Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce *Mycobacterium bovis* BCG in infected macrophages

Janice J. Endsley,*[,1] Mark A. Endsley,† and D. Mark Estes*[,‡,†]

*Departments of *Pediatrics and Sealy Center for Vaccine Development and †Microbiology and Immunology, University of Texas Medical Branch, Galveston; and ‡Departments of Veterinary Pathobiology and Molecular Microbiology and Immunology, University of Missouri-Columbia*

Abstract: Bovine natural killer (NK) cells were recently identified by positive selection of a NK cell-activating receptor p46 (NKp46)$^+$ CD3$^-$ lymphocyte population, which expresses CD25 and CD8 and lyses tumor cell lines following stimulation with recombinant interleukin-2. In the current work, we characterize the cytotoxic/effector potential of a CD3$^-$CD8$^-$CD11b$^-$ population isolated through negative selection of bovine peripheral blood leukocytes. This population is CD25^{lo}CD62^{hi} when isolated and becomes CD25^{hi}CD62L^{lo} following cytokine stimulation. Activated bovine NK cells increase expression of granulysin, interferon-γ, and perforin and have cytotoxic activity against human tumor cells and *Mycobacterium bovis* bacillus Calmette-Guerin-infected alveolar and monocyte-derived macrophages. Expression of a bovine homologue of the CD56 neural adhesion molecule expressed by human NK cells was detected in mRNA from brain tissue but was not detected in peripheral blood mononuclear cells or purified NK cell mRNA. Analysis of mRNA from nonstimulated peripheral blood NK cells demonstrates the constitutive expression of homologues of human NK receptors NKp46, CD244, and CD94 and the granule proteins granulysin and perforin. Phorbol ester-stimulated CD8$^+$ T cells also expressed CD244 and CD94, and CD4$^+$ T cells expressed CD94. These NK cell receptors bearing T lymphocytes may represent memory subsets characterized in humans. The results of these studies demonstrate that bovine NK cells may play an important role in the innate immune responses of cattle. *J. Leukoc. Biol.* 79: 71–79; 2006.

Key Words: *cytotoxic granule proteins · natural killer receptors · tuberculosis*

INTRODUCTION

Natural killer (NK) cells are an important component of the innate immune system conserved among mammals, birds, and fish [1–5]. NK cells are rapidly recruited to sites of infection [6] and can be specifically recruited to lymph nodes by a CXC chemokine receptor 3- and CD62L-dependent mechanism following activation by dendritic cells (DC) or adjuvant [7]. Early in the immune response to pathogens, NK cells are an important source of interferon-γ (IFN-γ) and other cytokines and contribute to the bias [T helper cell type 1 (Th1) vs. Th2] of the acquired immune response [8, 9]. NK cells also control pathogen spread early in infection through release of cytotoxic granule proteins, which lyse infected cells and have antimicrobial activity [10]. Activation of NK cells may also impede vaccine efforts as a result of cytolytic elimination of antigen-presenting cells (APC), production of cytokines that interfere with development of an appropriate immune response, or activation/inhibition of T lymphocytes via interactions between receptors shared by NK cells and subsets of T lymphocytes [8, 11–13].

In cattle, cells with natural cytotoxicity to tumors or virally infected cells have long been observed in peripheral blood [14–16], but specific phenotypic markers, effector functions, and activation requirements were not characterized. The lack of a comprehensive understanding of NK cells may be a limiting factor in vaccine and diagnostic development efforts for protecting cattle populations and restrict the use of the cow as an important model for several human diseases [17]. The role of NK cells in the immune responses of cattle has recently begun to be more fully explored following the identification of a phenotypically defined cell subset in peripheral blood with NK cell characteristics [18, 19]. Peripheral blood cells, which are reactive with an antibody specific for the NK cell-activating receptor p46 (NKp46), represent ~5% of the mononuclear cell population in cattle and vary with donor and age [18, 20]. NK cells were also observed in single-cell suspensions derived from bovine liver, spleen, lung, and lymph node [18]. Upon IL-2 activation, the NKp46$^+$ cells contain punctate granules, lyse representative human and mouse NK-sensitive tumor cell lines, and significantly increase expression of CD25 and CD8 [18]. The NKp46$^+$ population may represent a subset of bovine NK cells, as expression of NKp46 by human NK cells varies

[1] Correspondence: 2.120 Children's Hospital, University of Texas Medical Branch, Galveston, TX 77555. E-mail: jjendsle@utmb.edu

Received May 4, 2005; revised September 29, 2005; accepted September 30, 2005; doi: 10.1189/jlb.0505239.

from 20% to 40% of the total NK cell population [21]. A transient population of CD11b$^+$ cells isolated from neonatal calves expresses IFN-γ upon exposure to *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-infected DC in the absence of previous antigen exposure [19]. Constitutive expression of homologues of granulysin (Bo-lysin) and perforin, found in human T lymphocyte and NK cell cytotoxic granules, is also observed in a bovine CD3$^-$ cell population with lymphoid characteristics [21].

The cytolytic and effector functions of NK cells are orchestrated by an increasingly complex repertoire of activating and inhibitory receptors, which recognize alterations in surface molecules induced by infection, stress, or transformation [23, 24]. NK receptors (NKRs), which recognize tumor ligands (NKp30, NKp44, NKp46), are specific to NK cells, while receptors associated with recognition of infection or stress are also expressed by subsets of memory T lymphocytes [8, 24]. Receptors that are shared among NK cells and T cells have direct stimulatory activity in NK cells and costimulatory activity in T cells [24]. Between mouse and human homologues, several NK cell receptor molecules, which are activating in one species, are inhibitory in the other [23]. Consistent with the human molecule, bovine NKp46 is expressed by NK cells and is an activating receptor [18]. Bovine gene homologues of the activating receptors CD94 and NKp46, as well as several members of the killer immunoglobulin (Ig)-like receptor gene family, have been identified by screening a bovine spleen cDNA library [25]. Bovine leukocyte populations which express other NK cell receptor homologue have not been identified to date, and the functional conservation of activity of these receptors have not yet been characterized.

In the current studies, we describe the expression of molecules associated with phenotype, activation status, and trafficking capability of the total population of bovine peripheral blood NK cells. In vitro exposure to recombinant cytokines associated with growth and activation of human NK cells alters the expression of CD25 and CD62L and further enhances the expression of cytolytic/effector proteins by bovine NK cells. We also demonstrate that bovine NK cells can reduce numbers of the intracellular pathogen *M. bovis* in infected macrophages. Collectively, these studies demonstrate the potential for an important cytolytic and effector role of bovine NK cells during innate immune responses to pathogens.

