

ORIGINAL ARTICLE

## Gene expression profile of Th1 and Th2 cytokines and their receptors in human and nonhuman primates

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### Keywords

chimpanzee – IFN- $\gamma$  – IFN- $\gamma$ R – IL-12 – IL-4 – IL-4R $\alpha$  – macaque – baboon

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### Abstract

**Background** To date comparative knowledge concerning gene expression profiles of T-helper 1(Th1)/Th2 cytokines and their receptors between human and non-human primates is scarce.

**Methods** We assessed the gene expression level of both Th1 [interleukin-4(IL-4)] and Th2 [IL-12, interferon- $\gamma$ (IFN- $\gamma$ )] cytokines and the receptors (IL-4R $\alpha$ , IFN- $\gamma$ R1, IFN- $\gamma$ R2) in peripheral blood mononuclear cells (PBMC) from humans, chimpanzee, baboon, and macaque by a quantitative real-time reverse transcriptase-polymerase chain reaction(RT-PCR).

**Results** The expression level of the IFN- $\gamma$  gene was markedly lower in humans than that in non-human primates. The IL-4 gene expression was significantly higher, whereas that of IL-12 was distinctly lower, in human/chimpanzee than in baboon/macaque. The IFN- $\gamma$ R2 gene expression was especially higher in the macaque than in the other three primates.

**Conclusions** These results indicate distinct gene expression of Th1/Th2 cytokines and their receptors in primates. These also suggest characteristic differences in Th1/Th2 immune responses affecting host defense and/or disease susceptibility among these primates.

### Introduction

Non-human primates are used as invaluable models for biomedical studies of immune diseases and/or their therapy, such as allergy [33], gene vaccine [16], and infectious disease [22]. To date comparative knowledge concerning gene expression profiles of cytokines and their receptors participating in T-helper 1 and T-helper 2 pathways between human and non-human primates is scarce.

T-helper (Th) cells are divided into two groups, Th type 1 (Th1) and type 2 (Th2) [27]. Th1 cells express/produce Th1 cytokines, interferon- $\gamma$ (IFN- $\gamma$ ), interleukin-2(IL-2), and tumor necrosis factor- $\beta$ (TNF- $\beta$ ) which participate in cell-mediated immunity, whereas, Th2 cells produce Th2 cytokines, i.e. IL-4, IL-5, IL-6, IL-10, and IL-13, associated with antibody-mediated immunity [1, 28, 30]. The Th1 and Th2 cells counter-regulate each

other through expression/production of their distinctive cytokines, which appear to be a regulatory network for the immune responses mediated by them. IL-12, which is mainly produced by macrophages and dendritic cells, favors the differentiation of Th1 cells and plays a central role in Th1 responses [17, 23, 40]. In this study, we examined the gene expression profile of cytokines (IL-4, IL-12, IFN- $\gamma$ ) and the receptors of two of them (IL-4R $\alpha$ , IFN- $\gamma$ R1, IFN- $\gamma$ R2) in humans and in three non-human primates (chimpanzee, baboon, macaque), which are widely used in biomedical research. We found several characteristic expression profiles: a distinct difference in expression of IL-4 and IL-12 genes between hominoids (human, chimpanzee) and monkeys (macaque, baboon), the suppressed expression of the IFN- $\gamma$  gene in humans compared with its expression in non-human primates, and the elevated expression of both IFN- $\gamma$ R1 and IFN- $\gamma$ R2 genes in the macaque.

## Materials and methods

### Collection of blood samples

Heparinized venous blood samples were obtained from human volunteers ( $n = 6$ ), chimpanzees (*Pan troglodytes*,  $n = 4$ ), baboons (*Papio hamadryas*,  $n = 3$ ), and macaques (*Macaca fascicularis*,  $n = 3$ ). All the non-human primates used were normal adults raised at the Kyoto University Primate Research Institute (KUPRI) in accordance with the guidelines of the KUPRI for the Care and Use of Laboratory Primates. This institutional guideline is based on the 'Guide for the Care and Use of Laboratory Animals' by the US National Research Council [5, 29].

### Preparation of PBMC

Blood samples were centrifuged at 1000  $g$  for 15 minutes at room temperature to remove plasma. The cellular fraction was suspended in the same volume of sterile phosphate-buffered saline (PBS). The suspensions of non-human primate and human cells were then mixed with one-fifth volume of 5% Dextran T-2000 and T-500, respectively, and then incubated at 37°C for 30 minutes to separate the erythrocytes. The supernatant obtained, i.e. the leukocyte fraction, was overlaid on a Ficoll-Conray solution (density, 1.077) and centrifuged at 400  $g$  for 30 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) accumulated on the top of the Ficoll-Conray layer, and the PBMC fraction was washed twice with cold sterile PBS to remove contaminating platelets. All solutions for PBMC preparation were pyrogen-free.

