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Molecular and Functional Characterization of NKG2D, NKp80, and NKG2C Triggering NK Cell Receptors in Rhesus and Cynomolgus Macaques: Monitoring of NK Cell Function during Simian HIV Infection[1]

Roberto Biassoni,[2]* Manuela Fogli,[2†] Claudia Cantoni,*‡§ Paola Costa,[†] Romana Conte,[¶] Gerrit Koopman,[‖] Aurelio Cafaro,[#] Barbara Ensoli,[#] Alessandro Moretta,‡§ Lorenzo Moretta,*‡§ and Andrea De Maria[3†§]

An involvement of innate immunity and of NK cells during the priming of adaptive immune responses has been recently suggested in normal and disease conditions such as HIV infection and acute myelogenous leukemia. The analysis of NK cell-triggering receptor expression has been so far restricted to only NKp46 and NKp30 in *Macaca fascicularis*. In this study, we extended the molecular and functional characterization to the various NK cell-triggering receptors using PBMC and to the in vitro-derived NK cell populations by cytofluorometry and by cytolytic activity assays. In addition, RT-PCR strategy, cDNA cloning/sequencing, and transient transfections were used to identify and characterize NKp80, NKG2D, CD94/NKG2C, and CD94/NKG2A in *M. fascicularis* and *Macaca mulatta* as well as in the signal transducing polypeptide DNAX-activating protein DAP-10. Both *M. fascicularis* and *M. mulatta* NK cells express NKp80, NKG2D, and NKG2C molecules, which displayed a high degree of sequence homology with their human counterpart. Analysis of NK cells in simian HIV-infected *M. fascicularis* revealed reduced surface expression of selected NK cell-triggering receptors associated with a decreased NK cell function only in some animals. Overall surface density of NK cell-triggering receptors on peripheral blood cells and their triggering function on NK cell populations derived in vitro was not decreased compared with uninfected animals. Thus, triggering NK cell receptor monitoring on macaque NK cells is possible and could provide a valuable tool for assessing NK cell function during experimental infections and for exploring possible differences in immune correlates of protection in humans compared with cynomolgus and rhesus macaques undergoing different vaccination strategies. *The Journal of Immunology,* **2005, 174: 5695–5705.**

Nonhuman primate models, including cynomolgus and rhesus macaques, represent an important tool for the development and validation of vaccine models and for the evaluation of immune responses to a considerable list of human pathogens including not only simian immunodeficiency virus/HIV chimera (SHIV),[4] but also severe acute respiratory syndrome-coronavirus (SARS-CoV), measles virus, monkeypox virus, *Plasmodium falciparum*, hepatitis B, C, and E viruses, and anthrax (1–14). With respect to the correct identification of the different protective elements that concur to immune protection after vaccination, recent evidences suggest that the evaluation of NK cell function could provide interesting and useful information in adaptive immune responses (15–20). NK cells express receptors characterized by opposite functions that finely regulate their activity. Among inhibitory receptors, some are specific for different groups of MHC class I alleles (KIR, Ly49) (21–23), whereas others are still orphan receptors. In contrast, various activating receptors are involved in the triggering of NK-mediated natural cytotoxicity with a non-MHC-restricted pattern (24).

So far, only a limited number of reagents and information for the study of NK cell function have been available in macaques compared with the knowledge of human NK cell biology. To this end, the recent characterization of two natural cytotoxicity receptors (NCR) NKp46 and NKp30 and their function in cynomolgus macaques provided a useful insight into the possible evolution of NCR in different species (25).

The innate immune system has been recently shown to play a potentially pivotal role during infectious and neoplastic diseases including HIV infection, acute myelogenous leukemia, bone marrow transplantation in humans, and CMV infection in mice (26–29). In this regard, a reduced function of NK cells correlated with reduced expression of NCR in patients with acute myelogenous leukemia and in HIV-infected viremic patients (26, 27). Furthermore, a role for MHC class I-specific NK cell inhibitory receptors

*Istituto Giannina Gaslini, Dipartimentos [†]Medicina Interna e [‡]Medicina Sperimentale, [§]Centro di Eccellenza per la Ricerca Biomedica Universita' di Genova, [¶]Istituto Scientifico Tumori, Genova, Italy; [‖]Department of Virology, Biomedical Primate Research Centre, Rijswijk, The Netherlands; and [#]AIDS Division, Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Rome, Italy

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[2] R.B. and M.F. contributed equally to the work.

[3] Address correspondence and reprint requests to Dr. Andrea De Maria, Department of Internal Medicine, School of Medicine, University of Genova Padiglione Maragliano, Largo R. Benzi 10, I-16132 Genova, Italy. E-mail address: de-maria@unige.it

[4] Abbreviations used in this paper: SHIV, simian immunodeficiency virus/HIV chimera; NCR, natural cytotoxicity receptor; DAP, DNAX-activating protein; DC, dendritic cell; ORF, open reading frame.

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has been shown in mismatched bone marrow transplantation resulting in eradication of residual acute myelogenous leukemia burden and in prevention of both graft-versus-host disease and graft rejection (30). Similarly along this line, the expression by mouse NK cells of the inhibitory MHC class I-specific Ly49I receptor instead of its triggering counterpart Ly49H exposes the animals to fatal murine CMV infection (29, 31–33).

In addition to NCR (i.e., NKp46, NKp30, and NKp44), human NK cells are equipped with other triggering receptors that contribute to cell activation and target cell killing, including NKG2D, NKp80, NTB-A, 2B4, DNAM-1, and NKG2C. Among these molecules, NKG2D represents a major triggering receptor that is known to specifically recognize the stress-inducible MHC class I-related chain (MIC) molecules MICA, MICB, and UL16-binding proteins 1–4 (24, 34). On the contrary, NKp80, NTB-A, 2B4, and NKG2C appear to synergize with NCRs and NKG2D in the NK-mediated cytolysis (24, 35, 36).

Thus, in view of the still incomplete knowledge of the multiple triggering pathways involved in macaque NK cell function, a more detailed analysis of the expression and function of other triggering receptors could provide additional insights in macaque NK cell function and help identify protective response elements of the innate immune system that could be targeted or usefully exploited by different vaccine candidates and schedules.

To this end, we investigated the presence and function of additional NK cell-triggering molecules also in healthy cynomolgus and rhesus monkeys and in macaques (*Macaca fascicularis*) infected with SHIV89.6P, a pathogenic SIV/HIV chimera (3) to provide an estimate of the feasibility of their characterization in SHIV-infected animals.

In this study we show with a complete molecular and phenotypic characterization that NKG2D, NKp80, and NKG2C, similar to NKp46 and NKp30, are expressed and functional on the surface of NK cells and that, different to what has been observed with human HIV-1 infection, SHIV-infected *M. fascicularis* could display similar NK cell phenotypic and functional profiles compared with uninfected macaques.

Materials and Methods

Animal subjects and human donors

Peripheral blood was obtained from 14 adult cynomolgus monkeys (*M. fascicularis*) and two rhesus monkeys (*M. mulatta*) housed at the Istituto Superiore di Sanità animal facility (Rome, Italy) and at the Biomedical Primate Research Center (Rijsuijk, Holland). Animal housing follows the European guidelines for nonhuman primate care (European Commission and Council directive no. 86-609). The chronically infected macaques (*M. fascicularis*) had been challenged i.v. over 30 mo before with 10–15 monkey ID_{50} of the highly pathogenic SHIV89.6P, a SIV/HIV-1 chimera expressing the Tat, Rev, Vpu, and Env of HIV-1 and were kept at Istituto Superiore di Sanità animal facility housed in single cages within level III biosafety facilities. Human peripheral blood was obtained from five healthy donors.

Monoclonal Abs and immunofluorescence analysis

The following panel of anti-human mAbs was used: anti-CD16: XA59 (IgM) and nu-D4 (IgG1); anti-CD56: Gpr165 (IgG2a); anti-CD94: XA185 (IgG1), XA88 (IgG3), Y9 (IgM), and 5A5 (IgG1); anti-p58.1: 11pb6 (IgG1) and XA141 (IgM); anti p58.2: GL183 (IgG1) and Y249 (IgM); anti-p140: Q66 (IgM); anti-p70: Z27 (IgG1); anti-NKG2A: Z199 (IgG2b); anti-NKG2A and anti-NKG2C: P25 (IgG1); anti-NKp30: 7A6 (IgG1), Z25 (IgG1), and AZ20 (IgG1); anti-NKp46: BAB281 (IgG1) and KL247 (IgM); anti-NKp80: MA152 (IgG1) and LAPI171 (IgG1); anti-NKG2D: BAT221 or ON72 (IgG1); anti-CD244: pp35 (IgG1); and anti-NTB-A: MA127 (IgG1). In addition, anti-monkey CD3 (FN-18 (IgG1); BioSource International) were purchased.

