according to the present invention have a bioactivity as measured by such a test of 50% or higher compared to wild-type IL-23. More preferably the protein has a bioactivity of 75% or higher, more preferably 90% or higher, yet more preferably 95% or higher, and potentially it could have a bioactivity which is 100% or higher compared to wild-type IL-23.

The relative percentages bioactivities mentioned above can be in respect of any one of the markers, i.e. levels of IL-12 p70 or IFN-γ in BM-DC, or IL-17A, IL-17F, IL-21 and/or IL-22 in splenocytes. Alternatively, in a very rigorous assay, the relative percentage figure can the minimum relative percentage determined in respect of all of these markers.

IL-23 proteins can suitably be generated via expression vectors containing both p19 and p40 encoding DNA sequences separated by a sequence encoding a suitable linker sequence. Alternatively, separate expression vectors containing either the p19 or p40 encoding DNA sequences under control of separate promoters can be used to generate chicken IL-23.

In another aspect the present invention provides a polynucleotide which encodes a protein according to the present invention.

Thus the present invention provides an isolated polynucleotide encoding a protein having an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 1 or a functional fragment thereof.

The isolated polynucleotide may further comprise a polynucleotide encoding a protein having an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 3 or a functional fragment thereof.

A polynucleotide encoding an avian IL-23 protein may comprise both of these sequences and the sequences can be linked by a sequence encoding a linker.

Fragments of the provided nucleic acid sequence that encode a functional fragment of the protein also form part of the present invention. For example, a polynucleotide encoding such a functional fragment of the protein may be fused to polynucleotides encoding transmembrane regions and/or suitable signal sequences.

Specific exemplary nucleic acids which encode functional fragments of avian p19 and p40 are SEQ ID NOS 12 and 13, respectively. These nucleic acid sequences encode protein fragments in which the signal peptide has been removed.

The polynucleotides of the present invention include polynucleotides having variations in the nucleic acid sequence when compared to the identified nucleic acid sequence or having polymorphic sites. The term "variation" or related terms are intended to cover polynucleotides that differ from the identified nucleic acid sequence but which still encode a protein that has a biological activity similar to that of p19 and/or IL-23. Bioassays to determine the biological activity of any particular protein are described herein.

Variants may be natural or non-natural variants. Natural variants include homologues in various avian species or allelic variants. An allelic variant is one of several alternate forms of a gene occupying a locus on a chromosome of an organism. Sometimes, a gene is expressed in a certain tissue as a splicing variant, resulting in an altered 5'or 3' mRNA or the inclusion or exclusion of one or more exon sequences. These sequences, as well as the proteins encoded by these sequences, all are expected to perform the same or similar functions and thus also form part of the invention. Non-naturally occurring variants may be introduced by mutagenesis.

An isolated cDNA sequence may be incomplete due to incomplete transcription from the corresponding mRNA, or clones may be obtained containing fragments of the complete cDNA. Various techniques are known in the art to complete said cDNA sequences, such as RACE (Rapid Amplification of cDNA Ends).

Polynucleotides that have a nucleic acid sequence that is a variant of the identified nucleic acid sequence may be isolated by a method comprising the steps of:

a) hybridizing a DNA comprising all or part of the identified sequence as reflected in SEQ ID NO 2 or 4, under stringent conditions against nucleic acids being (genomic) DNA or cDNA isolated from avian cells which highly express the polynucleotide of interest; and

b) isolating said nucleic acids by methods known to a person skilled in the art.

The hybridization conditions are preferably highly stringent.

According to the present invention the term 'stringent' means washing conditions of 1 x SSC, 0.1% SDS at a temperature of 65 °C; 'highly stringent' conditions refer to a reduction in SSC towards 0.3 x SSC, more preferably to 0.1 x SSC. Preferably the first two washings are subsequently carried out twice each during 15-30 minutes. If there is a need to wash under highly stringent conditions an additional wash with 0.1x SSC is performed once during 15 minutes. Hybridization can be performed overnight in 0.5 M phosphate buffer pH 7.5 with 7% SDS at 65 °C. Such hybridization methods are disclosed in any standard textbook on molecular cloning, for example: Molecular Cloning: a laboratory manual, 3rd ed.; editors: Sambrook *et al.*, CSHL press, 2001.

As an alternative the isolation method might comprise nucleic acid amplification methodology using primers and/or probes derived from the nucleic acid sequence provided in the present invention. Such primers and/or probes are oligonucleotides that are at least 15 nucleotides in length; preferred oligonucleotides have about 25-50 nucleotides.

Variants or other avian homologues of the sequences depicted in SEQ ID NO 2 and 4 may also be identified by comparing the sequence *in silico* to other avian sequences that may be comprised in a computer database. Sequences may be compared with sequences in databases using a BLAST program (BLASTF 2.1. 2 [Oct.-19-2000]) (Altschul, SF, TL Madden, AA Schaffer, J Zhang, Z Zhang, W Miller, and DJ Lipman, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 1997,25: 3389-3402).

Preferred embodiments of the invention are polynucleotides encoding proteins having at least 95% identity with SEQ ID NO 1 or SEQ ID NO 10, and more preferred are those polynucleotides encoding proteins having at least 97% identity with SEQ ID NO 1 or SEQ ID NO 10, with those encoding proteins having at least 98 or 99% being more preferred. Most preferred are polynucleotides encoding the protein of SEQ ID NO 1 or SEQ ID NO 10.

Due to the degeneracy of the genetic code, polynucleotides encoding an identical or substantially identical amino acid sequence may utilize different specific codons. All polynucleotides encoding the polypeptides as defined above are considered to be part of the invention.

In particular preferred embodiments the polynucleotides according to the invention are isolated polynucleotides comprising a sequence having at least 80% identity with the nucleic acid sequence of SEQ ID NO 2 or SEQ ID NO 12. Such polynucleotides can, of course, contain additional sequences, such as expression control sequences or sequences encoding other proteins or protein subunits.

More preferred are those polynucleotides comprising a sequence having at least 90% identity, and yet more preferred at least 95, preferably 99% identity, most preferred 100% identity to the entire sequence of SEQ ID NO 2 or SEQ ID NO 12.

Such polynucleotides include polynucleotides comprising the nucleic acid sequence depicted in SEQ ID NO 2 or SEQ ID NO 12. In a further preferred embodiment of the invention the polynucleotide consists of the nucleic acid sequence as depicted in SEQ ID NO 2 or SEQ ID NO 12.

The polynucleotides according to the invention may be DNA or RNA, preferably DNA. DNA according to the invention may be obtained from cDNA. Alternatively, the coding sequence might be genomic DNA, or prepared using DNA synthesis techniques. If the polynucleotide is DNA, it may be in single stranded or double stranded form. The single strand might be the coding strand or the non-coding (anti-sense) strand.

Also included within the definition of polynucleotides are modified RNAs or DNAs. Modifications in the bases of the nucleic acid may be made, and bases such as inosine may be incorporated. Other modifications may involve, for example, modifications of the backbone.

"% identity" defines the relation between two or more polynucleotides or polypeptides on the basis of a comparison between their aligned sequences.

Identity can be calculated by known methods. Identity, or homology, percentages as mentioned herein are those that can be calculated with the GAP program, running under GCG (Genetics Computer Group Inc., Madison, WI, USA).

Parameters for polypeptide sequence comparison included the following:
Algorithm: Needleman and Wunsch, J. Mol. Biol. 1970, 48: 443-453.

As a comparison matrix for amino acid alignments the Blosum62 matrix is used (Henikoff and Henikoff, supra) using the following parameters:

- Gap penalty: 8
- Gap length penalty: 2
- No penalty for end gaps.

Parameters for nucleotide comparison that may be used:
Algorithm: Needleman and Wunsch (supra).

- Comparison matrix: matches = +10, mismatch = 0.
- Gap penalty: 50.
- Gap length penalty: 3.

Nucleic acids, especially DNA, according to the invention will be useful for *in vivo* or *in vitro* expression of the encoded protein. When the polynucleotides according to the invention are used for expression of the encoded polypeptide, the polynucleotides may include, in addition to the coding sequence for the protein or functional fragment thereof, other coding or non-coding sequences, for example,

leader sequences or fusion portions, linker sequences, marker sequences, promoters, enhancers and the like.

In a preferred embodiment the present invention provides isolated polynucleotides comprising a sequence having at least 80% identity with the nucleic acid sequence of SEQ ID NO 5. This sequence encodes a fusion protein comprising p19 and p40 linked by a linker sequence. Variants and modified forms of this sequence are, of course, also provided by the present invention.

Cytokines based on p19, such as IL-23, can be used to ether enhance an ongoing microorganism-induced immune response or a vaccination-induced immune response. Such responses can be based on both cellular and humoral immunity.

The polynucleotides according to the invention may be used in the production of recombinant proteins according to the invention. This can be achieved through expression of the proteins in a suitable host cell or multicellular organism. When used for expression of an encoded polypeptide, the polynucleotides may advantageously include, in addition to the coding sequence for the polypeptide, other coding sequences, for example, signal sequences, leader sequences, targeting sequences, fusion portions, marker sequences, sequences to assist in purification and the like.

The polynucleotides of the present invention may be used as DNA or vector vaccines, particularly when delivered together with nucleic acid sequences encoding, for example, immunogenic proteins derived from an avian pathogen.