MATERIALS AND METHODS

Leukocyte isolation and cytokine activation

Peripheral blood mononuclear cells (PBMC) were isolated as described previously [22] from healthy bovine donors. A lymphocyte population enriched for NK cells was obtained by negative selection of PBMC using AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany). The PBMC population was depleted of T lymphocytes, B lymphocytes, monocytes, and major histocompatibility complex (MHC) class II$^+$ cells using antibody to bovine CD3 ε, CD4, CD14, CD21, γδ T cell receptor (TCR), and MHC II (MM1A, CACT138A, MMG1A, GB25A, GB21A, and TH14B from VMRD, Pullman, WA) and magnetic bead-conjugated rat anti-mouse IgG1 and rat anti-mouse IgG2^{a+b}, by AutoMACS sorting. The remaining negative PBMC fraction was further enriched by repeating the depletion procedure described above. Purity of the doubly depleted population was assessed using primary antibodies MM1A fluorescein isothiocyanate (FITC; CD3), 1653 FITC (CD4, Serotec, Oxford, UK), BAQ111A (CD8, VMRD), and GB21A (γδ TCR, VMRD) as described above. Primary antibody was detected using FITC-labeled rat antibody to mouse IgG2b, IgG1, and IgM (BD Biosciences PharMingen, San Diego, CA). PBMC and NK populations were cultured at 10^6cells/ml in complete RPMI (cRPMI; RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin) with no stimulation or stimulation with recombinant human (rhu) cytokines (R&D Systems, Minneapolis, MN) IL-2 (500 pg/ml), rhu IL-12 (100 pg/ml), or rhu IL-15 (1 ng/ml). Proliferation of cytokine-activated NK cells was determined by incorporation of ^{3}H- thymidine (TdR; MP Biomedicals, Irvine, CA). Triplicate cultures of NK cells were stimulated with exogenous cytokines for 72 h, as described above, and pulsed with 1 μCi ^{3}H/well for the final 16 h of culture. Cells were harvested 72 h post-stimulation, and ^{3}H-TdR incorporation was determined using a 1450 Microbeta liquid and luminescence counter (PerkinElmer, Boston, MA). PBMC stimulated with rhu IL-2 (500 pg/ml) served as postive controls for proliferation.

Peripheral blood monocytes were positively selected by AutoMACS from PBMC using primary antibody to CD14 (MMG1A, VMRD) and magnetic bead-conjugated rat anti-mouse IgG1 (Miltenyi). Macrophages were derived from CD14$^+$ monocytes by 3-day culture with 1400 U/ml rhu granulocyte macrophage-colony stimulating factor (GM-CSF; Leukine, Immunex, Seattle, WA). Alveolar macrophages (AM) were obtained from bronchoalveolar lavage of bovine lungs obtained immediately after exsanguination from a local abbatoir and cultured in cRPMI with 1400 U/ml rhu GM-CSF. Twelve hours prior to infection with *M. bovis* BCG, macrophages were removed from tissue-culture flasks with Dulbecco's balanced salt solution (Cellgro, Herndon, VA) without $Ca^{+2}Mg^{+2}$ and containing 1 mM EDTA. Macrophages were counted, washed twice in cRPMI, and allowed to recover in 96-well flat-bottomed tissue-culture plates at 1×10^5 cells/well in the presence of 1400 U/ml rhu GM-CSF.

Flow cytometric analysis of surface markers and intracellular proteins

Phenotype markers were evaluated for PBMC and NK cell populations using antibody to CD3 (MM1A), CD2 (Serotec), CD8α (CACT80C), CD5 (B29A), CD11b (MM12A, CC94), CD25 (CACT116A), and CD62L (BAQ92A). In preparation for intracellular staining, lymphocytes were exposed to Brefeldin A (BD Biosciences PharMingen) during the last 5 h of culture. Staining was performed using Cytofix/Cytoperm intracellular staining reagents (BD Biosciences PharMingen) according to the protocol supplied by the manufacturer. Intracellular proteins were detected using phycoerythrin (PE)- or FITC-conjugated monoclonal antibody (mAb) to human perforin (BD Biosciences PharMingen) and bovine IFN-γ (Serotec). Samples were fixed using 2% buffered paraformaldehyde prior to analysis by flow cytometry.

Confocal microscopy

Intracellular perforin and IFN-γ were detected following 72 h of culture with rhu IL-12 (100 pg/ml) and rhu IL-15 (1 ng/ml) with Brefeldin A added during the last 5 h of culture. Staining was performed using Cytofix/Cytoperm intracellular staining reagents according to the protocol supplied by the manufacturer. Intracellular proteins were detected using PE-conjugated mAb to human perforin (BD Biosciences PharMingen) and FITC-conjugated antibody to bovine IFN-γ (Serotec). Additional samples were also stained with FITC- and PE-conjugated isotype-matched controls. Samples were fixed using 2% buffered paraformaldehyde and adhered to poly-L-lysine-coated slides by cytospin. Nuclei were counterstained with Slowfade 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR), and flourescently labeled proteins were visualized using a Zeiss laser-scanning confocal microscope [Optical Imaging Core, University of Texas Medical Branch (UTMB), Galveston].

Cytotoxicity measurements

NK cell lysis of human tumor targets was determined using a modification of the live/dead cell-mediated cytotoxicity assay (Molecular Probes). The K562 cell line (American Type Culture Collection, Manassas, VA) used as NK cell targets was grown in Iscove's modified Dulbecco's medium (IMDM), and targets were prepared by incubating overnight in dihexyloxacarbocyanine iodide (DIOC; Molecular Probes). NK cells were isolated by negative selection as described above and activated for 12 h with rhu IL-12 (100 pg/ml) and rhu

IL-15 (1 ng/ml). Following overnight labeling, K562 targets were washed twice in IMDM, and 2×10^4 cells were exposed to various ratios of NK cells in the presence of 75 μM propidium iodine. Following a 2-h exposure to IL-12/IL-15-activated NK cells, the percent of K562 targets positive for propidium iodine was determined by flow cytometric analysis of 10,000 DIOC-gated events (Flow Cytometry and Cell Sorting Core Laboratory, UTMB). Reduction of *M. bovis* BCG-infected macrophages by NK cells was determined using autologous monocyte-derived macrophages and allogeneic bovine AM as *M. bovis*-infected targets. Monocyte-derived macrophages and AM were infected at a multiplicity of infection of 1 with a frozen stock of *M. bovis* BCG Pasteur [1×10^7 colony-forming units (CFU)/ml] obtained from the United States Department of Agriculture (USDA) National Animal Disease Center (Ames, IA). Following infection, extracellular bacteria were removed with three washes of antibiotic-containing medium, and NK cells were added at various effector-to-target (E:T) ratios in a total of 200 μl antibiotic-free cRPMI/well. Following 24 and 72 h of exposure to NK cells, supernatants were collected, and adherent macrophages were washed twice in antibiotic-free medium. The remaining macrophage monolayer was lysed in 100 μl 0.067% sodium dodecyl sulfate as described previously [26]. Tenfold dilutions of supernatants and lysates in phosphate-buffered saline were plated on Middlebrook 7H11 agar plates (REMEL, Lenexa, KS), and CFU were determined at 3 weeks.