### RNA isolation and reverse transcription

Total RNA was extracted from PBMC by using an RNeasy micro kit (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's protocol in conjunction with on-column DNase treatment (RNase-Free DNase Set, Qiagen). The yield and purity of the obtained RNA were determined spectrophotometrically at 260 and 280 nm. cDNA was synthesized from 1  $\mu$ g of total RNA by reverse transcription with AMV reverse transcriptase XL (Takara Bio, Otsu, Japan) and primed with oligo (dT)12–18 primer (Invitrogen, Carlsbad, CA, USA).

### Real-time PCR for gene expression analysis of cytokines and their receptors

Real-time polymerase chain reaction (PCR) was performed with the ABI Prism 7700 Sequence Detector

System (Applied Biosystems, Foster City, CA, USA) using an SYBR detection system. PCR primers for cytokine (IL-4, IL-12, IFN- $\gamma$ ), receptor (IL-4R $\alpha$ , IFN- $\gamma$ R1, IFN- $\gamma$ R2), and GAPDH genes are listed in Table 1. PCR conditions were as follows: briefly, PCR proceeded in a 10- $\mu$ l reaction mixture containing 5  $\mu$ l of 2x SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), 0.2  $\mu$ l of 50x ROX Reference Dye (Takara Bio), 0.2  $\mu$ l of a mixture of forward and reverse primers (initial concentration of 10  $\mu$ M for each), 1  $\mu$ l of the reverse transcriptase (RT) product corresponding to 40 ng of RNA, and 3.6  $\mu$ l of distilled water. Samples were pre-incubated for 10 s at 95°C, and then subjected to 40 cycles of amplification at 95°C for 5 s for denaturing and at 63°C for 30 s for annealing-extension. A dissociation curve was generated at the end of the PCR cycles to verify that a single gene product had been amplified. GAPDH was chosen as an internal control for each RT product, and the standard curve for GAPDH cDNA within  $10^5$  to  $10^{10} \times 15$  copies was generated to quantify the control GAPDH mRNA level. The semi-quantitative assessment of the mRNA of interest was done by dividing with the expression level of the internal GAPDH and hence expressed as the so-called 'relative expression to GAPDH'. All assays were conducted in duplicate, and the mean value was used as the gene expression level. For interspecies comparison among primates the relative expression of each target gene was calculated vs. that of the human corresponding gene. All PCR amplicons were subjected to sequence analysis with ABI Genetic Analyzer 310 and their sizes (bp) estimated from the sequence data.

## Results

As to the relative gene expressions of IL-4 and IL-12, a distinct difference in the expression level of these genes was observed between human/chimpanzee (hominoid) and baboon/macaque (monkey). As shown in Fig. 1A, the expression level of the IL-4 gene in hominoids was markedly higher than that in monkeys; i.e. the human IL-4 gene expression was 89- and 30-fold higher than that of the baboon and macaque genes, respectively. In contrast, the expression level of the IL-12 gene was significantly lower in the hominoids than that in the monkeys (Fig. 1B). Interestingly, the expression level of the human IFN- $\gamma$  gene was clearly distinct from that for non-human primates including the chimpanzee (Fig. 1C), being 12–24 times lower.

Figure 2 shows the relative gene expression levels of the cytokine receptors examined, i.e. IL-4R $\alpha$ , INF-

