Identification, molecular cloning and functional characterization of NKp46 and NKp30 natural cytotoxicity receptors in *Macaca fascicularis* NK cells

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Natural killer (NK) cell recognition and function in humans is regulated by multiple cell surface receptors. The "on" signal leading to NK cell triggering is primarily mediated by natural cytotoxicity receptors (NCR). Analysis of NK cells in primate animal models is of particular relevance because NK cells may play an essential role in host defenses against infections. We analyzed *Macaca fascicularis* PBMC and *in vitro*-derived NK cell populations and clones by cytofluorometry, using a wide panel of mAb, and by cytolytic activity assays. In addition, RT-PCR strategy and transient transfections were used to isolate *M. fascicularis* NCR. NCR-specific mAb reactivity (anti-NKp46 and anti-NKp30) was present on *M. fascicularis* PBMC and on NK cell cultures. Macaque NCR were functional in both redirected killing and in mAb-mediated masking assays. Cloning of macNKp46 and macNKp30 NCR homologous genes showed a high sequence similarity (86 % and 88 %, respectively) with their human counterparts. Attempts at identifying NKp44 surface reactivity and at cloning the macaque homologue were unsuccessful. NKp46 and NKp30 NCRs, but not NKp44, are highly conserved in *M. fascicularis* NK cells. This suggests the possibility of a staged appearance of the NCR during phylogenesis and provides a useful tool for the study of natural immunity correlates of protection in primate SIV/SHIV infection models.

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1 Introduction

NK cells are involved in primary host defenses against pathogen-infected and tumor cells [1–3]. NK cell recognition and function in humans is regulated by multiple cell surface receptors. Inhibitory NK receptors (iNKR) bind to HLA class I and deliver inhibitory signals to NK cells [1–3]. The "on" signal leading to NK cell triggering is primarily mediated by natural cytotoxicity receptors (NCR) upon binding to as yet undefined cell surface ligand(s) on both normal and pathogen-infected or tumor cells [4, 5].

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Abbreviations: NCR: Natural cytotoxicity receptors **SIV:** Simian immunodeficiency virus **iNKR:** Inhibitory NK receptor **KIR:** Killer inhibitory receptor **SHIV:** Simian/human immunodeficiency virus

Analysis of NK cells in primate animal models is of particular relevance because NK cells may play an essential role in host defenses against certain infections as well as in graft rejection. Due to similarities of biologic and genetic features, the infection of macaques with simian immunodeficiency virus (SIV) serves as the most suitable animal model for human immunodeficiency virus type 1 (HIV-1) infection [6–11]. Accordingly, this animal model is of major importance for the development of vaccines and antiviral therapies. In this respect, relatively little is known on the role played by the innate immunity during HIV-1/SIV infection and on its contribution to the mechanisms underlying vaccine-induced protection from HIV-1 challenge [6, 7].

In monkeys, the available information on receptors responsible for NK cell triggering or inhibition is limited, with the exception of recent analysis of MHC class I-specific iNKR in chimpanzees and macaques. These

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studies showed relevant degrees of divergence in killer inhibitory receptors (KIR) and iNKR sequences, with more pronounced differences found in macaques [12–14]. Recent analysis of primate NK cells showed that CD16 is expressed on both NK cells and a subset of monocytes [15]. In addition, there is still debate on whether CD56 is expressed on non-human primate NK cells only or also on monocytes [16]. Thus, at the present time, in the absence of truly NK-specific receptors, two- or three-color cytofluorometric analysis is required for the phenotypic identification of NK cells in non-human primates after exposure to pathogenic SIV or to simian/human immunodeficiency virus (SHIV).

The use of monoclonal antibodies (mAb) that recognize human NCR molecules could provide further insights on the phenotypic and functional properties of macaque NK cells and could therefore provide useful information in vaccine trials. The partial similarity of iNKR sequences between human and chimpanzee, together with the finding that one of the NCR homologues (NKp46) is conserved in rodent NK cells [17–19], suggest that a fair degree of sequence identity may exist between human and macaque NCR. Accordingly, available reagents (mAb) specific for molecules expressed on human NK cells may also recognize *Macaca fascicularis* NCR.

In the present study we show that NKp46 and NKp30 NCRs are highly conserved in *M. fascicularis*, as documented by comparative analysis of cDNA sequences, by reactivity with a panel of specific mAb and by functional assays. These data may also provide useful tools for an appropriate monitoring of the function and the involvement of NK cells in monkey SHIV/SIV vaccine models.

2 Results

2.1 Analysis of iNKR- and NCR-specific mAb reactivity on *M. fascicularis* PBMC

Human NK cells are usually identified by the surface expression of CD16, CD56 and iNKR molecules. However, other cell types, including T cells or monocytes may also express these surface markers. In contrast, the recently identified NCR are selectively expressed by human NK cells. In a first attempt to define the reactivity of the human NK cell-specific reagents available in our laboratory with NK cells from *M. fascicularis*, we analyzed freshly drawn PBMC obtained from six animals. Cytofluorometric analysis showed that several of the currently available anti-CD16 mAb, including XA59, νD4, Sus142, A6.96 and KDS1, stained monkey PBMC with a bimodal pattern of distribution of fluorescence intensity. A variable proportion of $CD16^+$ PBMC could be detected in all animals with a range between 10 % and 35 % of the cells, showing mainly interindividual differences when the same mAb was tested on PBMC from different *M. fascicularis*. Similarly, four of the anti-human CD56 mAbs available in our laboratory, including Gpr156, A6.220, FS280 and c218, and the human CD8-specific mAb XA41 reacted with monkey PBMC, although with different fluorescence intensity as compared to human PBMC (Fig. 1 A, Panel A).