Gene expression

The expression of activating/inhibitory receptors and cytotoxic proteins in NK cells and T lymphocyte populations was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA from enriched PBMC populations isolated from healthy bovine donors. NK cells were enriched by negative selection of PBMC as described above, and purified CD3, CD4, CD8, and γδ T cells were isolated as described previously [22] following 48 h of stimulation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin. Brain tissue was obtained from a healthy cow at necropsy (kindly provided by Dr. Ray Waters, USDA National Animal Disease Center), and RNA was extracted from homogenized tissue following pulverization in liquid nitrogen using an RNeasy kit (Qiagen, Valencia, CA). The extracted RNA was treated with DNase (Ambion, Austin, TX) to remove potentially contaminating DNA. RT-PCR (Titan One Tube, Roche Laboratories, Indianapolis, IN) was performed according to the manufacturer's instructions using bovine-specific primers as follows: CD56, forward 5′-AGT GGC CGG AGA TGC CAA AG-3′ and reverse 5′-TTG TCC ACC TTC CTG ATG GTC AGC-3′; CD94, forward 5′-CCT TGA ACA AGC ATC CAC AG-3′ and reverse 5′-TCT TTT CAC AGT ATG CAT CC-3′; CD244, forward 5′-GGC AGA CAC AGCTGT CTG CTC-3′ and reverse 5′-ATA TCA TCT CCA GAC AAG GAA-3′; NKp46, forward 5′-AGA TGC TCT CGA AAC CTG CCA TCT-3′ and reverse 5′-AAG AAC GTG CTT GTA GCT GTT GCC-3′; glyceraldehyde 3-phosphate dehydrogenase (G3PDH), forward 5′-ACT GGC ATG GCC TTC CG-3′ and reverse 5′-CCC TGT TGC TGT AGC CAA AT-3′; Bo-lysin, forward 5′-CTG CTG CTC CAA GGA GAA GA-3′ and reverse 5′-GCA GTG GAG GGA GTT TGG T-3′; and perforin, forward 5′-GAT GCC AAC TTC GCC GCC CA-3′ and reverse 5′-TGT CAG TCA CGT ACT TGC TC-3′. Primers were designed from known bovine sequence data for NKp46 [25], CD94 [25], Bo-lysin [22], perforin [22], predicted bovine CD56 sequence data (gi 61878446), and a bovine expressed sequence tag with significant nucleotide identity to CD244 (TC217789, www.tigr.org). Bo-lysin expression by NK cells following cytokine stimulation was also detected by real-time PCR. Bovine sequence data for variants 62 and 89 [22] were used to design bovine-specific primers, forward 5′-GGT TTC ATT TGA TTC CCT GGG TCC-3′, reverse 5′-AGG GAG TTT GGT GAG AGA AAC TGG-3′, and probe 5′-AAA GCA CAG AAA CTC CAG CAT CCT CG-3′ labeled with 3'-carboxyfluorescein (Applied Biosystems, Foster City, CA). Effects of cytokine stimulation on relative Bo-lysin gene expression were determined using an ABI PRISM 7000 sequence detector (Real-Time PCR Core Facility, UTMB).

RESULTS

Cytokine activation alters the phenotype and cytotoxic/effector potential of bovine NK cells

Bovine NK cells isolated by negative selection from peripheral blood are $CD3^-CD11b^-CD8\alpha^-$ (**Fig. 1**) and account for ~3% of the PBMC population in adult animals (n=15, range of 0.5–10%) and 6% of the PBMC population in calves less than 5 months of age (n=6, range of 2–10%). Expression of CD11b and CD8α by NK cells did not increase following 72 h stimulation with IL-2 (data not shown). Thirty percent to 40% (varies by donor) of negatively selected NK cells from adult animals expressed CD2 and the activation marker CD5 when freshly isolated (Fig. 1). Forward- and side-scatter (FSC and SSC, respectively) characteristics indicate that NK cells stimulated with IL-2 or IL-15 become larger and more granular following 72 h of in vitro culture (**Fig. 2a**), and nonstimulated NK cells and IL-12-stimulated NK cells are unaffected. Incorporation of ^{3}H-TdR following 72 h of cytokine stimulation indicated that significant proliferation only occurred following stimulation with IL-15 (Fig. 2b), and the proliferative effect of IL-15 appeared to be further enhanced by addition of IL-12. An inverse relationship was observed for expression of CD62L and CD25 depending on activation status of the NK cell. In the absence of cytokine stimulation, NK cells expressed low levels of CD25 but markedly up-regulated expression of CD25 in the presence of IL-2, IL-15, IL-2/IL-12, or IL-15/IL-12 (**Table 1**). Expression of CD62L decreased following cytokine activation, and the effect was most pronounced in the presence of IL-15 (**Fig. 3**). Perforin was constitutively expressed by 30–50% of NK cells (varied with donor) in the absence of cytokine stimulation, and expression could be further enhanced by exposure to IL-15, IL-2/IL-12, or IL-12/IL-15. Constitutive IFN-γ expression varied from 3–10% by donor and could be markedly enhanced with IL-15, IL-2/IL-12, or IL-12/IL-15. The effect of the cytokine stimuli on expression of surface markers and intracellular perforin and IFN-γ was observed at 24 h and most pronounced at 72 h (Table 1). The expression of intracellular proteins and surface expression of CD25 and CD62L were similar among cells analyzed by flow cytometry immediately following isolation and cells analyzed after 24 h in culture in the absence of cytokine stimulation. Stimulation for longer than 72 h, especially in cytokine cocktails including IL-12, resulted in significant cell death (data not shown). The expression of phenotype markers and intracellular cytotoxic/effector proteins in response to cytokine stimulation by NK cells isolated from calf donors did not appear to differ from adult donors (Table 1, Figs. 2 and 3). The significant increase in intracellular perforin and IFN-γ could be observed by confocal microscopy by 72 h post-stimulation with IL-12/IL-15 (**Fig. 4, a–c**). Intracellular perforin and IFN-γ could not be detected by confocal microscopy, above background levels, in nonstimulated NK cells or T cells stimulated for 72 h with IL-12/IL-15 (data not shown).

Bovine NK cells have cytotoxic and antimicrobial activity

NK cells isolated by negative selection retain natural cytotoxicity characteristics and lyse the K562 erythroleukemic cell line (**Fig. 5a**). CFU of *M. bovis* BCG in infected autologous macrophages are dramatically reduced by 24 h in the presence of NK cells, regardless of the cytokines used as stimuli (Fig. 5b). The culture supernatants prior to lysis were also plated for CFU determination to ensure that the reduction of *M. bovis* was a result of NK cell effects (release of lytic granules or IFN-γ activation of macrophages) on the mycobacteria as opposed to

