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Identification, cloning, and sequencing of different cytokine genes in four species of owl monkey

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Abstract Non-human primates could prove to be suitable models for the study of infectious diseases such as malaria, tuberculosis, and hepatitis; the molecules of their immune systems are in the process of being fully characterized. Due to the relevance of cytokines in the modulation of the immune response, a molecular analysis of these proteins in non-human primates from the *Aotus* genus was carried out. Peripheral blood mononuclear cells from four species of *Aotus* monkey were obtained and their mRNAs for interleukin-2 (IL-2), IL-4, IL-6, IL-10, interferon-γ (IFN), and tumor necrosis factor (TNF)-α were characterized. This study shows a high degree of conservation between nucleotide and amino acid sequences of cytokines from different *Aotus* species and those from humans. The TNF-α molecules were identical in amino acid sequences for both.

Keywords Animal model · Cytokines · Human and monkey identity · Owl monkeys · Primate sequences

Introduction

The use of non-human primates as experimental models has represented a key tool in biomedical investigation. Examples of the use of the model cover several medical disciplines such as neurobiology, immunology, pathology, teratology, reproductive biology, tumor biology, infectious diseases, and biology transplants (King et al. 1988; Nowak 1994).

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One aim of our institute is to use suitable experimental models that allow the comparison of different immune responses at the molecular level. Non-human primates represent a very convenient experimental model for testing vaccine candidates or active components for the development of new drugs. Different laboratories have been extensively promoting the use of non-human primates from the *Aotus* genus as an experimental model in the development of malaria vaccines and possible immune response against different antigens of this pathogen (Collins 1994; Duque et al. 1998; Rodriguez et al. 1990; WHO 1988). We have thus established an *Aotus* colony in Leticia (in the Amazon Jungle) where we keep three different native species of *Aotus: A. nancymaae, A. vociferans,* and *A. nigriceps*. All the monkeys tested have a similar susceptibility for developing malaria after an in viv*o* intravenous challenge with *Plasmodium falciparum* parasites.

There are great limitations in the characterization of monkeys, due to restricted information about the functional subpopulation, cytokine regulation, and cellular activation markers. There are at least two different types of T helper (TH) lymphocytes that can be identified based not only on the cellular markers that they express on their surface, but also on the lymphokine profiles that they secrete (Cherwinski et al. 1987; Mosmann and Coffman 1989). The TH_1 lymphocytes secrete significant amounts of interleukin-2 (IL-2) (Taniguchi et al. 1983), tumor necrosis factor (TNF), and interferon-γ (IFN-γ), which augment the cell-mediated immune response. An immune response requiring optimal T cytotoxic lymphocyte activity or optimal macrophage activation thus benefits from the presence of such T cell help. Alternatively, TH_2 lymphocytes can secrete significant amounts of IL-4, IL-5, IL-6, IL-10, IL-13, and consequently, effectively augment humoral immune responses. Thus, protective immune responses that require production of antibodies benefit from the presence of these TH lymphocytes (Bost et al. 1997).

In this context, our group has been dissecting the *Aotus* immune system at the molecular level. In these studies we found a great similarity in structure and complexi-

ty between *A. nancymaae* and the human repertoire. At the amino acid sequence level in TCR-α (Favre et al. 1998) we found that the identity of amino acid sequences between *Aotus* and humans was greater than 80% and TCR-β (Vecino et al. 1999) was 77%–90% identical with their closest human counterparts. TCR-γδ (Daubenberger et al. 2001) exhibited smaller interspecies differences than intraspecies differences.

Other studies have reported the major histocompatibility complex (MHC) class II *DRB* genes from *Aotus nancymaae*. Thirty-four MHC *DRB* alleles were identified. Six allele lineages were detected; two have human counterparts (Niño-Vasquez et al. 2000). Studies of the MHC *DQA1* and the *DQB1/DQB2* polymorphic exon 2 segments in *A. nancymaae* identified 14 *DQB1* alleles that were grouped into two lineages. The essential amino acid residues contributing to MHC DQ peptide binding pockets 1 and 4 are conserved or semi-conserved between human and *Aotus* genera (Diaz et al. 2000a). Finally, the study of the immunoglobulin kappa light chain *V*, *J,* and *C* genes from *Aotus nancymaae* reported an identity of between 83% and 92% for junction regions, and 74% for the constant region between human and *Aotus* monkeys (Diaz et al. 2000b).

In the present study we amplified, cloned, and determined the complete nucleotide sequences of those genes encoding the cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α) of different *Aotus* species to provide a useful tool in the definition of the cellular immune response, its mediators, and role in protection against infectious diseases.

Materials and methods

Animals

Blood samples were obtained from 12 individuals, 3 from each of the following *Aotus* species: *A. nigriceps, A. nancymaae, A. vociferans*, and *A. lemurinus griseimembra.* The latter inhabits the Magdalena River valley, while the others have been held in the primate station in Leticia, Colombia. None of the individuals included in the study have been subjected to any antigenic stimulus. The *A. lemurinus griseimembra* samples were kindly provided by F. Ruiz from the NIH, Colombia. *Papio hamadryas* samples were obtained from a lymphoblastic S594 line infected with Herpes papio virus (ATCC) and were used as a source of DNA from the family *Cerpopithecidae* or Old World Monkeys (OWM) for comparison (Falk et al. 1976; Voss et al. 1992).