FITC- and PE-conjugated anti-isotype goat anti-mouse second reagent Abs were purchased from Southern Biotechnology Associates.

The reactivity of mAbs with PBMC populations was assessed by indirect immunofluorescence and flow cytometric analysis as described (25). Briefly, 10^5 cells were stained with the corresponding mAb, followed by an appropriate FITC- or PE-conjugated anti-isotype-specific goat anti-mouse antiserum (Southern Biotechnology Associates) as second-step reagent. Negative control reagents were murine mAbs directed against irrelevant surface molecules. All samples were analyzed on a flow cytofluorometer (FACSort; BD Biosciences). 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) vs number of events. For each analysis 10,000 events were counted.

Cell cultures

Human and monkey PBMC were isolated on Ficoll-Paque (Amersham Pharmacia Biotech). For macaque samples, mononuclear cells were collected following centrifugation and residual RBC were lysed using ACK ($NH_4Cl/KHCO_3$/EDTA) for flow cytometric analysis, and 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* and *Mulatta* 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 Biotech) for 30 min at 4°C (25, 26). In some experiments, proliferating cultures were further enriched using anti-CD56 (Gpr165) mAb for 30 min at 4°C followed by goat anti-mouse IgG microbeads (Miltenyi Biotec) 20 min at 4°C and immunoseparation by MS Columns (Miltenyi Biotec).

After immunomagnetic selection, $CD3^-CD4^-HLA\text{-}DR^-$ cells were either cultured on irradiated (5000 rads) feeder cells (PBMC and 221G cell line) in the presence of rIL-2 (100 U/ml, Proleukin; Chiron). The culture medium used was RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mM), and 1% antibiotic mixture (penicillin 5 mg/ml, streptomycin 5 mg/ml, and neomycin 10 mg/ml stock solution).

Cytotoxicity assay

A series of FcγR-positive and FcγR-negative target cells were used in the various cytolytic assays. The P815 murine mastocytoma ($Fc\gamma R^+$), the K562 human erythroleukemia ($Fc\gamma R^+$), and the FO1 human melanoma ($Fc\gamma R^-$) cell lines were used as targets.

NK cell-enriched populations were tested for cytolytic activity in a 4-h ^{51}Cr release assay as previously described (25, 26), either in the absence or in the presence of various mAbs. The concentration of the various mAbs was 10 μg/ml for the masking experiments and 0.5 μg/ml for the redirected killing experiments. The E:T ratio is indicated.

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

Total RNA was extracted using RNAClean (TIB MOLBIOL, Genoa, Italy) from NK cell clones or populations derived from *M. fascicularis* or macaca rhesus. Oligo(dT)-primed cDNA was prepared by standard technique.

PCR amplifications were performed with the following primers: NKp80, 5′-ACT CAC ATT GAA GAT GCA AGA TG-3′ (M up) and 5′-GCT AGA CCA GTG TCG ATG ATG G-3′ (M dw); NKG2D, 5′-ATT TGA TGG GGT GGA TTC-3′ (NKG2D mac up) and 5′-AGG TTG ATC ATC TTT ACA CAG-3′ (NKG2D mac dw); DNAX-activating protein (DAP)-10, 5′-CAG ACC CCA GTC CAC CAT G-3′ (DAP10 open reading frame (orf) up) and 5′-GTG CCA CCA CAC ACC ATC-3′ (DAP10 orf dw); CD94, 5′-CCT TCT CTA CTT CGC TCT TG-3′ (hCD94 up) and 5′-TTA CTC TCC ACC TTC TCT GC-3′ (hCD94 dw); NKG2A, 5′-ACA CTG CAG AGA TGG ATA ACC-3′ (hNKG2A up) and 5′-TTA CTT CTA AAG CTT ATG C-3′ (macNKG2A orf dw); NKG2C, 5′-ATG AAT AAA CAA AGA GGA ACC TT-3′ (macNKG2C up) and 5′-CAA ACG CAA ATG TTT TAC T-3′ (macNKG2C dw). Amplifications were performed for 30 cycles followed by a 7 min extension at 72°C, using AmpliTAQ (Applied Biosystems). PCR thermal conditions for NKp80 were 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C). For NKG2D, DAP-10, and NKG2C amplification, the 30 cycles were 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. For CD94 amplification, the 30 cycles were 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C. For NKG2A, touch down amplification cycling protocol has been used. In particular, the former 10 cycles were 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C and the latter 20 cycles were 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C.

All the complete ORF amplification products were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen Life Technologies). DNA sequencing was performed using BigDye Terminator Cycle Sequencing kit and a 377 Applied Biosystems Automatic Sequencer.

Transient transfections

293T cells were transiently transfected with, pcDNA3.1/V5-His-TOPO-macNKp80, pcDNA3.1/V5-His-TOPO-macfCD94, or cotransfected with pcDNA3.1/V5-His-TOPO-macrhCD94 plus pcDNA3.1/V5-His-TOPO-macrhNKG2A, pcDNA3.1/V5-His-TOPO-macfCD94 plus pcDNA3.1/V5-His-TOPO-macfNKG2C or pcDNA3.1/V5-His-TOPO-macrhCD94 plus pcDNA3.1/V5-His-TOPO-macrhNKG2C using Fugene 6 (Roche). Control transfections with the corresponding human cDNA constructs (pcDNA3.1/V5-His-TOPO-hNKp80, PCR3-hCD94, PCR3-hNKG2A, PCR3-hNKG2C) were performed in parallel experiments. Briefly, cells were seeded at 5×10^5/plate, and 24 h later they were incubated with 6 μg of each plasmid and 10 μl of Fugene 6 in DMEM/10% FCS. Chinese hamster ovary cells were transiently cotransfected with pcDNA3.1/V5-His-TOPO-macfNKG2D plus pcDNA3.1/V5-His-TOPO-macfDAP10 using GenePORTER 2 transfection reagent (GTS) following manufacturer's instructions. Control transfections with the corresponding human cDNA constructs (PCR3-hNKG2D and pCMV1FLAG-hDAP10) were performed in parallel experiments. After 48 or 72 h, transfected cells were used for cytofluorometric analysis. Cell transfectants were stained with the different specific mAbs followed by an isotype-matched PE-conjugated goat Ab and analyzed by flow cytometry using a FACSort (BD Biosciences).

Results

Analysis of coreceptor expression on PBMC and on NK cell populations derived from M. fascicularis *and rhesus macaques, and identification of NKp80 and NKG2D molecules*

We have previously shown that *M. fascicularis* NK cells express, similar to what is observed in human NK cells, functional NK cell specific markers, including NKp46 and NKp30 molecules (25). Because other triggering receptors in addition to NCR are involved in the modulation of human NK cell-mediated cytotoxicity, we wanted to determine whether a similar pattern of multiple triggering receptor expression was also found on NK cells from two animal models that are widely used in HIV vaccination protocols (i.e., cynomolgus and rhesus macaques) using reagents that are already available and specific for human NK cells.

To this end, we first analyzed the reactivity of mAbs specific for human NKG2D, NKp80, 2B4 (CD244), and NTB-A molecules using PBMC derived from *M. fascicularis* and *M. mulatta*. Cytofluorometric analysis of PBMC from 14 *M. fascicularis* and two *M. mulatta* animals revealed the presence of reactivity with the NKp80-specific LAPI171 or MA152 mAbs and with the NKG2D-specific BAT221 mAb in all the samples. The fluorescent intensity and the proportion of positive cells obtained in the macaque samples were comparable to the pattern usually observed in the cytofluorometric analysis of human PBMC (Fig. 1). In both macaque species, similar to what is observed in human PBMC, two-color fluorescence analysis using anti-CD3 and either anti-NKG2D or anti-NKp80 mAbs showed that these molecules are also expressed on the surface of $CD3^+$ PBMC in addition to NK cells (data not shown). In addition, the pattern of expression of NKp46 and of NKp30 on peripheral NK cells in rhesus macaques was superimposable to that previously described for *M. fascicularis* (data not shown) (25).

