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Technical report

Expressed gene sequences of the equine cytokines interleukin-17 and interleukin-23

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ABSTRACT

This report describes the initial cloning and characterization of the equine interleukin-17 (IL-17) expressed gene sequence from mRNA obtained from equine intestinal tissue and interleukin-23 (IL-23) expressed gene sequence from mRNA obtained from equine peripheral blood mononuclear cells. Equine IL-17 has 462 nucleotides in the translated region, determined by homology with known human and mouse sequences, and shares 84% and 75% identity, respectively. For the deduced amino acid sequences, the identity with human and mouse is 76% and 70%. Equine IL-23 has 579 nucleotides in the translated region. Homology with known human and mouse sequences was determined to be 89% and 77%. Deduced amino acid identities are 89% with the human sequence and 70% with the mouse sequence. The gene sequences were identified as part of the U.S. Veterinary Immune Reagent Network with a goal of developing reagents in order to aid veterinary researchers in the investigation of diseases in livestock species.

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Interleukin-17 (IL-17) family members are secreted by a novel subset of T helper cells known as Th17 and play an important role in organ-specific autoimmunity (mainly lungs, joints, and intestines [Hunter, 2005]) as well as defense against specific pathogens (Korn et al., 2007). Composed of six family members, i.e. IL-17A-F, IL-17 is a homodimeric molecule involved in local inflammation. IL-17A, the subject of this report, and IL-17F are most closely related and share a common receptor, IL-17RA (Hunter, 2005; Korn et al., 2007). Production of IL-17A and -F has been associated with CD8+ cells, γδ-T cells, neutrophils, memory CD4+ cells in order to induce expression of a variety of chemokines (CXCL10, CXCL1), cytokines (G-CSF, GM-CSF, IL-6, IL-8), as well as recruiting, activating, and influencing migration of neutrophils (Korn et al., 2007). While previously considered to be dependent on interleukin-23 (IL-23) for differentiation from naïve T cells into Th17 cells, recent work has indicated that initial differentiation is dependent on IL-6 and/or TGFβ with subsequent expansion of the Th17 lineage dependent on IL-23 (Ivanov et al., 2007; Korn et al., 2007; Stockinger and Veldhoen, 2007). In addition, IL-2, IL-4, IL-27, and IFN-γ prevent the differentiation of naïve T cells into the Th17 lineage (Ivanov et al., 2007).

Interleukin-23, a member of the IL-12 family of cytokines, is a heterodimeric molecule composed of the IL-12p40 subunit and a novel p19 subunit (Kleischek et al., 2006; Hunter, 2007). While similar to IL-12 both structurally and in the ability to increase production of IFN-γ by T cells, the ability of IL-23 to induce IL-17 provides a role unique compared to that of IL-12 in both the development and maintenance of autoimmune inflammations (Hunter, 2005). Recent studies have shown that in mice deficient in the p19 sub-unit IL-17 production is impaired and clearance of fungal infections is reduced (Kleischek, 2006), and a decreased resistance to *Mycobacterium tuberculosis* ocurred in patients where there was a reduced Th17 response coupled with a down-regulation of IL-23 (Khader and Cooper, 2008).While the interactions are not fully understood, the attainment and characterization of

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cytokines, such as IL-17 and IL-23, by collaborative projects like the U.S. Veterinary Immune Reagent Network provide valuable tools for veterinary immunologists to further the understanding of disease in important veterinary species.

To obtain the transcript sequence for equine IL-17, primers were designed corresponding to the nucleotides at the 3′ and 5′ end that include the initial methionines and stop codons based on previously published primer pairs (Debrue et al., 2005) used to amplify a partial cDNA sequence of equine IL-17 from bronchoalveolar lavage (sequence not published). The forward primer sequence was 5′-ATGGCTCCTCTGAGAACTTCATCCG-3′, and the reverse primer sequence was 5′-TTAACCCATGTGGCGGA-CAATG-3′. The IL-23 primers were designed based on GenBank acc # NM_001082522 (Kralik et al., 2005; Musilova et al., 2005) with a resultant forward primer sequence: 5′-AGAGATTCCACAGGGACTGACT-3′, and the reverse primer sequence: 5′-TAGCATTGCTGAGCCATA-GATGT-3′. The previous equine sequence was based on genomic DNA (Musilova et al., 2005) and then confirmed through RT-PCR amplification and sequencing (Kralik et al., 2005).