Peripheral blood lymphocyte isolation and culture

Non-human peripheral blood mononuclear cells were isolated from heparinized venous samples obtained by femoral bleeding. The cells were then separated by density gradient centrifugation using the Ficoll-Hypaque method (Sigma, St. Louis, Mo., USA). After isolation, cells were washed three times in phosphate-buffered saline 1× (Sigma) and grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (ICN), 100 units/ml human recombinant IL-2 (Hoffman La Roche, Nuttley, N.J.,USA) and 10 µg/ml phytohemagglutinin (Difco, Detroit, Mich., USA). They were incubated for 4 or 12 h at 37°C in 5% CO_2. The number of lymphocytes used ranged between 10 and 12×10^6 cells per flask. *P. hamadryas* lymphoblastic cells were harvested by centrifuging virus-infected culture and then cultured in medium supplemented with 15% supernatant from the P-388D1 line (ATCC TIB 63).

RNA isolation

Total RNA was isolated according to the Trizol one-step procedure (Gibco). Briefly, after cell homogenization in a single-phase phenol isothiocyanate solution, the mixture was extracted with chloroform and the aqueous upper phase was separated, the RNA being precipitated with 1 volume of isopropanol. RNA was washed with 75% ethanol, dried, and suspended in DEPC-treated water (Sambrook and Maniatis 1989). The product was quantified and its quality verified by gel electrophoresis.

Reverse transcription

Total RNA (200 ng) was denatured for 10 min at 70°C for reverse transcription. First-strand synthesis was performed at 37°C for 1 h in a 30-µl reaction mixture containing 1× RT buffer (Gibco), 10 mM dithiothreitol (Gibco), 4 units RNAsin (Promega), 0.5 mM dNTPs (Promega), 200 units MMLV-RT (Gibco), and 3 mM random primers (Gibco). The reaction was stopped by heating the tube at 95°C for 2 min.

Polymerase chain reaction

Each product was primary amplified with two commercial human β-actin specific primers (Clontech Laboratories Palo Alto, Calif., USA) to verify the suitability of the cDNA where an 838-bp band was expected. A set of commercial primers were used (Table 1)

Table 1 Oligoprimers used for amplification of the different cytokines from human and non-human primates

Oligonucleotide	Sequence	Expected size
Hum IL-2	5′ ATGTACAGGATGCAACTCCTGTCTT	458 bp
Hum IL-2	3′ GTCAGTGTTGAGATGATGCTTTGAC	
Hum IL-4	5′ CGGCAACTTTGACCACGGACACAAGTCCGTA	344 bp
Hum IL-4	3′ ACGTACTCTGGTTGGCTTCCTTCACAGGACAG	
Hum IL-6	5′ ATGAACTCCTTCTCCACAAGCGC	628 bp
Hum IL-6	3′ GAACAGCCCTCAGGCTGGACTG	
Hum IL-10	5′ AAGCTGAGAACCAAGACCCAGACATCAAGGCG	328 bp
Hum IL-10	3′ AGCTATCCCAGAGCCCCAGATCCGATTTTGG	
Hum IFNγ	5′ GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC	427 bp
Hum IFNγ	3′ CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG	
Hum TNFα	5′ GAGTGACAAGCCTGTAGCCCATGTTGTAGCA	444 bp
Hum TNFα	3′ GCAATGATCCCAAAGTAGACCTGCCCAGACT	
Hum βact	5′ ATCTGGCACCACACCTTCTACAATGAGCTGCG	838 bp
Hum βact	3′ CGTCATACTCCTGCTTGCTGATCCACATCTGC	

Table 2 Cytokine sequences analyzed

Species	Key	Cytokines					
		INF-γ	**TNF-α**	**IL-2**	**IL-4**	**IL-6**	**IL-10**
Callithrix jaccus	CalJac	X64659					
Aotus lemurinus	AotLem	**AF097327**	**AF097329**	**U88364**	**AF097321**	**AF097323**	**AF097325**
Aotus nancymaae	AotNan	**AF014512**	**AF014513**	**U88361**	**AF014509**	**AF014510**	**AF014511**
Aotus nigriceps	AotNig	**AF097326**	**AF097328**	**U88363**	**AF097320**	**AF097322**	**AF097324**
Aotus vociferans	AotVoc	**AF014507**	**AF014508**	**U88362**	**AF014504**	**AF014505**	**AF014506**
Cercocebus torquatus	CerTor	L26025		U19846	U19838	L26032	L26030
Macaca fascicularis	MacFas		AB000513	D63352	AB000515	AB000554	AB000514
Macaca mulatta	MacMul	L26024	U19850	U19847	L26027	L26028	L26029
Macaca nemestrina	MacNem	L26026		U19852			L26031
Paplo hamadryas	PapHam		AF019963	**U88365**			
Hylobates lar	HylLar	AF164787		K03174			
Homo sapiens	HomSap	AF375790	X02910	X01586	AF395008	AF372214	M57627
Gorilla gorilla	GorGor	AF164789					
Pan troglodytes	PanTro	AF164788					
Pongo pygmaeus	PonPyg	AF164786					
Mus musculus	MusMus	K00083	AF109719	AF399982	M25892	X54542	M84340

for each cytokine for amplifying different size fragments. The cDNAs obtained from *Aotus,* human, and *P. hamadryas,* as well as the PCR controls provided by Clontech, were denatured at 94°C for 5 min followed by 35 cycles of 55°C for 1 min., 72°C for 2.5 min, 94°C for 1 min, and a final extension cycle at 72°C for 7 min. PCR products were revealed on 1% agarose gels and visualized on a UV transilluminator.