Regarding the expression of 2B4 (CD244) and NTB-A coreceptors, as determined by cytofluorometric analysis, the available human-specific mAbs (pp35 and MA127, respectively) did not show any reactivity with PBMC derived from both macaque species (Fig. 1).

Thus, these results show that, in addition to NKp46 and NKp30 homologues, both macaque species express at least two additional surface Ags that are readily recognized by mAbs specific for human NKp80 and NKG2D NK cell-triggering receptors.

NKp80 and NKG2D on macaque NK cells are functional and trigger NK cell cytotoxicity

We next asked whether the surface molecules recognized by NKp80- and NKG2D-specific mAbs displayed the same functional properties of their human homologues. To this end, polyclonal NK cell cultures from cynomolgus PBMC were generated in vitro and analyzed for their cytolytic activity in a redirected killing assay using $Fc\gamma R^+$ P815 murine target cell lines, either in the presence or in the absence of anti-NKp80 (LAPI171) and anti-NKG2D

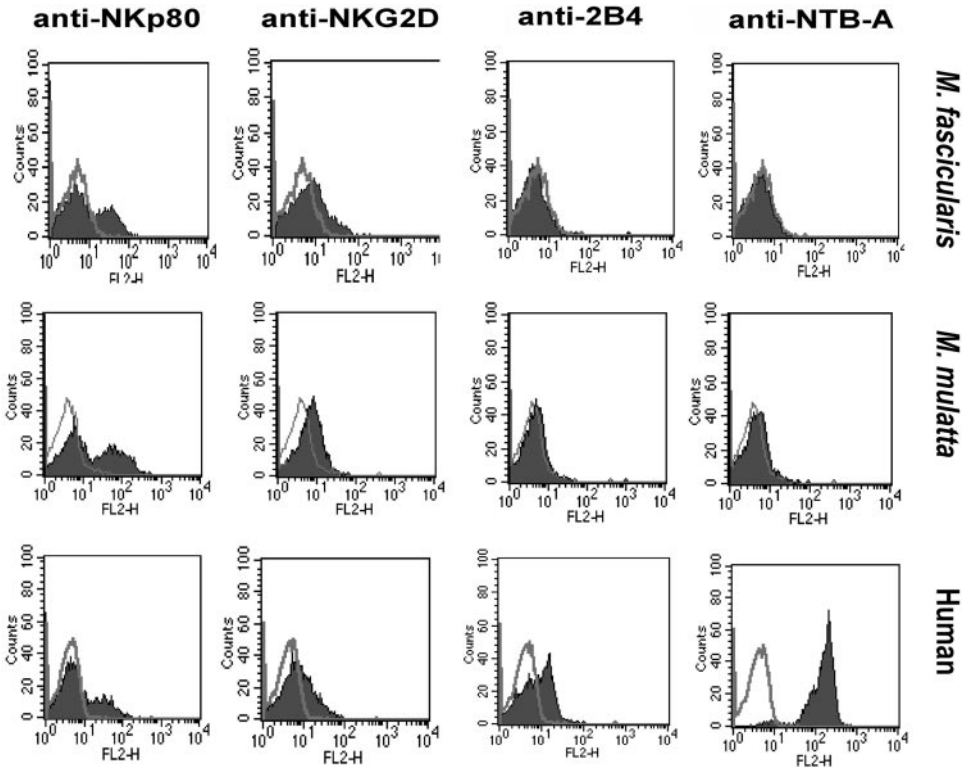

FIGURE 1. Comparative analysis of NKp80, NKG2D, 2B4, and NTB-A surface expression in *M. fascicularis*, *M. mulatta*, and human PBMC. Cytofluorometric analysis of NKp80, NKG2D, 2B4, and NTB-A expression on PBMC derived from *M. fascicularis* (*upper panels*), *M. mulatta* (*middle panels*), and human (*lower panels*) donors. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated surface molecules. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were acquired. The experiment is representative of 14 *M. fascicularis*, two *M. rhesus*, and five human samples processed.

(BAT221) mAbs. In all the experiments, in line with the results obtained on PBMC, NK cell populations derived from both species failed to show reactivity with anti-2B4 (CD244) or anti-NTB-A mAbs.

As shown in Fig. 2*A*, both anti-NKp80 and anti-NKG2D mAbs, as well as anti-CD16 and anti-NKp46, induced an efficient lysis of FcγR$^+$ P815 cells comparable to what is observed with human purified NK cell populations.

To further determine the relative role of both NKp80 and NKG2D in the lysis of tumor targets in cynomolgus macaques, we assayed NK cell populations against the human melanoma cell line FO1 in the presence or in the absence of specific mAbs (mAb-mediated masking of triggering molecules). As shown in Fig. 2*B*, mAb-mediated masking of the various triggering receptors, including NKp46, NKp30, NKp80, and NKG2D, resulted in a marked decrease of FO1 melanoma target cell lysis in both macaque and human NK cells upon masking with NKp46 and NKG2D, but not with NKp80. Similar to what is observed with human NK cells, macaque NKp46 and NKG2D play a major role in NK-mediated lysis of FO1 melanoma target cells, whereas NKp80 is not involved in the lysis of this cell line. The same cytolytic assays were performed using NK cells derived from rhesus macaques and could verify a functional pattern using the NKp80 and NKG2D mAbs discussed (data not shown). Due to the small cell numbers in only two rhesus monkeys, possible phenotypic and functional differences between the two species could not be evaluated. Larger animal populations and repeated assays will need to be assessed in future work to provide an overall accurate comparison of NK cell triggering receptor expression and function in the two species.

Thus, these results indicate that as in human, the molecules recognized in both macaque species by anti-human NKp80 and anti-human NKG2D mAbs are involved in NK cell-triggering and target cell lysis.

Cloning of NKp80, NKG2D, and DAP-10 cDNAs in cynomolgus and rhesus macaques

In previous work we had performed the molecular characterization of NKp30 and NKp46 homologous genes in *M. fascicularis* by

