International Immunology, Vol. 21, No. 3, pp. 245–255
doi:10.1093/intimm/dxn144

NKp44 expression, phylogenesis and function in non-human primate NK cells

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Keywords: cytotoxicity, evolution, molecular biology, natural cytotoxicity receptors

Abstract

Molecular and functional characterization of the natural cytotoxicity receptor (NCR) NKp44 in species other than *Homo sapiens* has been elusive, so far. Here, we provide complete phenotypic, molecular and functional characterization for NKp44 triggering receptor on *Pan troglodytes* NK cells, the closest human relative, and the analysis of NKp44-genomic locus and transcription in *Macaca fascicularis*. Similar to *H. sapiens*, NKp44 expression is detectable on chimpanzee NK cells only upon activation. However, basal NKp44 transcription is 5-fold higher in chimpanzees with lower differential increases upon cell activation compared with humans. Upon activation, an overall 12-fold lower NKp44 gene expression is observed in *P. troglodytes* compared with *H. sapiens* NK cells with only a slight reduction in NKp44 surface expression. Functional analysis of '*in vitro*' activated purified NK cells confirms the NKp44 triggering potential compared with other major NCRs. These findings suggest the presence of a post-transcriptional regulation that evolved differently in *H. sapiens*. Analysis of cynomolgus NKp44-genomic sequence and transcription pattern showed very low levels of transcription with occurrence of out-of-frame transcripts and no surface expression. The present comparative analysis suggests that NKp44-genomic organization appears during macaque speciation, with considerable evolution of its transcriptional and post-transcriptional tuning. Thus, NKp44 may represent an NCR being only recently emerged during speciation, acquiring functional relevance only in non-human primates closest to *H. sapiens*.

Introduction

In the absence of prior exposure, activating NK cell receptors evolved to sense and kill tumor- or virus-infected cells (1, 2). In healthy subjects, triggering NK cell receptor function is counterbalanced by MHC class I-inhibitory receptors

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Received 10 July 2008, *accepted* 8 December 2008

Transmitting editor: S. Romagnani

Advance Access publication 15 January 2009

[killer Ig-like inhibitory receptor, inhibitory NK receptor (iNKR)]. Upon cell transformation or virus infection, the iNKR protective role may be lost since transformation-dependent down-regulation of surface MHC class I expression takes place (3, 4). Thus, in the absence of sufficient MHC recognition following its overruling by licensing or tuning influences (5), triggering NK cell receptors are responsible for cell activation leading not only to target cell killing but also to dendritic cell (DC) editing or for the shaping of adaptive immune response and inflammatory cell recruitment (6).

The relevance of these functions is supported by the phylogenetic conservation of triggering receptor structure and function from mice through to humans. Several activating receptors (i.e. NKp46, NKp30, NKG2D and CD94/NKG2C) have been identified and characterized in different mammal species including rodents, bovines and primates [i.e. macaques, chimpanzees and humans (7–18)] revealing remarkable areas of shared structure and functional activity. In addition, it is now well accepted that reduced surface expression of natural cytotoxicity receptors (NCRs) is associated with marked impaired NK cell cytolytic functions (19, 20).

Exceptions occur, however, and are represented by NKp80 and NKp44 (15, 16). The human NKp80 molecule is a triggering receptor recognizing the TLR-induced AICL molecule (21) whose homologous locus is not found in rodents, but it is present in non-human primates (macaques and chimpanzees) (15, 16). The NKp44 molecule (encoded by the *NCR2* locus) is expressed on the surface of IL-2 cultured, but not on resting human NK cells and therefore is referred to as an activation-induced triggering receptor (22, 23).

So far, there is lack of information that could support the notion of NKp44 expression/function in species other than *Homo sapiens*. The *NCR2* gene encoding NKp44 could not be located in the mouse synthenic genomic region (mouse chromosome 17) of human chromosome 6, where the NKp44 locus has been mapped (23–25). The view of a lack of NKp44-genomic information in mice is further supported by the finding that the same genome region displays different orthologous genes known to closely map to the human *NCR2* locus, such as the triggering receptor expressed on myeloid cells that are reported to share sequences and structural homologies with NKp44 (25, 26), while it lacks the *NCR2* gene.

In the course of previous attempts at the phenotypic, functional and molecular characterization of triggering receptors in macaques, successful identification of other triggering receptors including NKp30, NKp46, NKp80 and NKG2D were not followed by NKp44 identification (10, 15, 16). These difficulties suggested that its locus could have possibly appeared more recently than the other NCRs or that its transcription was affecting cDNA isolation and cloning. It is also possible that it could represent a new member of the NCR family probably arisen after mouse speciation, ~65 to 85 million years ago (MYA) (27), facilitating additional regulation capacity of triggering NK innate immune responses (28).

Knowledge of NKp44 regulation, expression and function could be relevant to the understanding of non-human primate immune biology and their differential susceptibility to viral infections and cancer (29, 30). For instance, the peculiar benign course of HIV infection in chimpanzees (31) and the inducible expression of NKp30 in *Pan troglodytes* NK cells resembling NKp44 inducibility upon activation in humans (15) contribute to focus the interest on the presence, expression and regulation of NKp44 in the closest living relative of *H. sapiens*.

Full genomic sequence maps have very recently become available for *P. troglodytes* and *Macaca mulatta* (32, 33). These, however, represent raw information that does not provide additional insight on comparative transcription expression or function of NKp44 in these species.

Given the above areas of uncertainty, we decided to analyze in detail *NCR2* locus in the genome of macaques and of *P. troglodytes*. In view of the postulated human–chimpanzee speciation occurring between 4 and 6.3 MYA (34), as opposed to the human/ape lineage diverging from Old World monkeys ~25 MYA (35–37), and accounting for the report that *M. mulatta* may have originated from a *fascicularis*-like ancestor ~2.5 MYA after macaques branch point (38), we considered in this analysis *Macaca fascicularis*, *M. mulatta* and *P. troglodytes*. Accordingly, the study of the genomic arrangement, transcription and expression of NKp44 would allow to verify if this locus may represents a recent phylogenetic acquisition leading to a fine-tuning of the innate NK cell responses.

The current analysis reveals a spectrum of differences ranging from inefficient transcription in macaques to marked transcription with extensive post-transcriptional regulation in chimpanzees and indicates that the genomic organization and expression of the NKp44 evolved over the last 23–25 MYA. Differences in the surface expression of functional NKp44 receptors suggest differential regulation in non-human primates compared with human NK cells.