Southern blotting and hybridization

PCR products were electrophoresed in a 1% agarose gel and then alkaline transferred to a nylon membrane (Zeta-Probe). Afterwards, the membrane was washed in a 2× SSC solution and UV fixed for 30 s (Stratagene). Human cDNA PCR product was radiolabeled with ^{32}P (Rediprime Labelling RPN, Amersham) and used as a probe. Hybridization was performed in a solution of 100 μg/ml salmon sperm, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, 2× SSC, and the radiolabeled probe at a final concentration of 10^6 cpm/ml. Incubation was carried out at 65°C overnight. A 10-min room temperature wash was then performed in 2× SSC followed by a final wash step in 2× SSC at 65°C. Membranes were exposed to Kodak X-OMAT film at –70°C overnight.

Cloning

After excision of the corresponding bands and purification using Gene Clean protocol (US BioClean), PCR products were ligated into the pMOS Blue vector and competent cells were thermally transformed, according to manufacturers' recommendations (Amersham, UK).

Plasmid DNA from positive selected clones was isolated using the Wizard Miniprep protocol (Promega) and the presence of the desired insert was verified by restriction analysis, both with *Sma*I and with *Eco*RI enzymes (Promega).

Sequencing

The chain termination method with Delta *Taq* Polymerase Version 2.0 (Amersham, UK), using both T7 and U19 primers that flank the insert site in the pMOS vector, was used. At least two clones were sequenced in each case to verify the results.

GenBank accession number

The nucleotide sequence for all cytokines from *A. lemurinus*, *A. nancymaae*, *A. nigriceps*, and *A. vociferans* have been included in the GenBank database, as well as the IL-2 sequence from *P. hamadryas*. These accession numbers are shown in Table 2 in bold.

Construction of gene trees

Alignments were performed using program Clustal X (Thompson et al. 1997). Trees were generated with the neighbor-joining algorithm included in the MEGA 2.1 program (Kumar et al. 2001). Distances were estimated using the gamma amino acid distance method. Bootstrapping was performed with 1,000 replicates. GenBank accession numbers of those sequences used for this analysis are included in Table 2.

Results

Three blood samples from three different individuals from each species were mixed to collect a higher quantity of RNA. Interestingly, a higher quantity of total human RNA was obtained after 12 h stimulation (1,068 μg/ml) compared with 4 h of stimulation (260 μg/ml), in contrast with *Aotus m*onkey RNA where no significant variation was observed (260 μg/ml and 369 μg/ml respectively).

The reverse transcription was shown to be successful by amplification of mammalian β-actin-specific primers (data not shown; Clontech 1994). Amplification products from the different cytokines obtained with the primers provided by Clontech gave the expected results in all cases, as shown in Fig. 1. Southern blot hybridization using the respective human cytokine product as a probe showed strong recognition of the different non-human PCR amplification products from all species assayed (Fig. 2), and indicated a high sequence similarity among them.