We next analyzed the reactivity on *M. fascicularis* NK cells with a wide panel of mAb specific for various iNKR. None of the mAb specific for human iNKR, including p58.1, p58.2, p70, p140, CD94, NKG2A and LIR1/ILT2, reacted either with freshly drawn *M. fascicularis* PBMC or with *in vitro* cultured NK cell populations and clones (data not shown). On the contrary, mAb specific for human NCR, including NKp46 and NKp30, were found to react with PBMC of all the animals analyzed. In particular, 2–10 % of PBMC were stained by the NKp30-specific mAb AZ20 and Z25, while 1–10 % of the PBMC were stained by the NKp46-specific mAb BAB281 and KL247. In agreement with data on fresh human PBMC, NKp44-specific mAb (Z231, AZ140 and KS38) did not react with fresh *M. fascicularis* PBMC. A representative panel of cytofluorometric reactivities is shown in Fig. 1 B, Panel B.

To analyze the reactivity of the NCR-specific mAb also with activated NK cells from *M. fascicularis*, we further derived NK cell populations and clones. Analysis of activated NK cell populations derived from two animals and cultured *in vitro* in the presence of rIL-2 confirmed the presence of surface molecules recognized by mAb specific for human CD56 and CD16. In addition, these activated NK cell populations were stained by anti-NKp30 mAb (Z25, AZ20) to a similar extent as human NK cell populations (Fig. 2). Although anti-NKp46 mAbs (BAB281, KL247) stained a proportion of monkey NK cells, the reactivity was clearly lower than that obtained using human NK cells. On the other hand, no reactivity was observed with anti-NKp44 mAb (Z231, KS38), that have been previously demonstrated to react selectively with activated human NK cells [4] (Fig. 2).

2.2 NCR-mediated NK cell triggering in *M. fascicularis*

The lytic activity of NK cell ($CD16^+$) populations and of four representative NK cell clones was assayed in a 4 h ^{51}Cr-release assay against K562 target cells. Both the NK cell populations and clones lysed K562 cells at low effector/target cell ratios (40 % lysis at 10:1 E/T ratio for the polyclonal population and as much as 70–90 % at 2.5:1

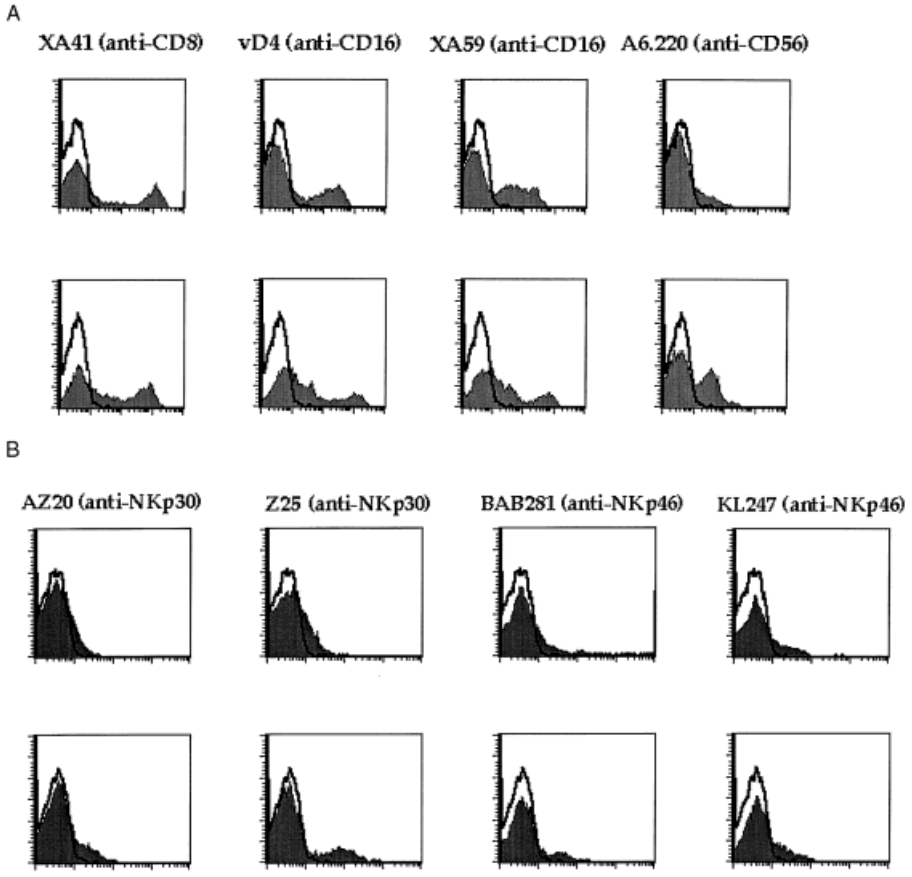

Fig. 1. (A) Comparative analysis of CD16, CD56 and CD8 surface expression in human and *M. fascicularis* PBMC. Cytofluorometric analysis of CD8, CD16 and CD56 expression on *M. fascicularis* PBMC (upper panels) and on human PBMC (lower panels) in two representative experiments. Representative diagrams are shown for 2 of the 6 anti-CD16 mAb and for 1 of the 5 anti-CD56 mAb that were tested. Green fluorescence intensity (horizontal axis) represents reactivity to the indicated mAb. Results are expressed as logarithm of green fluorescence intensity (arbitrary units) versus number of events. For each analysis 10^4 events were counted. (B) Comparative analysis of NKp30 and NKp46 expression in human and *M. fascicularis* PBMC. Cytofluorometric analysis of NKp30 and NKp46 expression on *M. fascicularis* PBMC (upper panels) and of human PBMC (lower panels) in two representative experiments. Green fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of green fluorescence intensity (arbitrary units) versus number of events. For each analysis 10^4 events were counted.