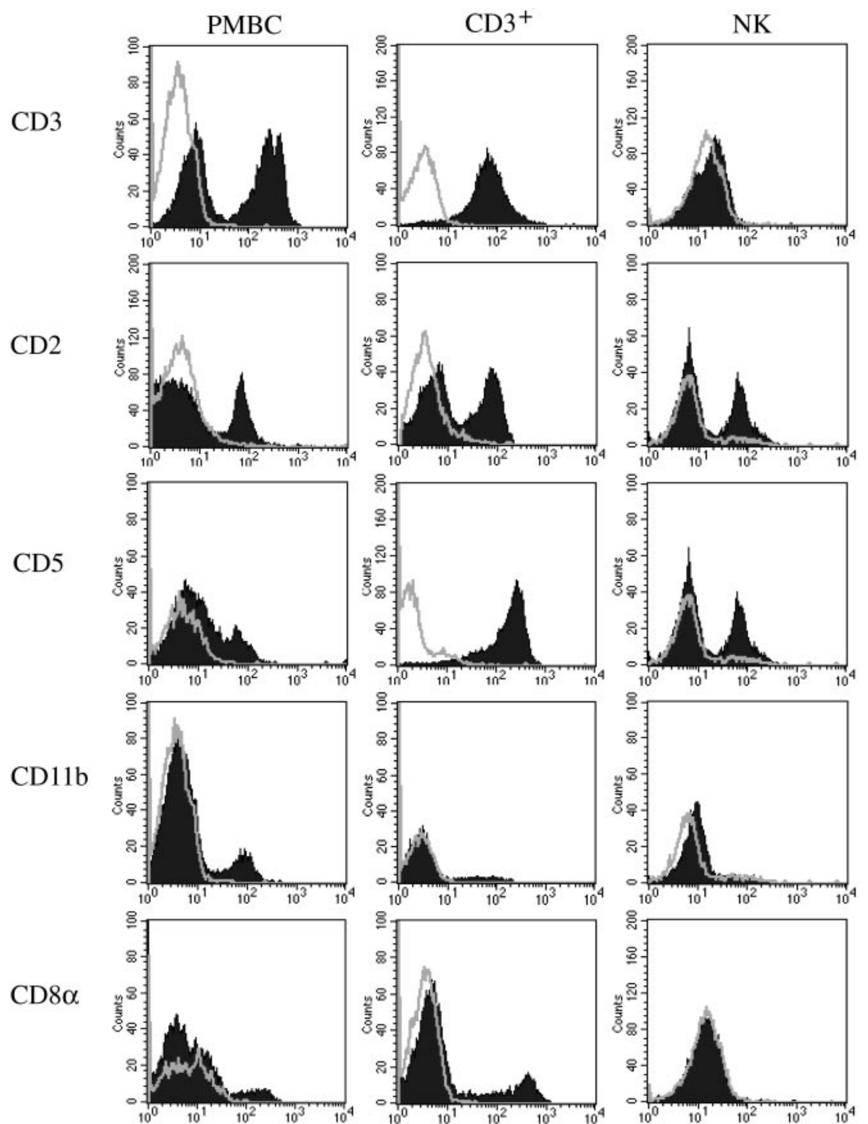

Fig. 1. Surface phenotype of bovine NK cells isolated by negative selection from peripheral blood. Nonstimulated PBMC, CD3$^+$ T lymphocytes, and NK cells from peripheral blood were labeled with bovine-specific antibodies to CD3, CD2, CD5, CD11b, and CD8α (solid) and isotype control antibody (open). Results shown are representative of data obtained from two healthy adult blood donors.

lysis of the infected cell and release of intracellular bacteria. CFU of *M. bovis* BCG in supernatants from infected autologous macrophage cultures were also reduced in the presence of NK cells (Fig. 5b). A further reduction of *M. bovis* BCG CFU in infected autologous macrophages was observed following 72 h of exposure to NK cells (data not shown), which from three donors reduced CFU of *M. bovis* BCG in infected allogeneic AM, and NK cells from a fourth donor were ineffective (Fig. 5c). The activity of NK cells isolated from calf donors against the K562 tumor cell line and *M. bovis*-infected macrophages demonstrates that NK cells from young calves have fully competent cytotoxic activity (Fig. 5, a–c).

NK receptors are expressed by bovine NK cells and subsets of T lymphocytes

To determine the expression of natural killer reception (NKR) genes which have previously been identified in genomic bovine DNA [24], RT-PCR was performed on purified NK cells and T cell subsets. As determined by flow cytometry, NK cells were ≤1% positive for CD3, CD21, or CD14, and T cell subsets (CD3, CD4, CD8, and γδ TCR) were ≥98% pure (data not shown). Expression of the NKp46 gene was detected in RNA from the NK cell population and absent in RNA from the CD3$^+$ population (**Fig. 6a**). Bovine granulysin and perforin were detected in NK and all T lymphocyte populations, and greater expression of perforin may be indicated by the results presented for NK and CD8 samples (Fig. 6a). Activation of NK cells with IL-15 increased relative expression of granulysin, as determined by real-time PCR by 80-fold within 14 h following stimulation as compared with non-stimulated samples (data not shown). NK cells and activated CD3$^+$ T lymphocytes expressed CD244 and CD94 genes. Consistent with human homologues, CD94 was expressed by CD4 and CD8 T cell populations, and CD244 expression was confined to CD8 T cells. A faint band observed for CD244 expression in the γδ T cell population may represent low-level expression or may indicate the presence of a minor NK or CD8 T cell contaminant. Expression of a homologue of CD56, an adhesion molecule expressed by human neural tissue and NK cells, was detected in bovine neural tissue and not detected in bovine NK cells (Fig. 6b).

DISCUSSION

NK cells, which are just beginning to be characterized in cattle, are an important component of the innate immune

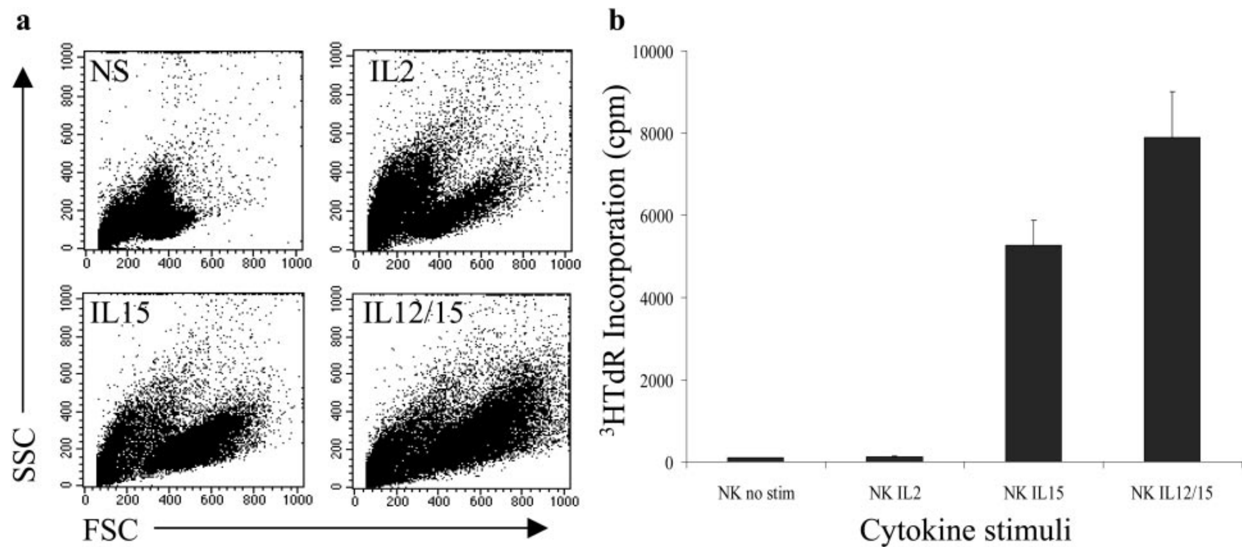

Fig. 2. NK cells proliferate following cytokine activation. NK cells were isolated by negative selection from peripheral blood and cultured in complete medium without cytokine [no stimulation (NS)] or with IL-2 (500 pg/ml), IL-15 (1 ng/ml), or IL-12 and IL-15. FSC and SSC results presented (a) are representative of experiments performed with four animal donors, two adult animals and two calves less than 5 months of age. Proliferation (b) was measured as 16 h uptake of ^{3}H-TdR following 72 h of in vitro culture with cytokine. Results are presented as average ± SD of triplicate samples. cpm, Counts per minute.

