The recovered human lymphocytes from the HIV-DC immune mice were depleted of human $CD8^+$ cells by the magnetic beads-negative selection method (Dynal, Oslo, Norway). These enriched population of human $CD4^+$ T cells (2×10^6 cells) were co-cultured with freshly obtained 2×10^5 autologous mitomycin- C treated adherent PBMC as APC, in the presence of AT-2 inactivated HIV-1 containing 50 ng p24 in a volume of 1 ml RPMI 1640 medium supplemented with 20 U/ml human IL-2 in individual wells of a 24 well plate (BD Pharmingen, San Diego, CA) at 37°C. Two days later, these $CD4^+$ T cells were assayed for HIV-1 peptide-specific responses as follows. An initial screening was performed utilizing pools of 10 sequential overlapping peptides and once a pool of peptides was shown to lead to the synthesis of the HIV-1 suppression factor, a second screening was performed to identify the individual peptide. Thus, enriched population of 2×10^4 autologous fresh adherent PBMC were first dispensed in a volume of 50 µl into individual wells of a 96-well microtiter plate and triplicate wells incubated with 50 µl of media containing the various individual pools of 10 HIV-1 peptides for the primary screen (each at 10 µg/ml) or an individual HIV-1 peptide for the secondary screen (10 µg/m) for 1 h at 37°C. A total of 2×10^5 HIV-1 primed and *in vitro* re-stimulated $CD4^+$ T cells were then added to these cultures in a volume of 100 µl of media and the co-cultures incubated at 37°C in a 5% CO^2 humidified atmosphere. Two days later, the culture supernatants were harvested and aliquots assayed for HIV-1 inhibition activity and levels of IFN-γ.

HIV-1 inhibition and IFN-γ assays

As previously reported (Yoshida et al. 2003), PBMC were activated with anti-CD3/anti-CD28 antibody-coated magnetic beads (Dynal, Oslo, Norway) at cell to bead ratio of 1:1 in 20 U/ml IL-2 containing RPMI medium in vitro for 3 days. These activated PBMC's (5×10^5 cells/well) were pre-incubated at 37°C for 1 h in a volume of 100 µl with either various dilution of serum from the HIV-DC immune mice or culture supernatants obtained from the $CD4^+$ T cells that were stimulated with various HIV-1 peptides as described above. Then a volume of 100 µl of virus suspension containing 500 $TCID_{50}$ of $HIV\text{-}1_{JR\text{-}CSF}$ or $HIV\text{-}1_{NL4\text{-}3}$ was added to these cultures for an additional 4 h. The microtiter plate was centrifuged and the supernatant fluid removed, and the procedure repeated for three times. The cultures were then incubated in 0.2 ml of 20 U/ml IL-2-containing RPMI medium for 5 days. The levels of HIV-1 p24 produced in the culture supernatants were determined by a commercial ELISA kit (Zepto Metrix, Buffalo, NY). Levels of human IFN-γ in the stimulated culture supernatants were assayed utilizing a commercial ELISA kit (R&D Systems.inc. Minneapolis, MN).

HLA typing
Donor PBMC's were HLA typed by a DNA typing method.

Results

In order to identify the lineage of cells that was the major producer of the R5 HIV-1 suppression factor *in vivo*, SCID mice were engrafted with PBMC depleted of either $CD4^+$ or $CD8^+$ cells, together with HIV-DC. Various dilutions of the immune sera from these mice were then examined for the R5 HIV-1 suppression activity *in vitro*. As seen in Fig. 1, as little as 5% of the sera from the mice engrafted with either unfractionated PBMC or CD8-depleted PBMC significantly ($p < 0.05$) inhibited R5 HIV-1 infection. In contrast, up to 20% of the sera from the CD4-depleted PBMC-engrafted mice did not show any detectable R5 HIV-1 suppressive activity. Similar assays conducted using X4 HIV-1 failed to show any inhibition, denoting that the inhibition was selective for R5 HIV-1 (data not shown). In these engrafted mice, the numbers of human $CD4^+$ and $CD8^+$ T cells existed at similar levels as in the mice engrafted with unfractionated PBMC (data not shown). These data indicate that $CD8^+$ T cells are not required for the production of the R5 HIV-1 suppression factor *in vivo*, and that the HIV-1-immune human $CD4^+$ T cell population is the major source for the R5 HIV-1 suppression factor *in vivo*.

In efforts to map the epitopes of HIV-1 antigens that lead to the activation of $CD4^+$ T cells and the subsequent release of the HIV-1 suppression factor, a large panel of 15-20 mer peptides spanning a wide range of HIV-1 proteins were screened using a re-stimulation assay as described in the methods section. In order to enrich HIV-1 antigen-responding $CD4^+$ Th cells *in vitro*, $CD8^+$ T cell-depleted human lymphocytes obtained from three groups of HIV-DC immune hu-PBL-SCID mice, which had been engrafted with PBMC's from different individuals, were first stimulated with inactivated whole HIV-1 in the presence of autologous APC *in vitro*. These HIV-1 primed enriched $CD4^+$ T cells were then re-stimulated by co-culture in the presence of a pool of 10 HIV-1 peptides with fresh APC for primary screening. After 2 days, culture supernatants were harvested and examined for the R5 HIV-1 suppression factor at a final dilution of 50%. Supernatant fluids giving values of > 50% reduction compared to controls (incubated with media alone) in p24 antigen synthesized in the R5 HIV-1 infected cells were considered positive and a secondary screening performed to identify the individual HIV-1 peptide inducing such HIV-1 suppression factor activity. In order to make sure that the R5 HIV-1 suppression was not mediated by CCR5-binding β-chemokines, the culture supernatants to be tested were individually pre-absorbed by incubation with Heparin-Sepharose followed by incubation with a pool of blocking mAbs against human RANTES, MIP-1α and

MIP-1β each at a concentration of 10 μg/ml. Fig.2 a, b and c show representative data from three independent experiments conducted on cells from the 3 donors, respectively. These data suggest that the ability of HIV-1 peptides to induce the HIV-1 suppressor factor resides in multiple HIV-1 peptides from a number of HIV-1 proteins. As expected, there were variations noted in the HIV-1 proteins and peptides that induce the HIV-1 suppression factor in the 3 individual donors. Thus, the peptides recognized by donor 1 $CD4^+$ T cells consisted of peptides from HIV-1 pol, gag and env antigens, while those recognized by donor 2 were of gag, env, vif and rev antigens, and those recognized by donor 3 were of gag, env, vif and rev antigens. None of the supernatant fluids examined showed detectable X4 HIV-1 suppression activity in the assay employed (data not shown).

There was specificity in the assay since the R5 HIV-1 suppression factor was not detected either in the culture supernatants from APC cultured with or without HIV-1 peptides or supernatant fluids from immune $CD4^+$ T cells cultured in the absence of APC or peptides (data not shown). The individual variations in the response to peptides may be due to HLA restriction of responding antigenic peptides, as the three donors were of different HLA class II type (Table 1). It is interesting to note that the production of the R5 HIV-1 suppression factor by the bulk $CD4^+$ T cells was accompanied by the production of human IFN-γ but not in all the cases.

Discussion

Our laboratory has previously documented the synthesis of a soluble factor by PBMC's isolated from SCID mice previously engrafted with a mixture of human PBMC's and HIV-1-pulsed autologous DC's which suppressed infection of R5 HIV-1 *in vitro* (Yoshida et al. 2003). In the present study we have identified the $CD4^+$ T cell lineage as a requirement for the synthesis of this factor. In addition, we have attempted to identify the HIV-1 epitopes that are recognized by $CD4^+$ T cells that lead to the synthesis of this factor. There are a number of issues that need to be addressed in light of these findings. Thus, first of all, we submit that the anti-viral factor is distinct from the other anti-viral factors that have been previously identified. The fact that the suppressor activity was not neutralized by the addition of antibodies against the chemokines RANTES, MIP-1α and MIP-1β suggest that such suppression is not likely due to the synthesis of these chemokines which are known inhibitors of R5 HIV-1 infection (Capobianchi et al. 1998; Verani and Lusso. 2002). The factor described herein is also distinct from the $CD8^+$ cells anti-viral factor (CAF) identified by the laboratory of Dr. Jay Levy (Mackewicz et al. 2003) since it is synthesized by $CD4^+$ T cells but not $CD8^+$ T cells and whereas the CAF is effective against all HIV-1, HIV-2 and select retroviruses, the factor reported herein appears to be specific for R5 but not X4 tropic HIV-1. In addition, it is not likely to be either lymphotactin (Van Coillie et al. 1999), alpha-defensins (Zhang et al. 2002), the heparin binding protein termed anti-thrombin III (Geiben-Lynn et al. 2002), the natural killer enhancing factors A and B (Geiben-Lynn et al. 2003), and some additional factors (reviewed by Levy et al. (Blackbourn et al. 1994)) since all of these have been shown to be synthesized primarily by $CD8^+$ but not $CD4^+$ T cells. There has been an anti-viral factor that is synthesized following the interaction of $CD4^+$ T cells with APC's (Butera et al. 2001), however, at present it is not clear whether this factor is similar to the factor described herein and studies are underway to determine the similarity if any between these 2 factors. It is clear though that there are a plethora of such anti-viral factors that have been and continue to be described. The biologic reasons for such a multitude of anti-viral factors is difficult to imagine except to state that these are likely due to redundancies that nature has bestowed on the vertebrate species to protest itself from such viral infections.

The second issue concerns the multiple epitopes of HIV-1 that induce the generation of this factor by $CD4^+$ T cells. These epitopes interestingly were not restricted to a single HIV-1 protein and included both viral structural and accessory proteins. The obvious individual variations in responding antigenic peptides suggests that this may be due to HLA-restriction of peptide recognition by the immune $CD4^+$ T cells, although additional studies are necessary to confirm this issue. Furthermore, the present study suggests that the CD4 factor-producing HIV-1-immune $CD4^+$ T cells consist of heterogeneous clones. Since the peptides tested in this study were overlapping 15~20 mers, an additional study is required to more precisely define the specific HIV-1 epitope that has specificity for the $CD4^+$ T cells. It is also unclear whether the suppressor factor synthesized by $CD4^+$ T cells in response to distinct HIV-1 peptides is identical or distinct. Biochemical identification of the factor (s) is needed to address this issue and such studies are in progress.

The next issue concerns the synthesis of IFN-γ by some but not all the $CD4^+$ T cell in the assays reported herein. Since the cultures performed consisted of a mixture of $CD4^+$ T cells, it is not clear whether the synthesis of the anti-viral factor and IFN-γ was the result of a single clonal population of multiple clones of $CD4^+$ T cells. Clonal analysis needs to be performed to address this issue.

It is important to note that the HIV-1 gag p17 antigen peptide (LERFAVNPGLLETSE) recognized by the HIV-DC immune $CD4^+$ T cells from two out of the three donors shares amino acid sequence with another gag p17 peptide (ERFAVNPGLLETSEGCR) that is widely recognized by IFN-γ-producible $CD4^+$ Th cells from at least 25% HIV-1-infected individuals (Kaufmann et al. 2004). These results strongly support the use of DC-based vaccination of hu-PBL-SCID mice with whole inactivated HIV-1 virion to stimulate and expand HIV-1-specific

$CD4^+$ T cells in efforts to study the effectiveness of these cells for anti-viral control. More importantly, it should be noted that the CD4 factor producing $CD4^+$ T cells generated in the hu-PBL-SCID mice recognize multiple HIV-1 proteins similar to the studies that have reported for HIV-1-specific IFN-γ producing $CD4^+$ T cells in HIV-1 infected individuals (Kaufman et al. 2004). Although the existence and clinical role of the CD4 factor in HIV-1-infected individuals remains unknown, the data reported herein suggest that HIV-1 vaccines containing multiple HIV-1 epitopes or proteins, rather than those with single HIV-1 protein or epitope, will be likely to be more effective in expanding a large number of multiple HIV-1-reactive $CD4^+$ Th cells that are capable of producing the CD4 factor and other helper and HIV-1-suppresing cytokines. This idea is partially supported by the recent findings by Lu et al. (2004) who showed the efficacy of a therapeutic dendritic cell (DC)-based whole HIV-1 virion vaccination for HIV-1 infection.