E/T ratio for the clones). The results demonstrated that $CD16^+$ NK cells from *M. fascicularis* display NK-mediated cytolytic activity.

We next analyzed whether the surface molecules recognized by human NCR-specific (NKp46, NKp30) mAbs on *M. fascicularis* NK cells displayed the same functional properties as their human homologues. To this end, we analyzed wether anti-CD16 (νD4, KD1), antiNKp46 (BAB281) or anti-NKp30 (Z25, AZ20) mAb could mediate cell triggering in a redirected killing assay. In these experiments, NK cell populations or clones were analyzed for their cytolytic activity against the $Fc\gamma R^+$ P815 murine target cell line, either in the presence or in the absence of the above mAb. As shown in Fig. 3 both anti-NKp30 and anti-NKp46 mAb, as well as anti-CD16mAb, induced lysis of $Fc\gamma R^+$ P815 cells. The lower cytolytic activity induced by anti-NKp46 mAb (25–35 %) in *M. fascicularis* NK cells as compared to human NK cells is not surprising since the surface expression of NKp46 in monkey $CD16^+$ NK cells was lower (5–8 %) than in human NK cells, and also lower than the expression of NKp30 and CD16 (Figs. 2 and 3 A).

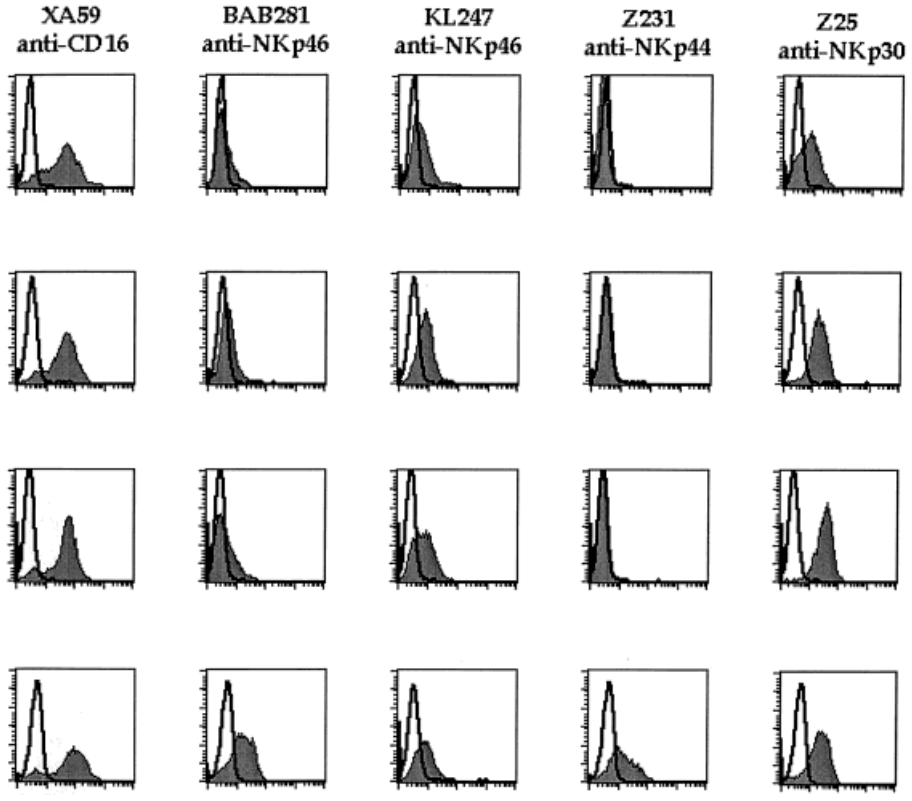

Fig. 2. Expression of CD16, NKp46, NKp30 and NKp44 on *in vitro* cultured human and *M. fascicularis* NK cells. Cytofluorometric analysis of CD16, NKp46, NKp44 and NKp30 expression on a representative *M. fascicularis* NK cell population (upper panels), on two representative *M. fascicularis* NK cell clones (middle rows) and on a representative human NK cell population cultured *in vitro* (lower panels). Green fluorescence intensity (horizontal axis) represents reactivity to the indicated mAb. Results are expressed as logarithm of green fluorescence intensity (arbitrary units) versus number of events. For each analysis 10^4 events were counted. Reactivities for mAb AZ20 (anti-NKp30) were similar to what is shown for mAb Z25.

2.3 mAb-mediated masking of NKp46 or NKp30 prevents lysis of NK-susceptible target cells by *M. fascicularis* NK cells

Previous data showed that the cytolytic activity of human NK cell clones against certain human targets could be inhibited by anti-NCR mAb [17, 20, 21]. To determine further the role of the surface molecules recognized by anti-NKp46 and anti-NKp30 mAb in *M. fascicularis*, NK cell populations or clones were assayed against the human melanoma cell line FO1 in the absence or in the presence of specific mAb. In the presence of anti-NKp46 or anti-NKp30 mAb, a partial inhibition of FO1 cell lysis could be observed in *M. fascicularis* populations or clones, with a more marked decrease when both both mAb were present at the same time (Fig. 3 B).