system. During the early immune response to pathogenic challenge, NK cells control pathogen load by cytotoxic activity and cytokine production, regulate activation and cytokine production by DC, and affect the bias (Th1 vs. Th2) of the acquired immune response [8–10, 27]. Functional activity and tissue localization of NK cells can be affected by antigen as well as vaccine adjuvant [7, 27, 28]. The primary roles attributed to NK cells in human and mouse immunity are early control of viral infection and tumor survellience via cytotoxic lysis of altered or infected cells. NK cells contribute to the immune response to other pathogens through production of cytokines, immunomodulation of DC and T lymphoctyes, and cytolytic and antimicrobial function [8, 11, 29]. The importance of NK cells in the immune response to mycobacteria is not fully delineated in human and murine tuberculosis. NK cells proliferate, increase expression of IFN-γ and perforin, and have cytolytic and antibacterial activity against infected target cells [26, 30, 31] following exposure to mycobacterial antigens. The IFN-γ produced following activation of NK cells by mycobacterial antigens enhances the development of a Th1 immune response [7, 9] and activates macrophages to kill intracellular bacteria through increased nitric oxide production [32]. A recent study in immunocompetent mice demonstrates that depletion of NK cells does not alter the clinical outcome of infection [31], indicating that the functional importance of NK cells during tuberculosis may be redundant in the absence of immunocompromising factors or coinfection. Studies of NK cell function in experimental bovine tuberculosis may help clarify the role of NK cells in human tuberculosis as a result of the full complement of relevant molecules (i.e., granulysin) expressed by cattle and not mice [22, 33].

Bovine leukocytes capable of nonantigen-restricted cytotoxicity against cells presenting tumor or viral antigens were described over a decade ago [14–16] in the absence of phenotypic characterization. Recently, the functional activity of phenotypically defined bovine NK cells has begun to be explored [18, 19, 22]. A transient $CD3^-CD8\alpha^+CD11b^+$ lymphocyte population, isolated from peripheral blood of neonatal

TABLE 1. Effects of Cytokine Activation on Surface Phenotype and Intracellular Effector Protein Expression of Bovine NK Cells[a]

	No stim	IL-2	IL-15	IL-2/IL-12	IL-15/IL-12
24 h					
$\%CD25^+$	10 (±3.1)	11 (±3.5)	26 (±10.6)	23 (±9.3)	38 (±9.2)
$\%CD62L^+$	33 (±8.9)	25 (±6.5)	18 (±3.9)	30 (±6.8)	25 (±4.8)
$\%Perforin^+$	20 (±4.9)	31 (±7.3)	47 (±3.2)	53 (±4.6)	60 (±4.7)
%IFN-γ^+	5 (+2.6)	7 (+3.1)	17 (+7.4)	13 (+3.5)	23 (+1.4)
72 h					
$\%CD25^+$	nt	9 (+5.1)	35 (+13.5)	40 (+8.6)	48 (+18.0)
$\%CD62L^+$	nt	27 (+1.7)	5 (+1.8)	10 (+5.3)	12 (+6.5)
$\%Perforin^+$	nt	50 (±9.4)	66 (±5.5)	77 (±3.0)	84 (±1.9)
%IFN-γ^+	nt	32 (±15.5)	44 (±12.0)	64 (±5.0)	81 (±6.0)

[a] Data presented as average ± SEM of four (three adults and one calf) individual blood donors.
nt = Not tested.

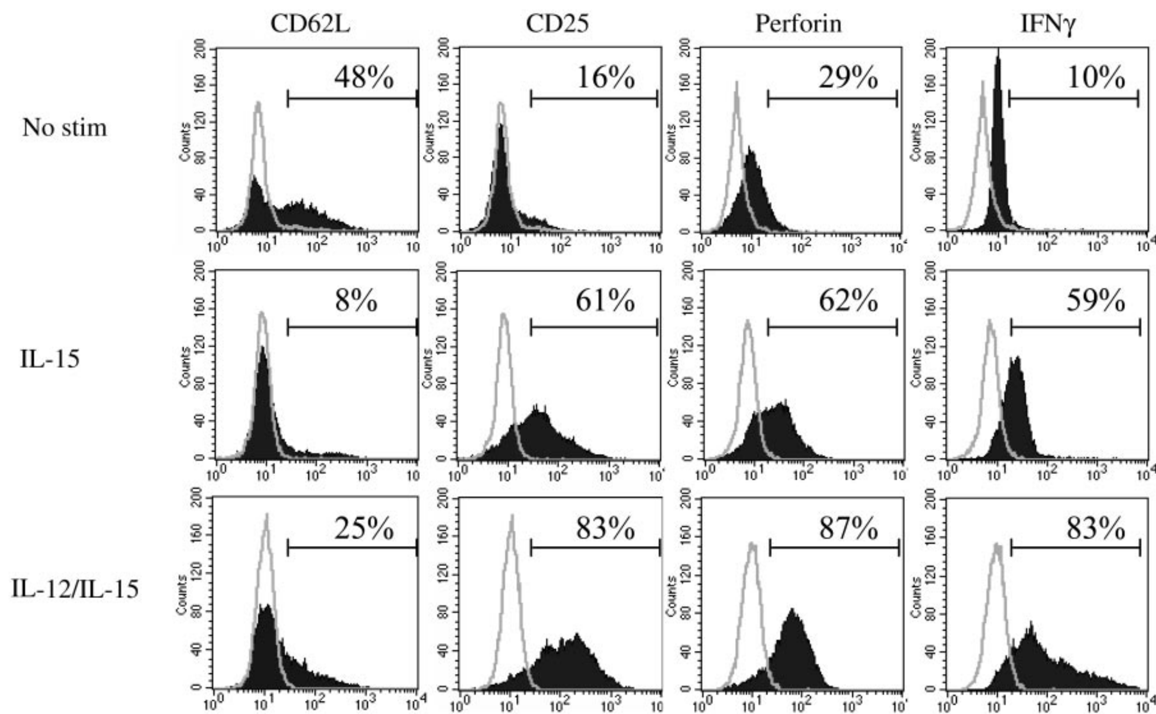

Fig. 3. Cytokine exposure affects NK cell expression of activation markers, trafficking molecules, and effector molecules. NK cells were isolated by negative selection from peripheral blood and stimulated with IL-15 (1 ng/ml) and IL-12 (100 pg/ml) and IL-15 (1 ng/ml), or nonstimulated (No stim). Specific detection with antibodies to bovine CD62L, CD25, antibody to human perforin, and IFN-γ is shown (solid). Nonspecific detection with isotype-matched control antibody is shown (open). Results shown are representative of data obtained from four healthy blood donors, three adults and one calf.