A wide variety of host cell and cloning vehicle combinations can be used for cloning and expression. Polynucleotides of the present invention may be cloned into any appropriate expression system. Suitable expression systems include bacterial expression system (e. g. *Escherichia coli* DH5α), a viral expression system (e.g. Baculovirus), a yeast system (e.g. *Saccharomyces cerevisiae*) or eukaryotic cells (e.g. COS-7, CHO, BHK, HeLa, HD11, DT40, CEF, or HEK-293T cells). A wide range of suitable expression systems are available commercially. Typically the

polynucleotide is cloned into an appropriate vector under control of a suitable constitutive or inducible promoter and then introduced into the host cell for expression. A selectable marker, such as glutamine synthetase or dihydrofolate reductase, is often used to allow selection of cells with high copy numbers of the vector to promote high expression levels.

In another aspect the present invention therefore provides a recombinant vector comprising a polynucleotide according to the invention. Suitable vectors include bacterial or yeast plasmids, cosmids, phagemids, fosmids, a wide host range plasmids and vectors derived from combinations of plasmid and phage or virus DNA. An origin of replication and/or a dominant selection marker can suitably be present in the vector.

The vectors according to the invention are suitable for transforming a host cell. Examples of suitable cloning vectors are plasmid vectors such as pBR322, the various pUC, pEMBL and Bluescript plasmids, or viral vectors such as HVT (Herpes Virus of Turkeys), MDV (Marek Disease Virus), ILT (Infectious Laryngotracheitis Virus), FAV (Fowl Adenovirus), FPV (Fowlpox Virus), or NDV (Newcastle Disease Virus). pcDNA3.1 is a particularly preferred vector for expression in animal cells.

When used in the expression of the proteins of the present invention, a vector according to the present invention typically comprises an expression control sequence operably linked to the nucleic acid sequence coding for the protein to control expression of the relevant polynucleotide. Such expression control sequences generally comprise a promoter sequence and additional sequences which regulate transcription and translation and/or enhance expression levels. Suitable expression control sequences are well known in the art and include eukaryotic, prokaryotic, or viral promoter or poly-A signal. Expression control and other sequences will, of course, vary depending on the host cell selected or can be made inducible. Examples of useful promoters are the SV-40 promoter (Science 1983, 222: 524-527), the metallothionein promoter (Nature 1982,296: 39-42), the heat shock promoter (Voellmy *et al.*, P.N.A.S. USA 1985,82: 4949-4953), the PRV gX promoter (Mettenleiter and Rauh, J. Virol. Methods 1990, 30: 55-66), the human CMV IE promoter (US 5,168,062), the Rous Sarcoma virus LTR promoter (Gorman

et al., P.N.A.S. USA 1982, 79: 6777-6781), or human elongation factor 1 alpha or ubiquitin promoter. Many other suitable control sequences are known in the art, and it would be routine for the skilled person to select suitable sequences for the expression system being used.

After the polynucleotide has been cloned into an appropriate vector, the construct may be transferred into the cell, bacteria, or yeast by means of an appropriate method, such as electroporation, $CaCl_2$ transfection or lipofectins. When a baculovirus expression system is used, the transfer vector containing the polynucleotide may be transfected together with a complete baculo genome.

These techniques are well known in the art and the manufacturers of molecular biological materials (such as Clontech, Stratagene, Promega, and/or Invitrogen) provide suitable reagents and instructions on how to use them. Furthermore, there are a number of standard reference text books providing further information on this, e.g. Rodriguez, R. L. and D. T. Denhardt, ed., "Vectors: A survey of molecular cloning vectors and their uses", Butterworths, 1988; Current protocols in Molecular Biology, eds.: F. M. Ausubel *et al.*, Wiley N. Y. , 1995; Molecular Cloning: a Laboratory Manual, supra; and DNA Cloning, Vol. 1-4, 2nd edition 1995, eds.: Glover and Hames, Oxford University Press).

In a further aspect the present invention also provides a cell capable of expressing a recombinant protein, characterised in that the cell comprises a polynucleotide according to the invention encoding the recombinant protein to be expressed. Suitably the cell is a host cell transformed with a polynucleotide or vector as described above. The polynucleotide or vector according to the invention can be stably integrated into the genomic material of the cell or can be part of an autonomously replicating vector. "Recombinant" in this context refers to a protein that is not expressed in the cell in nature.

Host cells can be cultured in conventional nutrient media which can be modified, e.g. for appropriate selection, amplification or induction of transcription and thus expression of the recombinant protein.

The host cells can be prokaryotic or eukaryotic. Preferably the cells are eukaryotic. Eukaryotic cells can suitably be of avian or non-avian origin. Cells that are of non-avian origin may be, for example, insect cells, HeLa, BHK, HEK-293T, CHO, or COS-7 cells. Suitable avian cells include CEF, HD11 or DT-40 cells.

Suitable culture conditions for various suitable cell types are well-known to the person skilled in the art.

In a further aspect the present invention provides a cell culture comprising cells according to the invention.

Expression of recombinant p19, and preferably also p40, enables the production of pure proteins, essentially free from contaminants such as other cytokines and other immunologically active agents.

According to a further aspect, the present invention provided antibodies which are specific for the proteins of the present invention. In particular the invention provides antibodies which are specific for avian, especially chicken, p19. Such antibodies can be generated via techniques generally available. Preferably the antibodies are monoclonal antibodies. In a preferred embodiment the antibodies are specific for avian p19 and thus bind specifically to avian p19 but do not bind specifically to non-avian p19. For example, such antibodies can bind to an avian p19 subunit with an affinity of at least 10^{-7} M but do not specifically bind to a non-avian p19 subunit, e.g. they bind with an affinity of not more than 10^{-4} M, preferably 10^{-3} M or less. The antibodies according to the invention are suitable for use in diagnostics or for isolation and purification of proteins such as avian chicken IL-23 from crude preparations. Moreover, the antibodies can be used to develop assays for quantitative analysis of protein production in vitro or for quantitative measurements of protein levels *in vivo*. The term antibody is intended to cover antibody variants such as minibodies, nanobodies, Fabs, SCFVs and the like.

Antibodies, including monoclonal, can be generated against epitopes of a target protein by well-known techniques which would be routine for the skilled person. Such techniques include, for example, phage display.

Vaccination against an infectious disease aims to elicit an immune response that limits infection and/or clinical symptoms associated with infection by a pathogen. It is important that the correct type of immune reaction is triggered, since many types of immune mechanisms that can be activated are inadequate for control of the particular pathogen. Low responsiveness to vaccine antigens can be overcome by administering the antigens in combination with adjuvants.

Adjuvants are defined as those components of a vaccine formulation other than the antigen which contribute to enhanced immune responsiveness to the antigen. Known adjuvants include aluminium salts, oil emulsions, derivatives of muramyl peptide, monophosphoryl lipid A, liposomes, QS21™,MF-59™ and Iscoms™.

The effects of vaccination are strongly influenced by the choice of adjuvant that is administered together with the vaccine antigen. There is a need for improved adjuvants to improve vaccination, especially in commercial bird production, as explained above.

Proteins according to the invention, in particular avian IL-23, may have a potent adjuvant effect on the immune response of a subject to a vaccine.

Thus in another embodiment the invention provides for an adjuvant composition comprising an effective adjuvant amount of a protein according to the invention, in particular an avian IL-23. The adjuvant composition can be administered concomitantly or sequentially with a vaccine formulation containing the antigen.

Proteins according to the invention can conveniently be included in the vaccine formulation. Thus in another embodiment the present invention provides for a vaccine comprising at least one active agent, an effective adjuvant amount of a protein according to the invention, and a pharmaceutically acceptable carrier or diluent.

A protein according to the present invention can be a molecule comprising the whole of the p19 and p40 subunits or fragments or variants thereof, provided said fragments or variants retain the ability to act as a cytokine.

An adjuvant composition according to the present invention comprises a protein according to the invention, preferably an avian IL-23 protein, and a pharmaceutically acceptable carrier.

Suitable pharmaceutical carriers are water, saline, and the like. Additionally, the adjuvant composition may comprise one or more other adjuvants such as oil emulsions, aluminium salts, derivatives of muramyl dipeptide, monophosphoryl lipid A, liposomes, QS21™, MF-59™, Iscoms™ and the like.

The proteins according to the invention may also be used in conjunction with other cytokines or adjuvants.

The adjuvant composition according to the invention may alternatively comprise a nucleic acid capable of expressing a protein according to the invention. For example, the nucleic acid could be a plasmid, e.g. a DNA plasmid. Nucleotide sequences encoding antigens and/other cytokines that are used in conjunction with a protein according to the invention can be present on the same DNA plasmid or on a separate plasmid.

Upon administration of such a DNA adjuvant composition to a subject, host cells take up and express encoded genes on the inoculated DNA, resulting in *in vivo* expression of the proteins according to the invention, for example, avian IL-23.

A vaccine composition according to one aspect of the present invention comprises at least one active agent (i.e. an antigenic agent) and an effective adjuvant amount of a protein according to the invention, i.e. in an amount which will cause the vaccinated subject to produce an enhanced immunological response as compared to the vaccine without said protein.

The required effective amount for an adjuvant composition or vaccine according to the invention is dependent on the type of active agent used, the type of pathogen immunized against, as well as the type of vaccinated subject. Determination of the effective amount is well within the routine skills of the practitioner, and will generally be from 0.001 to 500 µg/dose. Preferably the amount is from 0.01 to 50 µg/dose, and more preferably from 0.1 to 5 µg/dose.

The active agent for use in a vaccine according to the invention can be of viral, bacterial or parasitic origin. The active agent can be the whole pathogen which causes the disease or may consist of one or more components derived from said pathogen, e.g. antigenic proteins, protein fragments, protein-polysaccharides, protein-lipopolysaccharides, lipopolysaccharides, or suchlike. Where the active agent is a whole pathogen, said pathogen can be live or inactivated. Live pathogens are typically either attenuated or naturally occurring mild strains of the pathogen. Inactivated pathogens are typically pathogens which have been inactivated, e.g. killed, by chemical or physical means. Chemical inactivation means include formaldehyde, glutaraldehyde, β-propiolactone, and ethyleneimine. Physical inactivation means include UV radiation, γ-radiation, heat, and X-radiation.