Methods

Animal subjects and human donors

PBMCs were obtained from 13 adult healthy chimpanzees (*P. troglodytes*), 10 cynomolgus (*M. fascicularis*) and 4 rhesus (*M. mulatta*) monkeys. Animals were housed at the Biomedical Primate Research Centre in Rijswijk (*P. troglodytes* and *M. mulatta*), The Netherlands, and Istituto Superiore di Sanità, Rome (*M. fascicularis*). Animal housing was according to the International and European guidelines for non-human primate care (European Commission and Council directive no. 86-609, November 24, 1986). Human peripheral blood was obtained from five healthy donors.

mAbs and immunofluorescence analysis

The following panel of anti-human mAbs was used—anti-CD16: KD1 (IgG2a); anti-CD56: GPR165 (IgG2a) and NKp44: KS38 (IgM) and Z231 (IgG1) (20, 21). In addition, human CD3: SK7 FITC/APC; human CD16: 3G8 PE (Becton Dickinson, San Jose, CA, USA), IgG1 and anti-monkey CD3 [FN-18 (IgG1); BioSource International] and human NKp44 mAb (R&D Systems, Inc., Minneapolis, MN, USA), IgG2A and FITC-conjugated anti-isotype goat anti-mouse second reagent antibodies have been purchased from Southern Biotechnology (Birmingham, AL, USA).

The reactivity of mAbs with PBMC populations and transient transfectants was assessed by indirect immunofluorescence and flow cytometric analysis. Briefly, 10^5 cells were stained with the first mAb, followed by an appropriate FITC-conjugated secondary antiserum, as second-step reagent. Negative control reagents were murine mAbs directed against irrelevant surface molecules. All samples were analyzed on a cytofluorometer (FACSCalibur, Becton Dickinson, Mountain View, CA, USA) using CellQuest (version 3.3). Cells were gated by forward- and side-scatter parameters based on low scatter and small size. Results are expressed as logarithm of green fluorescence intensity (arbitrary units) versus number of events. For each analysis, 10 000 events were counted. Results are expressed as the proportion of the cells expressing a given surface antigen as compared with a negative control consisting of cells stained with irrelevant mAbs or as mean fluorescence intensity (MFI) channel that represents molecule density on the stained cells.

Cell cultures

Human and chimpanzee PBMCs were isolated using density gradient centrifugation. We prepared macaque mononuclear cells by centrifugation as previously described (10). PBMCs were depleted of plastic-adherent cells and incubated with anti-CD3 (JT3A and FN-18), anti-CD4 (HP2.6) and anti-HLA-DR (D1.12) mAb for 30 min at 4°C, followed by goat anti-mouse-coated Dynabeads (Dynal Biotech) for 30 min at 4°C (10, 36).

Human and chimpanzee PBMCs were enriched for NK cells using MACS NK Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). After immunomagnetic selection, cells were cultured on irradiated (5000 R) feeder cells (PBMC and 221G cell line) in the presence of r-IL-2 100 U ml^{-1} (Proleukin, Chiron Corp., Emeryville, CA, USA). The culture medium used was RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mM) and 1% antibiotic mixture (penicillin 5 mg ml^{-1}, streptomycin 5 mg ml^{-1} stock solution).

Cytotoxicity assay

Redirected killing was performed using the P815 murine mastocytoma ($FcR\gamma^+$) cell line as target. NK cell-enriched populations were tested for cytolytic activity in a 4-h ^{51}Cr-release assay as previously described (36), either in the absence or in the presence of 0.5 μg ml^{-1} Z231 (IgG1 anti-NKp44), BAB281 (IgG1 anti-NKp46) or AZ20 (IgG1 anti-NKp30) mAbs, respectively. Effector/target cell (E/T) dilutions were always performed, and E/T ratios are indicated in the text.

NCR2-genomic characterization in M. fascicularis

Genomic DNA from *M. fascicularis* cells was extracted with the DNA mini kit using standard protocol (Qiagen, Milan, Italy). Different amplifications have been performed to obtain a 780-bp amplicon for exon-1, intron-1, exon-2 and intron-2 flanking region using NKp44 orf frw (CCCACGAGCACACAGGAAAA) and NKp44 INT2B rev (GCTGCAGGCTTGGGAGTC) primers; a 691-bp amplicon for exon-3, intron-3, exon-4, intron-2 and intron-4 flanking regions with NKp44 INT2B frw (AGCCCAGGCCACAGGTGT) and NKp44 INT4B rev (GCCGATTCCCCTCTGACC) oligodeoxynucleotides and a 590-bp amplicon for exon-5 and flanking regions NKp44 INT4 frw (AGAGGGTGAACTTGGAGAC) and NKp44 3′ stop rev (ATAGAGGGCAAACTAGAGAAG) primers. PCRs using *Taq* recombinant DNA polymerase (Invitrogen, Carlsbad, CA, USA) followed by a denaturation step of 2 min at 94°C were performed for 40 cycles of 15 s at 94°C and 30 s at 58°C followed by a final extension step of 40 s at 72°C.

A 6.7-kb amplification product, containing exons 1–4, introns 1–3 and intron 4 flanking region, was obtained using a nested PCR approach. First, NKp44 orf frw and NKp44 INT4B rev have been amplified using the Expand High Fidelity PCR System (Roche, Milan, Italy) in 1.5 mM $MgCl_2$ containing buffer with the following cycling condition: 94°C for 2 min, followed by10 cycles each 15 s at 94°C, 30 s at 62°C and 4.5 min at 68°C and next 20 cycles each 15 s at 94°C, 30 s at 60°C, (4.5 min + an additional 5 s per cycle) at 68°C and then an extension step of 7 min at 72°C. The second PCR used NKp44 ATG frw (CAGGAAAAGGGCCACATGG) and NKp44 INT4C rev (TCCTGGGCAGAAGCTGTTC) using the same reagent and cycling condition as described above.

A 8.6-kb amplification product, containing exons 4 and 5, intron 4, 5′ and 3′ flanking regions, was obtained using a first amplification using 44-ex3-frw1 (CCAGACCCAGACCTCTTG) and 44-ex5-rev (AAATGTGGGGATGCAGACAG) followed by a second reaction with 44-ex4-frw2 (CCAGGCCACAGAACTCCA) in combination with 44-ex5-rev2 (CAAAGCGTGTTCATCATCATTA). Both amplifications were performed using the Expand High Fidelity PCR System (Roche) and the following cycling condition: 94°C for 2 min, 10 cycles each 15 s at 94°C, 30 s at 60°C and 8 min at 68°C and next 20 cycles each 15 s at 94°C, 30 s at 58°C, (7 min + an additional 20 s per cycle) at 68°C and then an extension step of 7 min at 68°C.