2.4 Cloning of the *M. faccicularis* NCR homologous genes

Searching of expressed sequence tag database (EST) with a BLASTN algorithm program using the human NKp30 cDNA sequence (Acc No. AJ223153) revealed an EST clone (Acc. No. AW308336) from a cDNA library made from pooled tissues of *Bov taurus*, sharing more than 80 % of local sequence identity with human NKp30 cDNA. Three different primers were designed in the region conserved between the two sequences. These primers were utilized in combination with a primer designed on the human sequence in three different RT-PCR reactions starting from RNA derived from a *M. fascicularis* polyclonal NK cell population. These reactions always resulted in amplification products of size comparable to those obtained from RNA derived from human NK cells. In view of these successful results, we attempted to amplify the cDNA containing the complete open reading frame (ORF) of the *M. fascicularis* NKp30

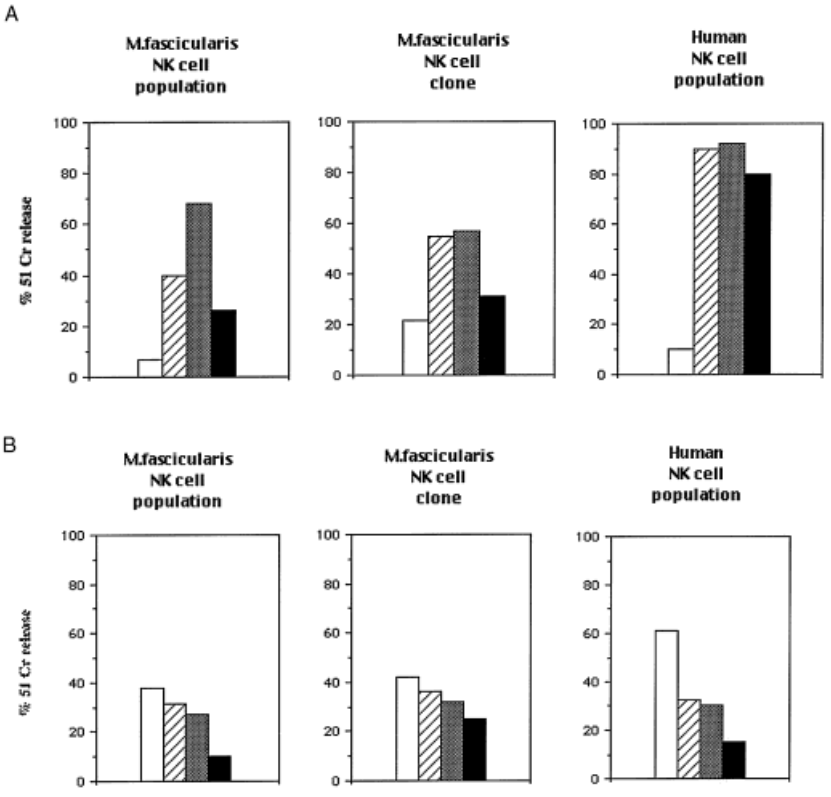

Fig. 3. (A) Triggering of cytolytic activity via CD16, NKp30 and NKp46 in *M. fascicularis* NK cells. Redirected killing assay of a representative *M. fascicularis* NK cell population (left panel) of a representative *M. fascicularis* NK cell clone (middle panel) and of a human NK cell population (right panel) against FcγR$^+$ P815 cells. NK cell effectors were assayed in a 4-h ^{51}Cr-release assay against P815 cells at the effector/target cell ratios indicated either in the absence (□) or in the presence of anti CD16 (▨), anti-NKp30 (▩) or anti-NKp46 (■) mAb (IgG). The E/T ratios were 5:1 for the NK cell populations and 0.6:1 for the NK cell clone. The increase in ^{51}Cr release over baseline (no mAb) reflects the ability of a given mAb to trigger the lytic machinery of the cells. (B) mAb-mediated masking of triggering receptors inhibits the cytolytic activity of *M. fascicularis* NK cells. A representative *M. fascicularis* NK cell population (left panel), a representative *M. fascicularis* NK cell clone (middle panel) and a human NK cell population (right panel) were assayed for cytolytic activity against the FO1 human melanoma cells either in the absence (□) or in the presence of anti NKp46 (▨), anti-NKp30 (▩) or anti-NKp30$^+$ anti-NKp46 mAb (■). The effector/target cell ratios were 5:1 for the NK cell populations and 2.5:1 for the NK cell clone. The decrease in ^{51}Cr release over baseline (no mAb) reflects the ability of a given mAb to mask the interaction of a specific activating molecule on the effector with its ligand(s) on the target cell.

homologue (macNKp30) using two primers designed on the human cDNA sequence. The 612-bp amplified product (M-2) shares 90 % nucleotide identity with the same region of human NKp30 cDNA, while the putative encoded protein product displays 88 % amino acid identity with human NKp30 (Fig. 4 A). The M2 cDNA contains a 531-bp ORF coding for a 176 amino acid protein belonging to the immunoglobulin superfamily (Ig-SF). An extracellular region of 120 amino acids with an Ig V-like domain, a transmembrane portion of at least 19 residues containing the positively charged amino acid Arg and a cytoplasmic tail of 33 amino acids characterize the putative protein. Since macNKp30 expressed by *M. fascicularis* NK cells is capable of transducing activating signals, it is possible that the Arg residue present in the transmembrane region is involved in the association with the signal transducing subunit CD3ζ, as previously shown for human NKp30 [21]. Moreover, both AZ20 and A76 anti-NK-p30 specific mAb stained macNKp30 protein after transient transfection of M2 cDNA in 293T cells (Fig. 4 B). Altogether, these data indicate that the cDNA isolated from *M. fascicularis* (Acc. No. AJ278389) NK cells encodes the homologue of the human NKp30 NCR.