The active agent may be a nucleic acid (e.g. a DNA plasmid) capable of *in vivo* expression of a pathogen or selected components derived from said pathogen. In addition, the vaccine may comprise a nucleic acid (e.g. a DNA plasmid) capable of expressing a protein according to the invention *in vivo*. The nucleic acid encoding said protein adjuvant and the DNA encoding said pathogen or selected components may be present on one and the same nucleic acid molecule, or may be present on separate nucleic acid molecules. Upon administration of the nucleic acid vaccine to a subject, host cells will take up and express *in vivo* said active agent as well as said protein according to the invention. DNA vaccines are described, for example, in US 5,580,859.

Pharmaceutically acceptable carriers or diluents that can be used to formulate an adjuvant composition or a vaccine composition according to the invention are sterile and physiologically compatible such as for example an aqueous buffer, a saline

solution and the like. In addition stabilizers, preservatives and the like may be added to these compositions.

The compositions of the present invention may take any form that is suitable for oral, parenteral or *in ovo* administration. Methods of *in ovo* administration are known in the art and are emerging as a preferred route for bird vaccination. Suitable methods are described in various articles such as Vaccine. 2008 Jan 24;26(4):522-31 and Vet Q. 2004 Jun;26(2):76-87. Other routes of administration which may be used include administration by inhalation or intranasal administration. For oral use, the adjuvant or vaccine compositions according to the invention may be formulated as solutions, syrups, suspensions, tablets, capsules and the like. For parenteral use, the active can be formulated as a solution, suspension, emulsion or lyophilized powder in association, or separately provided, with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 1-10% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g. sodium chloride, mannitol, etc.) and chemical stability (e.g. buffers and preservatives). The formulation is preferably sterilised, e.g. by known and suitable techniques.

In general, suitable carriers, excipients and diluents should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration.

The compositions can be prepared in unit dosage form for administration to a subject. The amount and timing of administration are at the discretion of the treating physician (e.g. veterinarian) to achieve the desired purposes. A typical pharmaceutical composition for intravenous or subcutaneous administration includes about 0.1 to 10 mg of active ingredient per subject per day.

Preparation of the compositions according to the present invention is carried out by means conventional for the skilled person. Methods for preparing administrable compositions, whether for intravenous or subcutaneous administration or otherwise, will be known or apparent to those skilled in the art and are described in more detail

in such publications as Remington: The Science and Practice of Pharmacy, Lippincott Williams and Wilkins; 21st Revised edition (1 May 2005).

In a further aspect the present invention provides a method of stimulating an immune response in a subject comprising administering a protein, polynucleotide, vector or a cell according to the present invention to said subject. The subject is preferably a bird, more preferably a chicken. The immune response stimulated is preferably generation of an immunological memory response by activating antigen presenting cells.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to the "antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

"Amino acid sequence", as used herein, refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragments or portions thereof, and to naturally occurring or synthetic molecules. "Amino acid sequence" and like terms, such as "polypeptide" or "protein" as recited herein are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

"Antibody" refers to intact molecules as well as fragments thereof that are capable of specific binding to an epitopic determinant. Antibodies that bind a polypeptide (for example, a polypeptide encoded by a nucleic acid of the present invention) can be prepared using intact polypeptides or fragments as the immunizing antigen. These antigens may be conjugated to a carrier protein, if desired.

"Cell culture", as used herein, refers to a proliferating mass of cells that may be in either an undifferentiated or differentiated state.

"Fusion protein", as used herein, refers to a protein containing amino acid sequences from each of two distinct proteins; it is formed by the expression of a recombinant

gene in which two coding sequences have been joined together such that their reading frames are in phase. Hybrid genes of this type may be constructed in vitro in order to label the product of a particular gene with a protein that can be more readily assayed (for example, a gene fused with lacZ in *E. coli* to obtain a fusion protein with β-galactosidase activity). Alternatively, a protein may be linked to a signal peptide to allow its secretion by the cell.

The term “isolated” means a biological component (such as a nucleic acid molecule or protein) that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extra chromosomal DNA and RNA, and proteins. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids, proteins and peptides.

“Nucleic acid sequence”, as used herein, refers to a polymer of nucleotides in which the 3′ position of one nucleotide sugar is linked to the 5′ position of the next by a phosphodiester bridge. In a linear nucleic acid strand, one end typically has a free 5′ phosphate group, the other a free 3′ hydroxyl group. Nucleic acid sequences may be used herein to refer to oligonucleotides, or polynucleotides, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin that may be single- or double-stranded, and represent the sense or antisense strand.

“Transformation”, as used herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection, and particle bombardment. Such “transformed” cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also

include cells that transiently express the inserted DNA or RNA for limited periods of time.

"Th cells" (T helper cells) are well known a sub-group of lymphocytes that play an important role in the immune system, particularly in the adaptive immune system. They help the activity of other immune cells by releasing T cell cytokines. They are essential in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages.

"Transfection", as used herein, refers to the introduction of foreign nucleic acid into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics. Transfection may, for example, result in cells in which the inserted nucleic acid is capable of replication either as an autonomously replicating molecule or as part of the host chromosome, or cells that transiently express the inserted nucleic acid for limited periods of time.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

The reader's attention is directed to all papers and documents which are mentioned in this application, and which are open to public inspection, and the contents of all such papers and documents are incorporated herein by reference.

Brief Description of the Figures

Embodiments of the present invention will now be described, by way of example only, with reference to the accompanying figures in which:

- Fig 1 shows a sequence comparison of human, mouse, chicken, and cow p19 subunits.
- Fig 2 shows the nucleotide sequence (229 nt) and amino acid sequence (76 aa) of an EST predicted to be chicken IL-23α. IL-23α F1 primer, IL-23α R1, and IL-23α R2 primer are indicated by arrows with reference numbers. Primer orientation is indicated by arrows.
- Fig 3 shows a pile-up of LOC100858694 with mammalian IL-23α sequences.
- Fig 4 shows the chicken IL-23 Flexi-Construct design.
- Fig 5 shows the quantification of chIL-17A mRNA levels in splenocytes stimulated with purified rchIL-23 for 3 days, expressed as fold change from levels in un-stimulated controls. Data are from 1 bird but are representative of 6 biological replicates.
- Fig 6 shows the quantification of chIL-17F mRNA levels in splenocytes stimulated with purified rchIL-23 for 1 day, expressed as fold change from levels in un-stimulated controls. Data are from 1 bird but are representative of 6 biological replicates.
- Fig 7 shows the quantification of chIL-22 mRNA levels in splenocytes stimulated with purified rchIL-23 for 6 hours, expressed as fold change from levels in un-stimulated controls. Data are from 1 bird, but are representative of 6 biological replicates.

Description of Specific Embodiments of the Invention

Identification and cloning of chicken IL-23α/p19

Il-23α/p19 is not annotated in the chicken genome and the sequence is not present. The platypus IL-23α amino acid sequence was BLAST queried against the NCBI database. This pulled out a single EST sequence (Genbank accession no. CK613546.1) which had not been annotated and was not included in the genome assembly, which, when translated and aligned with platypus, human and mouse amino acid sequences, showed high identity across a number of residues (including some conserved cysteines). The chicken amino acid sequence was subsequently BLAST queried against the Galgal 3.0 removed data reads (i.e. sequence reads from the genome sequencing projects that did not make it into the final assembly) and this yielded 3 putative exons (Contig122339.1,Contig293561.1 andContig91141.1), giving us further confidence that this was a partial sequence of chicken IL-23α.

Primer design for amplification of chIL-23α/p19 was based on the predicted EST sequence (see Figure 2).

Partial length chicken IL-23α cDNA was amplified by using 1 µl of the first PCR product as template in a second PCR reaction (second-round RT-PCR). Total RNA from splenocytes stimulated for 24 h with ConA was used as the initial template, with primers based on the predicted chIL-23α EST (see Figure 2).

GCACGAGGAGGACATGGA	F1 (SEQ ID NO 6)
CATCTTCGCCACACACCCA	R2 (SEQ ID NO 7)
CAGGTCCCCATGCGTTCC	R1 (SEQ ID NO 8)

The predicted band size was 229 nt using IL-23α R1, and 166 nt using IL-23α R2. PCR products of the right size were gel-extracted and TA cloned into pGEM-T Easy for sequencing. One clone was confirmed to be IL-23α by comparison to the EST.

A longer partial length chicken IL-23α cDNA sequence was amplified by RT-PCR from RNA isolated from chicken splenocytes stimulated with phytohaemagglutinin (PHA) for 4 h. First strand synthesis of the cDNA template used an oligo(dT)20 primer. The predicted band size of the amplified cDNA product, using gene-specific primers, was 229 nt using IL-23α F1 and R1, and 166 nt using IL-23α F1 and R2.

Again, one PCR product was cloned and sequenced and confirmed to be the full-length IL-23α cDNA.

Having confirmed that the chIL-23 EST can be amplified from RNA extracted from stimulated chicken splenocytes, gene-specific and nested primers were designed for 5' and 3' RACE-PCR to obtain the full length nucleotide sequence for chIL-23α. A 3' RACE-PCR product was readily amplified, cloned, sequenced and confirmed to be chicken IL-23α. Whilst efforts were underway to generate a 5' RACE product to confirm the rest of the sequence, the NCBI nucleotide database was searched with the sequence already cloned and sequenced.