All animal use was approved by institutional animal care and use committees. Equine peripheral blood mononuclear cells (PBMC) were isolated from whole blood as described previously (Loftus et al., 2006), and total RNA was isolated from PBMC using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA was synthesized from total RNA with the Reverse Transcription System using oligo-dT primers (Promega, Madison, WI). Intestinal tissue biopsies (approximately 1 cm^2) were obtained at necropsy from three regions of the intestinal tract; jejunum, ileum and cecum of a young (six-year old) horse that was euthanized for another study. Approximately one-half of the tissue from a site was placed in 1000 μL of RNA stabilization reagent (RNA-STAT, Fisher Scientific, Hampton, NH), minced with a sterile blade, and placed in a 5-mL snap-cap vial. The tissue was homogenized with a rotor–stator homogenizer (Power Gen 125, Fisher Scientific, Hampton, NH) and a sterile generator probe (Power Gen disposable 7 mm × 110 mm, Fisher Scientific, Hampton, NH). After vortexing to further homogenize the sample, 200 μL of chloroform was added and mixed vigorously for 15–20 s. Samples were allowed to stand for 3 min. Following centrifugation at 12,000 × g for 15 min at 4 °C, the upper 400 μL of the aqueous layer was transferred to a new 1.5-mL centrifuge tube and 320 μL of isopropyl alcohol was added and pulse-vortexed. Samples were incubated at 20 °C for a minimum of 30 min. Following centrifugation at 20,000 × g for 10 min at 4 °C, the supernatant was decanted, the rim of the tube blotted, and 800 μL of 75% ethanol added to the pellet. Following vortexing, samples were centrifuged at 14,000 × g for 5 min at 4 °C. The supernatant was carefully removed and discarded, and the tubes placed on their side to allow the pellets to dry while under the path of an air-flow hood for several minutes. A 60-μL volume of RNase-free water was added and the samples placed in a 60 °C water bath for 10 min, pulse vortexed and the concentration of RNA determined by measuring the absorbance at 260 nm. Samples were stored at −80 °C until needed for further assay.

A polymerase chain reaction (PCR) in a total volume of 50 μL was performed using PCR Master Mix (Promega) according to the manufacturer's protocol. The reaction was heated to 95 °C for 3 min, then run through 40 cycles as follows; 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. After verifying the PCR product via agarose gel electrophoresis, DNA bands of the predicted size were excised from the agarose gel and purified using QUIAprep Spin

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(A)   1 atggctcctc tgaagaacttc atccgtgtca ctgctgctgc tgctgagtct ggtggctatc
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(A)
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  1 atggctcctc tgagaacttc atccgtgtca ctgctgctgc tgctgagtct ggtggctatc
 61 gtgaaggcgg gaatagtaat cccacaaaat ccggaatgcc cgaacactgg ggacaagaac
121 ttccctcaga atgtgaagat caacctaaac gtccttaacc ggaaaacgaa ttccagaagg
181 gcctcagatt accacaaccg ctccacctcc ccttggaatc tccaccgcaa cgaggaccct
241 gagagatacc cctctgtgat ctgggaggcg aagtgccgcc acctgggctg tgtcaatgcc
301 gaagggaagg tagacttcca catgaactcc gtccccatcc agcaagagat cctggtcctg
361 cgcagggaat ctcagaactg cccccactcc ttccagctgg agaagatgct ggtggccgtg
421 ggctgcacct gcgtcacccc cattgtccgc cacatgggtt aa
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(B)
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  1 atgctgggga gcagagctgt gttgctgctg ctgctgctcc tgtggccccg gactgctcag
 61 gcccgggctg tgcctggagg cagtagccct gcctgggctc agtgccagca gctctcacag
121 aagctctgta cgctggcctg gagtgcacat ccaccaatgg gacatgtgga tctaccaaga
181 gaagagggag atgctgagac tacaaatgat gtcccccata tccagtgcga ggatggctgt
241 gatcctgaag gactcaggga caacagtcag ccctgcttgc aaaggatcca ccagggcctg
301 gtttttacg agaagctgct gggctcagac attttcacag gggagccttc tctactcccc
361 aatggccctg tggaccagct tcacgcctcc ctcctgggcc tcaggcaact cttgcagcct
421 gagggtcacc actgggagac tgagcagatt ccaagcccca gtcccagcca gccgtggcag
481 cgcctccttc tccgccccaa gatccttcgc agcctccagg cctttgtggc tgtagctgcc
541 cgggtctttg cccatggagc agcaaccctg accccttaa
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Fig. 1. (A) Nucleotide sequence of equine IL-17 cDNA (GenBank # EU276063). (B) Nucleotide sequence of equine IL-23 cDNA (GenBank # EU438773). Start and stop codons are underlined.