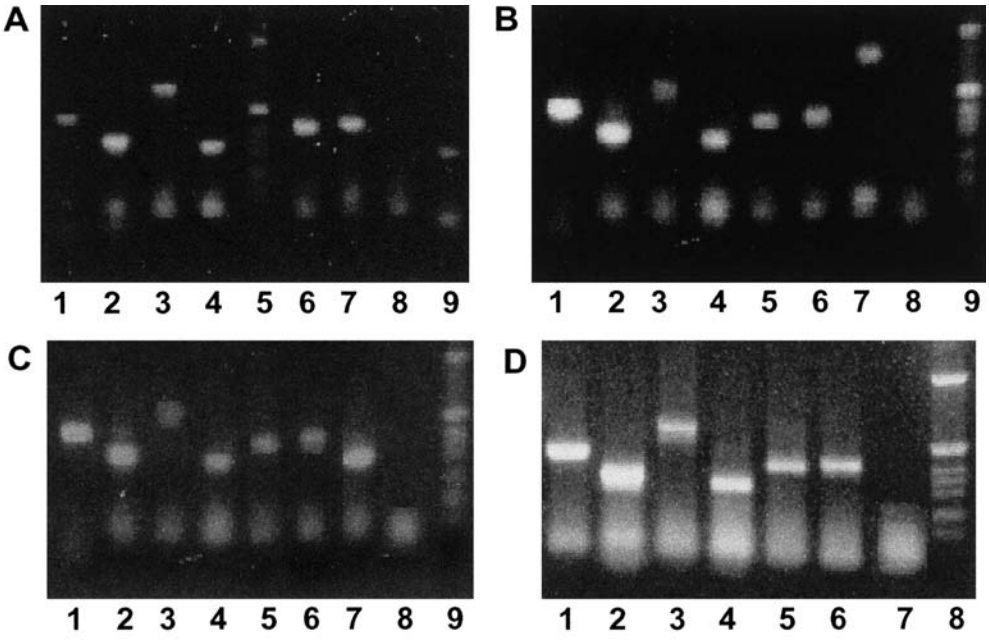

Fig. 1a–d Amplification products from different cytokines obtained from monkey cDNA. **a** *Aotus nancymaae* [*lane 1* interleukin-2 (IL-2), *lane* 2 IL-4, *lane* 3 IL-6, *lane* 4 IL-10, *lane* 5 molecular weight markers (MWM), *lane* 6 interferon-γ (IFNγ), *lane* 7 tumor necrosis factor-α (TNFα), *lane* 8 negative control, *lane* 9 positive control]. **b** *A. nigriceps* (*lane* 1 IL-2, *lane* 2 IL-4, *lane* 3 IL-6, *lane* 4 IL-10, *lane* 5 IFNγ, *lane* 6 TNFα, *lane* 7 positive control, *lane* 8 negative control, *lane* 9 MWM). **c** *A. vociferans* (*lane* 1 IL-2, *lane* 2 IL-4, *lane* 3 IL-6, *lane* 4 IL-10, *lane* 5 IFNγ, *lane* 6 TNFα, *lane* 7 positive control, *lane* 8 negative control, *lane* 9 MWM). **d** *A. lemurinus* (*lane* 1 IL-2, *lane* 2 IL-4, *lane* 3 IL-6, *lane* 4 IL-10, *lane* 5 IFNγ, *lane* 6 TNFα, *lane* 7 negative control *lane* 8, MWM)

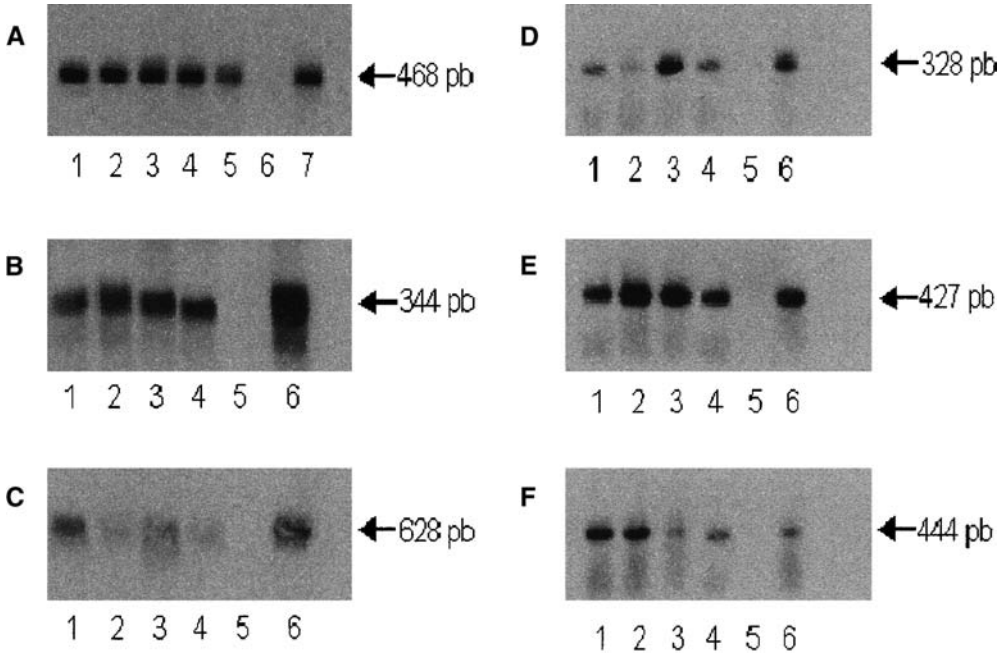

Fig. 2a–f Southern blot. Probes from human cytokines strongly recognized different monkey cytokine amplification products. **a** IL-2 (*lane* 1 *A. nancymaae*, *lane* 2 *A. nigriceps*, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 *P. hamadryas*, *lane* 6 negative control, *lane* 7 human). **b** IL-4 (*lane* 1 *A. nancymaae, lane* 2 *A. nigriceps*, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 negative control, *lane* 6 human). **c** IL-6 (*lane* 1 *A. nancymaae, lane* 2 *A. nigriceps*, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 negative control, *lane* 6 human). **d** IL-10 (*lane* 1 *A. nancymaae, lane* 2 *A. nigriceps*, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 negative control, *lane* 6 human). **e** IFNγ *lane* 1 *A. nancymaae*, *lane* 2 *A.* nigriceps, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 negative control, *lane* 6 human).**f** TNFα (*lane* 1 *A. nancymaae*, *lane* 2 *A. nigriceps*, *lane* 3 *A. vociferans*, *lane* 4 *A. lemurinus*, *lane* 5 negative control, *lane* 6 human)

Sequences from the different cytokines from the four *Aotus* species analyzed in this study and their homologues in other primates, including *P. hamadryas*, were compared against the human cDNA sequence; amino acid level changes are shown in Figs. 3, 4, 5, 6, 7, 8 (in order, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α). Interestingly, only IL-2 presented a new insertion codon between nucleotides 116 and 117 in all the monkey sequences analyzed. The common mouse (*Mus*) was also included as an outgroup. Nucleotide and amino acid sequence alignments were reported in EMBLALIGN alignment database (Lombard et al. 2002); IL-2 ALIGN000405 (nucleotides), ALIGN000406 (amino ac-