As far as we know up to present, there have been no reports on cytokines that are identical to the CD4 anti-viral factor described herein. Attempts to identify the biochemical nature of this novel anti-viral factor have been hampered by both our inability to prepare sufficiently large quantities that are required for both biochemical identification and detailed characterization of this molecule coupled with the labile nature of the molecule. Fortunately, in our recent attempts, we have succeeded in immortalizing CD4 factor-producing $CD4^+$ T cells by HTLV-I-mediated transformation (Yoshida et al. unpublished). These cells will be helpful for the identification of not only the factor itself, but also its putative receptor.

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Figure Legends

Fig.1 Requirement of human $CD4^+$ cells, but not $CD8^+$ cells, to generate the R5 HIV-1 suppression factor in the DC-HIV-immune hu-PBL-SCID mice.

Together with HIV-DC, PBMC or those depleted of $CD8^+$ or $CD4^+$ cells were engrafted into the spleen of four SCID mice per group. After 5 days, mice were boosted with HIV-DC. After 5 days, serum samples from each group were pooled and tested for the R5 HIV-1 suppression activity on activated PBMC. Bars show the means of HIV-1 p24 values in the PBMC culture supernatants on day 5 post infection. HIV-1 proliferation in medium control was 7.7 ng/ml.

Fig.2 Recognition of various HIV-1 peptides by HIV-1-immune human $CD4^+$ T cells from three different donors generated in the HIV-DC-immune hu-PBL-SCID mice.

HIV-DC immune CD4+ T cells (2×10^5 cells) were stimulated with various peptides at a concentration of 10 μg/ml in the presence of 2×10^4 fresh adherent PBMC as APC in a volume of 200 μl in individual wells of a 96 well plate. After 2 days, the culture supernatants were harvested and their activity of R5 HIV-1 inhibition and the levels of human IFN-γ were determined as described in the materials and methods. Percent inhibition of HIV-1 production was calculated using values obtained from HIV-1 infected PBMC that were pretreated with the medium alone. Representative data from three independent experiments for donor 1 (a), donor 2 (b), and donor 3 (c) were show.

Footnotes

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Abbreviations used in this paper: HIV-1, human immunodeficiency virus type I; DC, dendric cells; HIV-DC, inactivated HIV-1-pulsed DC: Th, helper T; hu-PBL-SCID mouse, severe combined immunodeficiency mouse engrafted with human PBMC; AT-2, aldrithiol-2; $TCID_{50}$, 50% tissue culture infectious dose.

Table I HLA class II typing

	DR	DQ
Donor 1	15 / -	6 / -
Donor 2	4 / 14	4 / 7
Donor 3	4 / 13	6 / 8

Cross-Talk between Activated Human NK Cells and CD4$^+$ T Cells via OX40-OX40 Ligand Interactions[1]

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It is important to understand which molecules are relevant for linking innate and adaptive immune cells. In this study, we show that OX40 ligand is selectively induced on IL-2, IL-12, or IL-15-activated human NK cells following stimulation through NKG2D, the low affinity receptor for IgG (CD16) or killer cell Ig-like receptor 2DS2. CD16-activated NK cells costimulate TCR-induced proliferation, and IFN-γ produced by autologous CD4$^+$ T cells and this process is dependent upon expression of OX40 ligand and B7 by the activated NK cells. These findings suggest a novel and unexpected link between the natural and specific immune responses, providing direct evidence for cross-talk between human CD4$^+$ T cells and NK receptor-activated NK cells. ***The Journal of Immunology*, 2004, 173: 3716–3724.**

For an effective T cell response at least two signals are needed: the first is delivered by TCR interaction with MHC and peptide, and the second involves ligation of costimulatory receptors. Costimulation can involve augmenting cell proliferation, cell survival, and/or the production of cytokines. Many receptors have now been described to be costimulatory, including receptors of the Ig superfamily, such as CD28 and ICOS, and receptors of the TNF superfamily. Interactions between TNF ligands and TNFR family members, including for example OX40 ligand (OX40L) and OX40, have been implicated in T cell costimulation (1). Expression of OX40L is inducible and has been reported on several hemopoietic cell types, including dendritic cells (2), B cells (3), T cells, and microglial cells, as well as on vascular endothelial cells (4). OX40L expression is induced on APCs several days after activation by CD40L-CD40 interactions or by inflammatory stimuli (1, 2). Recently, high levels of OX40L have been shown to be expressed on a new type of CD3$^-$CD4$^+$ accessory cell, located in B cell follicles, capable of promoting survival of Th2 cells through OX40-OX40L interactions (5). OX40 is expressed predominantly by activated CD4$^+$ T cells (6). OX40$^+$ cells are found in the T cell zones of lymphoid organs following priming with Ag (3), and also have been detected in situ in several inflammatory states, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, chronic synovitis, graft-vs-host disease, and on tumor-infiltrating lymphocytes (6–9). Ligation of OX40 on CD4$^+$ T cells by agonist reagents can increase clonal expansion and cytokine production (10), enhance memory T cell development (11), and augment anti-tumor immunity (12). OX40 has also been shown to play an important role in the stimulation of anti-viral CD4$^+$ T cell responses in vivo (13).

NK cells are lymphocytes that provide innate immunity against tumors and virus-infected cells. A balance of signals received from multiple activating and inhibitory receptors regulates their effector functions (14). These receptors allow NK cells to rapidly survey their environment for danger. When an imbalance in signaling favors activation, secretion of cytokines and/or release of cytotoxic granules occurs (14). In humans, NKG2D is one of the activating receptors that is expressed on NK cells, γδ T cells, and CD8αβ T cells (15). NKG2D recognizes as ligands UL16-binding protein 1 (ULBP1), ULBP2, ULBP3, ULBP4, and the MHC class I chain-related molecules, MICA and MICB (15, 16). These NKG2D ligands are generally absent or expressed at low levels on most healthy cells, but can be induced by viral (17) and bacterial infections (18, 19). In addition, they are frequently up-regulated in many epithelial tumors (20) and in "stressed" cells (21).

Several studies have focused on the ability of NK cells to regulate adaptive immune responses through the production of Th1-type cytokines early during infection (22) or through the activation of dendritic cells (23). In addition, by establishing cocultures of NK- and Ag-activated T cells, it has been shown that human NK cells can be induced to secrete IFN-γ in response to IL-2 produced by activated T cells (24). In contrast, much less has been reported about the physical interactions that may take place between NK cells and adaptive immune cells, in particular CD4$^+$ T cells.

In this study, we show that OX40L can be induced on human NK cells by stimulation through their activating NK receptors. In addition, we present direct evidence for cross-talk between CD4$^+$ T cells and NK cells in which OX40-OX40L and CD28-B7 interactions contribute to T cell proliferation and IFN-γ production in response to TCR-induced activation.

Materials and Methods

Reagents, cytokines, Abs, and flow cytometry

Human rIL-12 and IL-15 were purchased from BioSource International (Camarillo, CA). The National Cancer Institute Biological Resources

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[4] Abbreviations used in this paper: OX40L, OX40 ligand; cIg, control Ig; SEB, staphylococcal enterotoxin B; ULBP, UL16-binding protein; KIR, killer cell Ig-like receptor.

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Branch Preclinical Repository (Frederick, MD) generously provided human rIL-2. Staphylococcal enterotoxin B (SEB) and PHA were purchased from Sigma-Aldrich (St. Louis, MO). The following mouse anti-human mAbs were used: anti-killer cell Ig-like receptor (KIR)2DS2 (DX27), neutralizing anti-CD80 (L307), and anti-CD86 (IT2.2) (BD Pharmingen, San Diego, CA), FITC-conjugated anti-CD80 (BU63; Caltag Laboratories, Burlingame, CA), FITC-conjugated anti-CD86 (MEM-233; Caltag Laboratories), anti-CD8α (Leu2a; BD Pharmingen), anti-CD4 (Leu3a; BD Pharmingen), anti-HLA-DR (BD Pharmingen), anti-NKG2D (clone 149810; R&D Systems, Minneapolis, MN), anti-CD56 (DX32), neutralizing anti-OX40L (5A8) (2, 4), anti-CD16 (B73.1) (kindly provided by Dr. G. Trinchieri, Schering-Plough, Dardilly, France), and anti-CD3 (OKT3; American Tissue Culture Collection, Manassas, VA). PE-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), FITC-conjugated anti-mouse IgG was purchased from Zymed Laboratories (South San Francisco, CA), and goat anti-mouse IgG $F(ab')_2$ was from Cappel Laboratories (ICN Biomedicals, Opera, Milan, Italy). Cells were analyzed by using a FACSCalibur (BD Biosciences, San Jose, CA) or a small desktop Guava Personal Cytometer with Guava ViaCount and Guava Express software (Burlingame, CA). Viable lymphocyte populations were gated based on forward and side scatters and by propidium iodide staining.

Cell lines, plasmids, and transfectants

The NKL cell line, generously provided by Dr. Mike Robertson (25), was cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 U/ml human rIL-2. Cells were cultured at a density of 5×10^5/ml in a 37°C incubator with 5% CO_2. For all experiments, cells were grown at a density of 1×10^6/ml in medium containing IL-2. Generation of NKL stably expressing KIR2DS2 was described previously (26). Because mouse Ba/F3 pro-B cells are IL-3 dependent for their proliferation, the Ba/F3 cells used in these experiments were transfected with an expression plasmid containing the mouse cDNA IL-3 to provide for autocrine growth (kindly provided by Dr. S. Tangye, Centenary Institute, Sydney, Australia). MICA transfectants were established by retroviral transduction using the pMX-pie vector (27, 28) containing a MICA*0019 cDNA.

Preparation of NK cells and T cells

Small resting $CD4^+$ T lymphocytes were purified as follows: PBMC were isolated by lymphoprep density gradient centrifugation, monocytes and B cells were removed by adherence to nylon wool, then cells were labeled with anti-CD8, anti-CD56, anti-HLA-DR, and anti-CD19 mAbs, and these cells were mixed with magnetic beads coated with goat anti-mouse IgG (Dynal Biotech, Oslo, Norway). Thereafter, $CD8^+$, $CD19^+$, $HLA\text{-}DR^+$, and $CD56^+$ cells were removed by magnetic cell sorting. The remaining cells were >98% $CD4^+CD3^+$, as assessed by immunofluorescence and flow cytometric analysis. Polyclonal NK cell cultures were obtained by coculturing nylon nonadherent PBMC with irradiated (3000 rad) RPMI 8866 B cells for 9–10 days at 37°C in a humidified 5% CO_2 atmosphere, as previously described (29). NK cell cultures were >90% $CD16^+CD56^+CD3^-$, as assessed by immunofluorescence and flow cytometric analysis. Contaminating T cells were depleted by magnetic cell sorting, yielding a final NK population >98% $CD16^+CD56^+CD3^-$.

Stimulation of the cells, RNA preparation, microarrays, and data analysis

Twenty-four-well culture plates were coated with goat anti-mouse IgG (5 μg/ml, in carbonate buffer, pH 9.6) at 37°C for 4 h. Wells were washed three times with PBS and primary Abs were added to each well at 10 μg/ml, or amounts indicated in the figures, and incubated overnight at 4°C in PBS. When in combination with anti-NKG2D mAb, anti-KIR2DS2 mAb was used at 0.5 μg/ml. NKL cells were plated at 2×10^6/ml in each well in 500 μl of medium. Poly(A)$^+$ RNA was isolated using an mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Gene expression modulation between unstimulated and stimulated NKL cells was evaluated by using Incyte standard procedures (Palo Alto, CA), as described elsewhere (30). Briefly, poly(A)$^+$ RNA were labeled with Cy3 or Cy5 fluorescent labeling dyes using reverse transcription, followed by hybridization onto a Human Drug Target 1 microarray (Incyte) (31, 32). This microarray contained a total of 9129 elements representing a total of 8481 unique gene clusters whose identity was confirmed by stringent PCR verification during manufacturing. The Cy3/Cy5 ratio for each element was considered valid if the signal to background ratios for both dyes exceeded 2.5, and if the signal of either dye exceeded 250 fluorescence units. A total of 6125 elements returned valid Cy3/Cy5 ratios for all 20 hybridizations (10 treatments hybridized in duplicates). Elements were further selected based on a minimum Cy3/Cy5 ratio of 2-fold in either direction in at least one experimental condition, yielding 406 elements of interest. These elements of interest were then clustered using an agglomerative clustering algorithm (Ward's method, JMP; SAS Institute, Cary, NC). All data are expressed in $\log_2$, where negative values denote gene up-regulation (Cy3 < Cy5) and reciprocally, positive values represent gene down-regulation (Cy3 > Cy5).