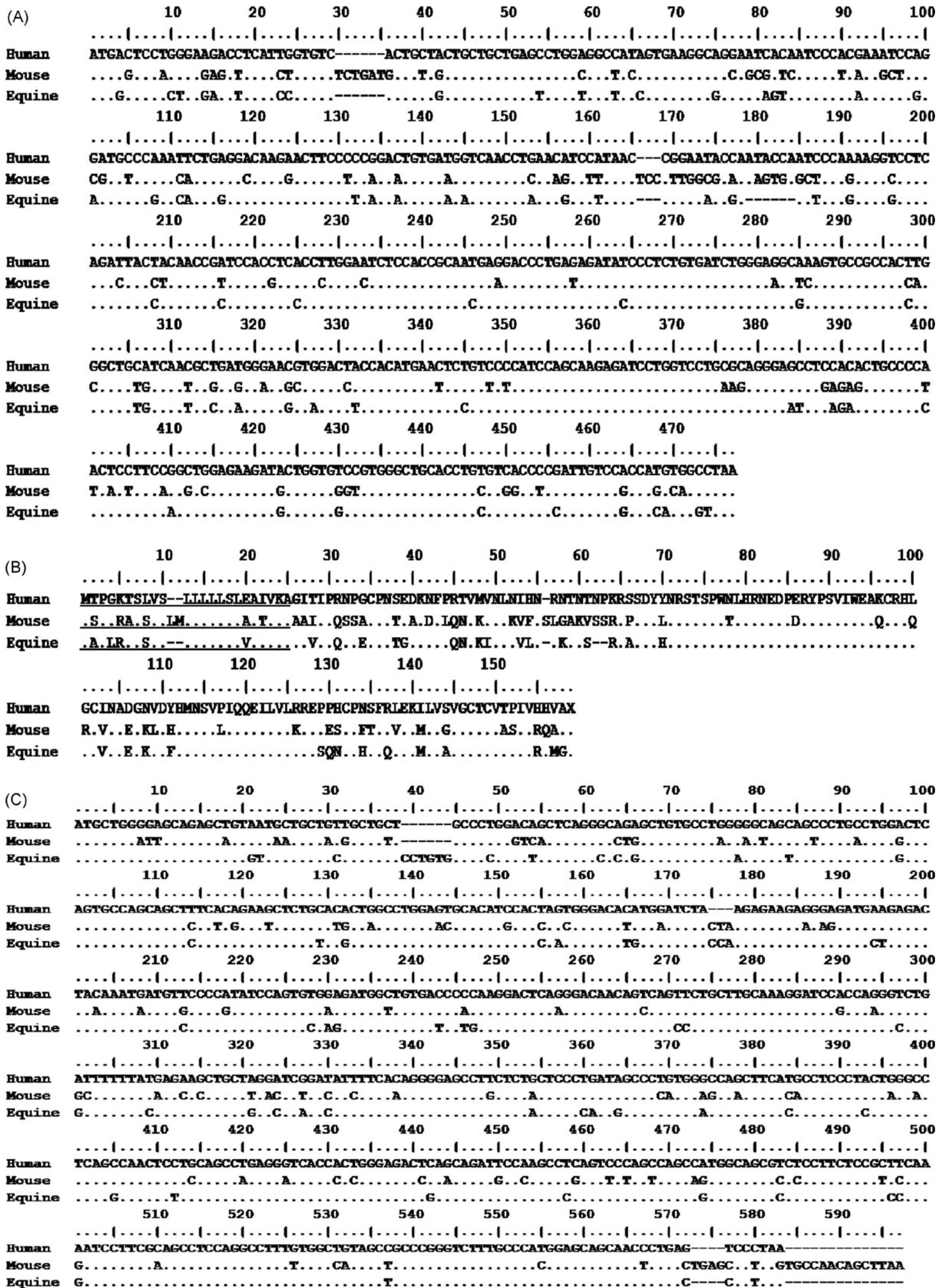

Fig. 2. (A) Alignment of the equine IL-17 nucleotide sequence with human and mouse. Dots indicate identity relative to the human sequence. Nucleotide sequence with position 1 in this alignment refers to the first base of the start codon. (B) Alignment of the equine IL-17 deduced amino acid sequence with human and mouse with the predicted signal sequence underlined. (C) Alignment of the equine IL-23 nucleotide sequence with human and mouse. (D) Alignment of the equine IL-23 amino acid sequence with human and mouse.

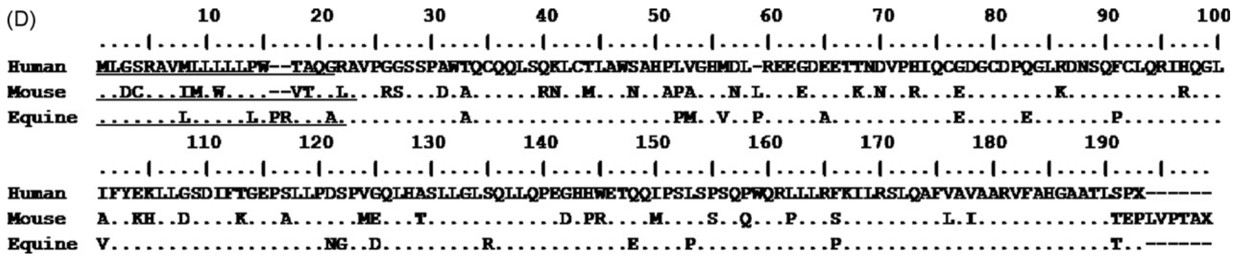

Fig. 2. (*Continued*).

mini-prep kit. Two microliters of the product was ligated into pCR2.1-TOPO vector using the TOPO-TA Cloning Kit and sequencing performed commercially by GeneWiz (South Plainfield, NJ) using M13R and T7 primers in separate reactions. All sequence analyses were performed with BioEdit software (Hall, 1999), unless mentioned otherwise.

The cDNA sequences for IL-17 and IL-23 are shown in Fig. 1, and were deposited in GenBank (accession numbers EU744563 and EU438773, respectively). Alignment of human, mouse, and equine IL-17 was performed (Fig. 2A), and showed that the coding region of equine IL-17 shares 84% identity with human IL-17 (GenBank acc # NM_002190, bases 46–513; Cha et al., 2008) and 77% identity with the mouse IL-17 coding region (GenBank acc # NM_010552, bases 58–334; Intlekofer et al., 2008). Fig. 2B shows deduced amino acid (aa) sequences of nucleotide sequences in Fig. 2A. Equine IL-17 deduced aa sequence had 88% with the human protein sequence (GenBank acc # NP_002181), and 70% identity with the mouse protein sequence (GenBank acc # NP_034682). Fig. 2C shows the alignment performed for equine IL-23 coding region (bases 68–646), human IL-23 and murine IL-23 coding regions. Equine IL-23 shares 89% identity with the human IL-23 (GenBank acc # NM_016584, bases 167–736; Kroening et al., 2008) and 77% identity with the murine IL-23 coding region (GenBank acc # NM_031252, bases 113–703; Ju et al., 2008). Deduced amino aa alignment of the nucleotide sequences shown in Fig. 2C are shown in Fig. 2D. Equine IL-17 shares 88% identity with human IL-17 (GenBank acc # NP_057668) and 70% identity with murine IL-17 (GenBank acc # NP_112542).