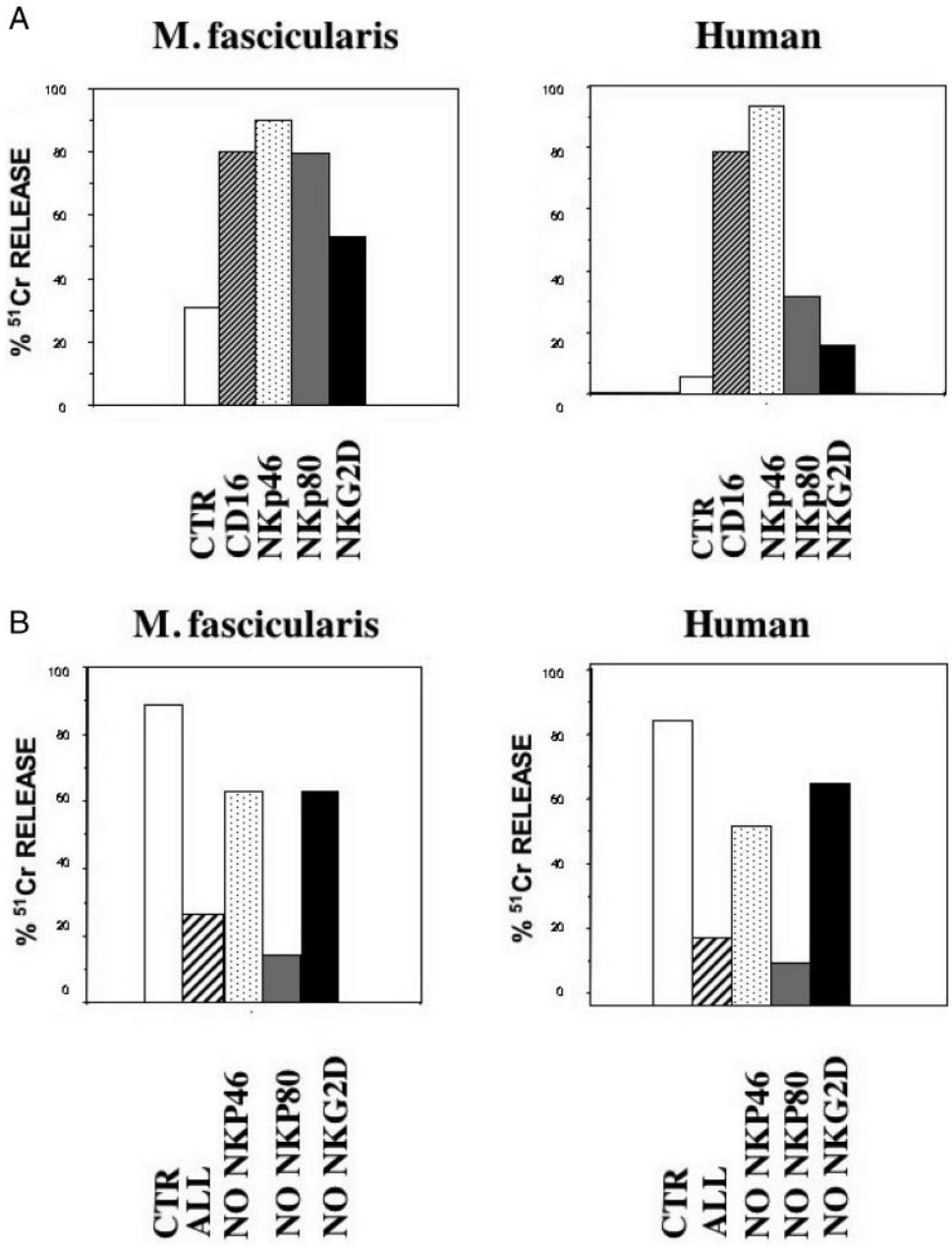

FIGURE 2. *A*, Triggering of cytolytic activity via CD16, NKp46, NKp80, and NKG2D in *M. fascicularis* NK cells. Two representative NK cell populations either derived from *M. fascicularis* (*left*) or human (*right*) were tested in a redirected killing assay against the FcγR$^+$ P815 cell line. Cytolysis was evaluated in a 4 h ^{51}Cr release assay at 5:1 E:T cell ratios, either in the absence (□) or in the presence of anti-CD16 (nD4) (▨), anti-NKp46 (BAB281) (dotted bar), anti-NKp80 (LAPA171) (▩), or anti-NKG2D (BAT221) (■) mAbs (IgG). The mAb-dependent increase in ^{51}Cr release over baseline (CTR is irrelevant mAb) reflects the ability of a given surface molecule to trigger the lytic machinery of the cells. Experiments are representative of seven performed. *B*, Monoclonal Ab-mediated masking of triggering receptors inhibits the cytolytic activity of *M. fascicularis* NK cells. Representative *M. fascicularis* (*left*) and human (*right*) NK cell populations were assayed for cytolytic activity against the FO1 human melanoma cells either in the absence (□) or in the presence of a combination of anti-NKp46, anti-NKp80, and anti-NKG2D (KL247, LAPI171, and BAT221, respectively) (▨) or in the presence of the same combination mentioned without anti-NKp46 (dotted bar), or anti-NKp80 (▩) or anti-NKG2D (■) mAbs. The E:T cell ratios were 5:1. 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 surface molecule expressed by effector cells with its ligand(s) present on the membrane of target cells. Experiments are representative of four performed.

using BLAST and Clustal analysis for the selection of primers able to cross-amplify the corresponding cDNA expressed in macaque NK cells (25).

In this study, we adopted the same approach to characterize NKp80, NKG2D, and DAP-10 homologous genes in both *M. fascicularis* and *M. mulatta*. In addition, we also isolated the *M. mulatta* NKp30 cDNA. Regarding *M. fascicularis*, we observed for NKp80 (AJ426430), NKG2D (AJ426429), and DAP-10 (AJ5543000) cDNA identity to the human nucleotide sequences of 94, 95, and 85%. The amplified products coded for proteins of 231, 216, and 78 amino acids, respectively, sharing 90, 94, and 84% identity with the human homologues, respectively.

Regarding *M. mulatta* cDNA, we observed for NKp80 (GenBank accession no. AJ622828), NKG2D (GenBank AJ554302), and DAP-10 (GenBank AF321610) a 94, 95, and 83% nucleotide identity, respectively, as compared with the human sequences. The amplified products were coding for proteins of 231, 216, and 79 amino acids respectively, sharing with human sequence 90, 94, and 82% identities. Of note, in comparison to the human protein sequence, both macaque DAP-10 protein sequences display an identical 14 amino acid deletion in the short extracellular region (Fig. 3*A*). This deletion corresponds in the human sequence to the 3′ portion of DAP-10 exon 2 and may originate in macaques by an alternative splicing probably due to the presence of a cryptic or alternative splice site. Moreover, both MA152 and LAPI171 anti-human NKp80 mAbs stained 293T cells transiently transfected with a *M. fascicularis* cDNA construct (Fig. 3*B*). In contrast, BAT221 an anti-human NKG2D mAb was able to detect surface expression of Chinese hamster ovary cells cotransfected with *M. fascicularis* NKG2D and either *M. fascicularis* or human DAP-10 cDNA constructs. Notably, human DAP-10 stabilizes the surface expression of macaque NKG2D less efficiently when compared with macaque DAP-10 (Fig. 3*C*). Regarding the rhesus macaque DAP-10 molecule, which was not used in cotransfection experiments, the difference from the cynomolgus sequence resides in only two amino acid residues that are located in the cytoplasmic domain (97% amino acid identities in rhesus vs cynomolgus). Thus, although it could be interesting to verify this point in future work, it is likely that the minimal differences (two residues) between rhesus and cynomolgus sequences do not affect the surface stabilization of NKG2D. In line with this observation, our finding of a more relevant difference between human and both macaque DAP-10 sequences (14 amino acid deletion) (Fig. 3*A*) could account for the less efficient stabilization on the cell surface of *M. fascicularis* NKG2D by human DAP-10 cotransfection (Fig. 3*C*).

Regarding the triggering receptors, we had previously characterized two NCRs (NKp30 and NKp46) in *M. fascicularis* and showed their high similarity with the human counterparts in both sequence and function. Given some species differences between *M. fascicularis* and *M. mulatta* and the involvement of both animals in vaccine programs, we attempted molecular and functional characterization of NKp30. In fact, using the same strategy, we successfully identified the *M. mulatta* NKp30 (GenBank accession no. AJ554301) homologue that compared with the human sequence, displays 97% nucleotide and 90% amino acid identity, respectively. Remarkably, the charged amino acid arginine present in the