This identified a single hit, LOC100858694 (www.ncbi.nim.nih.gov/gene/100858694), which identified an uncharacterised computer-predicted mRNA which is not present in the current genome build. An amino acid pileup of this predicted sequence with IL-23α from other species suggested that this mRNA was over-predicted, as there were many apparently extra residues in the chicken sequence (see Figure 3). This is not the norm for chicken cytokine genes. Usually, the gene structure of the chicken cytokine gene is very well conserved with those of its mammalian orthologues, in that there is the same number of exons and these are approximately of the same length. Based on this knowledge that has been accumulated over 20 years in the field, we hypothesised that the first exon of LOC100858694 was over-predicted (too long) and predicted what we thought would be the actual chicken IL-23α sequence. We then used primers designed to the extremities of the predicted chicken sequence (forward primer starting with the start codon, reverse primer starting with the stop codon), with RNA from PMA-stimulated chicken splenocytes as template, and generated a full-length IL-23α cDNA clone, which was confirmed by sequencing of multiple clones of the RT-PCR product as confirming our prediction.

The amino acid and DNA sequences of p19 (IL-23α) are shown below, and are annotated as SEQ ID NOS 1 and 2 respectively. The amino acid and DNA sequence of the p40 subunit are shown in the sequence listing below, and are annotated as SEQ ID NO 3 and 4 respectively.

SEQ ID NO 5 shown below is the sequence of a construct comprising the p19 and p40 subunits of IL-23 linked by a linker sequence. Both the nucleic acid and the encoded amino acid sequence are shown. This nucleic acid construct is suitable for expression in a suitable host cell to express a fusion protein with chicken IL-23 activity. The construct comprises sequences encoding, in sequential order, the mouse CD8α signal peptide, a V5HIS6 tag which comprises a precision protease site, the p40 mature protein, a flexi linker, and the p19 mature protein with stop codon (see Fig 4). The sequences of p19 and p40 have been modified to remove the signal peptide. Secretion of the fusion protein expression product of the construct is mediated by the mouse CD8α signal peptide.

The most obvious utility for the novel proteins provided by the present invention is in the form of an avian IL-23 cytokine, i.e. a multi-subunit protein comprising avian p19 and p40, or variants thereof that retain their biological activity. However, the p19 subunit has independent utility, i.e. without the p40 subunit being present. For example, p19 may be used in assays to screen for agents that can modulate avian IL-23 activity. It may also be used as in the manufacture of antibodies to avian IL-23. Furthermore, it may be useful in a therapeutic or research situation to restore p19 activity where native p19 activity is deficient. P19 may also have activity when combined with subunits other than p40.

Bioassays

In vitro assays can be used to test the activity of IL-23 and variants or derivatives thereof. It is, of course, possible that the results of such *in vitro* assays may not correspond exactly to activity *in vivo*, but they are nonetheless a practical and biologically relevant method of testing the activity of IL-23 variants and derivatives. Two cellular assays to measure the bioactivity of IL-23 are described below.

Stimulation of chicken BM-DC with purified IL-23 to increase expression of IL-12 p70 and IFN-γ

Chicken bone marrow-derived dendritic cells (BM-DC) are derived as follows:

1) Cut the ends off the femur bone and flush the bone marrow with a 21G needle and syringe of appropriate size using approx. 10 ml PBS into a sterile Petri dish. Rinse the Petri dish with a further 5 ml PBS to ensure removal of all the cells.

2) Pool any bone marrow from different birds (of the same line) together, mix gently by pipetting up and down. Transfer cells to a universal.

3) Centrifuge at 500 x *g* for 10 min, at room temperature. Discard supernatant and re-suspend cells in an appropriate volume of PBS (at RT).

4) In a 15 ml Falcon tube, carefully overlay Histopaque 1.083 (or 1.119, although there are usually more contaminating rbcs) with equal volume of diluted bone marrow cells. Centrifuge at 1200 x *g* (brakes OFF) for 20 min at RT.

5) Collect cells at the interface and transfer to a fresh tube and make up to 20 ml with PBS.Pellet cells by spinning at 500 x *g* for 10 mins at 4 °C.

6) Re-suspend cells in 10 ml of pre-warmed RPMI, count and adjust concentration to approx. 1x 10^6 cells/ml in Complete Media. Seed into 6 well plates at 3 ml per well with recombinant GM-CSF (1:250) and IL-4 (1:250), and incubate at 41 °C, 5% CO_2.

7) Replace 3/4 medium (with fresh, pre-warmed Complete Medium) every 2 days, taking care not to dislodge adherent cells (these are the DCs). Non-adherent cells (such as granulocytes and dead cells) will be removed at this stage.

8) With microscopic examination, cells become an irregular shape from about day 3 or 4, and dendrites can be seen from about day 4 onwards. From about day 4, DC colonies will be beginning to grow.

BM-DCs are stimulated with LPS (positive control) or recombinant IL-23 protein. Optimal concentrations are determined empirically. The appropriate culture period should also be determined, but will be in the period of 24-72 hours.

Following stimulation, the supernatant from the cultured cells will be collected and RNA will be isolated from the cells (IL-23-stimulated, LPS-stimulated (positive control) and un-stimulated (negative control)) using standard RNA isolation kits. The RNA will then be assayed for IL-12 p35, IL-12 p40 and IFN-γ mRNA expression

levels using quantitative RT-PCR. IL-12 p70 and IFN-γ protein levels in the supernatant will be assayed by capture ELISA.

The increase in IL-12 p35, IL-12 p40 and IFN-γ mRNA or IL-12 p70 and IFN-γ protein expression relative to the negative control is representative of the bio-activity of IL-23 or variants or derivatives thereof. Thus the relative bioactivity of a derivative or variant of IL-23 can be determined by comparing the bio-activity of the variant or derivative with wild type IL-23.

Stimulation of splenocytes with IL-23 to increase expression of IL-17A.

Chicken splenocytes are isolated as follows:

1) Aseptically remove spleen into cold DMEM or RPMI or PBS.

2) Prepare single cell suspension of lymphocytes in a Petri dish containing 15-20 ml cold media, by teasing apart tissue using blunt-end forceps. (*Alternatively: tissue can be mashed through a sieve using the end of a syringe plunger).* Transfer single cell suspension to 20 ml universal, avoiding any large clumps of tissue, and allow to settle.

3) Add 5-7 ml of Ficoll Paque/Histopaque 1.077 to a 50 ml Falcon tube. Slowly pipette the single cell suspension (~15-20 ml) to form a discreet layer above the Ficoll. *(Alternatively:Ficoll Paque/Histopaque can be underlaid the single cell suspension using a long-bore pipette/needle).*

4) Centrifuge at 400 *g* with the brakes OFF for 20 min at RT.

5) Carefully draw off lymphocytes from density gradient interface, and transfer to a universal.

6) Add media to ~20 ml, mix by inversion and wash by pelleting cells at 250 *g* for 10 min.

7) Tip off supernatant, re-suspend cell pellet in 20 ml media and remove 20 μl for cell count. Repeat wash in media to be used for culture.

8) Carry out viable cell count, using Trypan Blue.

9) Re-suspend cells at desired concentration, in appropriate media.

Culture of lymphocytes is typically at 5 x 10^6 cells/ml, in DMEM supplemented with 2 mg/ml BSA (or appropriate serum e.g. 10% FCS or 8% FCS + 2% CS) and Pen/Strep, with incubation at 41°C, 5% CO_2.

Splenocytes are stimulated with a mitogen such as ConA (positive control) or recombinant IL-23. Optimal concentrations should be determined empirically. The suitable culture period should be determined, but will typically be in the period of 24-72 hours.

Following stimulation, RNA is isolated from cells (IL-23-stimulated, ConA-stimulated (positive control) and un-stimulated (negative control)) using standard RNA isolation kits. The RNA is then assayed for IL-17A mRNA expression levels using quantitative RT-PCR.

The increase in IL-17A expression relative to the negative control is representative of the bioactivity of IL-23 or variants or derivatives thereof. Thus the relative bioactivity of a derivative or variant of IL-23 can be determined by comparing the bioactivity of the variant or derivative with wild-type IL-23.

Quantitative RT-PCR suitably uses appropriate TaqMan® probes specific for the sequence of interest, and suitable procedures for carrying out quantitative RT-PCR are well known in the art.

Expression of a Recombinant IL-23 Protein

The flexi-IL-23 construct (SEQ ID NO 5) is synthesised in the vector pcDNA3.1 and this is sequenced to confirm the structure. This vector is used to express the recombinant IL-23in mammalian cells. Suitable cells for expression include COS-7, CHO and HEK-293T cells.

Cells are transfected with the vector including the flexi-construct and grown in bulk (0.5-10 L). Supernatant is harvested, analysed by SDS-PAGE and Western blot. A two-step purification process (His-affinity purification followed by ion-exchange chromatography) follows to isolate the target expression product. Finally, the tag is

cleaved by protease. Suitable methods of achieving expression, including transfection methods, growth conditions and media, are well known to the skilled person and are described in the relevant textbooks, some of which are listed herein.

The flexi-IL-23 construct, and indeed other IL-23 expression constructs, may also be expressed in *E. coli*, baculovirus, yeast or any other appropriate expression system using the appropriate expression vector(s), etc. The person skilled in the art is able to readily determine the most suitable expression system depending on the desired properties and quantity of expression product.