**Table 1** Sequences of each primer and annealing site of target gene

	Forward	Reverse	Reference	PCR product (bp) *
IL-4 primer	5'-CGAAACTCTGAACAGCCTCACAGAG-3'	5'-TCAGCTCGAACACTTTGAATATTCTCTCAT-3'	L26027	358
Human	A.....T.....	.....	NM_000589	358
Chimpanzee	A.....T.....	.....	AY130260	358
Cynomolgus	.....T.....	.....T.....	AB000515	358
IL-4R $\alpha$ primer	5'-TGACCTGGAGCAACCCGTATC-3'	5'-GAAGGGCTCCCTGTAGGAGT-3'	NM_000418/AY459192	266
Human	.....	.....	NM_000418	266
Chimpanzee	.....	.....	XM_001135957	266
Cynomolgus	.....	.....	AY459192	266
IL-12 primer	5'-AGCAGCTGGTCATCTCTGGTT-3'	5'-CCAGCATCTCCAACCTTTTGA-3'	**	247
Human	.....T.....	.....	AF180563	247
Chimpanzee	.....T.....	.....	XM_527101	247
***Rhesus	.....	.....	U19841	247
IFN- $\gamma$ primer	5'-TGCAGAGCCAAATGTCTCTTTTAC-3'	5'-GGGATGCTCTCGACCTCGAAA-3'	NM_000619/D89985/NM_001032905	294
Human	.....	.....	NM_000619	294
Chimpanzee	.....	.....	XM_001151968	294
Cynomolgus	.....	.....	D89985	291
IFN- $\gamma$ R1 primer	5'-GAGACGAGCAGGAAGTCGATT-3'	5'-ACTGGAATCACTAAGTGGCACT-3'	NM_000416(for Forward)/AF227551 (for Reverse)	157
Human	.....	.....G.....	NM_000416	157
Chimpanzee	.....	.....G.....	XM_001171493	157
Cynomolgus	...A.....	.....	AB173721	157
IFN- $\gamma$ R2 primer	5'-AGCACCCGAAGATTCGCCT-3'	5'-GCTCAGCTCGAAGCGGTAGA-3'	NM_005534	255
Human	.....	.....	NM_005534	255
Chimpanzee	.....	.....	XM_525461	255
***Rhesus	.....	.....A-C.....	XM_001091864	255
GAPDH primer	5'-CCATGGAGAAGGCTGGGG-3'	5'-CAAAGTTGTCATGGATGACC-3'	NM_002046	246
Human	.....	.....	NM_002046	246
Chimpanzee	.....T.....	.....	XR_019963	246
Cynomolgus	...C.....	.....	AB220454	246

\*Size of PCR product was estimated from its sequence analysis.

\*\*Hofmann-Lehmann R *et al.* AIDS Res Hum Retroviruses 2002; 18:627-39.

\*\*\*Rhesus macaque sequences were referred because of little data of cynomolgus macaque ones.

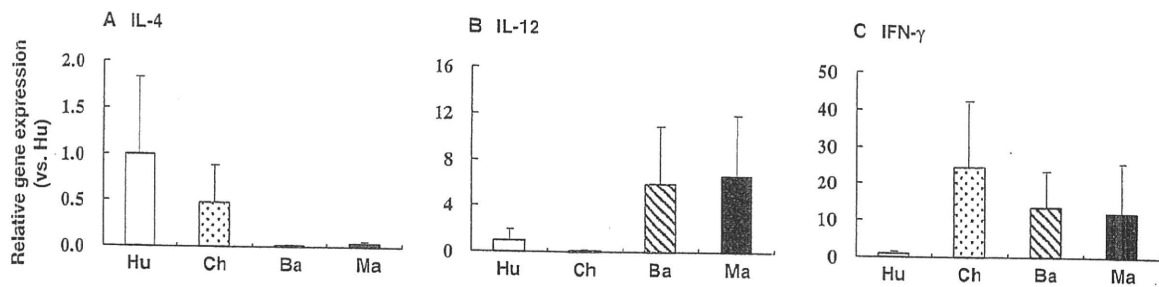


Fig. 1 Relative gene expression levels of Th1/Th2 cytokines in human (Hu; □), chimpanzee (Ch; ▨), baboon (Ba; ▩), and macaque (Ma; ■); preparation of peripheral blood mononuclear cells. IL-4 (A), IL-12 (B), and IFN- $\gamma$  (C) gene expression levels are shown as a ratio to those of GAPDH. Values are mean  $\pm$  SD relative to the levels of humans as controls, who were arbitrarily assigned a value of 1.