Both amplicons have been subcloned (Expand vector I, Roche) and the Mf-NKp44 gene sequence was obtained using BigDye Terminator Cycle Sequencing Kit and ABI3100 capillary electrophoresis automatic DNA sequencer.

5′ Rapid amplification of cDNA ends in P. troglodytes

To this end, the first-strand synthesis was carried out with AMV reverse transcriptase at 55°C for 1 h using the specific primer *NCR2*-SP1 (CTGGGCTTGGAGCTGGTGACTAAC) followed by the addition of homopolymeric A-tail to the 3′ end using terminal deoxynucleotidyl transferase. Tailed cDNA was amplified using *NCR2*-SP2 (CTTACACCAGCCTTTCTTCTCGTA) and oligo (dT)-anchor primer and finally a nested PCR amplification was performed using *NCR2*-SP3 (CTGCCCTGCCACACTTTGAAG) and a specific anchor primer. Amplicon was subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen) and checked for DNA sequence.

Primers and conditions used for the isolation of the NCR2 (NKp44) cDNA in primates

Total RNA was extracted starting from frozen cell pellet (5×10^5) using the acid guanidinium thiocyanate–phenol–chloroform extraction method (pepGLOD RNA-pure, PEQLAB, Erlangen, Germany) from either resting or IL-2-cultured NK cells derived from *P. troglodytes* and human donors. Analysis of *NCR2* mRNA expression was performed by reverse transcription (RT)–PCR using first-strand cDNA oligo (dT)-primed material (ReverAid H minus cDNA synthesis kit,

Fermentas International Inc., Burlington, Ontario, Canada) following the producer's indications. *NCR2* gene expression in humans and chimpanzees was evaluated with end-point PCR using Hs/ch 44 frw-716 (AGGAGGCTTCAGCACTTGTG) and Hs/ch 44 rev-916 (CGGACTTAGAGACAGAGTTG) primers, designed to cross-hybridize with *NCR2* in both species. Amplification reactions, utilizing *Taq* recombinant DNA polymerase (Invitrogen), have been performed denaturing the template for 10 min at 94°C and cycling 40 times for 30 s at 94°C and 45 s at 58°C followed by a 7-min extension step at 72°C. Amplified products have been separated and analyzed by electrophoresis on NuSieve® 3:1 Agarose (Lonza Group Ltd, Basel, Switzerland) and Photodocumentation has been acquired with ChemiDoc EQ and Quantity One Software version 4.5.0 (BioRad, Milan, Italy).

Quantitative PCR

A more precise analysis of NKp44 gene expression in humans, chimpanzees and macaques has been evaluated by real-time PCR using SYBR *Premix-Ex-Taq* (Takara Diatech, Jesi, Italy) following the manufacturer indications. The primers cross-reacting with the above-mentioned species and designed on different exons were for NKp44: UNI-44 frw (TCTAAGTCCGTCAGGTTCTATCTG) and UNI-44 rev (TGGAGCGTGGAGTTCTGTG) and for β-actin, used as internal reference gene: β-act UNI frw (CGCGAGAAGATGACCCAGA) and β-act UNI rev (CTCGTAGATGGGCACAGTGTG). Analysis was performed denaturing the template for 10 s at 95°C and cycling 40 times for 5 s at 95°C and 35 s at 55°C using the ABI7500 real-time PCR instrument. Relative quantification has been performed using $\Delta\Delta C_T$ methods using β-actin as reference gene.

Isolation of P. troglodytes NCR2 (NKp44) and DAP-12 cDNAs by RT–PCR

The 855-bp amplicon containing the complete open reading frame (ORF) amplification of *NCR2* was obtained, using the sequence information obtained by 5′ rapid amplification of cDNA end (RACE), using the NKp44 orf frw (CCCACGAGCACACAGGAAAA) and NKp44 orf rev (TCACAAAGTGTGTTCATCATCATCATCGCTTAT) denaturing for 10 min at 94°C followed by 40 cycles of 30 s at 94°C and 45 s at 55°C and a single extension step at 72°C for 7 min. The PCR product was subcloned into the pcDNA3.1/V5-His-TOPO vector and checked for DNA sequence as indicated above.

The 415-bp amplicon sequence containing the complete ORF for DAP-12 signal transducing polypeptide necessary for the NKp44 surface expression (21) have been obtained with Ptdap12-frw (CAGCATCCGGCTTCATGG) and Ptdap12-rev (GGTGGGCTTCAGGAATGG). PCR amplification was performed using the same cycling conditions for PtNKp44 and the amplicon was subcloned into pcDNA3.1/V5-His-TOPO vector and checked for DNA sequence as indicated.

Transient transfections

HEK293T cells were transiently co-transfected with p pcDNA3.1-PtNKp44 and pcDNA3.1-PtDAP12 utilizing Jet-PEI™ (PolyPlus-transfection, Illkirch, France). Control transfection with the corresponding NKp44 and DAP-12 human cDNA constructs was performed in parallel experiments. Briefly, cells seeded on six-well plate at 1×10^5 per well 24 h before transfection were incubated with 2 μg of each plasmid constructs and 4 μl of JetPEI using DMEM/10% FCS. After 48/72 h, transfected cells were analyzed by cytofluorometric analysis. Cell transfectants stained with anti-NKp44-specific mAbs followed by an isotype-matched PE-conjugated goat antibody were analyzed by flow cytometry using FACSCalibur cell analyzer (Becton Dickinson, Milan, Italy).

Results

NCR2 gene characterization in macaques

Since the *NCR2* locus is reported to lack in the mouse genome (24, 25) and since orthologous gene could not be identified in the available rat genome, appearance of *NCR2* locus may arise after the rodents branching point during phylogenesis. Thus, in view of the previous successful characterization of several triggering receptors in macaques and failure in identifying NKp44 expression on their NK cells (10, 16), we first examined evidence for the existence in *M. fascicularis* of the *NCR2* (NKp44) locus and its genomic organization.

Based on our original isolated cDNA sequence of human NKp44 (*NCR2*: AJ225109), as well as information of *H. sapiens* genome sequence and on preliminary informations (at the time of preparation of this manuscript) of *P. troglodytes* genome sequence assemblies, we designed deoxynucleotide primers able to cross-amplify human, chimpanzee and macaque NKp44 loci. Following this approach, we could successfully amplify the complete *M. fascicularis* NKp44 locus (Fig. 1A). The present results on *M. fascicularis* are in line with the recently characterized *M. mulatta* genome sequence (31). These results date back the appearance *NCR2* locus of ~2.5 MYA since cynomolgus-like monkeys represent the rhesus monkeys' ancestors (38) and confirm that the appearance of this locus dates back at least 25 million years during phylogenesis (37), at the time of macaque speciation.