Cytokine and proliferation assays

Homogeneous populations of cultured human primary NK cells were activated for 72 h with IL-2 (100 U/ml) and stimulated with anti-CD16 plate-bound mAb for 18 h. In some experiments, NK cells were preactivated with IL-15 (10 ng/ml) or IL-12 (10 U/ml). Dead cells were removed by Ficoll-gradient centrifugation. NK cells were fixed with 1% paraformaldehyde (in PBS, pH 7.4) for 7 min at room temperature. Different numbers of NK cells were plated with 1×10^5 highly purified autologous $CD4^+$ T cells, and cultured for 5 days in the presence of soluble anti-CD3 mAb (5 μg/ml) or SEB (0.5–25 ng/ml) or PHA (50 ng/ml). Blocking Ab against OX40L and/or CD80 and CD86 was added on day 0 at 5 μg/ml. Wells were pulsed with 0.5 μCi of [^{3}H]thymidine for the final 18 h of culture, and incorporated radioactivity was measured in a scintillation counter. Data are represented as the mean of cpm ± SD (triplicates). In some experiments, supernatants were collected at day 3 or 5, and the amount of IL-4 and IFN-γ was quantified by specific ELISA kits (BioSource International).

Results

Microarray analysis shows up-regulation of OX40L following triggering of NK-activating receptors on a human NK cell line

Microarray analysis was used to characterize genes up-regulated by the stimulation of NKG2D alone or in combination with the DAP12-associated KIR2DS2-activating receptor. As a model, we used a human NK cell line, NKL, which constitutively expresses the DAP10-associated NKG2D receptor (33), and was transfected with KIR2DS2 (26). Because NKG2D alone is an insufficient stimulus for the transcription-dependent production of IFN-γ (26, 34), this cell system is particularly useful because it provided the opportunity to evaluate the efficacy of NKG2D costimulation using as a read out the amplification of KIR2DS2-induced IFN-γ (Ref. 26 and data not shown). Poly(A)$^+$ mRNA from resting and stimulated NKL cells was extracted, and cDNA was prepared for the comprehensive analysis of gene transcription by using microarray technology. A Human Drug Target 1 Incyte microarray containing a total of 9128 elements was used. Analysis of data was performed using a hierarchical clustering algorithm to group genes with similar expression patterns across all the samples. We focused our attention on a group of seven genes that were amplified significantly following the simultaneous cross-linking of KIR2DS2 and NKG2D receptors (Table I). These genes included three chemokines (i.e., lymphotactin, MIP-1β, and CCL18), granzymes B and H, the platelet-activating receptor homologue (a seven transmembrane receptor of unknown function), and the TNF member OX40L (CD134L). Among this group of genes, OX40L mRNA was the only one that was up-regulated by NKG2D cross-linking alone (Table I). Previously, OX40L expression has been implicated predominantly in the function of APCs, such as activated monocytes, dendritic cells, and B cells. Thus, this unexpected finding prompted us to investigate the role of OX40L in human NK cell function.

Results from the microarray experiment were confirmed by showing that cross-linking KIR2DS2, NKG2D, and KIR2DS2 plus NKG2D indeed enhanced transcription of OX40L in NKL cells, as determined by quantitative RT-PCR analysis (data not shown). More importantly, KIR2DS2- and NKG2D-induced activation resulted in an increased expression of OX40L on the cell surface of NKL cells, as determined by using a specific anti-OX40L mAb

Table 1. *Microarray analysis of NKL cells stimulated through NKG2D and/or KIR2DS2*[a]

Gene Name	Accession Number	cIg	KIR2DS2	NKG2D	KIR2DS2 + NKG2D
Lymphotactin	AL031736	0.31	−0.85	0.48	−2.39
MIP-1β	AV758471	0.06	−1.03	−0.07	−2.14
Granzyme H	NM_004131	0.43	−0.58	0.96	−1.87
Granzyme B	M57888	0.12	−0.48	0.81	−1.74
PAR	NM_013308	0.32	−0.72	0.07	−1.63
OX40L	**BE349175**	**0.13**	**−0.68**	**−0.20**	**−1.26**
CCL18	NM_00298	0.48	−0.58	0.14	−1.42

[a] Differential expression ratios of control Ig (cIg)-treated NKL cells (Cy3) compared to anti-KIR2DS2 and/or anti-NKG2D-treated NKL cells (Cy5) expressed in $\log_2$. Negative values represent up-regulation of transcription compared with cIg-stimulated cells.

(Fig. 1*A*). Stimulation with high doses of anti-KIR mAb or anti-NKG2D mAb alone substantially up-regulated OX40L on the surface of NKL cells. In addition, anti-NKG2D mAb augmented up-regulation of OX40L on NKL cells stimulated with a suboptimal dose of anti-KIR mAb (Fig. 1*A*).

OX40 is expressed predominantly on activated $CD4^+$ T cells and prior studies have shown that interactions between OX40 on activated $CD4^+$ T cells and OX40L on APCs can augment T cell proliferation and cytokine production. Therefore, studies were performed to determine whether OX40L-bearing NK cells could costimulate $CD4^+$ T cell proliferation. NKL cells, which constitutively express OX40L (Fig. 1*A*), were cocultured with freshly isolated human $CD4^+$ T cells and were stimulated with anti-CD3 mAb or PHA. As shown in Fig. 1*B*, NKL indeed augmented $CD4^+$ T cell proliferation, and this activity was blocked, in part, in the presence of a neutralizing anti-OX40L mAb. These studies indicated that OX40L on NKL is functional and contributes to the proliferation of $CD4^+$ T cells. However, these studies were complicated by the necessity to use allogeneic $CD4^+$ T cells and also because NKL is a long-term NK cell line established from a patient with NK cell leukemia (25). Therefore, it was important to validate these findings using autologous NK cells and T cells from normal healthy individuals.

Both cytokines and NK receptor-mediated stimulation are required to induce OX40L on human peripheral blood NK cells

Freshly isolated, highly purified human peripheral blood NK cells do not express OX40L on the cell surface (data not shown), although a prior study had reported the presence of OX40L transcripts (35). Because the NKL cell line requires IL-2 for growth, we investigated whether OX40L could be induced on peripheral blood NK cells from healthy adults simply by culture in the presence of IL-2 or other cytokines known to stimulate NK cells, e.g., IL-12 and IL-15. As shown in Fig. 2*A*, culture of normal human peripheral blood NK cells in IL-2, IL-12, or IL-15 failed to induce OX40L. Therefore, based on the observation that OX40L was up-regulated in NKL cells stimulated through its activating receptors, we stimulated human polyclonal NK cells through CD16, an IgG FcR that signals via the ITAM-bearing FcεRIγ and CD3ζ adapter proteins. Whereas treatment with cytokines alone failed to induce OX40L, the majority (typically 60% or more) of normal NK cells stimulated by plate-bound anti-CD16 mAb together with IL-2, IL-12, and IL-15 expressed OX40L at high levels on the cell surface (Fig. 2*A*). Stimulation with anti-CD16 mAb in the absence of IL-2 (or IL-12 or IL-15) induced OX40L only on a small proportion of NK cells. A dose-dependent induction of OX40L was observed when NK cells were activated with anti-CD16 mAb in the presence of IL-2 (Fig. 2*B*). In contrast to OX40L, culture of peripheral blood NK cells in IL-2 only did induce expression of CD86 (Fig. 2*C*) and this was not enhanced by stimulation with anti-CD16 mAb (Fig. 2*D*). CD80, another ligand of the CD28 costimulatory receptor on T cells, was not induced by IL-2 (Fig. 2*C*), and there was only a very slight indication of CD80 induction when both IL-2 and anti-CD16 stimulation were combined (Fig. 2*D*).

Because studies using the NKL cell line indicated that stimulation through the NKG2D receptor up-regulated OX40L, we also investigated this using peripheral blood NK cells from healthy adults. Polyclonal populations of NK cells from healthy individuals were expanded in culture, preactivated with IL-2 and stimulated with a plate-bound mAb against NKG2D. Fig. 3*A* shows that NKG2D cross-linking induced OX40L on ~20% of the NK cells.

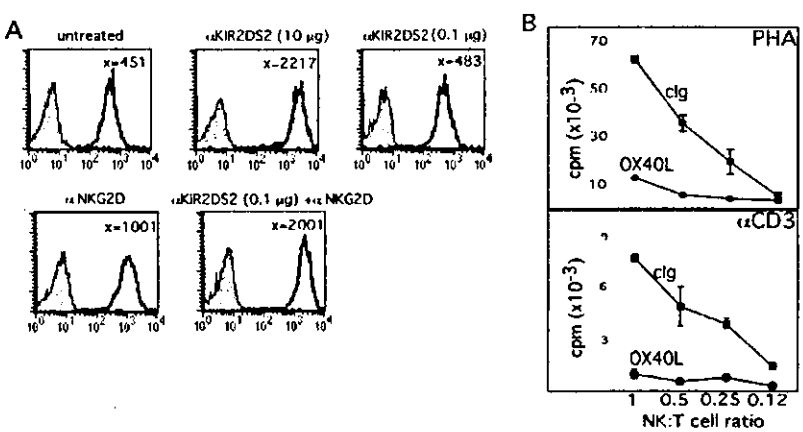

FIGURE 1. Up-regulation of OX40L on NKL by NK receptors and costimulation of $CD4^+$ T cell proliferation. *A*, NKL cells were stimulated with plate-bound mAb anti-NKG2D (10 μg/ml), anti-KIR2DS2 (10 μg/ml or 0.1 μg/ml), or both for 18 h. Cells were harvested and stained with PE-conjugated anti-OX40L mAb (open histograms) or with an isotype-matched cIg (filled histograms). *B*, Different amounts of paraformaldehyde-fixed NKL cells were cultured with 1×10^5 $CD4^+$ T cells in the presence of soluble anti-CD3 (5 μg/ml) or PHA (50 ng/ml). Neutralizing anti-OX40L mAb was added at day 0 and cocultures were harvested at day 5. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine for the final 18 h, and incorporated radioactivity was measured in a scintillation counter. A representative experiment of three is shown. Data are represented as the mean of cpm ± SD.