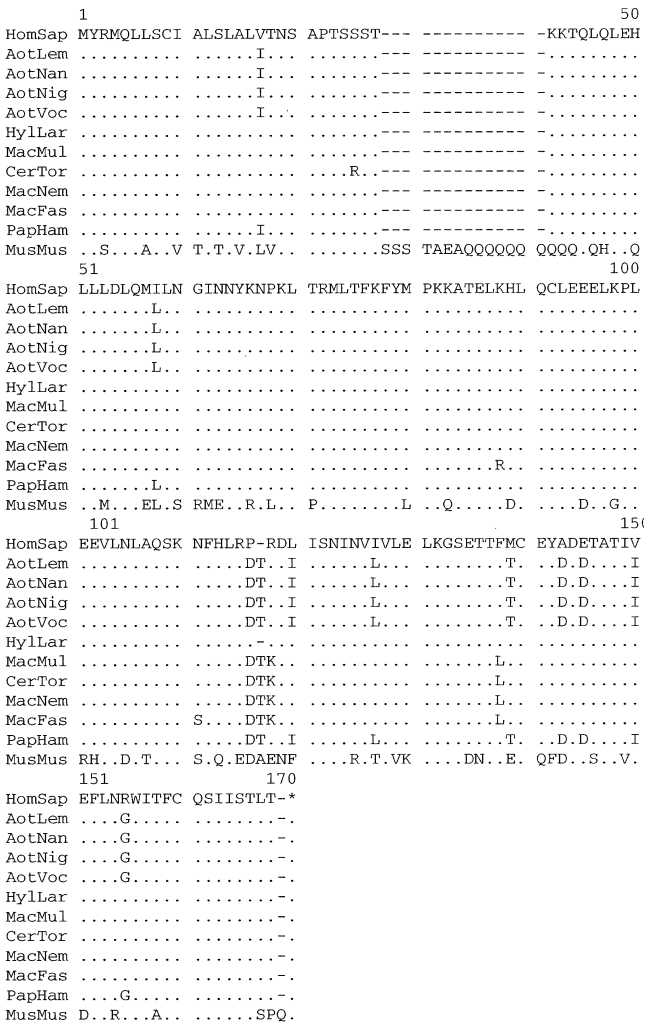

Fig. 3 Alignment of IL-2 for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

ids); IL-4 ALIGN000407 (nucleotides), ALIGN000407 (amino acids); IL-6 ALIGN000409 (nucleotides), ALIGN000410 (amino acids); IL-10 ALIGN000411 (nucleotides), ALIGN000412 (amino acids); IFN-γ ALIGN000402 (amino acids), ALIGN000401 (nucleotides); TNF-α ALIGN000404 (nucleotides), ALIGN000403 (amino acids); the EMBL database (accession number being assigned). They include sequence accession number. The alignments can be retrieved from the SRS databank at http://srs.ebi.ac.uk/.

A tree was generated for all cytokine nucleotide and protein sequences, using all primate species available from the GenBank data base (only protein trees are shown in Fig. 9). There is no difference between the trees from both classes of sequences. There was marked sequence convergence with *Platyrrhini* (NWM) in the case of IL-2 (*Papio* and NWM), IL-6 (*A. nancymaae* and *Catarrhini*), IL-10 (*A. nancymaae* and *Catarrhini*), and TNF-α (*A. nancymaae* and *Catarrhini*). The level of homology between all primates was notably high.

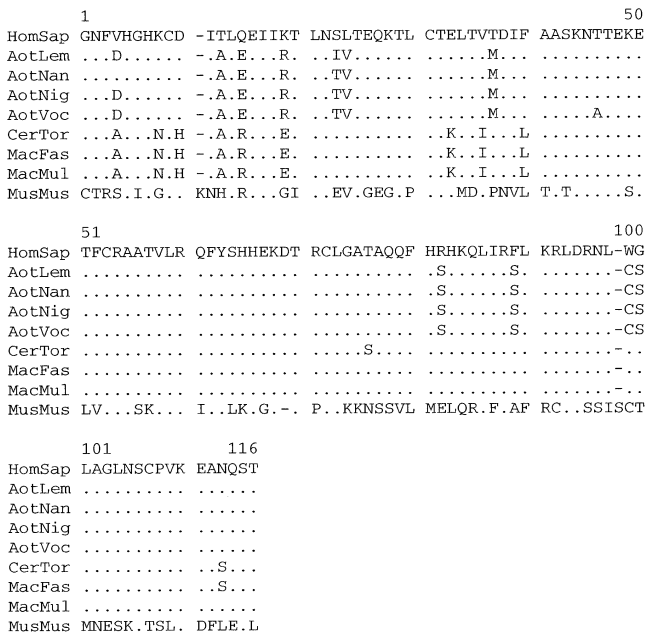

Fig. 4 Alignment of IL-4 for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