Based on the deduced amino acid sequence the molecular weight of equine IL-17 is a 17.26 kDa protein backbone. The equine IL-23 protein backbone has a predicted molecular weight of 21.04 kDa. In addition, analysis of the equine IL-17 sequence with Signal-P (Bendtsen et al., 2004; Emanuelsson et al., 2007) predicted cleavage between amino acids 23 and 24 with 91% probability, in close agreement with the predictions for the human (between aa 23 and 24) and mouse (between aa 25 and 26) proteins. The predicted cleavage for the equine IL-23 sequence fell between amino acids 21 and 22 with 86% probability, also in close agreement with human (between aa 19 and 20) and mouse (between aa 21 and 22). No other cleavage sites were predicted by Signal-P.

The nucleotide sequences of the equine IL-17A coding region were compared with sequences encoding other

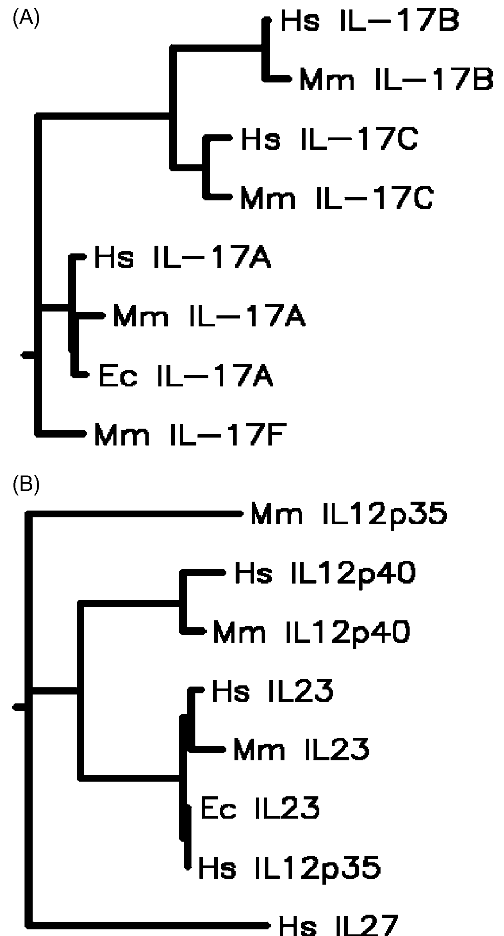

Fig. 3. (A) Cladogram showing relationship of equine IL-17 to human and mouse Th17 cytokine family members. The analysis used coding sequences only. The GenBank accession numbers for the sequences used are: Hs IL-17A: NM_002190; Hs IL-17B: NM_014443; Hs IL-17C: NM_013278; Hs IL-17D: NM_138284; Mm IL-17A: NM_010552; Mm IL-17B: NM_019508; Mm IL-17C: NM_145834; Mm IL-17D: NM_145837; Mm IL-17F: NM_145856; Ec IL-17A: EU744563. (B) Cladogram showing the relationship of equine IL-23 to other human and mouse IL-12 cytokine family members. The analysis used coding sequences only. The GenBank accession numbers for the sequences used are: Hs IL-12p35: NM_000882; Hs IL-12p40: NM_002187; Hs IL-23: NM_016584; Hs IL-27: NM_145659; Mm IL-12p35: NM_008351; Mm IL-12p40: NM_008352; Mm IL-23: NM_031252; Ec IL-23: EU438773. Hs: *Homo sapiens*; Mm: *Mus musculus*; Ec: *Equus caballus*.

Th17 cytokine family members in humans and mice using the Kitsch program (component of the PHYLIP package in Bioedit [Felsenstein, 1989; Hall, 1999]). The coding sequence for mouse IL-17F was used as an outlier for the generation of the tree (GenBank acc # NM_145856). The resulting tree (Fig. 3A) indicated that the IL-17A family members group closely with mouse IL-17F, which are structurally related and share a receptor IL-17RA. Fig. 3B compares the coding sequences of equine IL-23 with other IL-12 cytokine family members in humans and in mice, also using the Kitsch program. Human IL-27 was used as the outlier for the generation of the tree. Equine, murine, and human IL-23 appear to be more closely related to the human IL-12p35 subunit than IL-12p40 of human or mouse as expected but were not close to the mouse IL-12p35; the heterodimeric molecule IL-23 is composed of the IL-12 p40 and IL-23 p19 subunits [image provided through the Bioweb server maintained at the Institut Pasteur, Paris, France (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=drawtree)].