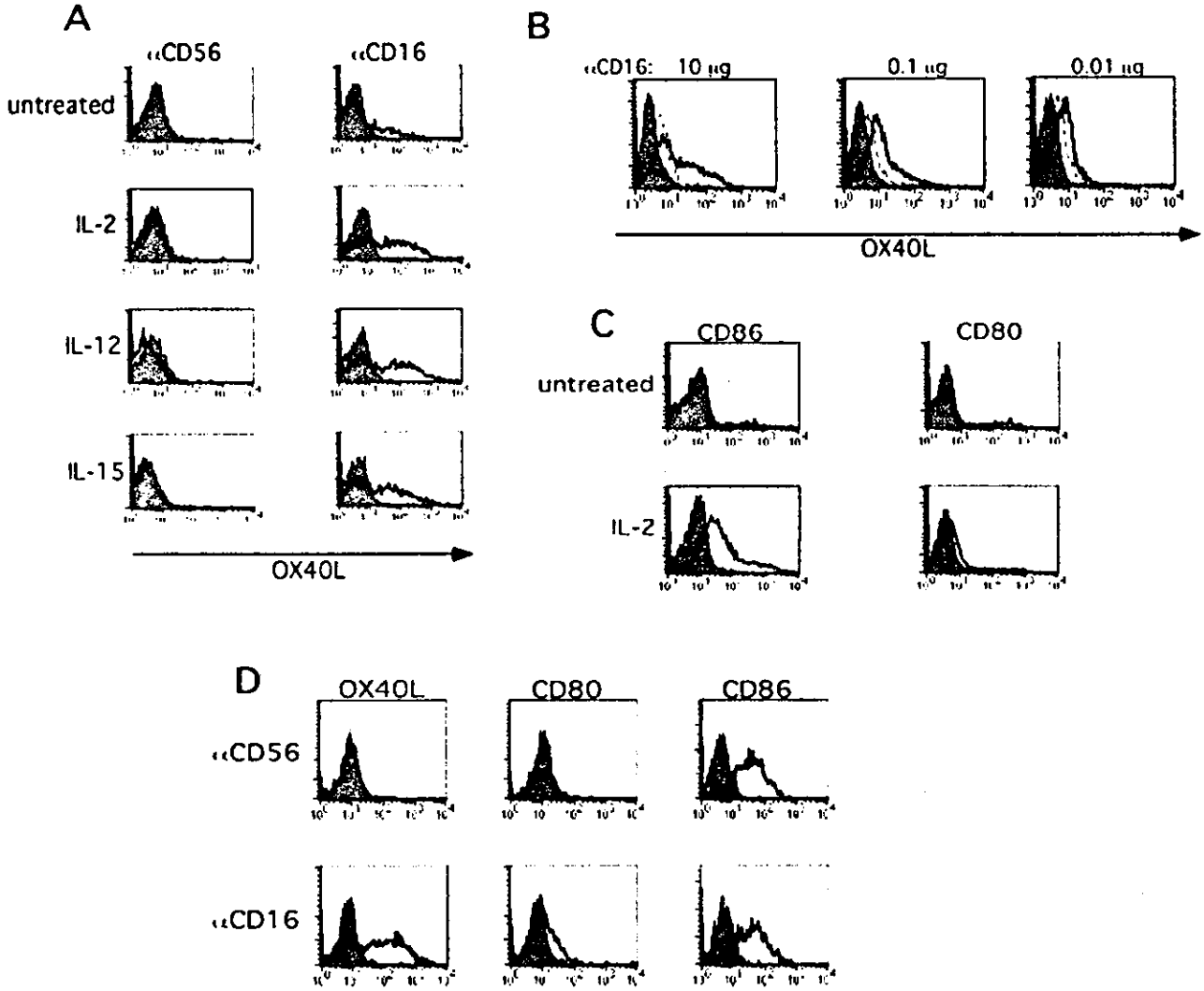

FIGURE 2. Induction of OX40L and B7 family members on human NK cells. *A*, Polyclonal human NK cells were preactivated with IL-2 (200 U/ml), IL-15 (10 ng/ml), or IL-12 (10 U/ml) for 48 h, and stimulated with plate-bound anti-CD16 mAb (saturating concentration). Plate-bound anti-CD56 mAb was used as a negative control. After 18 h of culture, cells were harvested and stained with PE-conjugated anti-OX40L mAb (thick line, open histograms) or with a cIg (filled histograms). *B*, IL-2-activated polyclonal NK cells were stimulated with different amounts (10 μg/ml, 0.1 μg/ml, and 0.01 μg/ml) of plate-bound anti-CD16 mAb (thick line). Plate-bound anti-CD56 mAb (dotted line) was used as a negative control for stimulation. Cells were harvested after 18 h and stained with FITC-conjugated anti-OX40L mAb (open histograms) or a cIg (filled histograms). A representative experiment of five is shown. *C*, Peripheral blood NK cells were cultured in the presence of IL-2 (200 U/ml) for 72 h and stained with FITC-conjugated anti-CD80 (thick lines, open histograms), FITC-conjugated anti-CD86 (thick lines, open histograms), or a cIg (filled histograms). *D*, IL-2-activated polyclonal NK cells were stimulated for 18 h with anti-CD16 or anti-CD56 (negative control) plate-bound mAbs. Cells were stained with PE-conjugated anti-OX40L mAb, FITC-conjugated anti-CD80 (thick lines, open histograms), FITC-conjugated anti-CD86 (thick lines, open histograms), or a cIg (filled histograms). In this experiment, CD86 was induced on these NK cells by coculture in IL-2, but was not further increased by stimulation with the anti-CD56 mAb used as a control.

As observed with anti-CD16 stimulation, induction of OX40L required both pretreatment with IL-2 and NKG2D activation because neither condition alone induced OX40L (data not shown). The ability of NKG2D stimulation to induce OX40L on NK cells was further validated by activation using stimulator cells bearing MICA, a physiological ligand of the NKG2D receptor. IL-2-preactivated peripheral blood NK cells were cocultured for 18 h with different ratios of the mouse pro-B cell line Ba/F3 or Ba/F3 cells stably expressing human MICA. As with anti-NKG2D mAb stimulation, OX40L was induced on ~20% of the IL-2-activated NK cells cocultured with MICA$^+$Ba/F3 cells, but not the untransfected Ba/F3 cells (Fig. 3*B*). Analysis of the kinetics of OX40L expression on human NK cells following stimulation with MICA-bearing cells showed that OX40L expression was transient; it was expressed rapidly after 5 h, peaked at 18 h, and then declined between 32 to 48 h poststimulation (Fig. 3*C*). These IL-2-activated NK cells were able to efficiently kill the MICA$^+$Ba/F3 cells, but not the untransfected Ba/F3 cells, demonstrating that the NKG2D receptor on the NK cells was specifically activated (data not shown).

Therefore, both by stimulation with anti-NKG2D mAb and by interaction with MICA$^+$Ba/F3 cells, we observed induction of OX40L on a subset comprising ~20% of IL-2-activated peripheral blood NK cells (Fig. 3). An examination of the phenotype of the NK cells stimulated by either anti-NKG2D or MICA$^+$Ba/F3 cells revealed that OX40L was induced on both the CD56^{bright}CD16$^{-/low}$ and on the CD56^{int}CD16^{high} peripheral blood NK cell subsets, although within these subsets a relatively higher fraction of the CD56^{bright}CD16$^{-/low}$ NK cells expressed OX40L (our unpublished observation). Therefore, the subset of peripheral blood NK cells presenting OX40L after NKG2D stimulation was not

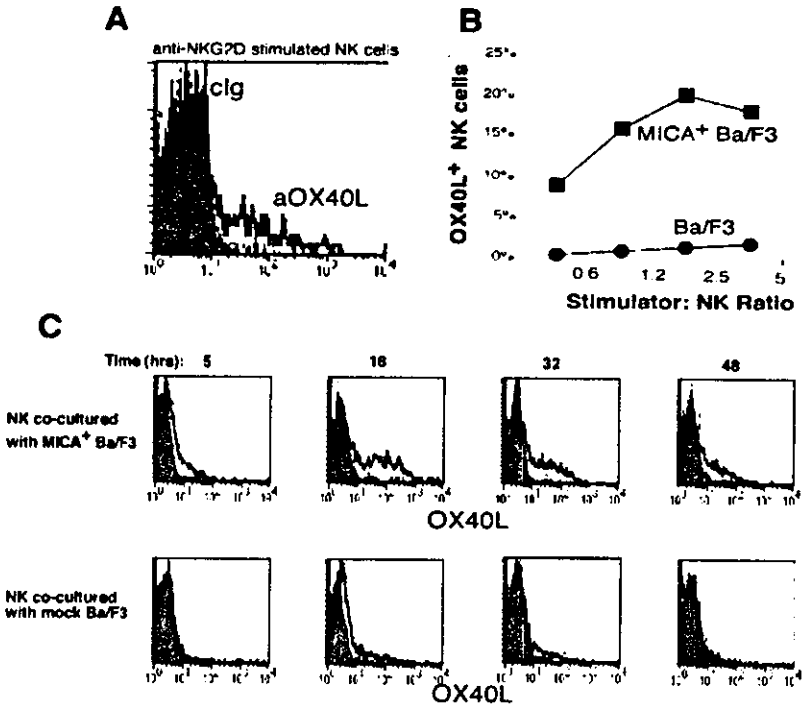

FIGURE 3. NKG2D stimulation induces OX40L on human polyclonal NK cells. *A*, Polyclonal human NK were activated with IL-2 for 72 h and stimulated with plate-bound anti-NKG2D mAb (thick line). Anti-CD56 mAb was used as a negative control of stimulation (dotted line). After 18 h of culture, cells were harvested and stained with FITC-conjugated anti-OX40L mAb (thick line, open histogram) or with cIg (filled histogram). A representative experiment of six is shown. *B*, IL-2-activated polyclonal NK cells were cultured with different numbers of mock Ba/F3 (●) or human MICA$^+$Ba/F3 (■) transfectants. After 18 h of coculture, NK cells were stained with anti-OX40L or control mAbs and the percentage of OX40L$^+$ NK cells is shown. A representative experiment of three is shown. *C*, Kinetics of OX40L induction on NK cells by coculture with NKG2D ligand-bearing cells. IL-2-activated polyclonal NK cells were cultured at a 1:2.5 stimulator:NK cell ratio with mock Ba/F3 (*lower panels*) or MICA$^+$Ba/F3 (*upper panels*) transfectants. Cells were harvested after 5, 18, 32, and 48 h of coculture, and stained anti-OX40L mAb (thick line, open histograms) and cIg (filled histograms). A representative experiment of three is shown.

restricted to either of these functionally distinct subsets defined by levels of CD56 and CD16 expression. In experiments combining both anti-NKG2D and anti-CD16 mAb stimulation (using optimal and saturating concentrations of both mAbs), the proportion of peripheral blood NK cells that expressed OX40L was equivalent to using optimal stimulation with anti-CD16 alone (data not shown).

NK cell costimulation of TCR-dependent CD4$^+$ T cell proliferation via OX40L-OX40 interactions

Our preliminary studies demonstrated that the OX40L$^+$ NKL leukemic cells were able to augment the proliferation of allogeneic human resting peripheral blood CD4$^+$ T cells stimulated with anti-CD3 mAb or PHA. The proliferation was partially, but substantially, inhibited by using a neutralizing anti-OX40L mAb (Fig. 1*B*). To address the potential interactions between NK cells CD4$^+$ T cells in a more physiological context, we performed additional experiments using autologous NK cells and CD4$^+$ T cells. We assayed proliferation induced not only by anti-CD3 mAb, but also by using autologous activated human NK cells (that express HLA-DR) to present SEB to autologous resting CD4$^+$ T cells. Because we had determined that anti-CD16 was more efficient than anti-NKG2D for inducing OX40L on peripheral blood NK cells, this system was chosen to evaluate the role of OX40L in the interactions between NK cells and autologous CD4$^+$ T cells. Highly purified, IL-2-preactivated peripheral blood NK cells were stimulated with anti-CD16 mAb, the NK cells were paraformaldehyde-fixed to prevent their proliferation or secretion of cytokines, and these cells were cocultured at varying ratios with highly purified resting autologous CD4$^+$ T cells in the presence of soluble anti-CD3 mAb. As shown in Fig. 4*A*, CD16-activated autologous NK cells efficiently costimulated anti-CD3-induced proliferation of CD4$^+$ T cells. This TCR-induced T cell proliferation was in part dependent upon OX40–OX40L interactions, because the proliferation was inhibited on average 60% (based on experiments using NK and T cells from seven different blood donors), in cultures containing the anti-OX40L specific neutralizing mAb 5A8. IL-2-activated NK cells that did not express OX40L were also able to costimulate the anti-CD3-induced proliferation of autologous CD4$^+$ T cells; however, this was always of a lower magnitude (approximately one third) than when the NK cells expressed OX40L as a consequence of prior stimulation via CD16 (Fig. 4*B*). An analysis of cytokines produced in these cultures revealed that the NK cell-costimulated T cells produced IFN-γ, but not IL-4 (Fig. 4*C*). Similar to the effects observed in the proliferation assays, anti-OX40L partially, but substantially, inhibited IFN-γ secretion induced by NK cell costimulation. In these experiments, fixed activated NK cells were used for costimulation to avoid the proliferation of the NK cells in response to IL-2, confirming that CD4$^+$ T cells were the responding population in the cultures, and to exclude that NK cell-derived cytokines were required for costimulation. We also established autologous NK:T cell cocultures with irradiated NK cells, and similar to fixed activated NK cells, irradiated activated NK also efficiently costimulated T cell proliferation in a OX40–OX40L dependent manner (data not shown).