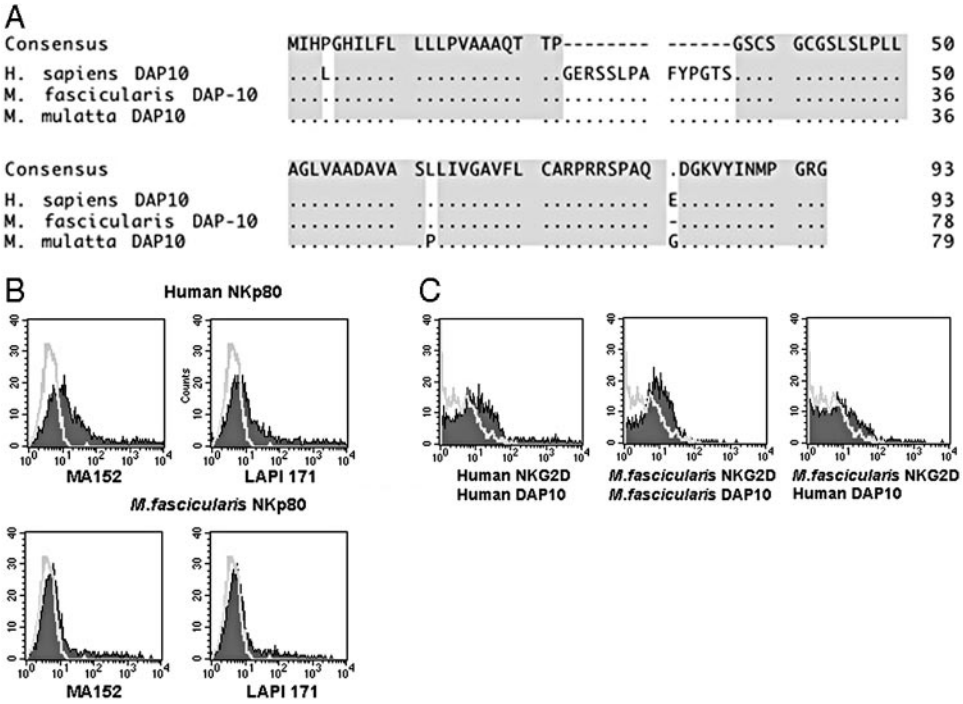

FIGURE 3. *A*, Amino acid sequence alignment of human, *M. fascicularis*, and *M. mulatta* DAP-10. Amino acid sequence alignment of DAP-10 polypeptide from human, *M. fascicularis*, and *M. mulatta* origin. Consensus sequence (*top*) is indicated, dashes were introduced to maximize homologies, and residues identical with the consensus are indicated by dots. Overall identity regions are shaded. *B*, Surface expression of NKp80 from *M. fascicularis* or human origin in transiently transfected HEK293T cells. Cytofluorometric analysis of 293T cells transiently transfected either with *M. fascicularis* NKp80 (*top panels*) or human NKp80 (*bottom panels*) cDNA constructs using two mAbs (MA152 and LAPI171) originally selected for human NKp80 reactivity. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were acquired. *C*, Surface expression of *M. fascicularis* and human NKG2D in DAP-10 cotransfected HEK293T cells. Cytofluorometric analysis of 293T cells transiently cotransfected either with human NKG2D and human DAP-10 cDNA constructs (*left*), *M. fascicularis* NKG2D, and *M. fascicularis* DAP-10 (*middle*), or *M. fascicularis* NKG2D and human DAP-10 cDNA constructs (*right*). Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were acquired.

transmembrane region, which is involved in the formation of a salt bridge with ITAM-bearing CD3ζ and FcϵRIγ polypeptides, is conserved both in human and in the two macaque species. As shown in Fig. 4, all the available mAbs specifically recognized 293T cells transiently transfected with human, cynomolgus, and rhesus NKp30 cDNA constructs. Thus, the present findings show that in these two macaque species there is a high degree of identity between not only NKG2D and NKp80 but also NKp30 sequences. In addition, we provide evidence that NKG2D and NKp80 sequences in both macaque species are encoding the homologous receptors to the human counterparts

CD94/NKG2C heterodimeric NK receptor in cynomolgus and rhesus macaques react with anti-human NKG2A mAb, but not by an anti-human NKG2A/NKG2C mAb

To characterize nonclassical MHC class I-specific receptors, involved in the fine-tuning of macaque NK cell cytolysis, we isolated the cDNA encoding CD94, NKG2A, and NKG2C receptor chains from both *M. fascicularis* and *M. mulatta* NK cells. In humans, the CD94/NKG2A heterodimer mediates inhibitory responses through Src homology protein 1 phosphatase association, whereas CD94/NKG2C triggers NK cell function using ITAM-bearing polypeptides such as DAP-12.

After the isolation cloning and sequencing of the CD94, NKG2A, and NKG2C target sequences of both *M. fascicularis* and *M. mulatta* CD94 (AJ585526 and AJ585527, respectively), NKG2A (AJ585528 and AJ585529, respectively), and NKG2C (AJ585530 and AJ585531, respectively) at 95, 92, and 94% nucleotide identity was found compared with human sequences. In both species, the amplified products coded for proteins of 179, 233, and 231 amino acids, respectively, sharing 92, 86, and 89% amino acid identity when compared with the human counterparts. Interestingly, in macaques the P25 mAb is unable to detect surface expression of both CD94/NKG2A and CD94/NKG2C in transiently cotransfected cells (Fig. 5*A*), whereas in human cells it recognizes specifically both heterodimeric molecules (36) (Fig. 5, *A* and *B*). On the contrary, the Z199 mAb specifically recognizes both macCD94/NKG2A and macCD94/NKG2C surface molecules in transiently transfected cells, whereas it recognizes only the human CD94/NKG2A molecules on cell transfectants (Fig. 5, *A* and *B*).

To further confirm the binding and recognition pattern of Z199 mAb, we next evaluated the functional properties of the NKG2A and NKG2C molecules using polyclonal macaque NK cell populations. To this end, we used a redirected killing assay with P815 cells and in vitro cultured NK cells of both human and macaque origin. Using human NK cells, upon binding of Z199 mAb in the presence of the anti-NKp30 7A6 mAb, the CD94/NKG2A molecule delivered an inhibitory signal, as previously reported (37) (Fig. 6*A*). In contrary in the same experimental setting using cynomolgus or rhesus NK cells, the same mAb triggered NK cell cytotoxicity (Fig. 6*B*). Thus, these data confirm the transfection results and show that the Z199 mAb recognizes CD94/NKG2C in peripheral blood macaque NK cells (both rhesus and cynomolgus) and that it can be used to explore NKG2C function in these animals.

Taken together these experiments show that in macaques the CD94/NKG2C molecule displays the same triggering functional activity as in human NK cells Interestingly, however, the same mAb (Z199) that, on human NK cells recognizes only the CD94/NKG2A inhibitory molecules, in macaques also recognizes the CD94/NKG2C receptor.

Analysis of NK cell-triggering receptor expression in infected and naive M. fascicularis *peripheral NK cells*

We recently described that during viremic HIV infection a decreased NK cell cytolytic activity is associated to a reduced surface expression of the NCR molecules (NKp46, NKp30 and NKp44), whereas no significant involvement of NKG2D and of NKp80 has been reported. In addition, the decreased NCR density was not only present on freshly separated purified NK cells, but persisted also after in vitro activation and culture of human NK cell populations.

Following the present characterization of additional triggering receptors in both cynomolgus and rhesus macaques, there was the full availability of mAbs specific for at least four different triggering receptors expressed by NK cells. Therefore, we next analyzed, a small number of naive and SHIV-infected cynomolgus monkeys to determine whether perturbations of NK cell receptor expression

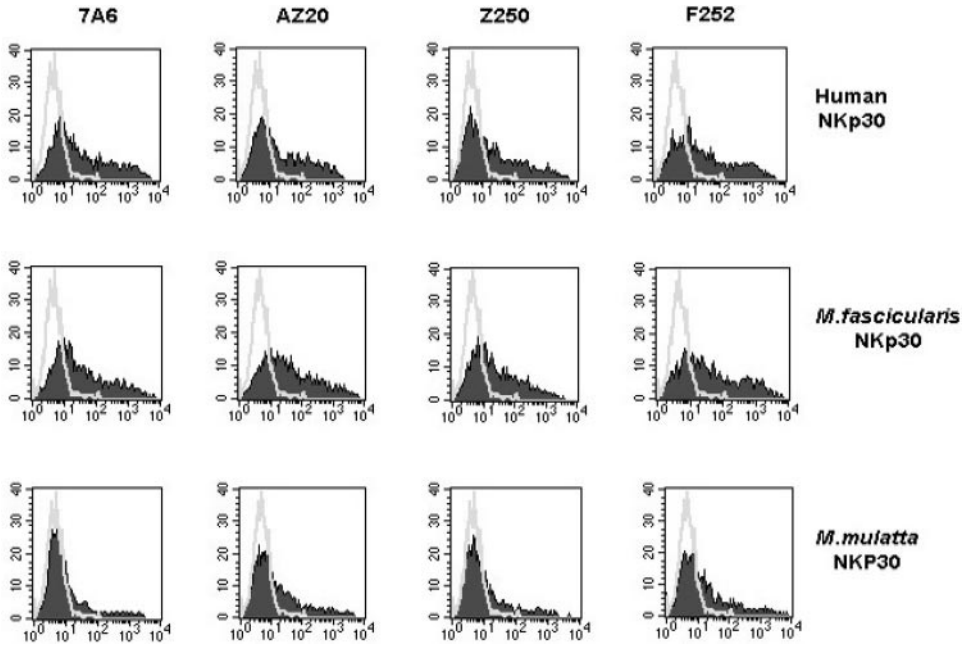

FIGURE 4. Surface expression of human, *M. fascicularis* and *M. mulatta* NKp30 cDNA construct in transfected HEK293T cells. Cytofluorometric analysis of 293T transiently transfected cells either with NKp30 cDNA constructs derived from NK cells of human (*upper panels*), *M. fascicularis* (*middle panels*), or *M. mulatta* (*lower panels*) origin using four anti-NKp30 mAbs (7A6, AZ20, Z250, and F252) originally selected for human NKp30 reactivity. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events.