As part of the US-VIRN project (www.vetimm.org), whose goal is to address the dearth of available reagents needed for immunology and infectious disease studies in livestock, poultry and fish, these genes will be expressed and assessed for bioactivity as well as used to produce monoclonal antibodies.

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References

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.
Cha, C.S., Chew, G.Y., Simpson, N., Priyadarshi, A., Wong, M., Grimbacher, B., Fulcher, D.A., Tangye, S.G., Cook, M.C., 2008. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J. Exp. Med. 205, 1551–1557.
Debrue, M., Hamilton, E., Joubert, P., Lajoie-Kadoch, S., Lavoie, J.P., 2005. Chronic exacerbation of equine heaves is associated with an increased expression of interleukin-17 mRNA in bronchoalveolar lavage cells. Vet. Immunol. Immunopathol. 105, 25–31.
Emanuelsson, O., Brunak, S., von Heijne, G., Nielsen, H., 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. Nat. Protocols 2, 953–971.
Felsenstein, J., 1989. PHYLIP—Phylogeny Inference Package (Version 3.2). Cladistics 5, 164–166.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98.
Hunter, C.A., 2005. New IL-12 family members: IL-23 and IL-27, cytokines with divergent functions. Nat. Rev. Immunol. 5, 521–531.
Intlekofer, A.M., Banerjee, A., Takemoto, N., Gordon, S.M., Dejong, C.S., Shin, H., Hunter, C.A., Wherry, E.J., Lindsten, T., Reiner, S.L., 2008. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science 321, 408–411.
Ivanov, I.I., Zhou, L., Littman, D.R., 2007. Transcriptional regulation of Th17 cell differentiation. Semin. Immunol. 19, 409–417.
Ju, J.H., Cho, M.L., Moon, Y.M., Oh, H.J., Park, J.S., Jhun, J.Y., Min, S.Y., Cho, Y.G., Park, K.S., Yoon, C.H., Min, J.K., Park, S.H., Sung, Y.C., Kim, H.Y., 2008. IL-23 induces receptor activator of NF-kappaB ligand expression on CD4+ T cells and promotes osteoclastogenesis in an autoimmune arthritis model. J. Immunol. 181, 1507–1518.
Khader, S.A., Cooper, A.M., 2008. IL-23 and IL-17 in tuberculosis. Cytokine 41, 79–83.
Kleischek, M.A., Muller, U., Brodie, S.J., Stenzel, W., Kohler, G., Blumenschein, W.M., Straubinger, R.K., McClanahan, T., Kastelstein, R.A., Alber, G., 2006. IL-23 enhances the inflammatory cell response in *Crytococcus neoformans* infection and induces a cytokine pattern distinct from IL-12. J. Immunol. 176, 1098–1106.
Korn, T., Oukka, M., Kuchroo, V., Bettelli, E., 2007. Th17 cells: effector T cells with inflammatory properties. Semin. Immunol. 19, 362–371.
Kralik, P., Matiasovic, J., Horin, P., 2005. Genetic evidence for the existence of interleukin-23 and for variation in the interleukin-12 and interleukin-12 receptor genes in the horse. Comp. Biochem. Physiol. D 1, 179–186.
Kroening, P.R., Barnes, T.W., Pease, L., Limper, A., Kita, H., Vasello, R., 2008. Cigarette smoke-induced oxidative stress suppresses generation of dendritic cell IL-12 and IL-23 through ERK-dependent pathways. J. Immunol. 181, 1536–1547.
Loftus, J.P., Belknap, J.K., Black, S.J., 2006. Matrix metalloproteinase-9 in laminae of black walnut extract treated horses correlates with neutrophil abundance. Vet. Immunol. Immunopathol. 113, 267–276.
Musilova, P., Kubickova, S., Vychodilova-Krenkova, L., Kralik, P., Matiasovic, J., Hubertova, D., Rubes, J., Horin, P., 2005. Cytogenetic mapping of immunity-related genes in the domestic horse. Anim. Genet. 36, 507–510.
Stockinger, B., Veldhoen, M., 2007. Differentiation and function of Th17 cells. Curr. Opin. Immunol. 19, 281–286.