On the basis of the high degree of NKp30 sequence identity, cloning of the NKp46 *M. fascicularis* cDNA (macNKp46) was directly attempted using primers able to amplify the human cDNA sequence. By this approach,

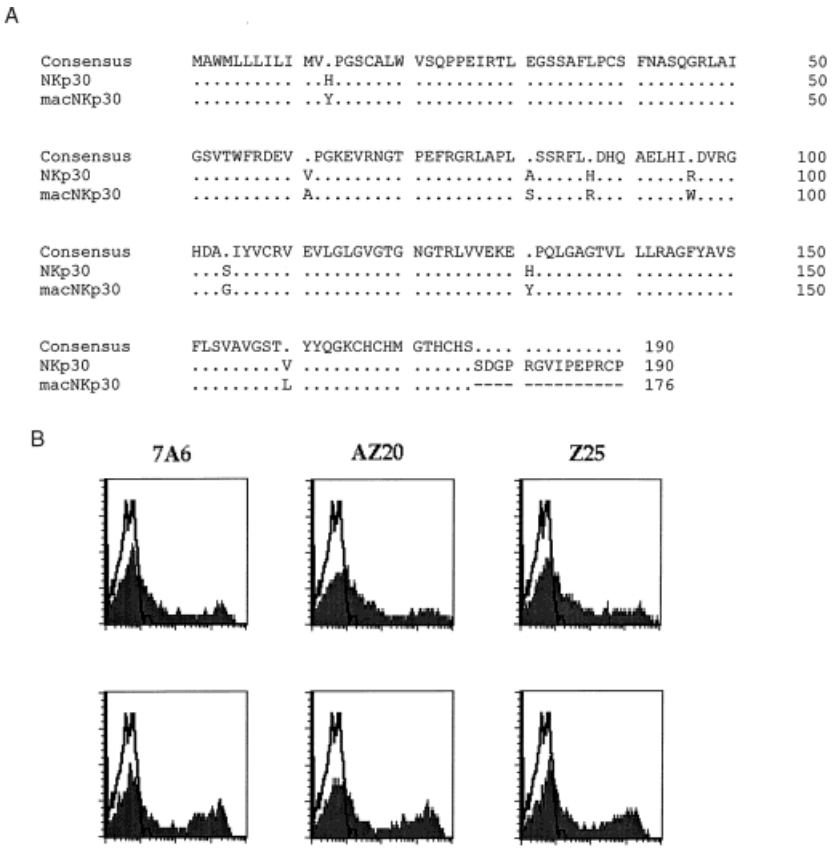

Fig. 4. (A) Alignment of macNKp30 and human NKp30 proteins. Amino acid sequences alignment between macNKp30 and human NKp30 proteins. Dashes were introduced to maximize homologies. Identical amino acids are indicated by dots. The EMBL accession numbers of these sequences are the following: macNKp30 (AJ278389) and human NKp30 (AJ223153). (B) Surface expression of macNKp30 and human NKp30 in transfected 293T cells. Cytofluorometric analysis of 293T cells either transfected with macNKp30 (upper panels) or with human NKp30 constructs (lower panels) using three different anti-NKp30 mabs (A76, AZ20 and Z25). Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAb. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) versus number of events.

a 983-bp cDNA (termed M-d), containing an ORF of 920 bp was amplified. The putative macNKp46 protein is characterized by a 21-residue signal peptide, an extracellular region of 232 amino acids with two Ig-like domains of the C2-type, a transmembrane portion of 20 residues containing the positively charged amino acid Arg and a cytoplasmic tail of 33 amino acids that does not contain any tyrosine residue. The ORF fragments of *M. fascicularis* cDNA and human NKp46 cDNA sequences were found to share 92 % identity, whereas the putative proteins displayed 86 % amino acid identity (Fig. 5 A). Cells transfected with M-d cDNA were brightly stained by both BAB281 and KL247 mAb, that are specific for human NKp46 receptor molecules (Fig. 5 B). This indicates that M-d cDNA encodes the *M. fascicularis* homologue of human NKp46. Altogether, when comparing human, cynomolgus monkey, murine and rat NKp46 cDNA, they display 64 % nucleotide sequence identity, and the corresponding putative proteins share 53 % amino acid identity.

Attempts aimed at the isolation of the *M. fascicularis* homologue of human NKp44 were unsuccessful, even when more exhaustive strategies than those applied for the isolation of macNKp46 and NKp30 were used (see Sect. 4.5). These data are in line with the failure to demonstrate anti-NKp44 mAb-reactive molecules on activated polyclonal NK cell populations or clones derived from *M. fascicularis*.