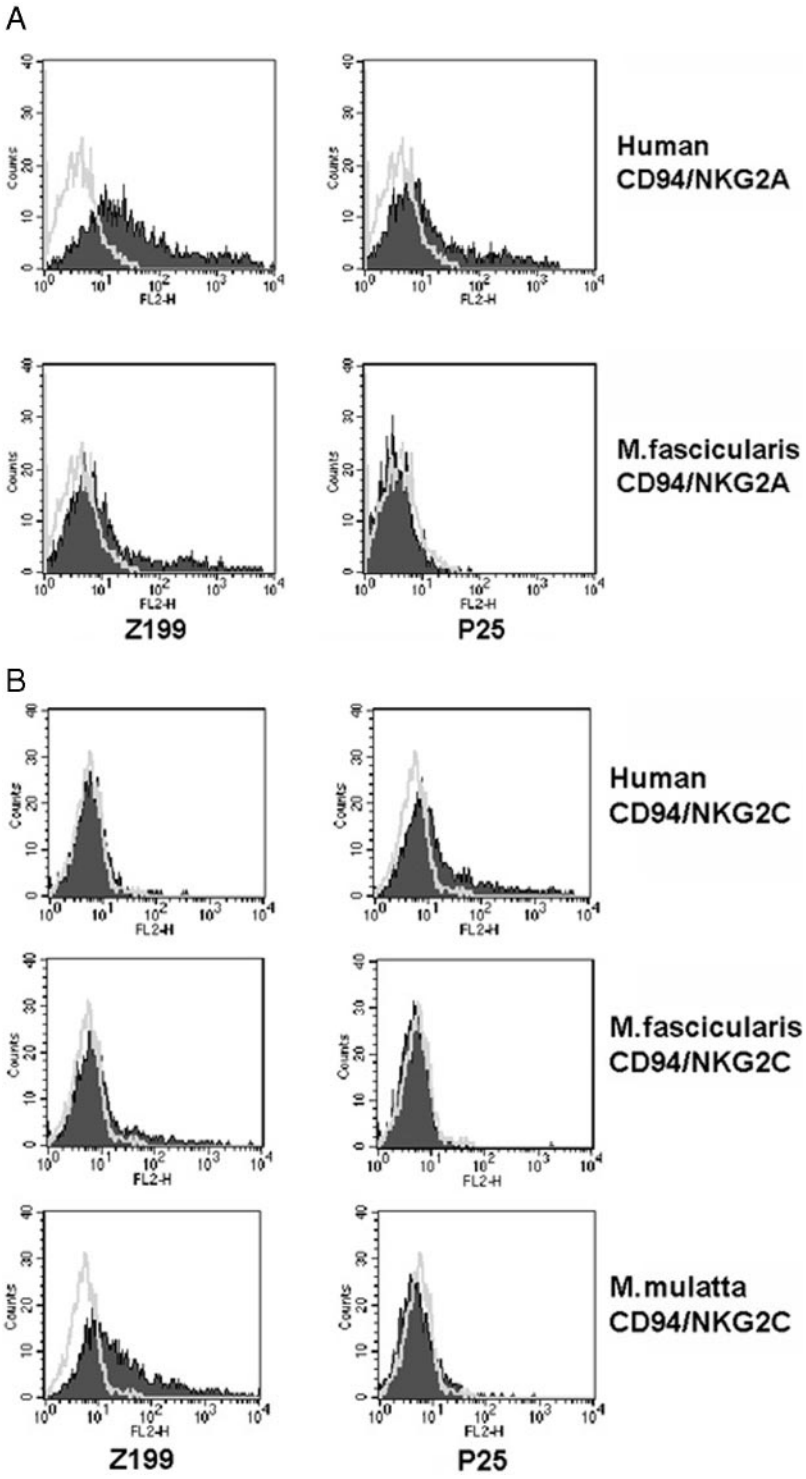

FIGURE 5. *A*, Surface expression of cotransfected human and *M. fascicularis* CD94 and NKG2A cDNA constructs in HEK293T cells. Cytofluorometric analysis of 293T transiently cotransfected cells either with human CD94 and NKG2A cDNA constructs (*top panels*) or with *M. fascicularis* CD94 and NKG2A cDNA expression vectors (*bottom panels*) using mAbs specific for human CD94/NKG2A (Z199) and human CD94/NKG2A+CD94/NKG2C (P25). Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. *B*, Surface expression of cotransfected human, *M. fascicularis* and *M. mulatta* CD94 and NKG2C cDNA constructs in HEK293T cells. Cytofluorometric analysis of 293T transiently cotransfected cells either with human CD94 and NKG2C constructs (*upper panels*), with *M. fascicularis* CD94 and NKG2C cDNA expression vectors (*middle panels*), or with *M. mulatta* CD94 and NKG2C (*lower panels*) constructs using mAbs originally selected to be specific for human CD94/NKG2A (Z199) or human CD94/NKG2A plus CD94/NKG2C (P25) surface receptors. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events.

and function could be detected studying the expression of the presently characterized molecules on their NK cells using mAbs recognizing macNKp30, macNKp46, macNKp80 and macNKG2D.

To this end we first evaluated whether differences of expression of these receptors could be detected on PBMC of seven chronically SHIV-infected and four uninfected *M. fascicularis* by cytofluorometric analysis using the specific mAbs. Cytofluorometric analysis of NCR (NKp46 and NKp30) mean fluorescence intensity showed that there was no significant difference in the density of surface molecules in infected or uninfected cynomolgus monkeys. (Fig. 7*A*). Single-color analysis of NKG2D or NKp80 expression does not provide useful information regarding their expression as these receptors may be expressed on both T lymphocytes and NK cells (data not shown).

Next, we derived in vitro cultured NK cell populations in the presence of rIL-2 from one naive and one chronically infected *M. fascicularis*. As determined by cytofluorometric analysis of fluorescence intensity (Fig. 7*B*), a decreased surface density of NKG2D molecules was present in the infected macaque, whereas NKp46, NKp30 and NKp80 were expressed to the same extent. To further verify whether the decreased expression of at least one of the triggering receptors in the infected animal was associated to a