Similar to the human homolog (AL136967), the NKp44 gene in *M. fascicularis* is organized in five exons (AM183302). The overall intron/exon organization in these two species is similar, with only one difference in the length of exon 3 that in *M. fascicularis* is 54-nucleotide longer (190 bp compared with 136 bp). This additional nucleotide sequence does not affect the NKp44 ORF and its presence and correct splicing was further confirmed by the analysis of a cDNA sequence fragment (AM402952) obtained using forward and reverse primers specific for exons 2 and 4, respectively. These additional nucleotides found on exon 3 could be envisaged to encode for an 18-amino acid longer 'stalk region'. Other additional cDNAs using retained fragments of intron 2 have been isolated in *M. fascicularis*. Although the use of alternative/cryptic splice sites have been already described in humans (NKp44RG2 variant; AJ010100) coding for a 36-nucleotide longer NKp44 ORF, all the alternatively spliced products characterized in *M. fascicularis* generate sequence frame shifts resulting in abortive transcripts. In particular, two representative cDNAs are shown in Fig. 1(B), both retaining 100 bp (AM402951) or 86 bp (AM408172) producing out-of-frame transcripts.

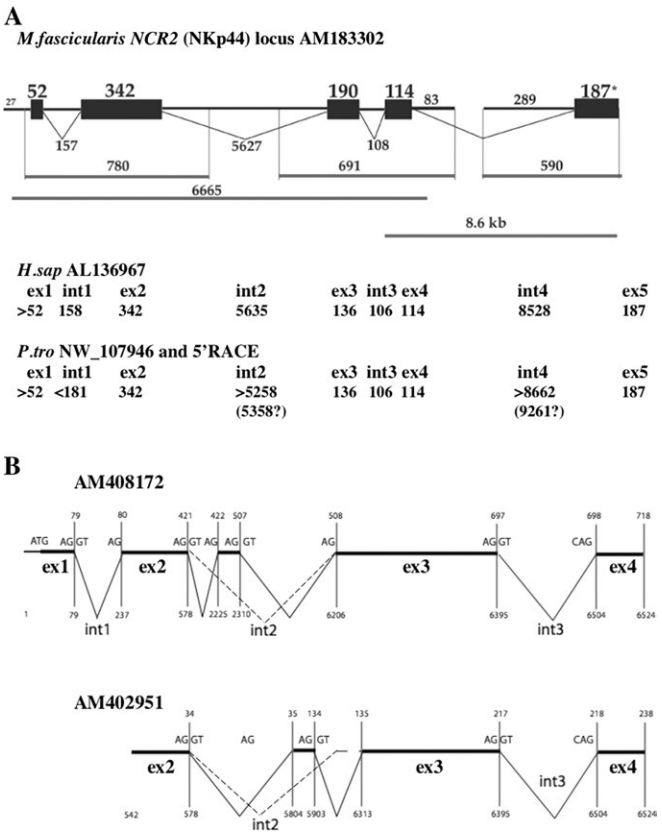

Fig. 1. *NCR2* gene organization in primates. Panel (A) Exons are shown as black boxes and the length of exons and introns are indicated. Lines under the gene representation indicate the length and the location of the amplified fragments used for sequence analysis. On the bottom are indicated briefly information regarding the sizes of equivalent region of the human and chimpanzee NKp44 gene loci. Panel (B) Frequently retained fragment of intron 2 in *Macaca fascicularis* cDNAs. Solid lines represent cDNA sequences of two unfunctional (out of frame) alternatively spliced clones (AM408172, AM402951) obtained from *M. fascicularis*. Exon/intron splice sites and nucleotide residue position in the cDNA (upper residues) and in the genomic organization of *M. fascicularis* NKp44 locus (AM183302) (lower residues) are indicated.

Although the transcription of the *NCR2* locus in *M. fascicularis* often resulted in the isolation of incorrectly spliced non-functional transcript sequences, we were able to identify NKp44 cDNA sequence containing the complete ORF, also. This cDNA sequence of 885 nucleotides (AM404415) shares 89% nucleotide identity with its human homologs and encodes a protein sequence displaying 84% amino acid identity compared with the human NKp44 receptor (Fig. 2).

NCR2 transcription in *M. fasciculari*-activated NK cells is very weak and resulted to be 3-fold less abundant than in resting human NK cells, which already do not express surface NKp44, and >350 times less the transcription level found on activated human NK cells (Fig. 3). Therefore, although these results warrant further analysis, they provide for the first time evidence that *M. fascicularis* shares overall gene organization similar to the human homolog, with very weak transcription levels, and usage of incorrect acceptor/donor splice sites suggesting a not-yet-fine-tuned transcription.

NCR2 gene expression in P. troglodytes NK cells

In view of the inefficient transcription of the *NCR2* locus in *M. fascicularis*, we next studied whether non-human primates closer to *H. sapiens* develop a functional NKp44 receptor.

At the time of manuscript preparation, the information available on the assembled *P. troglodytes*-genomic *NCR2* sequences (NW_107946) was aberrant in the 5′ NKp44 gene region. We therefore decided to perform rapid amplification of *P. troglodytes NCR2* 5′ cDNA end (RACE kit ends, Roche, Mannheim, Germany) using total RNA extracted from IL-2-cultured NK cells of a healthy chimpanzee animal donor. In particular, RNA–pure total RNA was used to generate a 182-bp 5′ cDNA fragment by RT–PCR using primers located on exon 2 and sharing identities between the *H. sapiens*- and *P. troglodytes*-genomic *NCR2* sequences AL136967 and NW_107946, respectively.

Using RT–PCR on activated *P. troglodytes* NK cells, we isolated an 855-bp amplified cDNA fragment (AM087959) containing an 831-bp complete NKp44 ORF. Sequence alignment between chimpanzee and human NKp44 showed a 99% nucleotide and 97% amino acid identity, respectively (Fig. 2B). Comparison of the NKp44 sequences of chimpanzees and macaques with the known human sequence showed that the NKp44 transmembrane-embedded lysine and the putative ligand-binding site, which is rich in basic residues (26), are both conserved in these non-human primates. A difference, however, is detected in the cytoplasmic tail, where *H. sapiens* and *P. troglodytes* both display a non-functional immune tyrosine-based inhibitory motif-like sequence (23, 39), while this is not present in the putative cynomolgus-encoded polypeptides (Fig. 2A).