Demonstration of Bioactivity of Recombinant Chicken IL-23

Introduction

Having confirmed the full-length chicken IL-23 p19 cDNA sequence, a chicken IL-23 flexi-construct was designed for recombinant protein expression, as discussed above. The chIL-23 flexi-construct design (schematic shown in Fig 4) is based on the mammalian IL-23 flexi-construct and contains the mouse CD8α signal peptide, an NH_2-terminal V5HIS6 Tag for purification, a precision protease site and the p40 mature protein flexi-linked to the p19 mature protein, followed by a stop codon. The chIL-23 flexi-construct was then cloned into the vector pcDNA3.1. Production of the chIL-23 flexi-construct and expression and purification of recombinant chIL-23 (rchIL-23) were carried out by Dundee Cell Products (Dundee, UK). In mammals, an IL-23 bioassay has previously been described (Aggarwal *et al.*, 2003), as discussed above. Briefly, spleen cells are cultured in the presence of recombinant IL-2 and various doses of recombinant IL-23 for up to 6 days, after which mRNA expression of IL-17A and IL-17F is measured as well as IL-17A protein expression. The following bioassays were used to demonstrate bioactivity of rchIL-23.

Materials and Methods

Cells were isolated from the spleen of 6-week-old J line birds for stimulation and subsequent RNA extraction. A single cell suspension was created by pushing the tissue through a 100 µM cell strainer into 15 ml of PBS. In a 50 ml Falcon tube, 7 ml

Histopaque 1.077 (Sigma) was carefully pipetted under the single cell suspension and centrifuged at 400 x *g* for 20 min at room temperature. Lymphocytes were drawn off the density gradient interface with a wide mouth pipette and washed with 20 ml media before centrifugation at 300 x *g* for 10 min. The wash step was repeated and the cells re-suspended at 5 x 10^6 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) containing 2 mg/ml bovine serum albumin (Sigma), 1% L-glutamine (Gibco), 1 U/ml penicillin (Sigma) and 1 µg/ml streptomycin (Sigma), following a viable cell count with trypan blue.

The cell suspension was added to 96-well U-bottomed plates (100 µl/well) containing six 10-fold serial dilutions (0.1-1000 ng/ml) of purified rchIL-23 +/- ex-COS recombinant chicken IL-2 (rchIL-2) diluted 1:500, in a final volume of 200 µl/well. Medium alone was used as the negative control and the mitogen Concanavalin A (ConA) (Sigma) at 1 µg/ml was used as a positive control. Assays were conducted in triplicate. Cells were incubated at 41°C in 5% CO_2 for between 6 h and 6 d with purified rchIL-23. RNA was isolated to assess expression of Th17 cytokines (IL-17A, IL-17F and IL-22) by real-time quantitative RT-PCR (qRT-PCR).

Results

Following stimulation of splenocytes with rchIL-23, chIL-17A mRNA expression was measured at 6 h, 1, 2, 3, 4 and 6 d post-stimulation using Taqman qRT-PCR. Expression of chIL-17A mRNA was the same from stimulated and un-stimulated splenocytes across all concentrations of rchIL-23 and all the time-points measured (Figure 5).

Recombinant chIL-23, when used to stimulate chicken splenocytes, increased mRNA expression of chIL-17F (Figure 6) and chIL-22 (Figure 7) in a dose-dependent manner. The data shown are from 1 bird and 1 time-point but are representative of 6 biological replicates and stimulation from 6 h – 6 d. Both bioassays (+/- IL-2) show a typical prozoan effect, with highest concentrations less than maximal and titrating out with increasing dilution to background levels.

Recombinant chIL-23 alone increased expression of chIL-17F compared to un-stimulated controls (dark bars). Recombinant chIL-23 in combination with rchIL-2 synergistically increased chIL-17F mRNA compared to stimulation with rchIL-23 alone (light bars).

Recombinant chIL-23 alone increased expression of chIL-22 compared to un-stimulated controls. Chicken IL-22 mRNA expression was less compared to chIL-17F mRNA expression at all time points measured from 6 h – 6 d (Figure 7).

Discussion

In mammals, IL-23 drives expression of IL-17A and IL-17F from splenocytes (Aggarwal *et al.*, 2003). We have demonstrated that chicken IL-23 drives expression of IL-17F and IL-22 in chicken splenocytes. Whilst this difference at first seems discordant, phylogenetic analyses show that chicken IL-17A and IL-17F look equally like the two mammalian molecules (Min and Lillehoj, 2002 and Kim *et al.*, 2012) and indeed they may be misnamed in the chicken. Nevertheless, we have demonstrated that rchIL-23 stimulates the expression of at least two signature Th17 cytokines, as do mammalian IL-23s.

References

- Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem. 2003; 278: 1910-4.
- Kim WH, Jeong J, Park AR, Yim D, Kim YH, Kim KD, Chang HH, Lillehoj HS, Lee BH, Min W. Chicken IL-17F: identification and comparative expression analysis in Eimeria-infected chickens. Dev Comp Immunol. 2012; 38: 401-9.
- Min W, Lillehoj HS. Isolation and characterization of chicken interleukin-17 cDNA. J Interferon Cytokine Res. 2002; 22: 1123-8.

Sequence Information

IL-23α/p19 amino acid sequence (SEQ ID NO 1) – signal peptide underlined:

MAPLRRLLLALCLPAMLLPPAVPFPAPSTDWAACRDLSQRLSRLLGTMKESHRVLSGVRLGG
EEDMEGECAPRIRCSDACDPSTLDTNSTLCLQRILQGLQHYQARLGSDIFATHPQPELKAVL
EELLSLVQVPMRSCRPPPPPQADSWAQPLLQHGTLERLRSFTAVMSRVFTHGASTR*

IL-23α/p19 DNA sequence (SEQ ID NO 2) – sequence encoding signal peptide underlined:

ATGGCCCCGCTCCGCCGCCTCCTGCTCGCACTCTGCCTCCCGGCGATGCTGCTGCCGCCCGC
GGTCCCCTTCCCGGCCCCGAGCACCGACTGGGCCGCCTGCAGGGACCTCTCTCAGCGGCTGT
CGCGGCTGCTGGGGACAATGAAGGAGTCGCACCGCGTTCTGAGCGGGGTCCGTCTGGGTGGA
GAGGAGGACATGGAGGGGGAGTGTGCCCCCCGCATCCGCTGCAGCGACGCCTGCGACCCCTC
CACGCTGGACACGAACAGCACGCTCTGCCTGCAGCGGATCCTGCAGGGGCTGCAGCACTACC
AGGCCAGGCTGGGCTCCGACATCTTCGCCACACACCCACAGCCGGAGCTGAAGGCGGTGCTG
GAGGAGCTGCTGAGCCTCGTGCAGGTCCCCATGCGTTCCTGCCGGCCCCCCCCGCCCCCCCA
AGCAGACTCCTGGGCTCAGCCACTGCTGCAGCACGGCACACTCGAACGGCTCCGCTCCTTCA
CCGCCGTCATGAGCCGCGTCTTCACCCACGGCGCCAGCACCCGCTGA

Chicken IL-23 p40 subunit (SEQ ID NO 3) – signal peptide underlined:

MSHLLFALLSLLSFAALLEAQWKLRENVYVIESEWNDETPAKKVKLTCDTSDEALPVYWKKG
TELKGTGKTLTTEVKEFPDAGNYTCLSAKTHEIISYSFFLITKVDSNGQMIRSILKSYKEPS
KTFLKCEAKNYSGIFTCSWMTENESPSVKFTIRSLKGSQGDVTCSSPVARTDKSVTEYTAQC
QKENYCPFAEEHQPTEMFLEVIDEVEYENYTSSFFIRDIIKPDPPQCQYASTNGTVTWTYPK
TWSTPKSYFPLTFRVKVESTKKYKSKVYDADEQSIQIPKTGPKDKISVQARDRYYNSSWSEW
STLCR

IL-23 p40 DNA sequence (SEQ ID NO 4) – sequence encoding signal peptide underlined:

ATGTCTCACCTGCTATTTGCCTTACTTTCATTACTTTCCTTTGCTGCCCTTCTGGAAGCACA
GTGGAAACTTAGAGAGAATGTGTATGTCATAGAATCTGAGTGGAACGATGAGACACCAGCTA
AAAAAGTGAAGCTCACCTGTGACACATCTGATGAAGCACTGCCAGTTTACTGGAAAAAGGGA
ACAGAACTGAAAGGAACTGGAAAGACTCTGACCACCGAAGTGAAGGAGTTCCCAGATGCTGG
CAACTACACCTGCCTGTCTGCTAAGACCCACGAGATTATCAGCTACAGTTTCTTTCTCATAA
CTAAAGTAGACTCCAATGGGCAAATGATACGGTCAATTCTGAAAAGCTATAAAGAGCCAAGC
AAGACGTTCTTAAAATGTGAGGCAAAGAACTACTCTGGAATTTTCACATGTTCATGGATGAC

AGAAAATGAGAGTCCAAGTGTGAAGTTCACAATTAGGAGCCTAAAAGGCTCTCAAGGAGATG
TAACCTGCAGCAGCCCTGTGGCTCGCACTGATAAATCTGTGACTGAATACACTGCCCAGTGC
CAGAAGGAAAACTACTGTCCATTTGCCGAAGAGCACCAGCCGACTGAGATGTTCCTGGAGGT
CATTGATGAGGTGGAATATGAGAACTACACTAGCAGCTTCTTCATCAGAGATATCATAAAGC
CAGACCCACCTCAATGTCAGTATGCAAGCACAAATGGAACTGTGACCTGGACATATCCCAAG
ACCTGGAGCACACCGAAGTCCTACTTCCCTTTGACTTTCAGGGTCAAAGTTGAAAGCACAAA
GAAATACAAAAGCAAGGTTTATGATGCTGATGAGCAGTCTATTCAGATTCCAAAGACTGGGC
CAAAAGATAAGATCTCTGTGCAGGCCAGGGATCGCTATTACAACTCATCCTGGAGTGAGTGG
TCCACGCTTTGCAGA

Flexi-IL-23 construct (SEQ ID NO 5):