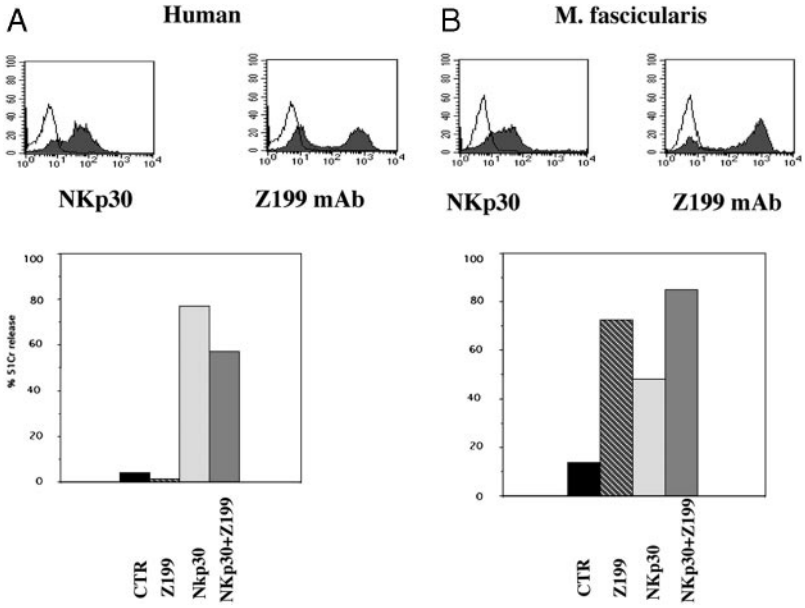

FIGURE 6. *A*, Functional assay of in vitro cultured polyclonal human NK cells expressing NKp30 and NKG2A/NKG2C surface receptors. Cytofluorometric analysis of NKp30 and NKG2A/NKG2C surface expression on in vitro cultured NK cells derived from human donors (*top panels*) in one of three representative experiments. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated molecules. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were acquired. Cytolysis was than evaluated in a 4 h ^{51}Cr release (*bottom panel*) redirected killing assay against FcγR$^+$ P815 cells, at 5:1 E:T cell ratio either in the presence of an irrelevant mAb (anti-CD56, Gpr165) (■) or in the presence of anti-NKG2A/NKG2C (Z199 mAb) (▧), anti-NKp30 (AZ20) (shaded bar), or anti NKp30 plus NKG2A/NKG2C (AZ20+Z199) (▨) mAbs (IgG). The increase in ^{51}Cr release over baseline (CTR is irrelevant mAb) reflects the ability of a given mAb to trigger the lytic machinery of the cells. The Z199 mAb triggering decreases NKp30-mediated target cell lysis upon binding to NKG2A. The experiments are representative of five performed. *B*, Functional assays of NKp30 and NKG2A/NKG2C on in vitro cultured NK cell populations derived from *M. fascicularis*. Cytofluorometric analysis of NKp30 and of NKG2A/NKG2C surface expression on in vitro cultured NK cells derived from *M. fascicularis* (*top panels*) in one of three representative experiments. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated surface molecules. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis, 10^4 events were counted. When NK cell effectors were assayed in a 4 h ^{51}Cr release (*bottom panel*) redirected killing assay against FcγR$^+$ P815 cells, at 5:1 E:T cell ratios either in the presence of an irrelevant mAb (■) or in the presence of anti-NKG2A/NKG2C (Z199 mAb) (▧), anti-NKp30 (shaded bar), or anti-NKp30+Z199 (▨) mAbs (IgG). The increase in ^{51}Cr release over baseline (CTR is irrelevant mAb) reflects the ability of a given mAb to trigger the lytic machinery of the cells. The Z199 mAb-mediated NK cell triggering induces lysis of target cells and increases NKp30-mediated target cell lysis upon binding to NKG2C. The experiments are representative of four performed.

decreased NK cell cytolytic activity, we performed cytotoxicity assays using K562 human erythroleukaemia, FO1 melanoma and P815 murine mastocytoma cell lines. As shown in Fig. 7*C*, a decreased lysis of K562 target cells as well as a decreased lysis of human FO1 melanoma cell targets was evident with NK cell populations derived from the infected animal. Further, a reduced activity was detected using the NK cell populations derived from the infected animal in the redirected killing of P815 cells in the presence of NKp30, NKG2D, and NKp80 mAbs. Finally, to provide an overall estimate of the NK cell triggering function in SHIV-infected cynomolgus macaques, we next compared the NK cell populations derived in vitro from five uninfected and six infected animals in a redirectd killing assay of P815 cells in vitro. As shown in Fig. 7*D*, the specific mAb mediated lysis was comparable in the two groups of monkeys (Mann-Whitney *U* test), with a nonsignificant trend toward decreased lysis mediated by NKG2 or NKp80 triggering, and a considerable level of variability that could be observed particularly in uninfected macaques

These experiments show therefore that triggering receptor molecule monitoring on NK cells is possible in animal models of disease. Interestingly, contrary to human patients infected with HIV-1, chronically SHIV-infected *M. fascicularis* do not display reduced NCR (NKp30, NKp46) expression ex vivo. In addition, although some NK cell populations derived in vitro from infected *M. fascicularis* can display a reduced NK cell cytolytic activity, the overall triggering activity of NKp30, NKp46, NKG2D, and Nkp80 receptors on NK cell populations activated in vitro is conserved.

Discussion

Recent work has provided convincing evidence of a widening spectrum of NK cell involvement in the regulation of immune responses in addition to the well established surveillance function against invading pathogens and tumors (38). In this regard, it has recently been determined that different subsets of NK cells are present in peripheral blood and in secondary lymphoid tissues in humans (17, 39). Further, NK cells have been shown to lyse immature dendritic cell (DC), whereas this is not the case with mature DC (20). In addition, NK-DC interaction delivers activating signals to NK cells through the interaction with NCR molecules (notably NKp30) providing evidence of an ample interplay between adaptive and innate immunity (18, 19). Finally, differential expression of cytokines and in particular of IFN-γ and TNF-α by NK cells may be further involved in the modulation of DC maturation. Thus, when considered in the general context of mucosal immune response toward invading pathogens, these observations, suggest an "editing" function of NK cells in the generation of adaptive immune responses, and may have relevance also in the design and

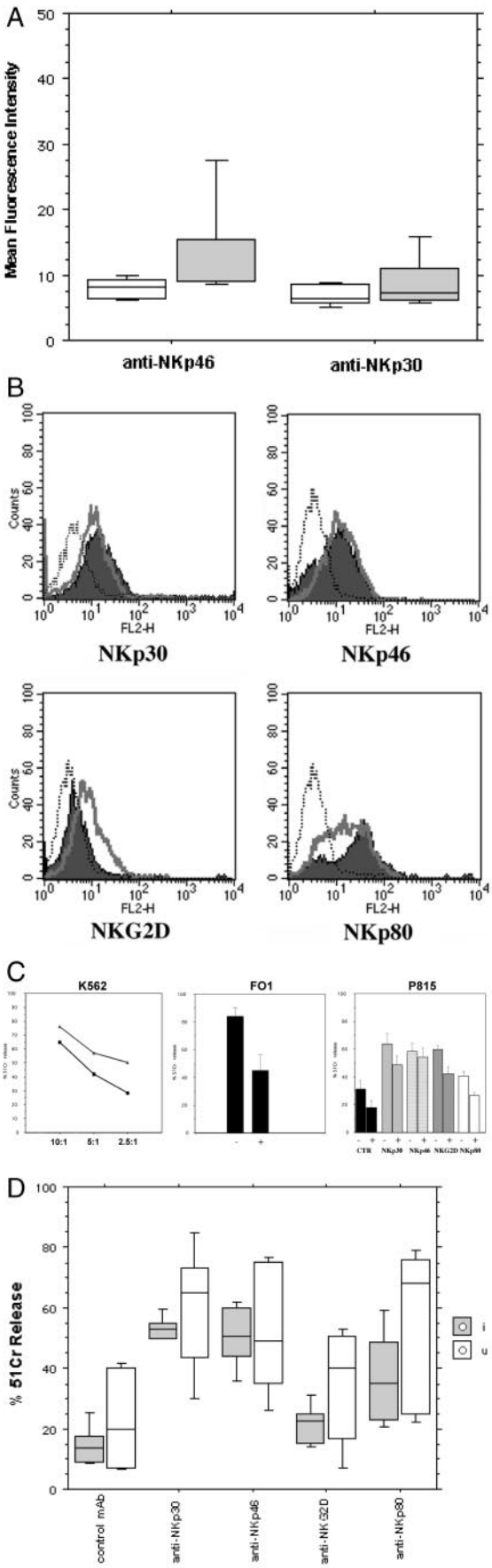

FIGURE 7. *A*, Mean fluorescence intensity analysis of NKp30, NKp46, NKG2D and NKp80 molecules expressed on PBMC in uninfected and SHIV-infected *M. fascicularis*. Cytofluorometric analysis of NKp46, NKp30, NKG2D, and NKp80 surface expression on PBMC from four uninfected (□) and seven chronically SHIV-infected (▨) *M. fascicularis* was performed. Results were expressed as logarithm of green fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were counted. Mean channel of fluorescence intensity was recorded from the green fluorescence intensity logarithmic scale and represents reactivity or relative density of surface Ag expression of the indicated surface molecules. Results are expressed as a box plot analysis of mean fluorescence intensity. The line indicates median expression, the boxes indicate 25–75 percentile, the vertical lines express the SD. Mann-Whitney *U* test analysis showed no significant differences. *B*, Cytofluorometric analysis of in vitro cultured NK cells derived from SHIV-infected and from control *M. fascicularis* shows reduced NKG2D fluorescence intensity in an infected animal. Cytofluorometric analysis of NKp46, NKp30, NKG2D, and NKp80 surface expression on purified NK cells cultured in vitro and derived from an uninfected (open histogram), and from one of the SHIV-infected *M. fascicularis* (filled histogram). Dotted lines represent negative control experiments using irrelevant mAbs. Red fluorescence intensity (horizontal axis) represents reactivity to the indicated surface molecules. Results are expressed as logarithm of red fluorescence intensity (arbitrary units) vs number of events. For each analysis 10^4 events were counted. *C*, In vitro activated purified NK cell populations derived from a SHIV-infected animal with decreased NKG2D expression have a decreased cytotoxic activity. NK cell populations (*B*) were assayed for their cytolytic activity against different NK cell susceptible tumor target cell lines (K562 erythroleukemia, FO1 melanoma) in 4 h ^{51}Cr release assays. When assayed against K562 target cells, NK cells derived from the SHIV-infected macaque (■) were less cytolytic than those derived from an uninfected animal (▲) at all E:T cell ratios tested. Similarly, against FO1 target cells NK cells derived from the infected animal (+) were compared with uninfected counterparts (−) at E:T 10:1 cell ratios. Redirected killing assays have been performed using the FcγR$^+$ P815 cell line using as effectors NK cells derived either from infected (+) or uninfected (−) *M. fascicularis* animals at 5:1 E:T ratio. Assays have been performed either in the presence of an irrelevant mAb (CTR) or in the presence of anti-NKp30, anti-NKp46, anti-NKG2D, or anti-NKp80 mAbs (IgG). *D*, Box plot analysis of redirected killing activity of in vitro cultured NK cell populations derived from uninfected and SHIV-infected *M. fascicularis*. Redirected killing assays have been performed using the FcγR$^+$ P815 cell line as ^{51}Cr-labeled targets and NK cells derived from six infected *M. fascicularis* (shaded box) or five uninfected *M. fascicularis* (□) as effectors at 5:1 E:T ratio. Assays have been performed either in the presence of an irrelevant mAb (CTR) or in the presence of anti-NKp30, anti-NKp46, anti-NKG2D or anti-NKp80 mAbs (IgG). Results are expressed as a box plot analysis of the percentage of ^{51}Cr release in the two groups of animals. The line indicates median lysis, the boxes indicate 25–75 percentile, the vertical lines express the SD. Mann-Whitney *U* test analysis showed no significant differences.