Next, we investigated the role of OX40-OX40L interactions in autologous NK:T cell cocultures in response to a physiological TCR ligand, rather than anti-CD3 mAb. Bacterial superantigens bind with high affinity to MHC class II Ags on APCs and with TCR β-chains on the responding T cells. This results in the T cell activation responsible for toxic shock syndrome and food poisoning. Activated NK cells express MHC class II molecules (36, 37) and present SEB to T lymphocytes (37). Thus, anti-CD16-activated MHC class II-positive NK cells and autologous freshly isolated resting CD4$^+$ T cells were cultured in the presence of different concentrations of SEB. As shown in Fig. 5*A*, activated NK cells efficiently present SEB to autologous CD4$^+$ T cells, stimulating T cell proliferation. Furthermore, OX40-OX40L interactions were required for optimal T cell proliferation, as shown in Fig. 5*B* by the ability of anti-OX40L mAb to substantially inhibit SEB-induced T cell proliferation. Collectively, these data indicate that CD16-activated NK cells can efficiently costimulate anti-CD3 or SEB-induced proliferation of autologous CD4$^+$ T cells, and that OX40L-OX40 interactions are critically involved.

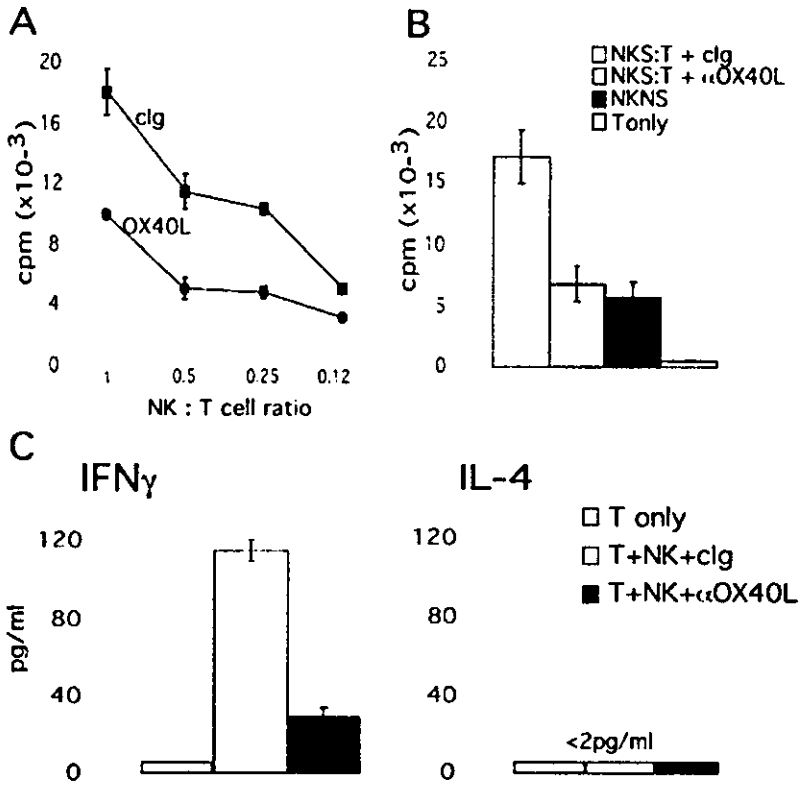

FIGURE 4. Anti-CD3 induced $CD4^+$ T cell proliferation and IFN-γ production costimulated by OX40L on autologous CD16-activated NK cells. *A*, IL-2-activated polyclonal NK cells were stimulated with plate-bound anti-CD16 mAb and fixed with 1% paraformaldehyde. Different numbers of anti-CD16-activated NK cells were plated with 1×10^5 autologous resting $CD4^+$ T cells in the presence of soluble anti-CD3 and cultured as described in Fig. 1*B*. Neutralizing anti-OX40L mAb or a cIg was added at day 0. A representative experiment of four is shown. Data are represented as the mean of cpm ± SD (triplicates). *B*, Cocultures of autologous activated NK cells and resting $CD4^+$ T cells at a ratio of 1:1 were established as described in Fig. 4*A*, using anti-CD16-stimulated NK cells (NKS) or cIg (anti-CD56 mAb)-treated NK cells (NKNS). Neutralizing anti-OX40L mAb or a cIg was added to the coculture of NKS and autologous $CD4^+$ T cells stimulated with anti-CD3, as indicated. Data are represented as the mean of cpm ± SE of seven independent experiments. *C*, Activated NK cell-resting $CD4^+$ T cell cocultures stimulated with anti-CD3 mAb were established as described in Fig. 4*A*. Neutralizing anti-OX40L mAb or a cIg was added to the cocultures, as indicated. Supernatants were collected after 72 h and tested for the presence of IFN-γ or IL-4. Data are represented as the mean ± SD (triplicates). A representative experiment of three is shown.

OX40L and B7 contribute to NK cell costimulation of $CD4^+$ T cell

We considered that the inability of anti-OX40L mAb to completely block $CD4^+$ T cell proliferation induced by activated NK cells may be due to the presence of CD86 (and perhaps CD80) on the activated NK cells (Fig. 2, *C* and *D*). Therefore, additional experiments were performed in which CD16-stimulated NK cells were cocultured with autologous $CD4^+$ T cells and anti-CD3 using a mixture of neutralizing mAbs against CD80 and CD86 (38) alone or in combination with anti-OX40L (Fig. 6). Interestingly, while mAbs against CD80 plus CD86 or OX40L individually partially inhibited NK cell-induced T cell proliferation, we observed that the combination of neutralizing mAbs against CD80, CD86, and OX40L completely blocked TCR-dependent $CD4^+$ T cell proliferation (results from two different blood donors are shown and are representative of five experiments). Collectively, these data show that CD16-stimulated NK cells efficiently costimulate TCR-dependent $CD4^+$ T cell proliferation through the expression of OX40L and B7-family members on the CD16-activated NK cells.

Discussion

Although it has been appreciated that NK cell production of IFN-γ and possibly other cytokines and chemokines can affect innate and adaptive immune responses, the potential role for direct cell-cell interactions between NK cells and T lymphocytes, in particular $CD4^+$ T cells, has not been explored. Roncarolo and colleagues (39) previously reported that human NK cell clones are able to stimulate autologous $CD4^+$ T cells, but the molecules involved in this process were not defined. Our unexpected finding that OX40L was up-regulated when NK cell receptors were stimulated on a transformed NK cell line prompted us to re-evaluate how activated NK cells are able to augment the TCR-dependent proliferation of resting autologous peripheral blood $CD4^+$ T cells. In this study, we provide evidence that activated human NK cells are able to help TCR-stimulated autologous $CD4^+$ T cells by a process that involves both OX40L and B7 costimulation.

Resting peripheral blood NK cells express neither OX40L nor B7, and different stimuli are required to induce these costimulatory molecules. Culture in IL-2 alone was sufficient to induce CD86, but not OX40L. By contrast, stimulation with IL-2 and activation through an NK receptor was required to induce OX40L. In addition to IL-2, IL-12 and IL-15 were also able to prime NK cells such that they up-regulated OX40L when subsequently stimulated via CD16. Because IL-12 and IL-15 are innate cytokines that may be more available at a site of inflammation or an ongoing immune response, these may represent the more physiologically relevant cytokines in vivo.

With respect to the NK receptors that induced OX40L, our first clues were derived from studies of the transformed NKL cell line. Although this cell constitutively expressed OX40L, it can be up-regulated by engaging either the DAP12-associated KIR2DS2 receptor that activates the Syk and ZAP70 tyrosine kinase pathways (40), or by stimulating the DAP10-associated NKG2D receptor that uses a PI3K-dependent activation pathway (33). We do not have Abs that can discriminate between the activating and inhibitory KIR; therefore, in studies of peripheral blood NK cells, we stimulated the NK cells with anti-CD16, which couples to the ITAM-bearing FcεRIγ and CD3ζ adapter proteins and activates Syk and ZAP70. When IL-2-preactivated peripheral blood NK cells were stimulated with either anti-CD16 or anti-NKG2D (or exposed to cells expressing the NKG2D ligand, MICA), OX40L was rapidly induced. Interestingly, only a subset comprising ~20% of the peripheral blood NK cells expressed OX40L after stimulating NKG2D, despite the fact that essentially all of the NK cells expressed NKG2D. Further studies are needed to determine why expression of OX40L was confined to a subset of the NKG2D-activated NK cells. By contrast, a much larger frequency of NK cells (typically 60% or more) expressed OX40L after CD16

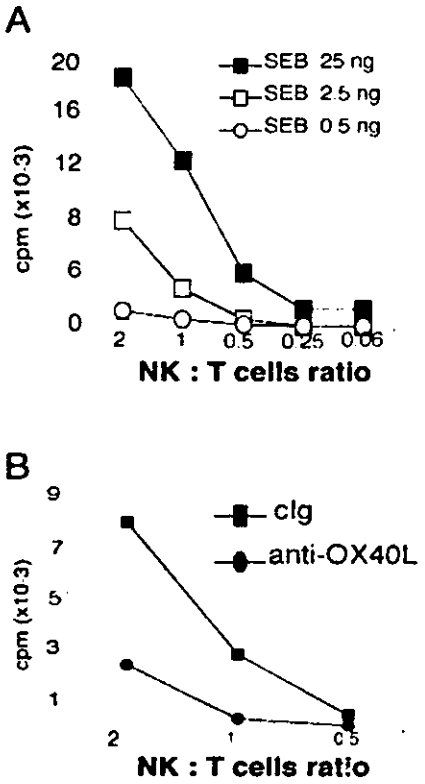

FIGURE 5. OX40L expressed on autologous NK receptor-activated NK cells is involved in SEB-induced proliferation of $CD4^+$ T cells. *A*, Anti-CD16-activated NK cells were prepared as described in Fig. 4*A*. Autologous resting $CD4^+$ T cells and activated NK cells were cocultured for 5 days in the presence of different concentrations of SEB, as indicated. Data are represented as the mean of cpm ± SD (triplicates). A representative experiment of two is shown. *B*, Autologous resting $CD4^+$ T cells and anti-CD16-activated NK cells at the indicated ratios were cocultured in the presence of 2.5 ng/ml SEB for 5 days. Neutralizing anti-OX40L mAb or cIg was added at day 0. Data are represented as the mean of cpm ± SD (triplicates). A representative experiment of three is shown.

activation. Many of the NK receptors, e.g., NKp30, NKp44, NKp46, CD16, and the activating KIR (41), use ITAM-based adapter proteins to activate the Syk/ZAP70 tyrosine kinases. Therefore, we suspect that OX40L may be induced when any of these diverse receptors are engaged because they use a common downstream signaling pathway. Together with the ability of IL-2, IL-12, or IL-15 to render the NK cells permissive for NK receptor induction of OX40L, our findings indicate that OX40L may be available in many different physiological situations for potential interactions with T cells bearing OX40.

Where might activated NK cells and $CD4^+$ T cells interact? This interaction might happen in peripheral tissues such as the liver in which both NK cells and T cells are resident (42) and accumulate following virus infection (43). Furthermore, a recent report has revealed that NK cells are relatively abundant in the human secondary lymphoid organs (44), and importantly, immunohistochemistry studies have detected NK cells in the parafollicular T cell areas of human lymph nodes (24), providing another possible location in which NK:T cell interactions might occur during an immune response. During a viral or bacterial infection, NK cells in the lymph nodes may be exposed to an environment containing IL-2, IL-12, or IL-15, and potential NKG2D ligands or immune complexes (that engage CD16), thereby providing the stimuli needed for induction of OX40L and allowing them to interact with activated $CD4^+$ T cell-expressing OX40.