3 Discussion

In the present study we have identified and molecularly characterized the *M. fascicularis* homologues of the NKp46 and NKp30 NCR. Both molecules are recognized

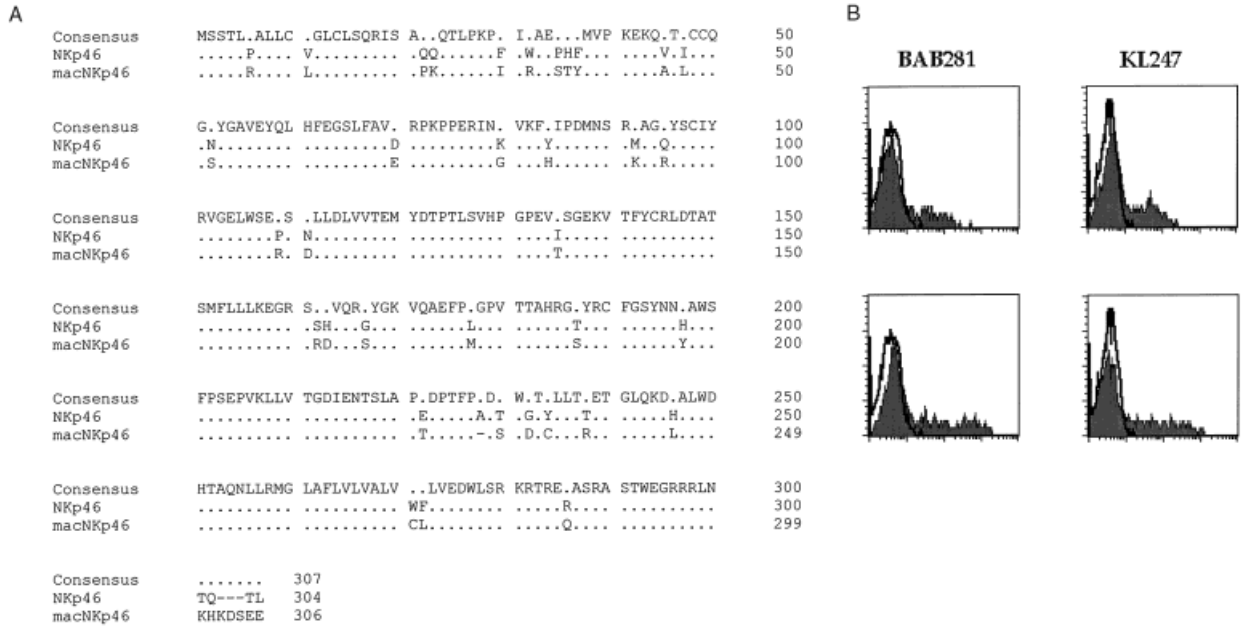

Fig. 5. (A) Alignment of macNKp46 and human NKp46-encoded protein sequences. Amino acid sequence alignment between macNKp46 and human NKp46 proteins. Dashes were introduced to maximize homologies. Identical amino acids are indicated by dots. The EMBL accession numbers are the following: macNKp46 (AJ278288) and NKp46 (AJ001383). (B) Surface expression of macNKp46 and human NKp46 in transfected 293T cells. Cytofluorometric analysis of 293T transiently transfected cells either with macNKp46 (upper panels) or human NKp46 constructs (lower panels) using two anti-NKp46 mAb (BAB281 and KL247). Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) versus number of events.

by mAb originally raised against human NK cells, thus providing an accurate tool for identification and functional analysis of macaque NK cells. This is particularly relevant, since nonhuman primates are the most useful animal model for HIV/AIDS vaccine trials. It is also noteworthy that the finding of NCR expression on $CD16^{+}56^{+}$ *M. fascicularis* cell populations and clones provides the demonstration that cells of NK cell lineage, based on NCR expression, express CD16 and CD56 in cynomolgus monkeys.

We were unable to detect any iNKR reactivity on macaque PBMC and also on activated NK cell populations and clones using a broad panel of anti-iNKR mAb. This is not surprising according to recent data provided by studies on chimpanzee and macaque iNKR and KIR cDNA sequencing. In fact, iNKR have been shown to display a high level of plasticity during evolution and are likely to match MHC class I divergence [12, 14, 22]. Indeed, recent work on chimpanzees has shown cross-reactivity only for a limited number of human iNKR-specific mAb, although the panel of mAb used was not exhaustive [12]. In addition, the sequence of macaque KIR markedly diverges from human KIR sequences [14]. Thus, based on these data and on the knowledge that chimpanzees and humans diverged approximately 5–6 million years ago while macaques and humans diverged approximately 20 million years [23], the lack of reactivity of this large panel of anti-human iNKR mAb on macaque NK cells is not surprising.

As far as NCR in *M. fascicularis* NK cells are concerned, we were able to detect both surface expression and function of NKp46 and NKp30, but not of NKp44 by using human NCR-specific mAb. Indeed, anti-NKp46 and anti-NKp30 mAb were able to trigger the cytolytic activity of both human and macaque NK cells in redirected killing assays and could also efficiently prevent lysis of NK-susceptible target cells in mAb-mediated masking experiments. The reactivity of anti-human reagents with macaque cells is in agreement with the finding that macNKp46 and macNKp30 display a high sequence similarity with their human counterparts (86 % and 88 % amino acid identity in the extracellular region, respectively). In this respect, different from the iNKR, NKp46 appears to be highly conserved during evolution, since mouse and rat homologous genes have been previously identified [18, 19], although no functional charac-

terization of the putative encoded proteins could be performed.