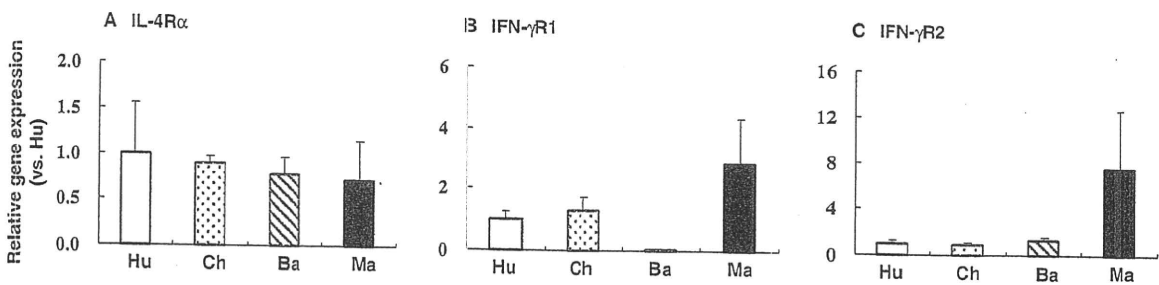


Fig. 2 Relative gene expression levels of Th1/Th2 cytokine receptors in humans (Hu; □), chimpanzee (Ch; ▨), baboon (Ba; ▩) and macaque (Ma; ■) PBMC. IL-4R $\alpha$  (A), IFN- $\gamma$ R1 (B), and IFN- $\gamma$ R2 (C) gene expression levels are shown as a ratio to those of GAPDH. Values are mean  $\pm$  SD relative to the levels of humans as controls, who were arbitrarily assigned a value of 1.

$\gamma$ R1, and INF- $\gamma$ R2. Among primates, unique gene expression levels of both IFN- $\gamma$  receptors, INF- $\gamma$ R1 and INF- $\gamma$ R2, were observed in the macaque, whose expression levels of IFN- $\gamma$ R1 and IFN- $\gamma$ R2 genes were approximately three- and eight-fold, respectively, higher than those of humans or other non-human primates (Fig. 2B,C). The IFN- $\gamma$ R1 gene was expressed at an exceptionally low level in the baboon, being 37-, 48-, and 107-fold lower than its level in humans, chimpanzee, and macaque, respectively (Fig. 2B). The level of IL-4R $\alpha$  gene expression appeared to be little different among the primates examined here (Fig. 2A).

## Discussion

Comparative studies of the physiological status of the immune system, especially Th1 and Th2 cytokines and their receptors, in human and non-human primates, give us invaluable information to perform immune-related biomedical examinations using non-human models. The gene expression profile on steady-state mRNA level in immune tissues or cells is a powerful genomics to assess some self-defense systems in physiological and/or pathological conditions in human and

non-human primates [13, 24, 37]. The comparative studies of protein level using antibodies are limited to cross-related species [25, 42], as there is much more difference in binding affinity or reactivity between target proteins and antibodies than that of RNA/DNA sequences. For this reason it is hard to find available antibody to examine quantitative analysis of target cytokines and/or their receptors between humans and monkey.

In this line we performed interspecies comparison on gene expression of cytokines and their receptors in unstimulated PBMC from humans, chimpanzee, baboon, and macaque. Gene expression profiling using unstimulated PBMC gives us information concerning potential homeostasis of Th1 and Th2 cytokines and their receptors under physiological conditions. These are also essential to know the *in vivo* counter-balance between Th1 and Th2 response in primates, while studies of stimulated PBMC provided more insights [3, 7].

Our new findings indicate a distinct difference in the expression level of Th1 (IL-12) and Th2 cytokine (IL-4) genes between hominoids (human, chimpanzee) and monkeys (baboon, macaque) and IFN- $\gamma$  gene within the hominoids. Higher gene expression of both IFN- $\gamma$

receptors, IFN- $\gamma$  R1 and IFN- $\gamma$  R2, was also observed in the macaque. In this study, each PCR primer was designed based on the DNA sequence of the target human and/or macaque gene. On sequence comparison of IL-4 gene around both primer binding sites, cynomolgus macaque had one mismatch near the 3'-end of the reverse primer (Table 1), while no mismatch in the sequence of rhesus macaque was observed. Using the IL-4 primers we obtained almost the same level of IL-4 gene expression between both macaques (data not shown), indicating that the apparent lower expression level of IL-4 gene in cynomolgus was not a result of PCR efficiency but the amount of its mRNA. Additionally, the nucleotide sequences of examined cytokine and receptor genes had very high homology with 93% to 100% among the four species (data not shown). Thus, the apparent differences in the gene expression level among primates are attributable not to the differential efficiency of the PCR reaction but to actual differences in the level of their steady-state mRNAs.

Regarding the expression level of the IL-4 gene, it appeared to be higher in hominoids than in monkeys, whereas that of the IL-12 gene was markedly lower in the hominoids than in the monkeys. Although we have no direct evidence to account for these differences yet, it was reported earlier that IL-4 downregulated the transcription of the IL-12 gene by reducing the promoter activity of this gene as well as destabilizing IL-12 mRNA [35]. Those findings suggest that IL-4 regulates IL-12 gene expression at both transcriptional and post-transcriptional steps. Such regulation is a plausible reason for the very low level of IL-12 gene expression in hominoids, which showed a very high expression level of their IL-4 gene.