Key to Components:
Mouse CD8α signal peptide
V5HIS6 tag
Precision protease site
p40 mature protein
flexi linker
p19 mature protein with stop codon

ATGGCCTCACCGTTGACCCGCTTTCTGTCGCTGAACCTGCTGCTGCTGGGTGAGTCGATT
M A S P L T R F L S L N L L L L G E S I

*ATCCTGGGGAGTGGAGAA***GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG**
I L G S G E **G K P I P N P L L G L D S T**

CGTACCGGTCATCATCATCATCATCATCTGGAAGTTCTGTTCCAGGGGCCCGCCCTTCTG
R T G H H H H H H L E V L F Q G P A L L

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E A Q W K L R E N V Y V I E S E W N D E

ACACCAGCTAAAAAAGTGAAGCTCACCTGTGACACATCTGATGAAGCACTGCCAGTTTAC
T P A K K V K L T C D T S D E A L P V Y

TGGAAAAAGGGAACAGAACTGAAAGGAACTGGAAAGACTCTGACCACCGAAGTGAAGGAG
W K K G T E L K G T G K T L T T E V K E

TTCCCAGATGCTGGCAACTACACCTGCCTGTCTGCTAAGACCCACGAGATTATCAGCTAC
F P D A G N Y T C L S A K T H E I I S Y

AGTTTCTTTCTCATAACTAAAGTAGACTCCAATGGGCAAATGATACGGTCAATTCTGAAA
S F F L I T K V D S N G Q M I R S I L K

AGCTATAAAGAGCCAAGCAAGACGTTCTTAAAATGTGAGGCAAAGAACTACTCTGGAATT

```
S  Y  K  E  P  S  K  T  F  L  K  C  E  A  K  N  Y  S  G  I

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F  T  C  S  W  M  T  E  N  E  S  P  S  V  K  F  T  I  R  S

CTAAAAGGCTCTCAAGGAGATGTAACCTGCAGCAGCCCTGTGGCTCGCACTGATAAATCT
L  K  G  S  Q  G  D  V  T  C  S  S  P  V  A  R  T  D  K  S

GTGACTGAATACACTGCCCAGTGCCAGAAGGAAAACTACTGTCCATTTGCCGAAGAGCAC
V  T  E  Y  T  A  Q  C  Q  K  E  N  Y  C  P  F  A  E  E  H

CAGCCGACTGAGATGTTCCTGGAGGTCATTGATGAGGTGGAATATGAGAACTACACTAGC
Q  P  T  E  M  F  L  E  V  I  D  E  V  E  Y  E  N  Y  T  S

AGCTTCTTCATCAGAGATATCATAAAGCCAGACCCACCTCAATGTCAGTATGCAAGCACA
S  F  F  I  R  D  I  I  K  P  D  P  P  Q  C  Q  Y  A  S  T

AATGGAACTGTGACCTGGACATATCCCAAGACCTGGAGCACACCGAAGTCCTACTTCCCT
N  G  T  V  T  W  T  Y  P  K  T  W  S  T  P  K  S  Y  F  P

TTGACTTTCAGGGTCAAAGTTGAAAGCACAAAGAAATACAAAAGCAAGGTTTATGATGCT
L  T  F  R  V  K  V  E  S  T  K  K  Y  K  S  K  V  Y  D  A

GATGAGCAGTCTATTCAGATTCCAAAGACTGGGCCAAAAGACAAGATCTCTGTGCAGGCC
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GCGGTGCTGGAGGAGCTGCTGAGCCTCGTGCAGGTCCCCATGCGTTCCTGCCGGCCCCCC
A  V  L  E  E  L  L  S  L  V  Q  V  P  M  R  S  C  R  P  P

CCGCCCCCCCAAGCAGACTCCTGGGCTCAGCCACTGCTGCAGCACGGCACACTCGAACGG
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P P P Q A D S W A Q P L L Q H G T L E R

CTCCGCTCCTTCACCGCCGTCATGAGCCGCGTCTTCACCCACGGCGCCAGCACCCGCTGA
L R S F T A V M S R V F T H G A S T R *

The amino acid sequence of chicken IL-23α/p19 with signal peptide absent (SEQ ID NO 10):

PAVPFPAPSTDWAACRDLSQRLSRLLGTMKESHRVLSGVRLGGEEDMEGECAPRIRCSDACD
PSTLDTNSTLCLQRILQGLQHYQARLGSDIFATHPQPELKAVLEELLSLVQVPMRSCRPPPP
PQADSWAQPLLQHGTLERLRSFTAVMSRVFTHGASTR*

The amino acid sequence of chicken IL-23 p40 subunit with signal peptide absent (SEQ ID NO 11):

ALLEAQWKLRENVYVIESEWNDETPAKKVKLTCDTSDEALPVYWKKGTELKGTGKTLTTEVK
EFPDAGNYTCLSAKTHEIISYSFFLITKVDSNGQMIRSILKSYKEPSKTFLKCEAKNYSGIF
TCSWMTENESPSVKFTIRSLKGSQGDVTCSSPVARTDKSVTEYTAQCQKENYCPFAEEHQPT
EMFLEVIDEVEYENYTSSFFIRDIIKPDPPQCQYASTNGTVTWTYPKTWSTPKSYFPLTFRV
KVESTKKYKSKVYDADEQSIQIPKTGPKDKISVQARDRYYNSSWSEWSTLCR

Nucleic acid sequence encoding chicken IL-23α/p19 with signal peptide absent (SEQ ID NO 12):

GCCCGCGGTCCCCTTCCCGGCCCCGAGCACGACTGGGCCGCCTGCAGGGACCTCTCTCAGCG
GCTGTCGCGGCTGCTGGGGACAATGAAGGAGTCGCACCGCGTTCTGAGCGGGGTCCGTCTGG
GTGGAGAGGAGGACATGGAGGGGGAGTGTGCCCCCCGCATCCGCTGCAGCGACGCCTGCGAC
CCCTCCACGCTGGACACGAACAGCACGCTCTGCCTGCAGCGGATCCTGCAGGGGCTGCAGCA
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TGCTGGAGGAGCTGCTGAGCCTCGTGCAGGTCCCCATGCGTTCCTGCCGGCCCCCCCCGCCC
CCCCAAGCAGACTCCTGGGCTCAGCCACTGCTGCAGCACGGCACACTCGAACGGCTCCGCTC
CTTCACCGCCGTCATGAGCCGCGTCTTCACCCACGGCGCCAGCACCCGC

Nucleic acid sequence encoding chicken IL-23 p40 subunit with signal peptide absent (SEQ ID NO 13):

GCCCTTCTGGAAGCACAGTGGAAACTTAGAGAGAATGTGTATGTCATAGAATCTGAGTGGAA
CGATGAGACACCAGCTAAAAAAGTGAAGCTCACCTGTGACACATCTGATGAAGCACTGCCAG
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GCTATAAAGAGCCAAGCAAGACGTTCTTAAAATGTGAGGCAAAGAACTACTCTGGAATTTTC
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GAGATGTTCCTGGAGGTCATTGATGAGGTGGAATATGAGAACTACACTAGCAGCTTCTTCAT
CAGAGATATCATAAAGCCAGACCCACCTCAATGTCAGTATGCAAGCACAAATGGAACTGTGA
CCTGGACATATCCCAAGACCTGGAGCACACCGAAGTCCTACTTCCCTTTGACTTTCAGGGTC
AAAGTTGAAAGCACAAAGAAATACAAAAGCAAGGTTTATGATGCTGATGAGCAGTCTATTCA
GATTCCAAAGACTGGGCCAAAAGATAAGATCTCTGTGCAGGCCAGGGATCGCTATTACAACT
CATCCTGGAGTGAGTGGTCCACGCTTTGCAGA

Claims

1. Protein comprising an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 1 or a functional fragment thereof.

2. Protein according to claim 1 further comprising a subunit having a sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 3 or a functional fragment thereof.