It should be appreciated that activated human NK cells express high levels of MHC class II (36, 37), which provides them the potential to present Ag to human $CD4^+$ T cells. Indeed, in these studies, we have shown that activated NK cells have the capability to directly stimulate $CD4^+$ T cell proliferation by presenting SEB to $CD4^+$ T cells. Therefore, activated human NK cells possess not only the required costimulatory molecules (e.g., OX40L and B7) for potential interaction with activated $CD4^+$ cells, but they also, in theory, have the capacity of present Ags via MHC class II. Collectively, our in vitro experiments provide compelling evidence that human NK cells and autologous $CD4^+$ cells can interact and that OX40L is an important participant in this process. It is difficult to provide formal proof of this interaction in vivo in humans. Unfortunately, because activated mouse NK cells (unlike human NK cells) do not express MHC class II, mice do not provide a relevant or appropriate model to examine MHC class II TCR-dependent $CD4^+$ cell interactions with NK cells. Although dendritic cells are considered the most potent APCs, the fact that activated NK cells express MHC class II, CD86, and OX40L strongly suggests the possibility that they may also communicate directly with $CD4^+$ cells. Otherwise, for what purpose would NK receptor-activated human NK cells express MHC class II, CD86, and OX40L?

Our findings demonstrate that human NK cell costimulation of TCR-induced $CD4^+$ T proliferation depends in a large part on OX40–OX40L interactions. Studies conducted using OX40-deficient mice have shown that OX40-deficient $CD4^+$ T cells initially become activated to secrete IL-2 (albeit at slightly lower levels than wild-type mice), but they are unable to sustain proliferation (45). Other studies performed on $OX40^{-/-}$ mice reported that the impaired in vitro proliferative response to anti-CD3 stimulation

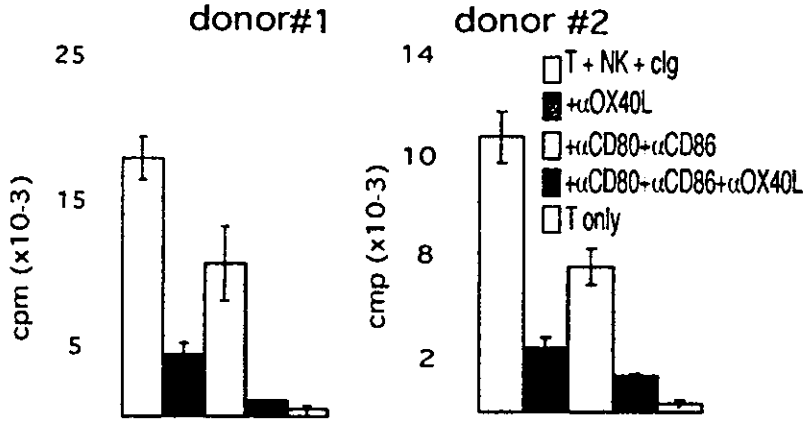

FIGURE 6. NK cell costimulation of TCR-dependent $CD4^+$ T cell proliferation involves B7 family members. Cocultures of anti-CD16-stimulated autologous NK and $CD4^+$ T cells at a ratio of 1:1 were established and stimulated with anti-CD3 as described in Fig. 4*A*. Neutralizing mAbs against CD80, CD86, and/or OX40L or cIg, as indicated, were added on day 0 at 5 μg/ml. Data are represented as the mean ± SD (triplicates). Two representative experiments of five are shown.

could not be corrected by the addition of exogenous rIL-2 (46). Most significantly, it has been shown that OX40 is a major regulator of anti-apoptotic proteins, such as Bcl-xL and Bcl-2 (45), and strongly promotes the survival of Ag-activated primary $CD4^+$ T cells (11). Similarly, the contribution of OX40-OX40L interactions to T cell proliferation that we have observed may favor T cell survival by the induction of Bcl-xL and Bcl-2, although this awaits further evaluation.

Previous studies reported that OX40L expressed on mouse B cells induce a Th2-type response, leading to the expansion of IL-4-producing mouse T effector cells and inhibiting IFN-γ expression (47, 48). In humans, a role for OX40L in the development of Th2 effector cells has also been reported (49). However, other studies do not support a differential role for OX40L in inducing Th1 vs Th2 differentiation (11, 13, 50, 51). suggesting that it only enhances the pre-existing response. In our studies using activated human NK cells to costimulate autologous $CD4^+$ T cells, we observed the production of IFN-γ, but not IL-4 secretion, by the TCR-activated T cells. These findings suggest that activated, mature human NK cells may preferentially promote T cell IFN-γ production.

We believe that the induction of OX40L on NK cells by NKG2D ligand-expressing cells might have important implications in the context of tumor surveillance and infectious diseases. It has been shown that the NKG2D ligand MICA is up-regulated on several human tumor cells and, interestingly, soluble MICA has been found in the serum of patients affected by different progressive tumors (52). In addition, several studies have reported that MICA is induced on cells infected with *Mycobacteria tuberculosis* (18), *Escherichia coli* (19), or cytomegalovirus (17). Thus, initial interactions between NK cells and NKG2D ligand-bearing cells or soluble NKG2D ligands may trigger killing and cytokine production and in the presence of IL-2, IL-15, or IL-12 may induce expression of OX40L on the NK cells. Subsequent interactions between $OX40L^+$ NK cells and $OX40^+$ T cells may amplify and sustain an adaptive ongoing immune response. At least under the experimental conditions used, we observed the induction of OX40L only on a subset of activated human peripheral blood NK cells. Further studies are necessary to resolve why some NK cells, but not others, expressed OX40L upon NKG2D stimulation, because all NK cells express NKG2D on the cell surface.

The OX40-OX40L interaction has been shown to induce bidirectional signals. For example, OX40L stimulation by OX40 transduces a signal in dendritic cells, which results in enhanced TNF-α and IL-1β production (2). Similarly, triggering of OX40L expressed on activated B cells results in B cell proliferation and Ig secretion (53). Finally, engagement of OX40L on vascular endothelial cells leads to the induction of c-*fos* and c-*jun* mRNA expression and the production of the chemokine RANTES (54, 55). Thus, while our present studies have focused on the potential role of OX40L on NK cell interactions with $CD4^+$ T cells, it will also be of interest to examine whether engagement of OX40L on NK cells might regulate their effector functions.

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Vaccination of Rhesus Macaques with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif

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Although the correlates of vaccine-induced protection against human immunodeficiency virus type 1 (HIV-1) are not fully known, it is presumed that neutralizing antibodies (NAb) play a role in controlling virus infection. In this study, we examined immune responses elicited in rhesus macaques following vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing an HIV-1 Env V3 antigen (rBCG Env V3). We also determined the effect of vaccination on protection against challenge with either a simian-human immunodeficiency virus (SHIV-MN) or a highly pathogenic SHIV strain (SHIV-89.6PD). Immunization with rBCG Env V3 elicited significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific. Sera from the immunized macaques neutralized primary HIV-1 isolates in vitro, including HIV-$1_{BZ167/X4}$, HIV-$1_{SF2/X4}$, HIV-$1_{CI2/X4}$, and, to a lesser extent, HIV-$1_{MNp/X4}$, all of which contain a V3 sequence homologous to that of rBCG Env V3. In contrast, neutralization was not observed against HIV-$1_{SF33/X4}$, which has a heterologous V3 sequence, nor was it found against primary HIV-1 R5 isolates from either clade A or B. Furthermore, the viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was markedly decreased after high-dose SHIV-MN challenge. In contrast, replication of pathogenic SHIV-89.6PD was not affected by vaccination in any of the macaques. Thus, we have shown that immunization with an rBCG Env V3 vaccine elicits a strong, type-specific V3 NAb response in rhesus macaques. While this response was not sufficient to provide protection against a pathogenic SHIV challenge, it was able to significantly reduce the viral load in macaques following challenge with a nonpathogenic SHIV. These observations suggest that rBCG vectors have the potential to deliver an appropriate virus immunogen for desirable immune elicitations.

Development of a preventive vaccine against human immunodeficiency virus type 1 (HIV-1) is urgently needed to control the spread of the virus worldwide. Although the immunological parameters that correlate with protective immunity against natural infection with HIV-1 are not fully known, it is assumed that a preventive vaccine must elicit potent, broadly reactive immunity against divergent strains of HIV-1 (25, 36, 42). Several recent studies have demonstrated that induction of virus-specific T-cell responses can confer protective immunity in nonhuman primate models, and these responses may also play a role in controlling HIV-1 replication in humans (6, 18, 19, 31, 33, 34, 38, 45, 48). Vaccine constructs containing viral *env* genes, in addition to *gag* and *pol*, have been shown to effectively control replication of challenge viruses (2, 5, 10), suggesting that neutralizing antibody (NAb) responses might also contribute to protection against pathogenic infection or disease progression. Passive transfer of serum immunoglobulin from chimpanzees experimentally infected with several different HIV-1 isolates has been shown to block the establishment of a simian immunodeficiency virus (SIV)-HIV chimeric simian-human immunodeficiency virus (SHIV) infection in pig-tailed macaques (37, 46). It is not known, however, whether vaccines that actively elicit a potent NAb response can provide protection in nonhuman primates challenged with SHIV.

Previously, we demonstrated that recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG), which secretes a chimeric protein consisting of the V3-neutralizing epitope of HIV-1 and α-antigen (rBCG Env V3), can induce HIV-1-specific NAb in a small-animal model (9, 15, 16). BCG was selected as a vaccine vehicle because it has several characteristics that are considered efficacious for developing a candidate

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HIV-1 vaccine (1, 49), including the ability to induce long-lasting immune responses (7). It is generally accepted that a candidate vaccine against HIV-1 must also be easily administered and affordable in developing countries, and it must be compatible with other commonly administered vaccines (35). If effective, a BCG-based recombinant HIV-1 (rBCG–HIV-1) vaccine would fulfill many of these critical requirements.

Results using other vaccine modalities, in particular, live attenuated SIV vaccines, have raised concerns about the potential for reversion to pathogenicity (3, 4), suggesting that many SIV strains may be potentially virulent. In this study, we used two distinct strains of challenge virus: SHIV-MN (29), which contains V3 sequences homologous to rBCG Env V3, and SHIV-89.6PD (12, 20, 28, 41), which is heterologous in the V3 region and highly pathogenic. We examined whether vaccination with rBCG Env V3 could effectively elicit NAb responses in rhesus macaques and whether it might effectively induce protective immunity against challenge with either SHIV-MN or SHIV-89.6PD.

MATERIALS AND METHODS

Animals. The macaques (*Macaca mulatta*) used in this study originated from China and were purchased through Japan SLC Ltd., Shizuoka, Japan. The animals were maintained according to standard operating procedures established for the evaluation of human vaccines at the Tsukuba Primate Center, National Institute of Infectious Diseases, Tsukuba, Ibaragi, Japan. The study was conducted in the P3 facility for monkeys in the Murayama Branch, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan, and in accordance with requirements specified in the laboratory biosafety manual of the World Health Organization.

Construction of the rBCG Env V3 immunogen. rBCG substrain Tokyo was produced by transfection of BCG-Tokyo 172 cells with plasmid pSO246 as described previously (21, 22, 30). The XhoI site of this plasmid was used to insert a mycobacterial codon-optimized DNA fragment encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence (NTRKSIHIGPGRAFYATGS), which has a neutralization sequence identical to that of HIV-1_{MN} (16, 23, 39, 52). The resulting rBCG vector was designated rBCG Env V3. By semiquantitation of a chimeric protein consisting of the V3 peptides and α-K protein (9), the concentration of the secreted protein was estimated to range from 1 to 3 μg/ml in the culture filtrate of rBCG Env V3 (data not shown).

Viruses. Viruses used in challenge experiments were kindly provided by Y. Lu, Harvard AIDS Institute, Cambridge, Mass. The SHIV-MN virus stock was prepared in concanavalin A-activated macaque peripheral blood mononuclear cells (PBMC) from normal animals, and the amount of virus was quantified by SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) (Coulter Co., Hialeah, Fla.). The tissue culture infective dose (TCID) of the stock was measured on CEMx174 cells (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, Md.). Stocks of HIV-1_{MN} and HIV-1_{IIIB} (AIDS Research and Reference Reagent Program) were prepared by propagating 100 50% TCID ($TCID_{50}$) of each virus in phytohemagglutinin-activated normal human PBMC, as described previously (17). The primary isolate, HIV-1_{MNp}, was kindly provided by J. Sullivan, University of Massachusetts Medical School, Worcester, Mass. All other viruses were obtained from the AIDS Research and Reference Reagent Program. Cell-free virus stocks were stored at −130°C until they were used.