We next studied whether differences in transcription regulation could be found in different species and could help explain different surface protein expression. Analysis of NKp44 transcription in *P. troglodytes* revealed the presence of consistent mRNA levels in both resting and activated NK cells (Fig. 3A). Similar results with basal NKp44 transcription were obtained also on human NK cells. Figure 3(B) shows a quantification of NKp44 transcripts, indicating that transcription of NKp44 is clearly inducible upon NK cell activation in *H. sapiens*, while its expression increased less efficiently in *P. troglodytes*. Although NKp44 transcription is clearly detectable in resting NK cells in both *H. sapiens* and *P. troglodytes*, its up-regulation upon activation is essentially observed in human NK cells, with >130-fold transcription levels compared with only 2-fold increments typical of activated chimpanzee NK cells (Fig. 3C). Interestingly, analyzing the absolute transcription level, NKp44 transcription is consistently higher (5-fold) in *P. troglodytes* compared with *H. sapiens*, while upon activation human NK cells transcribed >12 times the amount expressed in activated chimpanzee NK cells (Fig. 3B and C). These observations account for the >10-fold higher levels of NKp44 transcription in human-activated NK cells compared with chimpanzee-activated NK cells. This effect is unlikely to be attributed to the use of human IL-2 and to differences in its activity. Several reasons support this view, including 100% IL-2 protein sequence identity in chimpanzees and humans and the known capacity to efficiently induce NKp30 transcription and expression in

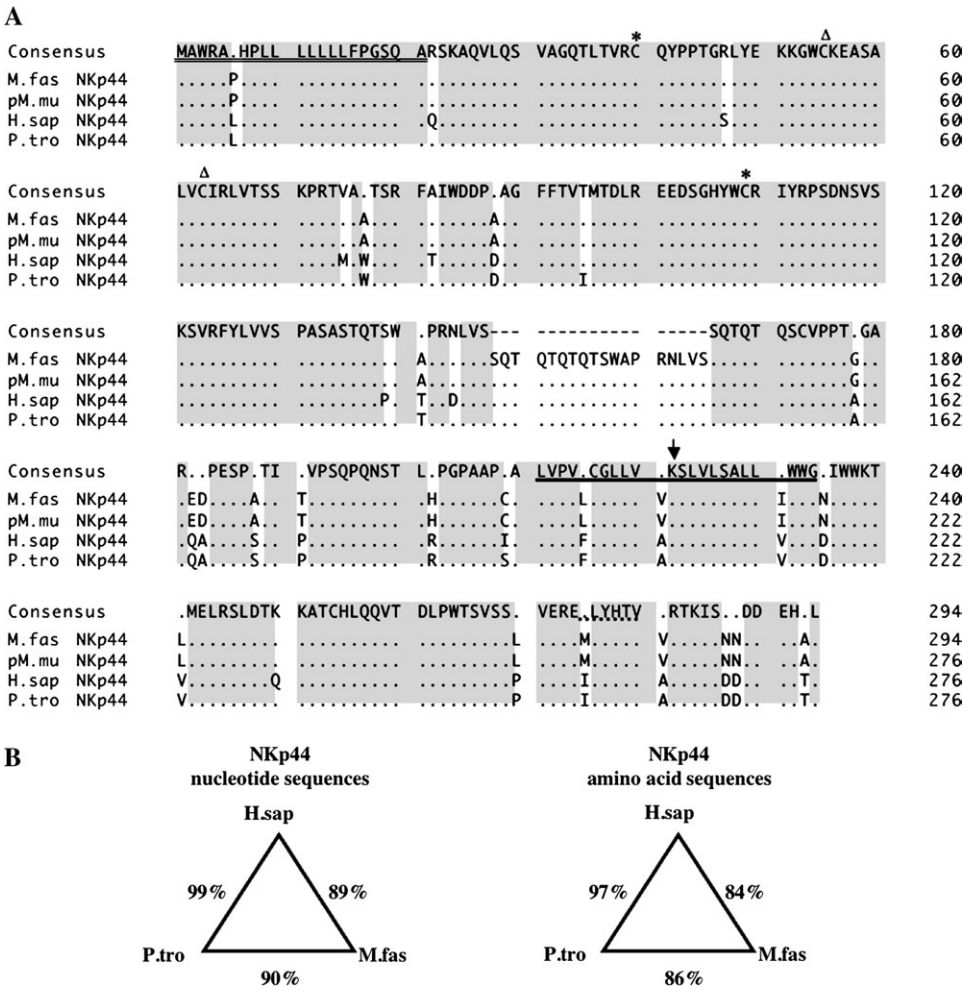

Fig. 2. NKp44 protein sequence in primates. Panel (A) Sequence alignment of chimpanzee, human and macaques NKp44 receptors. *Pan troglodytes*, *Homo sapiens* and putative *Macaca fascicularis* and rhesus protein sequences have been aligned using GeneWorks suite (Oxford Molecular Group Ltd, Oxford, UK). Consensus sequence is shown on top, residues identical with the consensus are indicated by dots; dashes were introduced to maximize homologies. Overall identity regions are shaded. Protein sequence analysis have been performed with Signal IP 3.0 and TMHMM servers (www.cbs.dtu.dk/services), transmembrane region (underlined) and leader sequence (double-underlined) are indicated under the consensus sequence, while an arrow points to the conserved lysine (K) residue, inside the transmembrane region, involved in the association with TYROBP/DAP-12 signal transducing polypeptide. Asterisks indicate the cysteines involved in the formation of the Ig-V domain disulphide bridge, while open triangles characterize an additional disulphide bridge typical of the NKp44 V domain and defining a novel Ig-V domain subset. Dashed line indicates an unfunctional intracytoplasmic immune tyrosine-based inhibitory motif-like motif. Panel (B) Graphical representation of primate NKp44 nucleotide or amino acid pairways sequence homology. Numbers indicate % of identical residues between the receptors of the indicated species, H. sap: *Homo sapiens*, P. tro: *Pan troglodytes* and M. fas: *Macaca fascicularis* (cynomolgus).

P. troglodytes-activated NK cells (15). In addition, the generalized and efficient use of human IL-2 in chimpanzee is reported in T-lymphocytes and NK cell studies. These results show that *NCR2* transcription occurs in resting NK cells both in human and chimpanzee and that there is a limited induction in chimpanzees resulting in >1 log lower accumulation of transcripts compared with human-activated NK cells.

Sequence alignment and phylogenesis of NKp44

In consideration of the presently identified NKp44 mRNA sequences of *P. troglodytes*, *M. fascicularis* and *M. mulatta* (inferred transcript from the assembled genome) and of the observed differences in transcription, we performed a detailed sequence analysis to study evolutionary relationship.