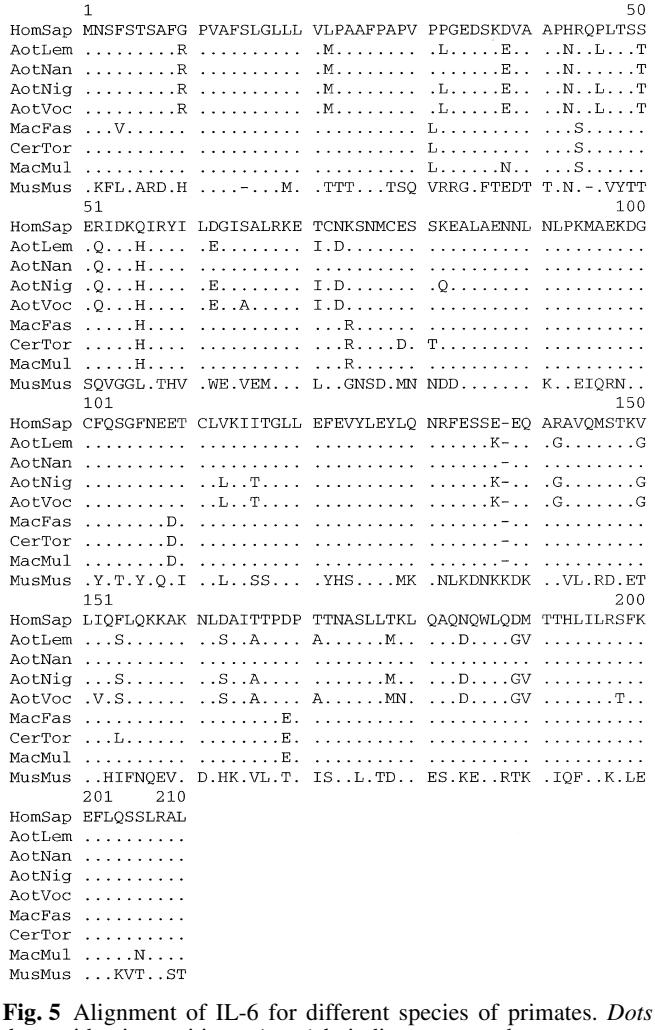

Fig. 5 Alignment of IL-6 for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

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       1                                                  50
HomSap QAENQDPDIK AHVNSLGENL KTLRLRLRRC HRFLPCENKS KAVEQVKNAF
AotLem .......... ........K. ..F..... .......... ...A.....V
AotNan .......... .......... ..F....P.. .......... ...A.....V
AotNig .......... ........K. ..F..... .......... ...A.....V
AotVoc .......... ........K. ..F..... .......... ...A.....V
MacMul ....H..... E......... .......... .......... ..........
CerTor ....H..... E......... .......... .......... ..........
MacNem ....H..... E......... .......... .......... ......M...
MacFas ....H..... E......... .......... .......... ..........
MusMus ...KHG.E.. E.L.....K. ....M..... .......... .......SD.

       51                                  83
HomSap NKLQEKGIYK AMSEFDIFIN YIEAYMTMKI RN*
AotLem S.......H. .........D .........T Q..
AotNan S......... .........D .......... ...
AotNig S......... .........D .........T Q..
AotVoc S......... .........D ...P.....T Q..
MacMul S......V.. .......... .......... Q..
CerTor S......V.. .......... .......... Q..
MacNem S......V.. .......... .......... Q..
MacFas S......V.. .......... .......... ...
MusMus ....DQ.V.. ..N....... C.....MI.M KS.
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Fig. 6 Alignment of IL-10 for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

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       1                                                  50
HomSap QDPYVKEAEN LKKYFNAGHS DVADNGTLFL GILKNWKEES DRKIMQSQIV
AotLem ..S....... ........D. .......... N..RT.R..G .........I
AotNan ..S....... .......... H......... N..RT.R..G .........I
AotNig ..S....... ..E.....D. .......... N..RT.R..G .........I
AotVoc ..S....... ........D. .......... N..RT.R..G .........I
CalJac .......... ........D. .......... D..RT.R..G .........I
MacMul .......... ........DP .......... D..R...... ..........
MacNem .......... ........DP .......... D..R...... ..........
CerTor .......... ........DP .......... D..R...... ..........
GorGor .......... .......... .......... .......... ..........
PanTro .......... .......... .......... .......... ..........
HylLar .......... ........D. ...N...... .......... ..........
PonPyg .......... ........D. .......... .......... ..........
MusMus HGTVIESL.S .NN...SSGI ..EEK.S... D.WR..QKDG .M..L....I

       51                                                 100
HomSap SFYFKLFKNF KDDQSIQKSV ETIKEDMNVK FFNSNKKKRD DFEKLTNYSV
AotLem .......... ..N......M .......... ......R.Q. ...R......
AotNan .......... ..N......M .......... ......R.Q. ...R......
AotNig .......... ..N......M .......... ......R.Q. ...R......
AotVoc .......... ..N......M .......... ......R.Q. ...R......
CalJac .......... ..N......M .......... ......R.Q. ...R......
MacMul .......... ....R..... ......I... .......... ..........
MacNem .......... ....R..... ......I... .......... ..........
CerTor ........S. ....R..... ......I... .......... ..........
GorGor .......... .......... .......... .......... ..........
PanTro .......... .......... .......... .......... ..........
HylLar .......... .......... .......... .......... ..........
PonPyg .......... .......... .......... .......... ..........
MusMus ...LR..EVL ..N.A.SNNI SV.ESHLITT ..SNS.A.K. A.MSIAKFE.

       101                             131
HomSap TDLNVQRKAI HELIQVMAEL SPAAKTGKRK R
AotLem N......... .......... .......... .
AotNan N......... .......... .......... .
AotNig N......... .......... .......... .
AotVoc N......... .......... .......... .
CalJac N......... .......... ...P.I...R .
MacMul ..S......V .......... .....I.... .
MacNem ..S......V .......... .....I.... .
CerTor .........V .......... .....I.... .
GorGor .......... .......... .......... .
PanTro .......... .......... ...V...... .
HylLar .......... .......... .......... .
PonPyg .......... .......... .......... .
MusMus NNPQ...Q.F N...R.VHQ. L.ESSLR... .
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Fig. 7 Alignment of IFNγ for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

When *Aotus* and humans were compared, our findings showed 95% homology in nucleotide sequences for IL-2, and 92% homology in amino acid sequences. Identity intervals were 95%–100% in nucleotide sequences

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       1                                                  50
HomSap PSDKPVAHVV ANPQAEGQLQ WLNRRANALL ANGVELRDNQ LVVPSEGLYL
AotLem .......... .......... .......... .......... ..........
AotNan .......... .......... .......... .......... ..........
AotNig .......... .......... .......... .......... ..........
AotVoc .......... .......... .......... .......... ..........
MacFas .......... .......... .........V ......T... ..........
MacMul .......... .......... .......... ......T... ..........
PapHam ......V... .......... .......... ......T... ..........
MusMus S......... ..H.V.E..E ..SQ...... ...MD.K... ....AD....