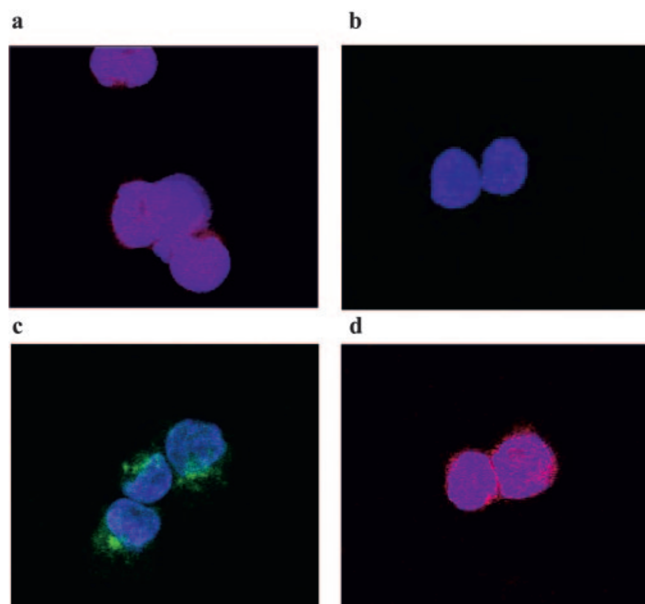

Fig. 4. Confocal microscopy detection of intracellular expression of IFN-γ and perforin by activated bovine NK cells. NK cells were isolated from peripheral blood of an adult blood donor by negative selection and cultured for 3 days in medium alone or medium containing 1 ng/ml rhu IL-15 and 100 pg/ml rhu IL-12. Following permeation, intracellular proteins were detected in nonstimulated cells (a), with PE-labeled antibody to human perforin, and in IL-12/15-stimulated cells using (b) FITC- and PE-labeled, irrelevant control antibodies, (c) FITC-labeled antibody to bovine IFN-γ, and (d) PE-labeled antibody to human perforin.

calves by positive selection of CD8, proliferates and produces large amounts of IFN-γ following in vitro stimulation with *M. bovis*-infected DC [19]. Bovine NK cells expressing a $NKp46^+CD8^+CD25^+$ phenotype proliferate in response to IL-2 stimulation and lyse human and mouse cell lines [18]. We have previously demonstrated that $CD3^-$ NK-like cells isolated by negative selection from bovine peripheral blood constitutively express bovine gene homologues of the cytotoxic granule proteins perforin and granulysin, and T lymphocytes require activation to express these molecules [22]. In the current study, a $CD3^-CD8^-CD11b^-$ population of bovine NK cells isolated by negative selection from adult and calf peripheral blood displays cytotoxic activity against a human tumor cell line and dramatically reduces CFU in *M. bovis* BCG-infected AM and monocyte-derived macrophages. Furthermore, we demonstrate that the proliferative capacity, the production of IFN-γ, and relative levels of perforin and granulysin expression by bovine NK cells can be affected by immunomodulatory cytokines produced by T lymphocytes (IL-2) and are especially affected by cytokines produced by APC (IL-12, IL-15). These results demonstrate that the cytokine microenvironment resulting from infection or vaccination of cattle may have important effects on expansion and functional activity of NK cells during an immune response.

The surface phenotype of bovine NK cells isolated by negative selection in the current studies differs from those of NK cells isolated by positive selection using an antibody-to-bovine NKp46 [18]. $NKp46^+$ bovine NK cells are $CD8^-CD25^-$ upon isolation and are induced to express a predominate $CD8^+$ $CD25^+$ phenotype following activation with recombinant bo-

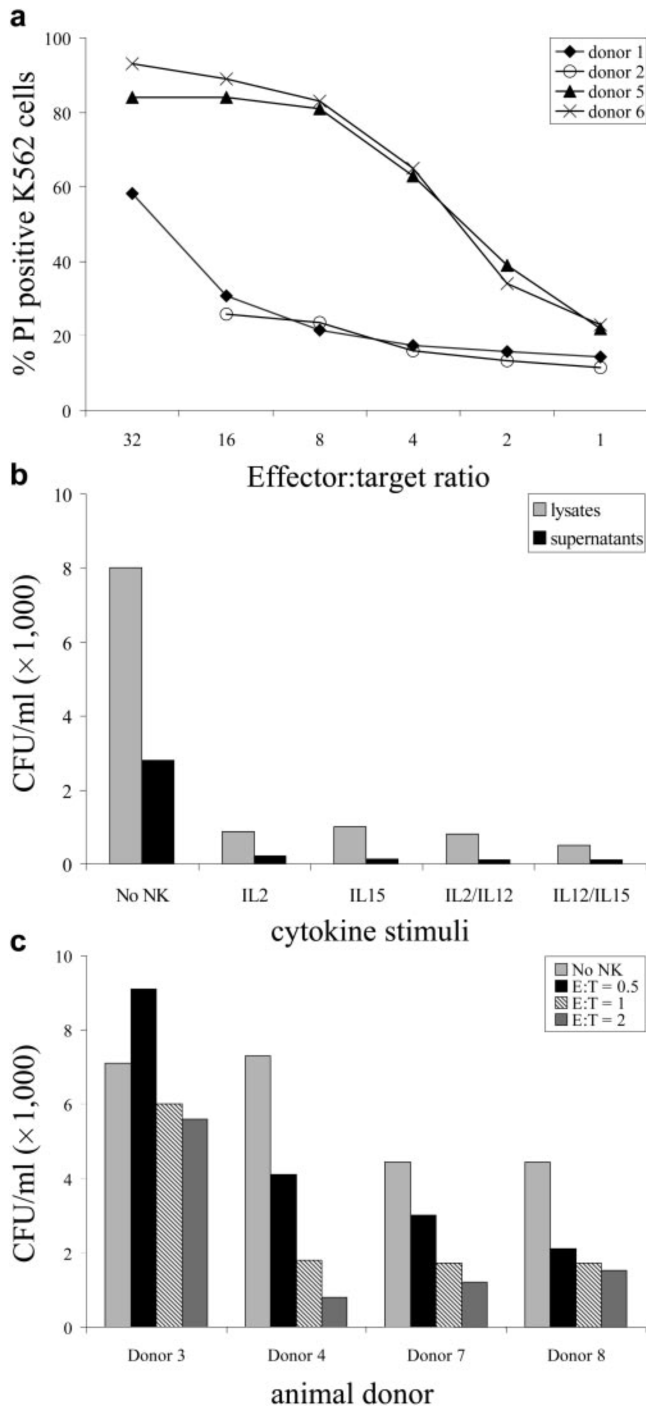

Fig. 5. Bovine NK cells lyse human tumor cells and reduce *M. bovis* numbers in infected macrophages. (a) Cytotoxicity of bovine NK cells against human K562 targets following 12 h of activation with 1 ng/ml rhu IL-15 and 100 pg/ml rhu IL-12. Cytoxicity was determined by flow cytometric analysis of DIOC-labeled targets positive for propidium iodide (PI) following exposure to NK cells. (b) Reduction of CFU of *M. bovis* BCG in lysates from autologous-infected macrophages and macrophage supernatants collected prior to lysis, following 24 h activation of NK cells with IL-2 (500 pg/ml), IL-15 (1 ng/ml), IL-2/IL-12, and IL-12/IL-15. Results are representative of experiments performed with three individual animal donors (two adults and one calf). (c) Reduction of CFU of *M. bovis* BCG in allogeneic-infected AM following 24 h activation with IL-12/IL-15. Donors 1–4 were adult animals, and Donors 5–8 were calves less than 5 months of age.