Nucleotide alignment and phylogram analysis of primate NKp44 sequences showed 95% mean identity when comparing exons, with the only exception of exon 3, where a 54-bp insertion is found typically only in *M. fascicularis*. A slightly weaker intron identity (87%) was found. Some nucleotides both in exons and intron sequences were found to be shared exclusively either between human and chimpanzee or between macaque species.

A preliminary analysis of exon sequences for species-specific nucleotide substitutions indicates a higher rate in humans compared with chimpanzees and *M. fascicularis* (9, 1, 1, respectively). The same analysis performed on intron sequences (intron 2) that are known to be less exposed to evolutionary pressure shows a higher rate of nucleotide

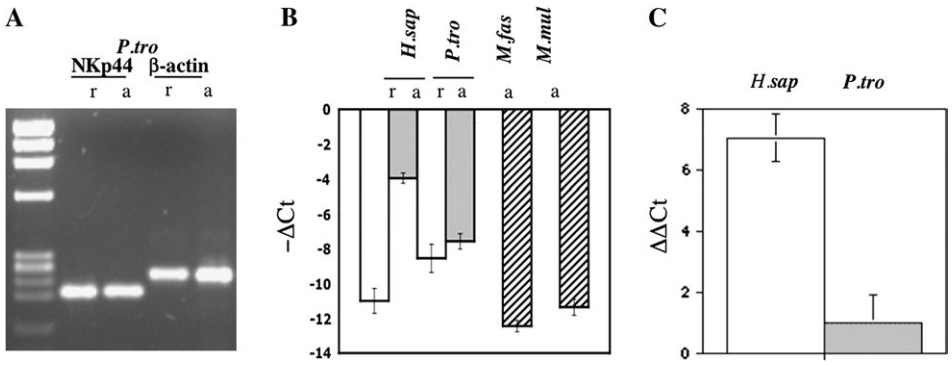

Fig. 3. Analysis of NKp44 mRNA expression in primates. Here, we show a representative test performed on two different donors for each primate species analyzed. Panel (A) PCR amplification products obtained from resting (r) and activated (a) chimpanzee NK cells show that NKp44 mRNA is transcribed both in resting and in activated NK cells. As control, the same RNA samples have been tested for control β-actin amplification as indicated. The first lane has been loaded with the Roche DNA molecular weight marker IX (0.072–1.35 *kbp*). Panel (B) NKp44 transcript expression levels normalized (ΔC_T) with the amount of an internal reference gene (β-actin) have been analyzed on material extracted from resting and activated NK cells of human and chimpanzee origin, while the same analysis has been performed on RNA obtained from activated macaque NK cells, only. Panel (C) Bars represent exponential-fold increase ($\Delta\Delta C_T$) of NKp44 mRNA molecules over resting conditions (standardized using the internal reference β-actin gene) after *in vitro* activation of purified NK cells. Open bars indicate *Homo sapiens* transcription behaviors, while dotted bars represent *Pan troglodytes*, standard deviation (+/−) values are indicated with lines.

substitution in *P. troglodytes* compared with *H. sapiens*, *M. fascicularis* and *M. mulatta* (105, 58, 50, 11, respectively). In addition, human and chimpanzee sequences appear to be evolved introducing species-specific nucleotides at a higher rate (in exons for human and in introns for chimpanzees) compared with macaques. It should be noted that this analysis is derived from sequences obtained from four chimpanzees and two macaques, respectively. Although the speculations based on this analysis are intriguing, they may represent donor-specific mutations rather that species-specific drifts and additional studies are required to conclusively address this issue.

Overall, the sequences of the putative encoded proteins of the four species analyzed display 84% amino acid identity. *M. fascicularis* and the predicted *M. mulatta* cDNA sequences share 100% amino acid identity and are characterized by an 18-residue longer sequence. Human and chimpanzee NKp44 share 97% amino acid identity. Analyzing the sequences of the four species, we identified 21 conserved amino acid pairs that are shared exclusively either between macaques or between human and chimpanzees. In addition, eight other residues (six in the extracellular region) are expressed only by the human NKp44 sequence, while two are unique only for chimpanzees (Fig. 2A). One of the *H. sapiens*-specific amino acid residues (S26R) is located in an exposed loop, probably available for ligand interaction, characterizing the CDR1 region of the NKp44 crystallographic structure (1 HKF) (26).

Molecular and phenotypic characterization of NKp44 expressed on chimpanzee NK cells

Using mAbs that were previously raised against human NKp44 (22), we next investigated whether mAb reactivity could be identified for homologous NKp44 molecules in non-human primates. Cytofluorometric analysis of resting and activated *M. fascicularis* NK cells using the available mAbs failed to identify specific mAb binding. Although the mAb specificity may not be optimal, lacking cross-reactivity with putative macaque molecules, these data might agree with the very weak NKp44 transcription in cynomolgus and rhesus macaques. Since NKp44 transcript production in activated macaque NK cells is ~3-fold less than the one found in resting human NK cells (which nevertheless do not express surface proteins), it is possible that macaques do not express surface NKp44 molecules at detectable levels (10).

As far as chimpanzees are concerned, similar to human resting NK cells, no surface expression of NKp44 could be detected on resting chimpanzee NK cells despite detectable basal mRNA transcription (Fig. 4, upper panels). Upon NK cell activation *in vitro* in the presence of IL-2, NKp44 mAb reactivity could be detected in purified NK cell cultures although at lower density (MFI) compared with human-activated NK cells (Fig. 4, lower panels). In order to further confirm that the apparently weaker staining was related to specific mAb recognition of chimpanzee NKp44 molecules, we next performed transient transfection experiments. As shown in a representative experiment (Fig. 5), cells transfected with either chimpanzee or human NKp44 constructs could be stained at comparable levels and efficiency by anti-human NKp44 mAbs of both γ1 and μ isotypes. Therefore, the lower cytofluorometric signal observed on chimpanzee NK cells was not attributable to different mAb affinity for NKp44 molecules but rather reflected lower molecule density.

Thus, inefficient transcription is likely associated to defective NKp44 expression in macaques (*M. fascicularis*), while mAbs (both γ1 and μ isotypes) specific for human NKp44 efficiently recognize chimpanzee NKp44.