V3-specific ELISA. HIV-1 V3 peptide-based ELISAs were used for titration and quantification of serum antibodies for detection as described previously (14). In brief, 96-well ELISA plates (MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μl of peptide MN (DKRIHIGPGRAFYTT)/well in 50 mM carbonate buffer (pH 9.3) at 5 μg/ml overnight at 4°C. The wells were washed and treated with 5% nonfat milk in phosphate-buffered saline for 1 h at 37°C. Duplicate samples containing either control or test macaque serum at appropriate dilutions were then added at 100 μl/well, and the plates were incubated for 1 h at 37°C. The wells were washed and incubated with a detection antibody solution consisting of peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) antibody (EY laboratories Inc., San Mateo, Calif.) at 100 μl/well for 1 h at 37°C. After final washes with 0.05% Tween-20–phosphate-buffered saline (PBST), peroxidase substrate was added, and the reaction was stopped by the addition of 0.5 M H_2SO_4.

IFN-γ ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were performed using the method developed by Mothe and Watkins of the Wisconsin University Primate Center and described elsewhere (18, 33). In brief, 96-well flat-bottom plates (U-CyTech-BV, Utrecht, The Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody before being washed with PBST and blocked with bovine serum albumin. Freshly isolated PBMC were mixed with either concanavalin A or 2 μM V3 peptide and were then incubated for 16 h at 37°C in 5% CO_2 in anti-IFN-γ-coated plates. Once the plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibodies were added, and the plates were incubated. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the plates after they were washed with PBST. The plates were then incubated for 1 h at 37°C. Developed wells were imaged, and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (33).

In vitro virus neutralization assays. GHOST cell neutralization assays were performed as previously described (8). Briefly, GHOST cells expressing either CXCR4 or CCR5 were used as targets for HIV-1 infection (50, 54). The cells were analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, Calif.), and 15,000 events were scored. The mean number of fluorescent GHOST cells determined from negative controls plus 2 standard deviations was considered the cutoff for a positive sample. Purified human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) and saline were included as additional controls.

M8166 cell-based virus neutralization assays were also performed as described previously (16, 47). In brief, the in vitro neutralization activity of purified macaque IgG was determined using 100 $TCID_{50}$ of either HIV-1_{MN} or SHIV-MN in cultures of M8166 cells. The results were compared with parallel cultures to which preimmune serum IgG was added. Neutralization was expressed as percent inhibition of HIV-1 p24 or SIV p27 antigen production in the culture supernatants. Purified normal macaque IgG was used as a control.

Quantification of cell-associated viral load. Levels of cell-associated virus were quantified by limiting dilution of PBMC (from 10^6 to 1 cells), and the virus was cocultured with M8166 cells as described previously (17). Virus released into the culture supernatant was measured by SIV p27 antigen ELISA (Coulter). The smallest number of PBMC required to produce a positive culture was considered the end point, and the titer of infectious virus was expressed as $TCID_{50}$ per 10^6 PBMC.

PCR detection of proviral HIV-1 infection of rhesus macaques. PBMC with SHIV were detected by DNA PCR using a primer pair that spans the C2-V3 sequence of HIV-1_{IIIB}, followed by Southern blotting with an SE1 probe, 5′-G CAGAAGAAGAGGTAGTAATTAGAT-3′ (nucleotides 7019 to 7043) (47). The positions of the oligonucleotides are numbered relative to the HIV-1_{HXB2} isolate in the ENTREZ database (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.). Viral DNA was quantified by comparison with standards derived from 8E5/LAV cells, which contain one copy of HIV-1 proviral DNA per cell (AIDS Research and Reference Reagent Program).

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative, competitive reverse transcription-PCR was performed using a competitor RNA and a DNA template as previously described (18, 32, 44). The detection limit of this assay was 500 RNA copies/ml in monkey plasma (18, 32).

Sequencing of HIV-1 Env C2-V3 sequence. To determine the sequence of the HIV-1 Env C2-V3 region, mRNA was extracted from stock virus and cDNA was synthesized using a Micro-FastTrack version 2.0 kit (Invitrogen, Carlsbad, Calif.) and a cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. The PCR products were cloned into a pCR II vector with a dual promoter using a TA cloning kit (Invitrogen) (47). Sequence analysis was performed using a Big Dye terminator cycle-sequencing FS kit (Perkin-Elmer, Foster City, Calif.) and automated ABI 310 sequencer (Perkin-Elmer) with Sp6 and T7 sequence primers (Invitrogen). Sequence data were compared with published HIV-1 sequences in GenBank (National Center for Biotechnology Information, National Institutes of Health).

Statistical analysis. Calculations of the geometric mean ± standard deviation (SD) were carried out with a microcomputer. Significance was defined as a P value of <0.05.

RESULTS

Vaccination protocol. Twenty-four male rhesus macaques (R-01 through R-24) were enrolled in the study. Of these, 15 were subcutaneously immunized for 24 weeks with 10 mg of

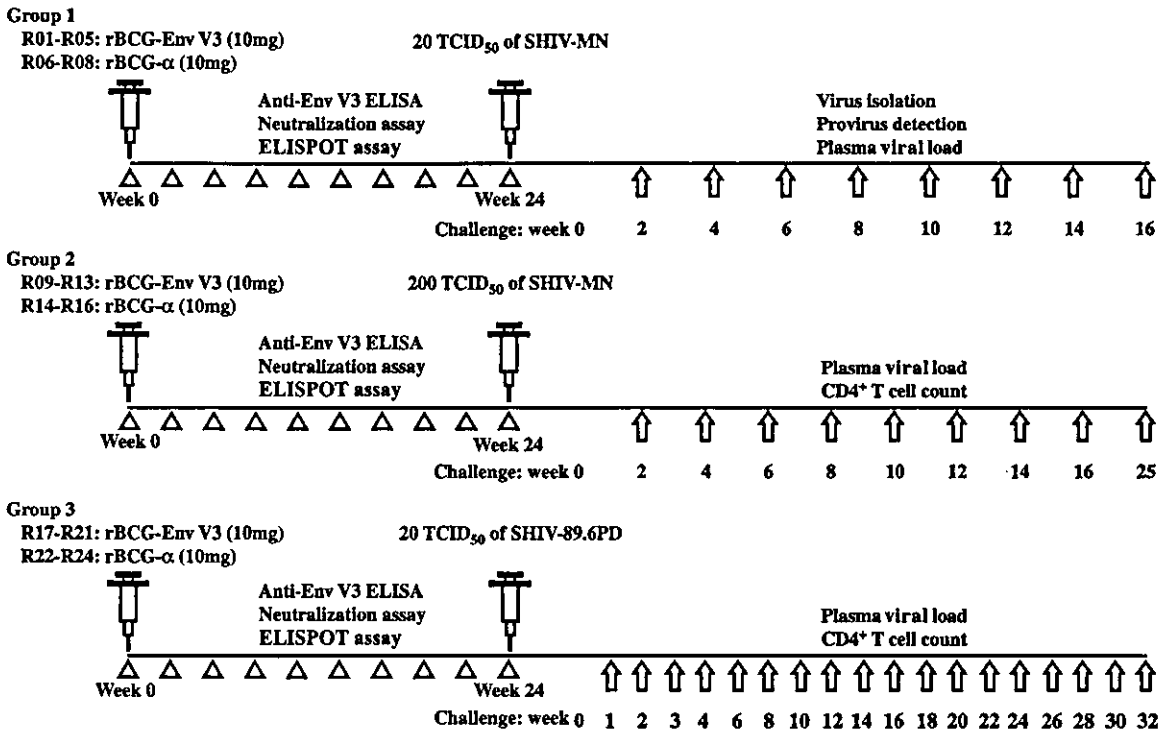

FIG. 1. Schematic representation of the experimental protocol for immunization of rhesus macaques with rBCG Env V3 and challenge with either SHIV-MN or SHIV-89.6PD. A total of 24 macaques were assigned to either the rBCG Env V3 vaccine or rBCG vector control group. The animals each received a single subcutaneous injection and were then split into three groups prior to challenge with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-98.6PD.

rBCG Env V3 (16), which expresses and secretes a chimeric protein consisting of α-antigen and the Env V3 region of HIV-1_{MN}. The remaining nine macaques were immunized by the same route and with the same dose of rBCG α-antigen and served as vector controls. All macaques inoculated with rBCG Env V3 remained in good health following vaccination. Three of the 15 immunized macaques experienced transient redness with slight erosion localized at the injection site; however, the reaction spontaneously resolved within 3 months. Following immunization, the 24 macaques were divided into three groups, each group consisting of five immunized animals and three vector controls. The macaques within each group received an intravenous challenge with either SHIV-MN (20 or 200 $TCID_{50}$) or SHIV-89.6PD (20 $TCID_{50}$) (Fig. 1).

Vaccine-induced HIV-specific immune responses following rBCG Env V3 immunization. (i) Neutralizing antibodies. As described above, 15 rhesus macaques were vaccinated with a single subcutaneous inoculation of 10 mg of rBCG Env V3. Induction of HIV-1-specific immunity was measured 24 weeks later in blood samples obtained pre- and postvaccination. All 15 immunized macaques exhibited HIV-1 Env V3 peptide-binding antibody activity by ELISA at serum dilutions ranging from 1:640 to 1:10,240 (Fig. 2). Antibody responses were monophasic, peaking at 4 to 6 weeks and then gradually declining. Serum samples obtained from naïve macaques were consistently negative by ELISA, while postvaccination sera did not react with a control fusion peptide of HIV gp41 (data not shown).

Antibodies were purified from the macaque sera to remove factors that might interfere with certain bioassays (51). The purified antibodies were then tested in vitro for the ability to neutralize SHIV-MN infection in M1866 cells (Fig. 3). Antibodies induced in macaques vaccinated with rBCG Env V3 strongly neutralized both the challenge SHIV-MN (grown in rhesus PBMC) and a T-cell line-adapted (TCLA) laboratory strain, HIV-1_{MN}. A mean 50% inhibitory concentration (IC_{50}) of 0.05 to 0.5 μg of IgG/ml was measured against SHIV-MN, and a mean IC_{90} of ~3.0 μg of IgG/ml was observed against HIV-1_{MN}. Neutralizing activity was detected in serum samples obtained 4 to 6 weeks after vaccination and was maintained for at least 24 weeks. Preimmune serum IgG from nine macaques immunized with vector alone, and IgG from three additional naive macaques (data not shown), did not neutralize either virus.