The conservation of the NKp30 sequence and function in macaques is more intriguing. In fact, we provide here the first evidence that macNKp30 represents a functional homologue of human NKp30 during evolution, since previous analysis of the genomic organization in mice only allowed the detection of a pseudo-gene [24, 25]. In this respect, the EST clone derived from *B. taurus*, used to isolate the macNKp30 cDNA, might represent a partial sequence of a non-primate NKp30 homologue. Thus, the present analysis supports the hypothesis that NKp30 appeared later than NKp46.

Three different anti-NKp44 mAb did not display reactivity with freshly isolated PBMC and with *in vitro* activated macaque NK cell cultures. Moreover, we were unable to identify a putative NKp44 transcript in *M. fascicularis* cells. It may perhaps be surprising that out of three NCR serving a similar function, two are highly conserved in humans and cynomolgus macaques, while NKp44 is absent in *M. fascicularis*. This would support a more recent appearance of NKp44 during evolution. Further studies are clearly needed in order to determine whether NKp44 has appeared only in humans, or if it is already present in primates that are reported to be phylogenetically more related to humans, including chimpanzees and gorillas. In this respect, the present study suggests the possibility of a staged appearance of the various NCR during phylogenesis with the following progression: NKp46-NKp30-NKp44. This progressive NCR appearance may reflect an increase in the complexity and in the fine-tuning of the innate immune response.

Finally, the present study may have a relevance on primate monkey models, which represent a very important tool for the development of vaccines and particularly of an HIV-1 vaccine. Indeed, a relevant part of the HIV-1 vaccine research has concentrated on immunization schedules, on protocols and on correlates of protection in monkey. African green monkeys are regarded as a model of naturally occurring tolerance to simian immunodeficiency virus and to foamy virus [26–28], while macaques have been extensively adopted to prove the possibility of a preventive vaccine for HIV-1 using different immunogens and to study their immune response [6–11, 29, 30]. In the course of these studies, the monitoring has been mostly limited to the adaptive immunity while little information is available on natural immunity, mostly because of the lack of suitable reagents. In this context, our study provides important tools not only for a more precise identification of *M. fascicularis* NK cells, but also to perform functional analysis since, at least in humans, NKp46 and NKp30 have been shown to represent two major receptors responsible for NK cells triggering in the process of natural cytotoxicity.

4 Materials and methods

4.1 Animal subjects and human donors

Peripheral blood was obtained from six adult cynomolgus monkeys (*M. fascicularis*) housed at the Istituto Superiore di Sanità animal facility, Rome, Italy in single cages according to the European guidelines for non-human primate care (ECC directive No. 86–609, Nov 24, 1986). Human peripheral blood was obtained from two healthy donors.

4.2 mAb and immunofluorescence analysis

The following panel of anti-human mAb was used: anti-CD3: JT3a (IgG2a); anti-CD4: HP2.6 (IgG2a); anti-CD8: OKT8 (IgG2a), XA41 (IgG1), ASTRA (IgG1), B9.4 (IgG2B); anti-CD16; KD1 (IgG2a), XA59 (IgM), νD4 (IgG1), A6.96 (IgG1), Sus142 (IgG2B), c127 (IgG1); anti-CD56; c218 (IgG1), FLS280 (IgG2a), A6.90 (IgG2B), A6.220 (IgM), GPR165 (IgG2a); anti-CD94: XA185 (IgG1), XA88 (IgG3), Y9 (IgM), 5A5 (IgG1); anti-p58.1: 11pb6 (IgG1), XA141 (IgM); anti p58.2: GL183 (IgG1), Y249 (IgM); anti-p140: Q66 (IgM); anti-p70: Z27 (IgG1); anti-NKG2A: Z270 (IgG1), Z199 (IgG2b), anti-NKG2A$^+$C: P25 (IgG1); anti-NKp30: 7A6 (IgG1), Z25 (IgG1), AZ20 (IgG1); anti-NKp44: Z231 (IgG1), KS38 (IgM), AZ140 (IgG1); anti-NKp46: BAB281 (IgG1), KL247 (IgM); mAb anti-α/β TCR: WT31 (IgG1) [20, 21, 31–36]. In addition, mAb Leu-4 (anti-CD3 IgG1), Leu-2a (anti-CD8, IgG1), Leu-3a (anti-CD4, IgG1), Leu12 (anti-CD19, IgG1) all from Becton Dickinson (Mountain View, CA), and anti-monkey CD3 (FN-18, IgG1, Biosource, Camarillo, CA) were purchased.

Fluorescein isothiocyanate-(FITC) conjugated anti-isotype goat anti-mouse second reagent antibodies were purchased from Southern Biotechnology (Birmingham, AL).

The reactivity of mAb with PBMC populations was assessed by indirect immunofluorescence and flow cytometric analysis as described elsewhere [31]. Briefly, 10^5 cells were stained with the corresponding mAb, followed by an appropriate FITC-conjugated isotype-specific goat anti-mouse antiserum (Southern Biotechnology, Birmingham, AL) as second-step reagent. Negative control reagents were murine mAb directed against irrelevant surface molecules. All samples were analyzed on a flow cytofluorometer (FACSort, Becton Dickinson). Data were analyzed using Lysis II (version 1.1). 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.