Functional analysis of NKp44 on P. troglodytes NK cells

In order to verify the *P. troglodytes* NKp44 functional activity, we next analyzed its triggering potential in redirected killing assays. Serial NK cell dilutions were assayed against P815 $Fc\gamma R^+$ cells in a ^{51}Cr-release assay in the presence of mAbs

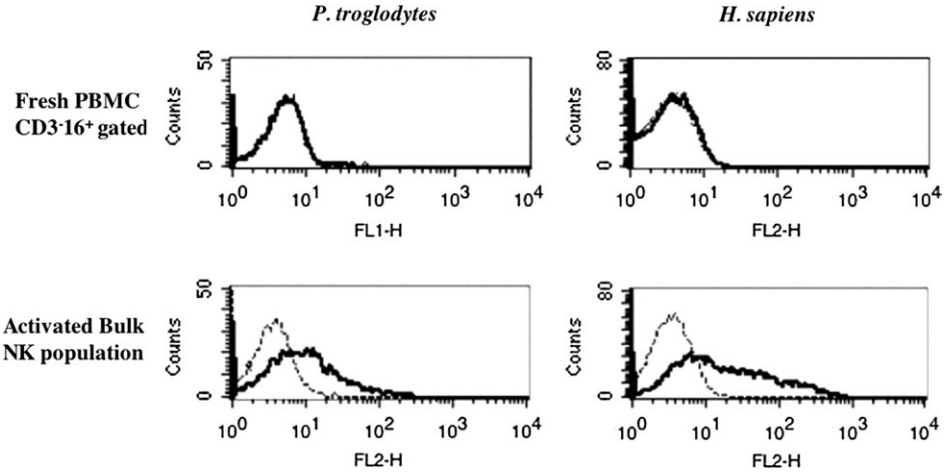

Fig. 4. Analysis of surface expression of NKp44 on resting or activated polyclonal NK populations. NK cells were gated according to lymphocyte forward- and side-scatter patterns, and a three-color flow cytometry was performed. Upper panels show the anti-NKp44 mAb (Z231) surface reactivity on freshly derived PBMC, gated on $CD3^{-}CD16^{+}$ cells, from chimpanzee cells (Juus on the left) and from human normal donor cells (CH4437 on the right). The same analysis (lower panels) has been performed on activated polyclonal NK cells as indicated on chimpanzee (left) and human (right) samples.

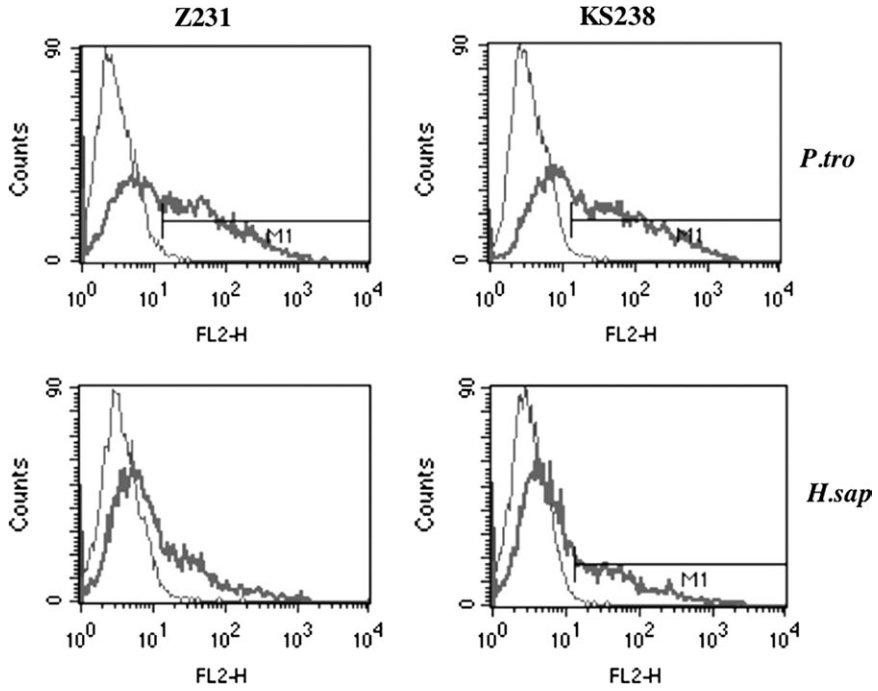

Fig. 5. Analysis of surface expression of chimpanzee- and human-derived NKp44 cDNA constructs in transiently co-transfected cells. Cytofluorometric analysis of HEK293T cells transiently co-transfected either with *Pan troglodytes* NKp44/DAP-12 (top panels) or human NKp44/DAP-12 (bottom panels) cDNA constructs using either Z231 or KS238 mAbs originally selected for human NKp44 reactivity. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) on *x*-axis plotted with number of events on *y*-axis. For each analysis, 10^4 events have been acquired.

specific for NKp44, NKp30, NKp46 or irrelevant mAbs. Effector NK cells were represented by chimpanzee or human 'in vitro' activated NK cell populations. As shown in Fig. 6, target cell killing occurred using an anti-NKp44 mAb similarly to NKp30 or NKp46 triggering with both human and chimpanzee effectors. In these experiments, differences in NKp44-mediated cytotoxic activity were observed, with lower lysis in NK cell populations derived from chimpanzees. The degree of target cell killing is known to be directly proportional to the cell-surface molecule density (39). The differences in the

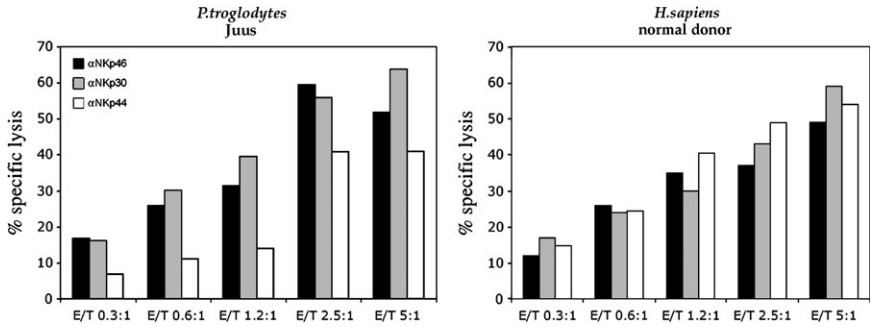

Fig. 6. Cytolytic activity of NK cell populations derived *in vitro*. NK cell effectors were assayed in a 4-h ^{51}Cr-release assay against P815 cells at different E/T ratios either in the absence (control) or in the presence of anti-NKp46, anti-NKp30 and anti-NKp44 mAbs (IgG). The increase in ^{51}Cr release over baseline (control) reflects the ability of a given mAb to trigger the lytic machinery of the cells. Results obtained from a representative experiment out of three are shown. Data obtained on chimpanzee Juus (left) and human donor (right) are shown as bar diagrams as % of specific lysis using E/T ratios from 0.3:1 to 5:1, filled bars represent data using anti-NKp46, while 'gray bars' and 'open bars' represent redirected killing adding anti-NKp30 or anti-NKp44, respectively.

extent of target cell lysis recorded in these experiments using NK cells from these two species could be associated to the observed differences in NCR expression.