3. Protein according to claim 2, comprising:
   - a subunit having an apparent molecular weight of approximately 19kD and having an amino acid sequence as depicted in SEQ ID NO 1 or SEQ ID NO 10, and
   - a subunit having an apparent molecular weight of 40kD and having the amino acid sequence as depicted in SEQ ID NO 3 or SEQ ID NO 11,

   said subunits being linked.

4. Isolated polynucleotide encoding a polypeptide having an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 1 or a functional fragment thereof.

5. Isolated polynucleotide according to claim 4 further encoding a polypeptide having an amino acid sequence showing at least 80% similarity with the amino acid sequence as depicted in SEQ ID NO 3 or a functional fragment thereof.

6. Isolated polynucleotide according to claim 4 or 5 comprising a polynucleotide sequence showing at least 80% identity with the sequence depicted in SEQ ID NO 2 or SEQ ID NO 12.

7. Isolated polynucleotide according to any one of claims 4 to 6 comprising a polynucleotide sequence showing at least 80% identity with the sequence depicted in SEQ ID NO 4 or SEQ ID NO 13.

8. Recombinant vector comprising a polynucleotide according to any one of claims 4 to 7.

9. Cell comprising a polynucleotide according to any one of claims 4 to 8 or vector according to claim 8.

10. Adjuvant composition comprising a protein according to any of claims 1 to 3, one or more polynucleotides according to any of claims 4 to 7, a vector according to claim 8, or a cell according to claim 9 and a pharmaceutical acceptable carrier.

11. Vaccine composition comprising an active agent derived from an avian pathogen and a protein according to any of claims 1 to 3, together with a pharmaceutically acceptable carrier.

12. Vaccine composition comprising an active agent derived from an avian pathogen and one or more polynucleotides according to any of claims 4 to 5, or a vector according to claim 6, together with a pharmaceutically acceptable carrier.

13. Protein according to any one of claims 1 to 3 for use as an immunomodulator.

14. Protein according to any one of claims 1 to 3 for use as an adjuvant.

15. Use of a protein according to any of claims 1 to 3 as an immunomodulator.

16. Use of a protein according to any of claims 1 to 3 as an adjuvant.

17. Pharmaceutical composition comprising a protein according to any one of claims 1 to 3 together with a pharmaceutical acceptable carrier.

18. Pharmaceutical composition according to claim 17 comprising an antigen.

19. An antibody which binds to a protein according to any one of claims 1 to 3 with an affinity of at least 10^{-7} M wherein the antibody does not specifically bind to a non-avian p19 subunit.

20. A method of stimulating an immune response in a subject comprising administering a protein according to any one of claims 1 to 3, one or more polynucleotides according to any of claims 4 to 7, a vector according to claim 8, or a cell according to claim 9.

21. The method of claim 20 wherein the subject is a bird.

Fig 1

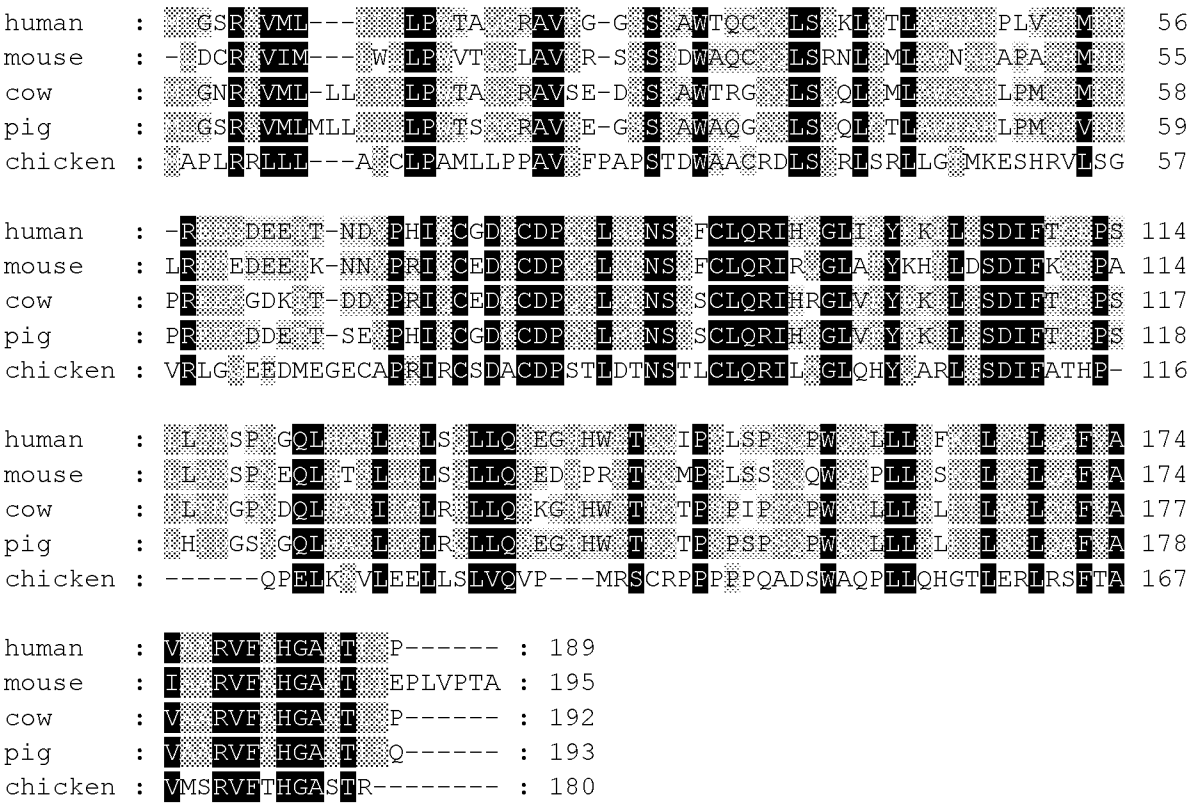

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mouse : -DCRVIM---WLPVTLAVR-SSDWAQCLSRNLML N APAM 55
cow : GNRVML-LLLPTARAVSE-DSAWTRGLSQLML LPMM 58
pig : GSRVMLMLLLPTSRAVE-GSAWAQGLSQLTL LPMV 59
chicken : APLRRLLL---ACLPAMLLPPAVFPAPSTDWAACRDLSRLSRLLG MKESHRVLSG 57
human : -R DEET-ND PHICGD CDP L NS FCLQRIH GLI Y K L SDIFT PS 114
mouse : LR EDEEK-NN PRICED CDP L NS FCLQRIR GLA YKH LDSDIFK PA 114
cow : PR GDKT-DD PRICED CDP L NS SCLQRIHRGLV Y K L SDIFT PS 117
pig : PR DDET-SE PHICGD CDP L NS SCLQRIH GLV Y K L SDIFT PS 118
chicken : VRLG EEDMEGECAPRIRCSDACDPSTLDTNSTLCLQRIL GLQHY ARL SDIFATHP- 116
human : L SP GQL L LS LLQ EG HW T IP LSP PW LLL F L L E A 174
mouse : L SP EQL T L LS LLQ ED PR T MP LSS QW PLL S L L E A 174
cow : L GP DQL I LR LLQ KG HW T TP PIP PW LLL L L L E A 177
pig : H GS GQL L LR LLQ EG HW T TP PSP PW LLL L L L E A 178
chicken : ------QPELK VLEELLSLVQVP---MRSCRPPPPQADSWAQPLLQHGTLERLRSETA 167
human : V RVF HGA T P------ : 189
mouse : I RVF HGA T EPLVPTA : 195
cow : V RVF HGA T P------ : 192
pig : V RVF HGA T Q------ : 193
chicken : VMSRVFTHGASTR-------- : 180

Fig 2

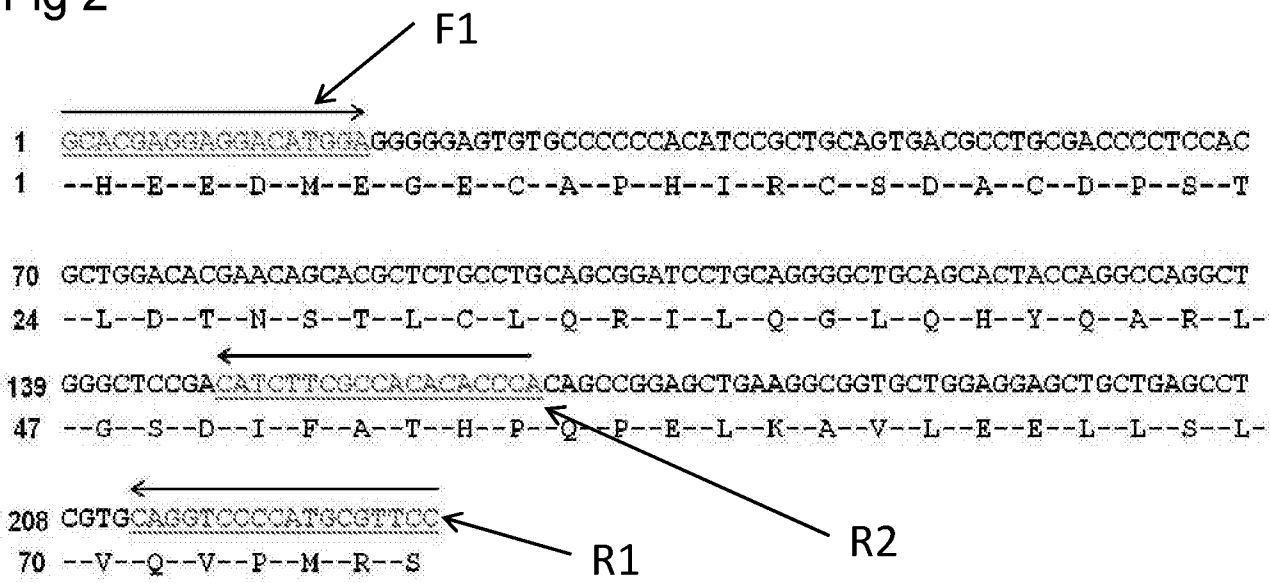

F1
1 GCACGAGGAGGAACATGGAGGGGGAGTGTGCCCCCCACATCCGCTGCAGTGACGCCTGCGACCCCTCCAC
1 --H--E--E--D--M--E--G--E--C--A--P--H--I--R--C--S--D--A--C--D--P--S--T
70 GCTGGACACGAACAGCACGCTCTGCCTGCAGCGGATCCTGCAGGGGCTGCAGCACTACCAGGCCAGGCT
24 --L--D--T--N--S--T--L--C--L--Q--R--I--L--Q--G--L--Q--H--Y--Q--A--R--L-
139 GGGCTCCGACATCTTCGCCACACACCCACAGCCGGAGCTGAAGGCGGTGCTGGAGGAGCTGCTGAGCCT
47 --G--S--D--I--F--A--T--H--P--Q--P--E--L--K--A--V--L--E--E--L--L--S--L-
208 CGTGCAGGTCCCCATGCGTTCC
70 --V--Q--V--P--M--R--S
R1
R2

Fig 3