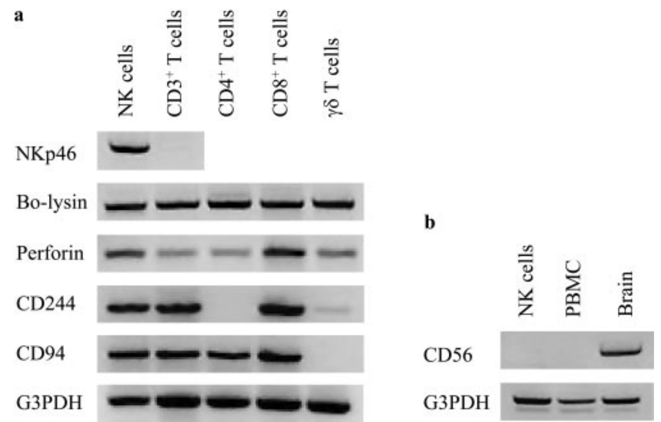

Fig. 6. Transcription of bovine homologues associated with activation and cytotoxicity by peripheral blood NK and T lymphocytes. (a) RT-PCR with bovine-specific primers was performed on RNA from nonstimulated NK cells and $CD3^+$, $CD4^+$, $CD8^+$, and γδ T lymphocyte populations from healthy adult blood donors following 48 h of stimulation with 10 ng/ml PMA and 1 μg/ml ionomycin. (b) RT-PCR with bovine-specific primers was performed on RNA from nonstimulated NK cells, PMA- and ionomycin-stimulated PBMC, and homogenized brain tissue. Equivalent RNA template was assessed by amplification of the G3PDH gene using bovine-specific primers.

vine IL-2 [18]. NK-like cells isolated from neonatal calves are $CD8\alpha^+CD11b^+$ following stimulation with *M. bovis*-infected DC [19], although the phenotype of this cell population has not been determined prior to in vitro culture. In the current studies, negatively selected bovine NK cells do not express CD8 or CD11b when freshly isolated or following cytokine stimulation. PCR analysis in the current trial indicates that NKp46 is expressed by cells within the total NK cell population. Negatively selected NK cells are $CD25^{lo}$ when freshly isolated and become $CD25^{hi}$ following activation with IL-15 or following activation with IL-12 in combination with IL-2 or IL-15. We also demonstrate that bovine NK cells express CD62L, and expression levels of CD62L are affected by cytokine activation. The observed inhibitory effect on CD62L expression by IL-15 and subsequent partial rescue by IL-12 concur with observations made in human NK cells [34]. An inverse relationship between expression of CD62L and CD25 may indicate that the stage of NK cell activation affects the ability of bovine NK cells to traffic to lymph nodes and sites of inflammation. Extravasation of highly activated NK cells from peripheral blood may be controlled in comparison with nonactivated NK cells.

Results of the current study demonstrate that NK cells from Donors 2–5 months of age are competent to express cytotoxic/effector proteins, lyse tumor targets, reduce CFU of mycobacteria, and alter expression of CD62L and CD25 in response to cytokine stimulation.

Tissue localization and functional attributes of human NK cells correlate well with expression levels of the neural cell adhesion molecule CD56. $CD56^{dim}$ NK cells, which are highly cytotoxic, predominate in peripheral blood, and $CD56^{bright}$ NK cells are primarily found in lymph nodes and extralymphoid sites and produce large amounts of IFN-γ, tumor necrosis factor α, and GM-CSF [35]. To date, expression of CD56 by NK cells has not been described in other species, and functional importance of the molecule on human NK cells has not been

determined. In the current studies, we demonstrate that bovine NK cells also do not express the CD56 molecule. Presently, the inherent functional properties of NK cell subsets await further study and reagent development.

The cytolytic and effector function of NK cells is regulated by signaling through a network of activating and inhibitory receptors, which recognize alterations in surface molecules induced by infection, stress, or transformation [23, 24]. Natural cytotoxicity receptors found on human NK cells (NKp30, NKp44, and NKp46) recognize ligands on transformed cells and transduce an activation signal to the NK cell that increases cytotoxicity and cytokine production [36, 37]. The percentage of human NK cells in peripheral blood, which expresses NKp46, varies dramatically by donor [21]. Expression of NKp46 homologues by human and bovine NK cells may be activation-dependent or represent expansion of distinct subsets of NK cells. Previous studies demonstrate that the percentage of $NKp46^+$ cells in peripheral blood of cattle decreases with age and may reflect a general decrease in NK cell numbers with aging, as observed in human populations [20]. In agreement, the percentage of NK cells isolated by negative depletion of peripheral blood in the current trials was lower in adult animals. Phenotypic characterization of negatively selected bovine NK cells for expression of NKp46 will reveal if $NKp46^+$ cells represent a significant component of the NK cell population and may help elucidate the functional significance of phenotypically defined subsets.

Several other NKRs also regulate activation or inhibition of NK cells. The human CD244 molecule is an activating receptor that is expressed on NK cells as well as memory CD8 T cells and γδ T cells [23]. In humans, CD244 ligation enhances cytotoxicity, granule release, and production of IFN-γ [38, 39]. Ligation of CD48 on T lymphocytes by CD244, in concert with antigen-specific TCR signaling, enhances cytotoxicity and IFN-γ release by T lymphoyctes [13]. CD94 is an important NKR, expressed by NK cells and subsets of αβ T cells, which can have activating or inhibiting function, depending on association with members of the NKG2 family [23]. Signaling through CD94 regulates NK cell cytotoxicity against cells expressing altered profiles of MHC I [23]. Levels of CD94 expression by NK cells and $CD8^+$ T cells appear be positively correlated with survival of effector cells [40]. We demonstrate that CD244 and CD94 are expressed by bovine NK cells and subsets of T lymphocytes analogous with their human counterparts. Studies are presently underway in our laboratory to delineate the functional importance of these molecules in reference to immunomodulatory strategies that enhance effector function and cytotoxicity by NK cells and memory CD8 T lymphocytes during infection.

Characterizing the contributions of NK cells to the immune responses of cattle, especially to viral and intracellular bacterial infection, is an important component for development of disease control measures to protect cattle populations. Therapeutic strategies that augment NK cell responses early in the immune response to viral infections, such as Foot and Mouth Disease Virus, may have potential for controlling pathogen spread prior to development of the aquired immune response. Effects of NK cells on DC and T lymphocyte function should be considered during evaluation of epitopes, adjuvants, and co-stimulatory molecules intended to optimize protective memory responses to vaccination. The contribution of NK cells to cyotoxicity and cytokine production should also be considered during diagnostic analysis of cell-mediated immune responses to antigen.