These experiments therefore confirmed that NKp44 molecules expressed on the surface of chimpanzee NK cells have similar function and triggering ability compared with their human homologs and with other NCRs already known to be expressed and functional in chimpanzee and humans.

Discussion

A relatively complete molecular characterization of NCRs is becoming available through extensive studies on non-human primates, rodents and other mammals (7–16, 36). One exception among NCRs is represented by NKp44. Previous attempts were unsuccessful in identifying NKp44-genomic sequences or transcripts in mice and macaques, respectively. This suggested a possible and recent phylogenetic appearance in humans. In order to verify this hypothesis, we performed an in-depth genomic, transcriptional and functional analysis of NKp44 in macaques and chimpanzees, among the closest humankind's living relatives.

Molecular analysis of genomic NKp44 sequences in *M. fascicularis* using cross-species reacting primers allowed the identification of the NKp44 locus in this species. Its organization is in line with the organization of the human locus, with only a major difference represented by a 54-bp insertion in the region coding for exon 3. During manuscript preparation, the complete *M. mulatta* genome became available (33). There is no mention in the macaque data of the 54-bp nucleotide insertion that was consistently found on repeated experiments in this work. This may indicate a difference between cynomolgus and rhesus NKp44 locus and could support the view of an evolutionary maturation.

A very low level of transcription of cynomolgus NKp44 and out-of-frame transcripts were found in this study, suggesting that macaque NKp44 could represent either a recently acquired or old obsolete gene. Although further work assessing the quantitative ratio of inefficient transcripts to full transcripts is needed to confirm the presence of prevalent inefficient transcription, which was not evaluated, the present qualitative findings suggest limited transcription tuning and probably with currently limited positive selection for its functionality. Together with very low full transcript levels, inefficient transcription also would explain previous failed attempts to identify NKp44 surface expression by cytofluorometric analysis and cDNA cloning (10). It is also in line with the reported absence of *NCR2* gene locus in phylogenetically distant species (i.e. mice) (24) and its presence in subsequently diverging species like *P. troglodytes* (as presently reported) and humans (23).

An unexpected and previously unreported observation derived from the present work is represented by the finding of detectable transcription with lack of surface expression of NKp44 in both resting chimpanzee and human NK cells. The amount of basal NKp44 transcription was 5-fold higher in chimpanzee compared with human, while its inducibility is much lower as compared with human NK cells. These results suggest that NKp44 may undergo post-transcriptional regulations in these species. The 12-fold differences in transcript expression between activated human and activated chimpanzee NK cells do not account for only slightly reduced molecule density on chimpanzee NK cells as determined by cytofluorometric analysis. Basal NKp44 transcription on resting NK cells in the absence of surface molecule expression bears similarity to the recent observation of chimpanzee NKp30 regulation (15) where basal RNA transcription is already present in resting NK cells that express very low or no surface molecule and increases upon IL-2 activation. Inadequate recognition of NKp44 molecules in chimpanzees could be ruled out by transfection experiments showing adequate recognition of chimpanzee NKp44. DAP-12-deficient patients fail to express cell-surface NKp44 molecules since the DAP-12 signal transducing polypeptide is known to be crucial for NKp44 cell-surface expression (23). Therefore, other mechanisms including failure of efficient DAP-12 association, defective DAP-12 transcription, altered ribosomal entry or other post-transcriptional mechanisms including RNA interference could be involved in NKp44 post-transcriptional regulation (23, 40, 41).

Overall, the present findings of an inefficient NKp44 transcription in *M. fascicularis*, of its modified inducibility in

chimpanzees and humans and the appearance of a higher rate of *H. sapiens*-specific amino acid substitutions contribute to the possible notion of an NCR which appeared more recently than the others (e.g. NKp46 and NKp30) during evolution. The unique amino acid substitutions in the human protein may represent structural adjustments aimed at the optimization of its function, possibly determined by a fine-tuning of NKp44 putative ligand recognition. These structural characteristics have not been found in a previously characterized NCR with similar extracellular Ig-V domain organization (NKp30) although in this case differences in transcription or post-transcriptional regulation also have been observed between *H. sapiens* and *P. troglodytes* (15). In this respect, the present finding of NKp44 transcription/expression in humans may reveal similarities to NKp30 regulation in chimpanzees and further suggest the view of a more recent appearance and speciation of *NCR2* molecules.

Finally, direct NKp44–pathogen interaction has been reported to be involved in HIV-1 recognition (41, 42). The present findings of a lower (~4-fold) NKp44 molecule density on activated chimpanzee NK cells, in addition to the recently observed difference in NKp30 regulation (15), could be involved in the more benign course of HIV infection in chimpanzee. A model of interactions where a dampened NKp44–ligand (pathogen, HIV) interaction would add to a dampened NK–DC cross-talk via NKp30 with reduced NK cell activation (15, 43, 44) could contribute to chimpanzee protection from generalized T and B cell activation and from progression to AIDS (29, 45). Although chimpanzees are our closest living creatures, the differences found in the regulation of triggering NK cell receptors, with two inducible surface NCR in chimpanzees (NKp44 and NKp30) instead of NKp44 only in humans, may have impact on innate and adaptive immune systems, as well as in pathogen-mediated immune responses.

In conclusion, the results of the present study support the view of a recent evolutionary acquisition of *NCR2*/NKp44 and underscore the relevance of NCR-mediated triggering in the possible modulation of immune responses. Slight differences in the mechanisms and in the regulation of NCR surface expression may result in different approaches to the containment of infectious diseases (e.g. HIV), thus these informations need to be carefully balanced with our present knowledge of the fine-tuning of immune responses to tumors and infections in primates (46). Different NK cell regulation in macaques and chimpanzees compared with humans may impact on the interpretation of animal models and of vaccine trials.

Funding

Associazione Italiana Ricerca sul Cancro to R.B.; Istituto Superiore di Sanità [Programma Nazionale AIDS, contratto n° 40F.55, Italian Concerted action for AIDS Vaccine (to A.D.M.); Accordi di Collaborazione Scientifica 40D61, 45D/1.13, 45F12] to A.D.M.; Ministero dell'Università e Ricerca Scientifica, Ministero della Salute (RF 2002/149) to A.D.M.; National Institutes of Health (POI A148225) to J.H.

Abbreviations

DC	dendritic cell
E/T cell	effector/target cell
iNKR	inhibitory NK receptor
ITIM	immune tyrosine-based inhibitory motif
MFI	mean fluorescence intensity
MYA	million years ago
NCR	natural cytotoxicity receptor
RT	reverse transcription

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