A marked difference in IFN- $\gamma$  gene expression was found between humans and chimpanzee. Its expression in humans was distinctly lower than that in chimpanzees as well as in baboons and macaques. It is well known that intestinal parasite infections affect Th1 or Th2 immune responses by regulating the expression of the genes encoding cytokines [2, 6, 32, 36]. Protozoan parasites are a potent stimulator of Th1 responses and of gene expression of the Th1 cytokine IFN- $\gamma$  [12, 21, 31]. Previous studies of helminth (S.Nakamura, unpublished data) and protozoal [38, 39] infections in chimpanzees and macaques (maybe in baboons also) indicated very strong and frequent infection in these non-human primates. This poor hygienic condition with high parasite infection of non-human primates is clearly different from that of the Japanese subjects examined here. Moreover, in previous study it was reported that African people under poor hygienic conditions had higher Th1 cytokine (IFN- $\gamma$ ) levels than

that of people in western countries [26]. The same trend was observed when comparing IFN- $\gamma$  gene expression in PBMC of normal subject(s) belonging to a developing country and those of Japan (S. N. unpublished data). Probably this difference in hygiene is a main factor responsible for the distinctly lower gene expression of IFN- $\gamma$  in humans than in non-human primates, though details of the mechanism regulating its gene expression remain to be clarified.

The IFN- $\gamma$  receptor consists of four subunit chains (two chains of ligand-binding subunit: IFN- $\gamma$ R1 and two chains of signal-transducing subunit: IFN- $\gamma$ R2) [34]. IFN- $\gamma$ R2 appears to be a limiting factor in the IFN- $\gamma$  signal pathway, as the gene expression of IFN- $\gamma$ R1 is ubiquitous during the cell cycle whereas that of IFN- $\gamma$ R2 is tightly regulated in accordance with cellular differentiation or activation [4, 8, 9, 41]. Interestingly, unique higher gene expression of both IFN- $\gamma$  receptor subunit chains, IFN- $\gamma$ R1 and IFN- $\gamma$ R2, was observed in the macaque. As we have little data concerning the mechanism regulating this unique gene expression of IFN- $\gamma$  receptors in the macaque yet, further studies on it are undoubtedly needed. This previously unknown finding should prompt comparative studies on the IFN- $\gamma$  signaling pathway in macaques, as they are widely used as a primate model for biomedical research in Th1 immunity-associated vaccine development and/or gene therapy.

Humans and chimpanzee had similar characteristics in gene expression profiles of some cytokines and their receptors. Especially in terms of the gene expression of IL-4 and IL-12, they were clearly distinct from monkeys. In recent studies [18, 43] some evolutionary considerations concerning neutral, positive, or negative selection on gene expression among primates were claimed. A genome-wide effect of positive selection was suggested in gene expression of immune system in primates [18]. These give an insight into the occurrence of a selective event in the gene expression of the cytokines during evolution of the hominoid lineage. This event would be caused by transcriptional and/or post-transcriptional regulation for cytokine gene expression. Bostik *et al.* reported distinct sequence differences in the promoter region of cytokine genes among humans, macaque and mangabey monkey [10]. Insertion of repeat sequences such as *Alu* or *ERV* is known to regulate gene expression [14, 15]. Epigenetic events such as DNA methylation or histone acetylation are also known to affect transcriptional regulation of gene expression [19, 20]. Non-coding (nc) RNA, especially micro-RNA, participates in post-transcriptional gene regulation [11]. These genome-wide effects could contribute

to the differently regulated expression of hominoid cytokine genes, although little has been reported about these repeat sequences, epigenetic event, or ncRNA in primate cytokine genes.

In conclusion, we have shown the characteristic differences in the gene expression profile of Th1/Th2 cytokines and their receptors among primates, which imply especially that humans are distinct from non-human primates. These differences suggest the species-specific nature implicated in the Th1/Th2 immune responses affecting host defense and/or disease susceptibility among these primates, which should be carefully considered in biomedical researches using non-human primates as experimental models instead of humans. Additionally, our SYBR Green real-time RT-PCR assay used here serves as a useful method to assess primate cytokines and their receptors in a homogenous and reproducible manner.

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