```
human   :  GSR VML  L--  TA  R  GG S AWTQ  LS KL TL----
mouse   : -DCR VIM W L--  VT  L  RS S DWAQ  LSRNL ML----
chicken :  APLRRLLLA CLPAMLLPPAVPF -APSTDWAA RDLS RLSRLLGTMK
cow     :  GNR VML  LLL  TA  R  SED S AWTRG  LS QL ML----

human   : ----------------------WS  PL----------------------
mouse   : ----------------------WN  A-----------------------
chicken : ESHRVLVSAGTGFGGAGRRGMLWERGA GALREPGVLRELEILRELEMLW
cow     : ----------------------WS  L-----------------------

human   : -------------------------------G M L- E  D-------
mouse   : -------------------------------AG M LL E ED-------
chicken : EPGEPGMLSEPGEMGMLGEAEVLQELGVLGE GELGMLLEP SGVRLGGE
cow     : -------------------------------G M LP E  G-------

human   : -EE T  PHI CGD CDP L NS FCLQRIH GLI Y K L SDIFT
mouse   : -EE K  P I CED CDP L NS FCLQRIR GLA YKH LDSDIFK
chicken : EDMEGECAP IRCSDACDPSTLDTNSTLCLQRIL GLQHY ARL SDIFA
cow     : -DK T  P I CED CDP L NS SCLQRIHRGLV Y K L SDIFT

human   :  PS  S GQL  L LS LLQ EG HW T IP LSP PW LL
mouse   :  PA  S EQL T L LS LLQ ED PR T MP LSS QW PL
chicken : THP-------QPELK VLEELLSLVQVP---MRSCRPPPPPQADSWAQPL
cow     :  PS  G DQL  I LR LLQ KG HW T TP PIP PW LL

human   : L F L L F AV RVF HGA T P------
mouse   : L S L L F AI RVF HGA T EPLVPTA
chicken : LQHGTLERLRSFTAVMSRVFTHGASTR--------
cow     : L L L L F AV RVF HGA T P------
```

Fig 4

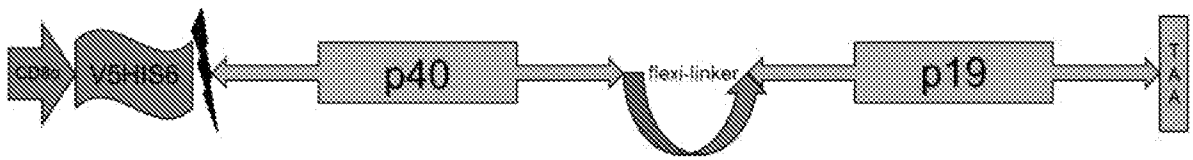


Fig 5

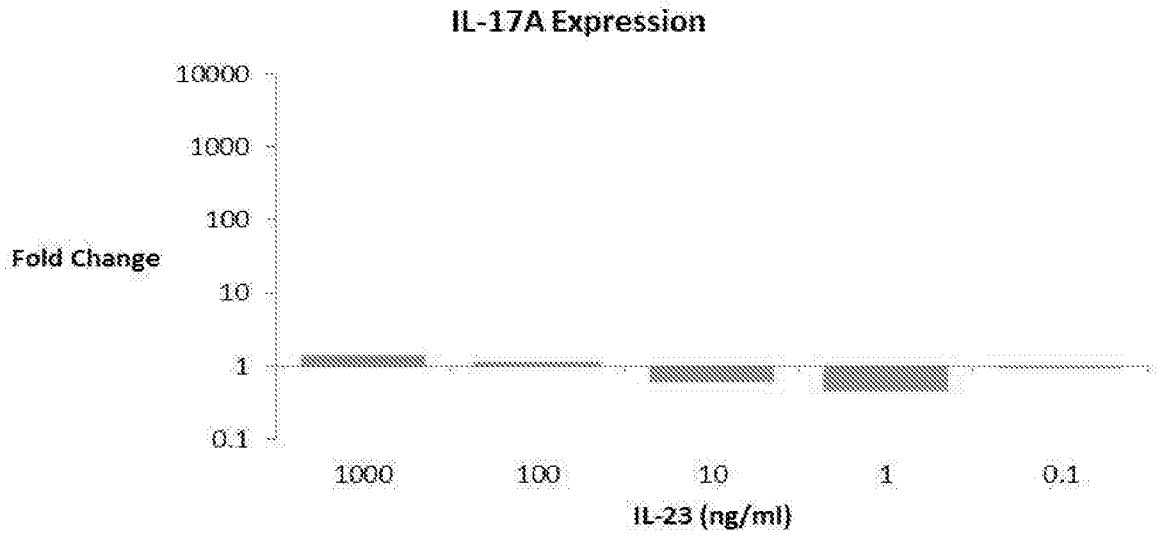

IL-17A Expression
10000
1000
100
Fold Change
10
1
0.1
1000
100
10
1
0.1
IL-23 (ng/ml)

Fig 6

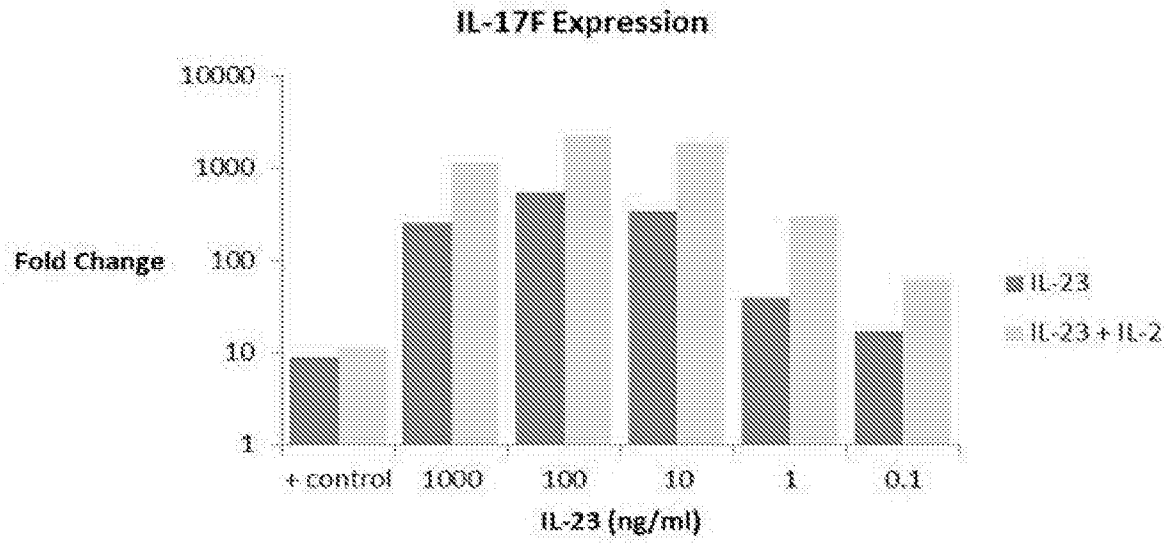
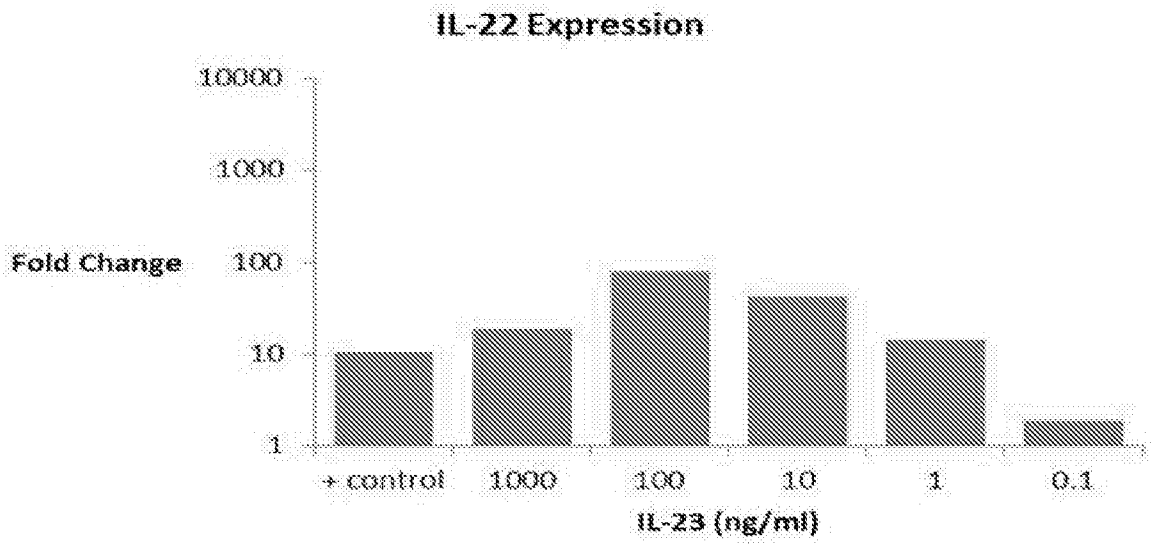

IL-17F Expression
10000
1000
Fold Change
100
10
1
+ control
1000
100
10
1
0.1
IL-23 (ng/ml)
IL-23
IL-23 + IL-2

Fig 7


IL-22 Expression
Fold Change
10000
1000
100
10
1
+ control
1000
100
10
1
0.1
IL-23 (ng/ml)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052197

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/54 C07K14/715
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/003017 A1 (AKZO NOBEL NV [NL]; DEGEN WILHELMUS GERARDUS JOHAN [NL]; SCHIJNS VIRGI) 8 January 2004 (2004-01-08)	7
A	whole document esp. seq id nos 1 and 3	1-6,8-21
A	US 5 994 104 A (ANDERSON ROBERT JAMES [GB] ET AL) 30 November 1999 (1999-11-30) the whole document	1-21
A	WO 2012/009760 A1 (CEPHALON AUSTRALIA PTY LTD [AU]; CLARKE ADAM WILLIAM [AU]; DOYLE ANTHO) 26 January 2012 (2012-01-26) the whole document	1-21

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2013	06/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brück, Marianne

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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