(ii) Neutralization responses against primary HIV-1 isolates. To further assess the specificity of antibodies in immune sera, neutralizing activity was evaluated against a panel of seven primary HIV-1 isolates using GHOST cells expressing either CCR5 or CXCR4 (Table 1). Purified IgG from macaques in each of the three immunization groups was able to effectively neutralize HIV-$1_{BZ167/X4}$, HIV-$1_{SF2/X4}$, and HIV-$1_{CI2/X4}$ (Table 1 and Fig. 4), with mean IC_{50} values of 5 to 7, 4 to 7, and 5 to 15 μg/ml, respectively. By comparison, neutralization of HIV-$1_{MNp/X4}$ required ~10-fold more serum IgG, with a mean IC_{50} of 50 μg/ml. Three additional isolates, HIV-$1_{SF33/X4}$, HIV-$1_{SF33/R5}$, and the clade A isolate HIV-$1_{VI313/R5}$,

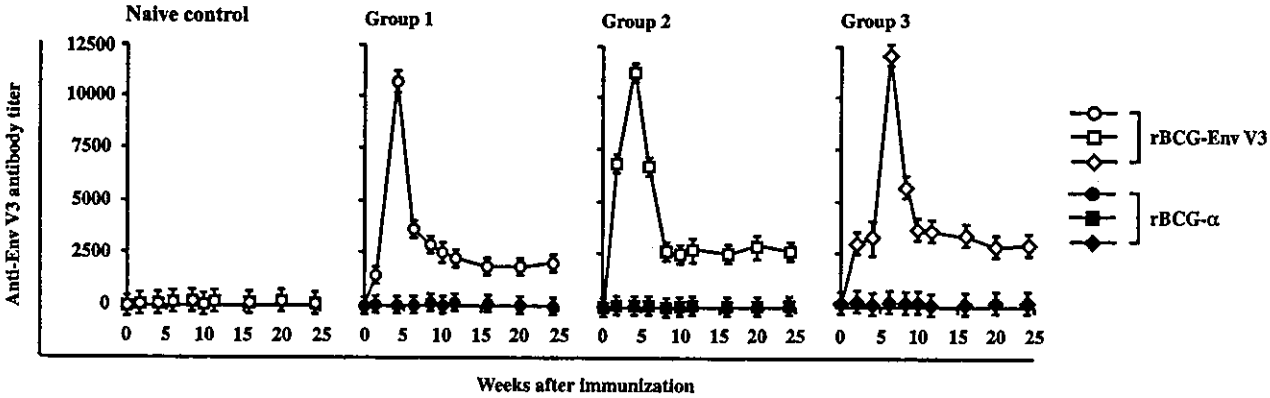

FIG. 2. Serum anti-V3 antibody titers determined by peptide-based ELISA. Preimmune and immune sera from macaques inoculated with rBCG Env V3 were collected and stored at −80°C until they were used. Sera from naïve macaques were used as controls. Data using preimmune sera were within the control levels (data not shown). The results are expressed as the means ± SD of four independent assays.

were not neutralized with serum IgG concentrations up to 50 μg/ml (Table 1). Preimmune sera had no neutralizing activity against any of the isolates. Thus, antibodies present in sera from the immunized macaques were able to neutralize primary HIV-1 isolates, including HIV-1_{BZ167}, HIV-1_{SF2}, and HIV-1_{CI2}, in assays using GHOST cells that express CXCR4 with 10- to 50-fold-higher sensitivity than that of the dual-tropic (X4-R5) TCLA strain HIV-1_{MNp}. Among the neutralization-sensitive viruses, the V3 sequence motifs of HIV-1_{BZ167} and HIV-1_{SF2} shown in Fig. 5 did not correlate with the observed neutralization profiles of HIV-1 Env V3.

(iii) V3 peptide-specific T-cell responses. Table 2 offers a comparison of the virus-specific T-cell response levels determined by IFN-γ ELISPOT analysis in immunized animals with the neutralization data provided in Fig. 2. Of the 15 animals immunized with rBCG Env V3 (180 and 160 SFC/10^6 PBMC at 6 weeks postimmunization [p.i.], respectively), only R-09 and R-10 showed very low levels of SFC activities at the time of

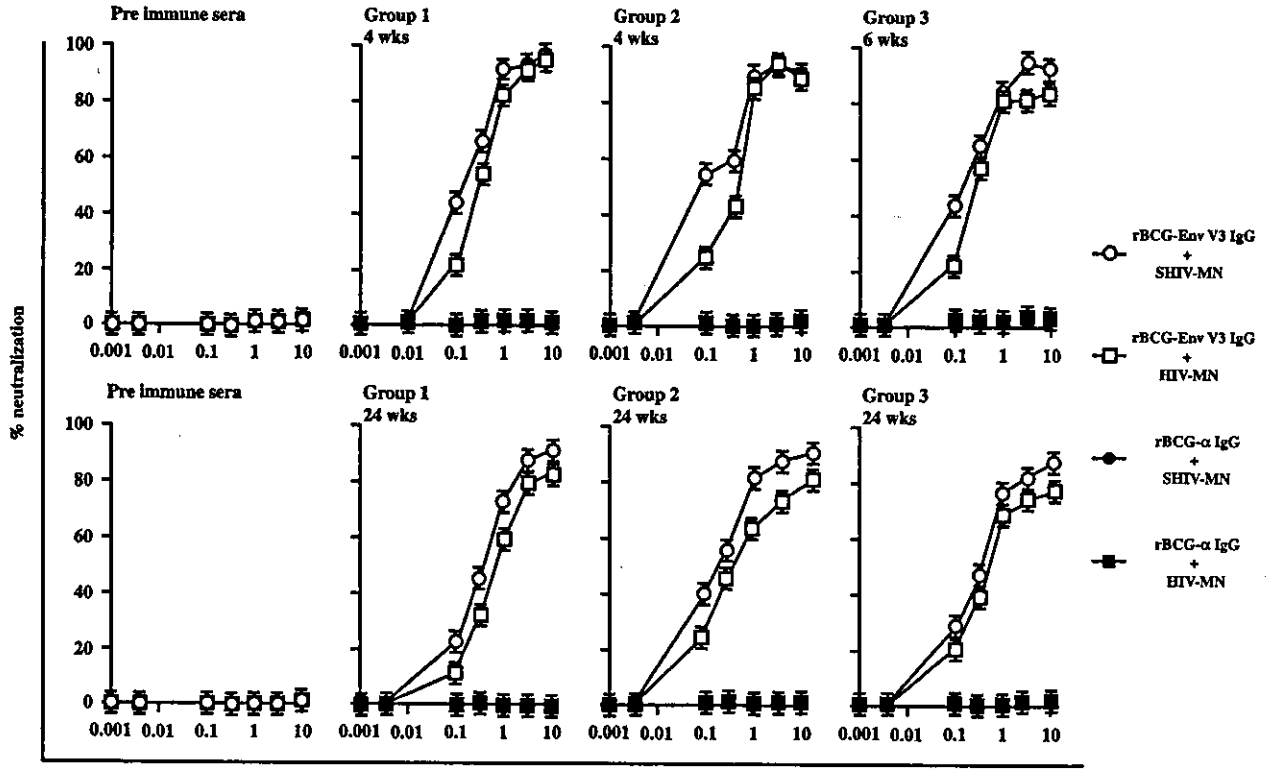

FIG. 3. HIV-1-specific neutralization antibody responses in macaques vaccinated with rBCG Env V3. Analysis of in vitro neutralization of SHIV-MN by anti-rBCG-HIV-1 antibodies using M8166 cell-based virus neutralization assays. Serum IgG was purified from preimmune or immune sera of macaques inoculated with rBCG Env V3 at the indicated times. The results are expressed as the means ± SD of four independent assays.

TABLE 1. 50% neutralization calculated on the basis of neutralization curves[a]

Serum sample	Neutralizing activity (μg)						
	BZ167/X4	MNp/X4	SF2/X4	SF33/X4	SF33/R5	V1313/R5	C12/X4
Group 1	6.5	50	7	>50	>50	>50	10
Group 2	5	50	4	>50	>50	>50	5
Group 3	7	50	6.5	>50	>50	>50	15
Pre immunization sera of groups 1, 2, and 3	>50	>50	>50	>50	>50	>50	>50

[a] The neutralization assays with the various viruses were carried out in GHOST cells expressing either CXCR4 (X4) or CCR5 (R5) as indicated in Fig. 4. BZ167, MNp, SF2, SF33, and CI-2 are HIV-1 clade B viruses. VI313 is an HIV-1 clade A virus.

SHIV challenge (120 and 110 SFC/10^6 PBMC at 24 weeks p.i., respectively) (Table 2). In contrast, <100 SFC/10^6 PBMC were observed in other immunized animals, and <20 SFC/10^6 PBMC were observed in controls. Thus, the V3 region antigen in the rBCG Env V3 proved unable to induce significant levels of virus-specific T-cell responses in immunized animals.

Challenge with low-dose SHIV-MN. The first group of eight macaques (R-01 through R-08), consisting of five animals that received rBCG Env V3 and three that received control rBCG α-antigen, were intravenously challenged with low-dose SHIV-MN (20 $TCID_{50}$) at 24 weeks p.i. The cell-associated virus load was measured in PBMC cocultures, and proviral copy numbers were estimated by DNA PCR using primary PBMC genomic DNA. The level of plasma viremia in each macaque was quantified by competitive reverse transcription-PCR to assess infection and virus replication for 16 weeks after virus challenge (Table 3).

Control macaques vaccinated with the vector alone (R-06 through R-08) were positive in all three viral-load assays 2 weeks after SHIV-MN challenge and remained positive for a follow-up period of 10 weeks. Because only low levels of viral RNA (<10^4 RNA copies/ml) were transiently detected 2 weeks postchallenge, all three assays (virus isolation, plasma RNA, and proviral DNA) were used for virus detection. Using these criteria, we observed that all three parameters remained negative after low-dose SHIV-MN challenge in three of five macaques vaccinated with rBCG Env V3 (R-02, R-04, and R-05). However, macaque R-01 was transiently positive in all three assays for virus infection at 4 weeks. Another macaque immunized with rBCG Env V3 (R-03) exhibited a sharp in-

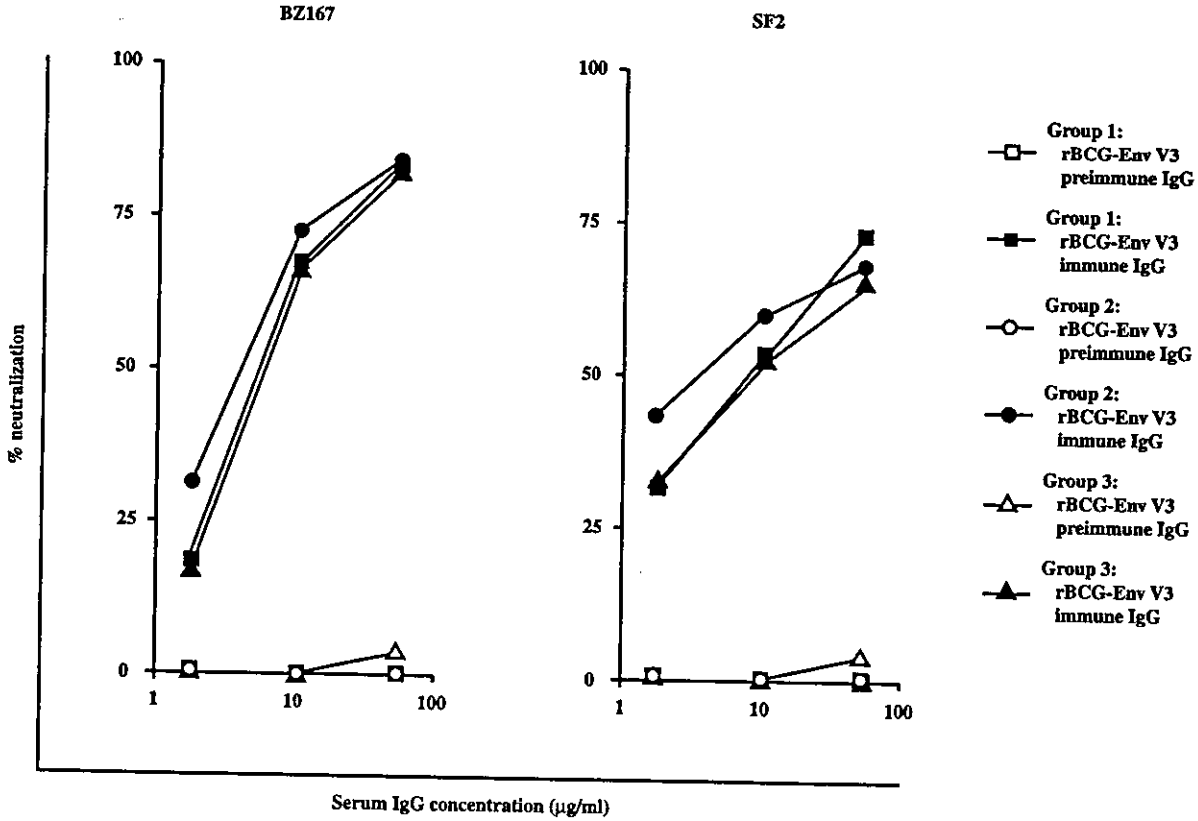

FIG. 4. Neutralization of HIV-1_{BZ167} and HIV-1_{SF2} in GHOST-X4 cells by immune sera from macaques vaccinated with rBCG Env V3. Dilutions of immune sera (closed symbols) and preimmune sera (open symbols) were tested in duplicate, and the percent neutralization was calculated using the mean value. The dose-response curves represent the means of three independent assays.