       51                                                 100
HomSap IYSQVLFKGQ GCPSTHVLLT HTISRIAVSY QTKVNLLSAI KSPCQRETPE
AotLem V......... .....FM... .S........ .A........ .........R
AotNan .......... .......... .......... .......... ..........
AotNig V......... .....FM... .S........ .A........ .........R
AotVoc V......... .....FM... .S........ .A........ .........R
MacFas .......... ....N..... .......... .......... ..........
MacMul .......... ....N..... .......... .......... ..........
PapHam .......... ....N..... .......... .......... ..........
MusMus V......... ...DYF.... ..V..F.I.. .E.......V ....PKD...

       101                                                148
HomSap GAEAKPWYEP IYLGGVFQLE KGDRLSAEIN RPDYLDFAES GQVYFGII
AotLem ..KTN..... .......... .......... L.....L... ........
AotNan .......... .......... .......... .......... ........
AotNig ..KTN..... .......... .......... L.....L... ........
AotVoc ..KTN..... .......... .......... L.....L... ........
MacFas .......... .......... .......... L.......... ........
MacMul .......... .......... .......... L......... ........
PapHam .......... .......... .......... L......... ........
MusMus ...L...... .......... ...Q....V. L.K....... ......V.
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Fig. 8 Alignment of TNFα for different species of primates. *Dots* denote identity positions. *Asterisks* indicate stop codons

and 91%–100% in amino acid sequences for all primates.

Our findings showed 94%–95% homology in nucleotide sequences and 89%–91% homology in amino acid sequences for IL-4; identity intervals were 92%–99% in nucleotide sequences and 85%–99% in amino acid sequences for IL-4 in all primates.

For IL-6, the identities ranged from 92% to98% and 88% to 96% for nucleotide and amino acid sequences respectively. For all primates the identity intervals were 91%–99% in nucleotide and 83%–98% in amino acid sequences.

For IL-10 the identities ranged from 93% to 94% for nucleotide sequence and 89% to 92% for amino acid sequence. For all primates, identity intervals were 92%–99% for nucleotide sequence and 86%–98% for amino acid sequence. A 93% homology was found in nucleotide sequences and 88%–89% homology in amino acid sequences for IFN-γ; identity intervals were 91%–100% for nucleotide sequences and 84%–100% for amino acid sequences for all primates.

Finally, for TNF-α the identities ranged from 94% to 99% for nucleotide sequence and 92% to 100% for amino acid sequence. For all primates the identity intervals were 93%–100% for nucleotide sequence and 91%–100% for amino acid sequences.

Discussion

This study shows, for the first time, the high nucleotide and amino acid sequence similarity between the different cytokines from various *Aotus* species and their human counterparts.