outcome of vaccination strategies (15). Along this line, with particular regard to the analysis of immune responses in animal models and to the expanding role of NK cells in the fine tuning of adaptive and innate immunity, the availability of additional reagents would greatly benefit the study of macaques, a nonhuman primate model that is widely used in the organ transplantation and pathogen infection/protection field (1–14, 16, 40).

In a previous paper we reported the functional and molecular characterization of NKp46 and NKp30 NCR in *M. fascicularis* (25). In the present study we characterized additional triggering receptor molecules in the two macaques species that are most commonly used in vaccination, transplantation and disease models (i.e., *M. fascicularis* and *M. mulatta*). We provide evidence that resting and "in vitro" activated macaque NK cells in both animal models express NKG2D and NKp80 that share the same triggering function as compared with their human homologues. In both species NKG2D, NKp80 and the signal transducing polypeptide DAP-10 showed an extremely high degree of amino acid sequence

homology compared with the already characterized human counterparts. These levels of identity are in line with what previously observed with *M. fascicularis* NKp46 and NKp30 molecules, and explain the cross-reactivity of mAbs that are known to specifically recognize these triggering receptors on human NK cells. Accordingly, failure to identify NTB-A and 2B4-like molecules using this approach may not exclude their existence in both species. Amino acid mutations in critical epitope residues recognized by the mAbs used in our study may explain the failure in detecting these molecules on macaque lymphocytes.

Our previous work focused on the study of NK cells from *M. fascicularis* (25). In the present work we add the molecular and phenotypic characterization of NKG2D, NKp80, and NKp30 expressed on NK cells derived from *M. mulatta* and show that they have an extremely high level of identity to those observed in *M. fascicularis*. Given the limited number of Rhesus macaques that were available further work will need to address the fine comparison of NK cell triggering receptor function using larger groups of *M. mulatta*. In fact, as far as the presently identified triggering receptors are concerned because both monkey species are widely used in the field of vaccination and organ transplantation (2, 4–6), it is of importance to know that these molecules are fully comparable in these two animal models also from the functional point of view.

The observation that there is a limited sequence divergence in the NCR triggering molecules was further confirmed by the present analysis of NKG2A, NKG2C and CD94 in cynomolgus and rhesus macaques. Interestingly, however, the Z199 mAb, which identifies only the inhibitory molecule CD94/NKG2A on human NK cells, triggers NK cell activation in macaques. This finding is in agreement with the mAb reactivity for both NKG2C and NKG2A in transfection experiments using cDNAs isolated from *M. mulatta* and *M. fascicularis*. Further work is needed to explain the apparent prevalence of triggering signals induced by Z199 mAb cross-linking on monkey NK cells. In fact, in similar experiments with human NK cells using a mAb specific for both NKG2A and NKG2C molecules (e.g., P25), a prevalence of inhibitory NKG2A-mediated signal is usually observed. To explain the triggering function of Z199 mAb in these NK cells, it will be interesting to verify whether, similar to what has been recently observed in CMV-experienced human donors (41), this is due to the prevalent expression of only CD94/NKG2C molecules on a large majority of cells whereas a minority of macaque NK cells coexpress both CD94NKG2A and CD94/NKG2C. In addition, the use of Z199 mAb will permit to elucidate the CD94/NKG2C function in macaque NK cells in pathological conditions particularly in conditions in which differences in other triggering receptor expression and function are less pronounced, including SIV or SHIV infection.

One possible aspect of concern when exploring the NK cell cytolytic activity in nonhuman primates could be considered the lack of available and suitable monkey-derived targets in experiments that explore macaque NK cell function. At present, however, there is no evidence yet that monkey-derived targets could behave in a different or "better" way compared with human (K562, FO1, etc.) or murine (P815) targets to explore triggering receptor function. Although one could agree, that monkey target cells could be considered more "physiological" when one is dealing with macaque NK cells as effectors, one has also to consider that the P815 assay used for both human and monkey NK cells uses murine target cells and is comparably useful. In addition, the extreme similarity of macaque triggering receptors to their human counterpart (our observations and Ref. (25)) suggests that there could be a shared ligand on target cells and therefore the time-consuming quest and validation of possible alternative cytolytic assays using monkey cells could be reasonably avoided. Conversely, macaque-derived target cells would be probably best used when the function of MHC-specific NK cell receptors on macaque NK cells should be addressed. In this case the difference between human and macaque targets could need an accurate evaluation if one considers the differences in MHC composition and sequence evolution in humans and in nonhuman primates.

Finally, in the present study, sets of experiments were designed to verify the feasibility of the study of NK cell triggering receptor monitoring in SIV/SHIV infected animals. It is known that in viremic HIV-infected human patients there is a prevalence of peripheral NK cells with NCR^{dull} phenotype, which helps to explain the reduced NK cell cytolytic activity that is observed during HIV infection (26). Using the mAbs specific for NKp46, NKp30, NKG2D, and NKp80 that are expressed on macaque NK cells, we did not find reduced levels of expression of NCR (NKp46 and NKp30) on fresh PBMC of infected animals. In addition, when derived in vitro, some NK cell populations from infected animals had reduced expression of NKG2D, which matched with a reduction in their cytolytic activity in vitro. Interestingly, the overall NKp30- and NKp46-redirected cytolytic function of NK cell populations derived in vitro from infected and uninfected macaques reflects their expression in peripheral blood cells and differs to some extent from the results obtained from HIV-1-infected human patients. The differences are surprising particularly if one considers that, as shown in the present work and previously (25), the expression and function of NKp30, NKp46, NKG2D, and NKp80 in NK cells from uninfected *M. fascicularis* is comparable to their uninfected human homologues. It is not clear whether these differences between infected macaques and infected humans could be attributed to intrinsic differences in NK cell function in response to different virus infections (HIV-1 vs SIV or SHIV) or to other as yet undescribed mechanisms. In fact, because the aim of this work was not a systematic analysis of NK cell function in SHIV-infected macaques, only a limited number of infected animals could be studied. Additional conditions including a larger cohort of infected and control animals, the evaluation during an acute vs a chronic infection setting, infection protocols using also different virus strains, and analysis of macaque NK cells in secondary lymphoid organs will be needed in this species and also to verify whether this can be observed in other experimentally lentivirus-infected nonhuman primates (e.g., rhesus macaques, chimpanzees).

In conclusion, particularly in view of the previously reported perturbation on the innate immune system in rhesus macaques that are acutely infected with a pathogenic SIV strain (SIVmac251) (16), the present characterization of functional NK cell-triggering receptors (NKp30, NKp46, NKp80, and NKG2D) and of functional NKG2C molecules could improve our understanding of NK cell function during vaccination studies, possibly favoring a comparison of the effects of vaccination in the different macaque models and allowing the dissection of the differences in innate immune responses to invading pathogens between human and macaque models of infection.

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Disclosures

The authors have no financial conflict of interest.

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