4.3 Cell cultures

Human and monkey PBMC were isolated on Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). For *M. fascicularis* samples, mononuclear cells were collected following centrifugation and residual red blood cells were lysed using FACS Lysing Solution (Becton Dickinson), for flow cytometric analysis, and using ACK ($NH_4Cl/KHCO_3$/EDTA) in order to further culture the cells *in vitro*. PBMC were depleted of plastic-adherent cells and incubated with anti-CD3 (JT3A and FN-18 for *M. fascicularis* cells), 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, Oslo, Norway) for 30 min at 4 °C (20,31). After immunomagnetic depletion $CD3^-4^-$ DR^- cells were either cultured on irradiated (5,000 rad) feeder cells in the presence of r-IL-2 100 U/ml (Proleukin, Chiron Corp., Emeryville, CA), and 1.5 ng/ml PHA (Gibco Ltd., Paisley, GB) in order to obtain activated polyclonal NK cell populations, or cultured under limiting dilution conditions in 96-well plates in order to obtain NK cell clones [32–34]. The culture medium used was RPMI 1640 supplemented with 10 % fetal calf serum, L-glutamine (2 mM) and 1 % antibiotic mixture (5 mg/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin stock solution).

4.4 Cytotoxicity assay

A series of FcRγ-positive and FcRγ-negative target cells were used in the various cytolytic assays. The P815 murine mastocytoma ($FcR\gamma^+$), the K562 human erythroleukemia ($FcR\gamma^+$) and the FO1 human melanoma ($FcR\gamma^-$) cell lines, were used as targets. NK cell-enriched populations and NK cell clones were tested for cytolytic activity in a 4 h ^{51}Cr-release assay as previously described [31, 32], either in the absence or in the presence of various mAb. The concentration of the various mAb was 10 μg/ml for the masking experiments and 0.5 μg/ml for the redirected killing experiments. The E/T ratios are indicated in the text.

4.5 RT-PCR strategy to isolate *M. fascicularis* NCR

The cDNA prepared by oligodT priming as described above was used for amplification of fragments of NKp30 homologue in macaque using the following primers: 5' GAC ATC TTC CGA CAT GGC (hb1C7 1 UP) or 5' GAC ATG GCC TGG ATG CTG (hb1C7 2 UP) or 5' ACC CAG ACC TCA CTG CT (1C7 for) and 5' CTT GGC TGG CAT TGA AGG (hb1C7 DOWN). All 3 amplifications (hb1C7 1 UP/hb1C7 DOWN – 149 bp), (hb1C7 2 UP/hb1C7 DOWN – 139 bp) or (1C7 for/hb1C7 DOWN – 187 bp) were performed for 30 cycles (30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C), followed by a 7 min extension at 72 °C, utilizing AmpliTAQ (Perkin Elmer-Applied Biosystems, Foster City, CA). The cDNA (macNKp30) containing the complete open reading frame (ORF) (612 bp) was obtained using the primers: 5' GAC ATC TTC CGA CAT GGC (hb1C7 1 UP) and 5' GAT TTA TTG GGG TCT TTT GAA G (7A6/38 rev). Amplification was performed with 25 pmoles of each primer for 30 cycles (30 s at 94 °C, 30 s at 58 °c, 30 s at 72 °C), followed by a 7-min elongation at 72 °C. Complete ORF sequence of macaque NKp46 (983 bp) was obtained by the use of the primers 5' CTGAGCGATGTCTTCCACAC (46A frw) and 5' CCGCCCAGGCTCAACACC (46P rev) under the following conditions: 30 cycles (30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C), followed by a 7 min elongation at 72 °C. All the complete ORF amplification products (macNKp30 and macNKp46) were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequencing was performed using BigDye Terminator Cycle Sequencing Kit and a 377 Applied Biosystems Automatic Sequencer (Perkin Elmer-Applied Biosystems).

Several attempts of NKp44 macaque sequence amplification were performed using four different primer combinations matching the human NKp44 sequence; in particular the sets of primers were the following: 15C FOR 1: 5'ACG AGC GCA CAG GAA AAG G and 15C REV 1: 5' GAG CAG GGC TGA CAG CAC; 15C FOR 2: 5' GCT AAC CGT GAG ATG CCA G and 15C REV 1: 5' GAG CAG GGC TGA CAG CAC; 15C FOR 3: 5' ACA AGC CCC TGA GTC TCC and 15C REV 3: 5' GTA GAT TCT ACA CCA GTA ATG; 15C FOR 3: 5' ACA AGC CCC TGA GTC TCC and P44 ORF DW: 5' TCA CAA AGT GTG TTC ATC ATC ATC ATC GCT TAT CTT AGT CC. Amplifications were performed for all four sets of primers using 30 cycles (30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C), followed by a 7 min elongation at 72 °C, with or without hot-start conditions.

4.6 Transient transfections

COS7 or 293T cells were transiently transfected with pCDNA3.1-macNKp30 or with pCDNA3.1-macNKp46 utilizing Fugene 6 (Roche, Monza, Italy). Control transfections with the corresponding human cDNA constructs were performed in parallel experiments. Briefly, cells were seeded at 5×10^5/plate, 24 h later they were incubated with 6 μg of each plasmid and 10 μl of Fugene 6 in DMEM/10 % FCS. After 48 or 72 h, transfected cells were used for cytofluorometric analysis. Cell transfectants were stained with the different mAb followed by an isotype-matched phycoerythrin-conjugated goat antibody and analyzed by flow cytometry using a FACSort (Becton Dickinson).

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