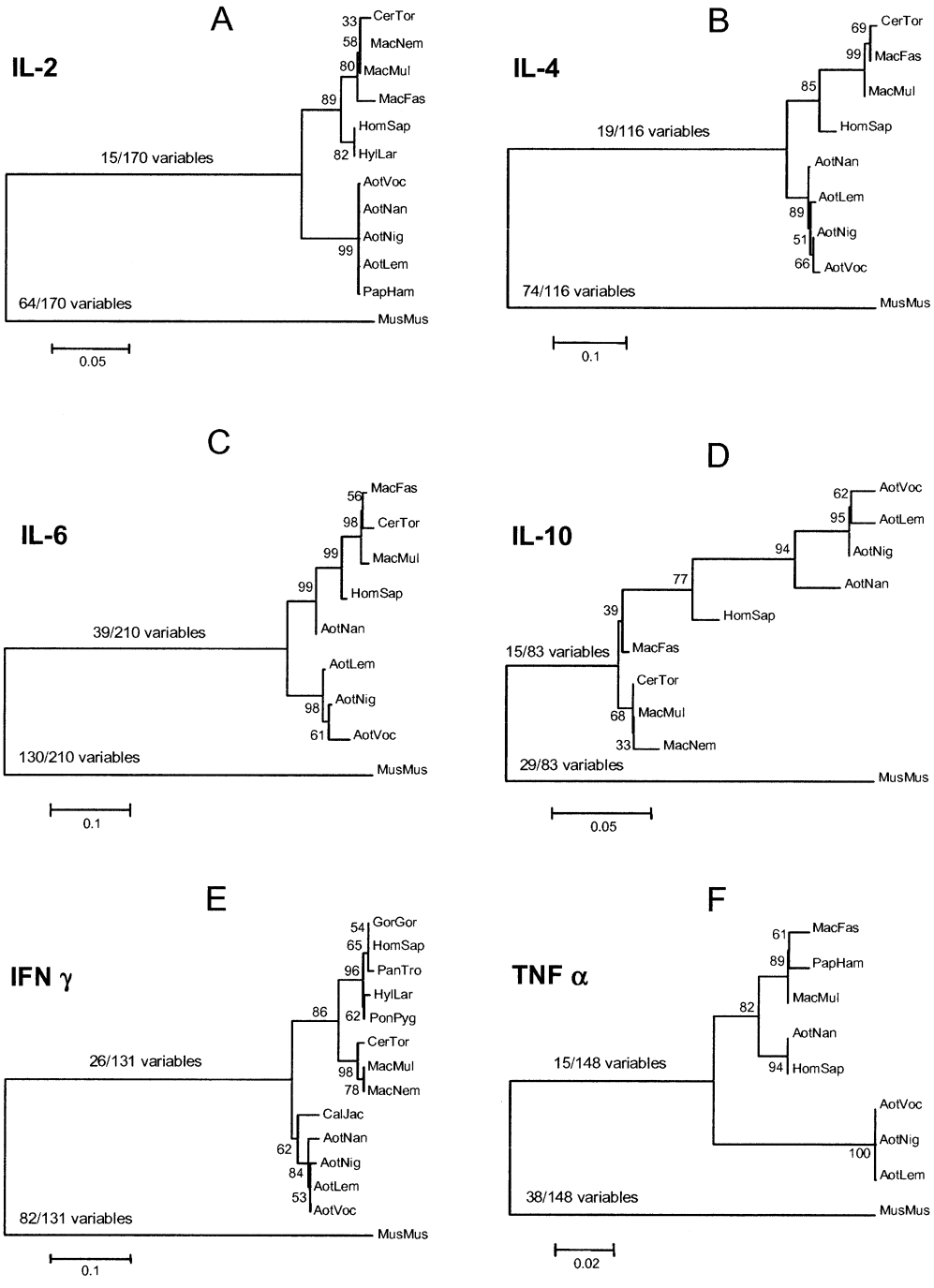

Fig. 9 Gene trees for cytokine amino acid sequences. Values above branches are bootstrap percentages derived from 1,000 replications. Neighbor-joining phylogenetic trees show the relationship between each cytokine type analyzed in this study and other primate cytokines. *Mus musculus* cytokines (common mouse) are used as outgroups. The values in the mouse branch correspond to the number of differences of the total positions in the alignment between primate group and mouse. The values in the primates branch correspond to the number of differences of the total positions in the alignment between primates. *Scale bar* represents substitutions per site. Distances were estimated by using gamma amino acid substitution model

The only previous study in this line of research compared mediators produced by *Catarrhini* such as the gibbon and the macaque, posing various phylogenetic hypotheses about possible evolutionary interspecies similarities between these non-human primates (Villinger et al. 1995). Other immunological comparative studies have focused mainly on the identification of T/B lymphocyte antigens and surface markers (Farrar et al. 1978; Letvin et al. 1983), as well as the presence of different MHC antigens in primate species, such as chimpanzees, gorillas, and orangutans (Balner et al. 1981; Geluk et al. 1993; Slierendregt et al. 1994; Ttrkova et al. 1993; Winton et al. 1985). These studies have revealed a high degree of polymorphism in some genes within these species, including human.

As we have shown in this study, there is a greater degree of homology between the non-human primate species when compared with human counterparts. Thus, some of the non-human primates cytokine sequences were identical to their human homologues (Fig. 9a and e) at the amino acid level, as in the case of TNF-α. However, their nucleotide sequence levels were slightly different (data not shown). To exclude possible cross contamination with human amplification product, this result was

confirmed with two completely independent cell and RNA samples. In this context it is also remarkable that the *A. nancymaae IL-6* gene is similar to the other *Aotus* genes (several substitutions in the first half of the sequence are present) and to the C-terminal part of the human genes. This cannot be explained easily with cross contamination and this result therefore supports the finding with the *TNF-α* gene.

Primates generally show great homology when different kinds of cytokines are compared; the number of variable positions inside the group is low when compared with the outgroup (*Mus musculus*), as shown in the different analyses (Fig. 9). Indeed, a convergent evolution was observed in several examples, as shown in the case of IL-2 (Fig. 9a) with the introduction of *Papio* into the NWM group. Interestingly, the IL-2 sequence in all *Aotus* species analyzed showed the addition of a new codon insertion that is not present in any of the *Catarrhini* sequences, including humans.

Furthermore, the *A. nancymaae* tree gene branch demonstrated the high relationship of this monkey with *Catarrhini* in the cases of IL-6, Il-10, and TNF-α (Figs. 9c, d, and f, respectively). These patterns of associations might well indicate a functional convergence between *Aotus nancymaae* and the *Catarrhini* (especially humans) in their humoral immune response. This hypothesis is supported by previous evidence showing a high rate of homology (Diaz et al. 2000b) at immunoglobulin gene amino acid sequence level, highlighting the owl monkey's fundamental role as an animal model for human infectious diseases.

Within the context of topology analysis, it is clear that the cytokines studied in *Aotus* showed high levels of interspecies and intraspecies identity, and thus the use of any species from this genus could be similar as viewed by cytokine homology.

The results shown here represent a first step in the study of cytokines in monkeys and can be used to analyze the role of these cellular mediators in protective immune response against infectious disease.

This experimental model has been used in the past for the evaluation of malaria vaccine candidates (Rodriguez et al. 1990). The understanding of the role of *Aotus* monkey cytokines in the regulation of the immune response will facilitate the prediction of human immune behavior when immunized with any vaccine candidate, as well as the protective response against the different human pathogens.

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