ACKNOWLEDGMENTS

J. J. E. was supported by a USDA-National Research Initiative postdoctoral research award (2002-35204-12307) and is currently supported by a National Institute of Allergy and Infectious Diseases fellowship for training in emerging and re-emerging infectious disease (T32 A107536). The Program for Prevention of Animal Infectious Diseases, ARS 58-1940-0-008, the Plum Island Animal Disease Center (USDA-Agriculture Research Service-Specific Cooperative Agreement #58-1940-4-438), and the Sealy Center for Vaccine Development provided partial support for this study. Additional funding was provided by the Committee on Research Animal Health budget funds and Animal Health Formula funds, College of Veterinary Medicine, University of Missouri-Columbia. We sincerely appreciate the efforts of Alan Patronella, Ronald Tessman, and Alexander Maue for providing the large supply of cattle blood necessary to complete these trials. A special thanks also goes to Elisabeth Shell for acquiring cattle lungs from local abbatoirs for collection of alveolar lavage.

REFERENCES

1. Domeika, K., Berg, M., Eloranta, M. L., Alm, G. V. (2002) Porcine interleukin-12 fusion protein and interleukin-18 in combination induce interferon-γ production in porcine natural killer and T cells. *Vet. Immunol. Immunopath.* **86,** 11–21.
2. Gobel, T. W., Chen, C. L., Shrimpf, J., Grossi, C. E., Bernot, A., Bucy, R. P., Auffray, C., Cooper, M. D. (1994) Characterization of avian natural killer cells and their intracellular CD3 protein complex. *Eur. J. Immunol.* **24,** 1685–1691.
3. Viveiros, M. M., Antczak, D. F. (1999) Characterization of equine natural killer and IL-2 stimulated lymphokine activated killer cell populations. *Dev. Comp. Immunol.* **23,** 521–532.
4. Tekin, S., Hansen, P. J. (2002) Natural killer-like cells in the sheep: functional characterization and regulation by pregnancy-associated proteins. *Exp. Biol. Med. (Maywood)* **227,** 803–811.
5. Shen, L., Stuge, T. B., Bengten, E., Wilson, M., Chinchar, V. G., Naftel, J. P., Bernanke, J. M., Clem, L. W., Miller, N. W. (2004) Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.* **28,** 139–152.
6. Natuk, R. J., Welsh, R. M. (1987) Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. *J. Immunol.* **138,** 877–883.
7. Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F. (2004) Induced recruitment of NK cells to lymph nodes provides IFN-γ for T(H)1 priming. *Nat. Immunol.* **5,** 1260–1265.
8. Raulet, D. H. (2004) Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* **5,** 996–1002.
9. Vankayalapati, R., Klucar, P., Wizel, B., Weis, S. E., Samten, B., Safi, H., Shams, H., Barnes, P. F. (2004) NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J. Immunol.* **172,** 130–137.
10. Smyth, M. J., Cretney, E., Kelly, J. M., Westwood, J. A., Street, S. E., Yagita, H., Takeda, K., van Dommelen, S. L., Degli-Esposti, M. A., Hayakawa, Y. (2005) Activation of NK cell cytotoxicity. *Mol. Immunol.* **42,** 501–510.
11. Cooper, M. A., Fehniger, T. A., Fuchs, A., Colonna, M., Caligiuri, M. A. (2004) NK cell and DC interactions. *Trends Immunol.* **25,** 47–52.

12. Assarsson, E., Kambayashi, T., Persson, C. M., Ljunggren, H. G., Chambers, B. J. (2005) 2B4 co-stimulation: NK cells and their control of adaptive immune responses. *Mol. Immunol.* **42,** 419–423.
13. Assarsson, E., Kambayashi, T., Schatzle, J. D., Cramer, S. O., von Bonin, A., Jensen, P. E., Ljunggren, H. G., Chambers, B. J. (2004) NK cells stimulate proliferation of T and NK cells through 2B4/CD48 interactions. *J. Immunol.* **173,** 174–180.
14. Palmer, L. D., Leary, T. P., Wilson, D. M., Splitter, G. A. (1990) Bovine natural killer-like cell responses against cell lines expressing recombinant bovine herpesvirus type 1 glycoproteins. *J. Immunol.* **145,** 1009–1014.
15. O'Reilly, K. L., Splitter, G. A. (1990) CD5dim peripheral blood lymphocytes proliferate to exogenous IL-2 in the absence of antigen and kill in an NK-like manner. *Cell. Immunol.* **130,** 389–400.
16. Campos, M., Ohmann, H. B., Rapin, N., Babiuk, L. A. (1991) Demonstration of the in vitro antiviral properties of bovine lymphokine-activated killer (LAK) cells. *Viral Immunol.* **4,** 259–268.
17. Hein, W. R., Griebel, P. J. (2003) A road less traveled: large animal models in immunological research. *Nat. Rev. Immunol.* **3,** 79–84.
18. Storset, A. K., Kulberg, S., Berg, I., Boysen, P., Hope, J. C., Dissen, E. (2004) NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. *Eur. J. Immunol.* **34,** 669–676.
19. Hope, J. C., Sopp, P., Howard, C. J. (2002) NK-like CD8(+) cells in immunologically naive neonatal calves that respond to dendritic cells infected with *Mycobacterium bovis* BCG. *J. Leukoc. Biol.* **71,** 184–194.
20. Kulberg, S., Boysen, P., Storset, A. K. (2004) Reference values for relative numbers of natural killer cells in cattle blood. *Dev. Comp. Immunol.* **28,** 941–948.
21. Sivori, S., Pende, D., Bottino, C., Marcenaro, E., Pessino, A., Biassoni, R., Moretta, L., Moretta, A. (1999) NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur. J. Immunol.* **29,** 1656–1666.
22. Endsley, J. J., Furrer, J. L., Endsley, M. A., McIntosh, M. A., Maue, A. C., Waters, W. R., Lee, D. R., Estes, D. M. (2004) Characterization of bovine homologues of granulysin and NK-lysin. *J. Immunol.* **173,** 2607–2614.
23. Lanier, L. L. (2005) NK cell recognition. *Annu. Rev. Immunol.* **23,** 225–274.
24. Snyder, M. R., Weyand, C. M., Goronzy, J. J. (2004) The double life of NK receptors: stimulation or co-stimulation? *Trends Immunol.* **25,** 25–32.
25. Storset, A. K., Slettedal, I. O., Williams, J. L., Law, A., Dissen, E. (2003) Natural killer cell receptors in cattle: a bovine killer cell immunoglobulin-like receptor multigene family contains members with divergent signaling motifs. *Eur. J. Immunol.* **33,** 980–990.
26. Brill, K. J., Li, Q., Larkin, R., Canaday, D. H., Kaplan, D. R., Boom, W. H., Silver, R. F. (2001) Human natural killer cells mediate killing of intracellular *Mycobacterium tuberculosis* H37Rv via granule-independent mechanisms. *Infect. Immun.* **69,** 1755–1765.
27. Moretta, A. (2002) Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat. Rev. Immunol.* **2,** 957–964.
28. Scharton, T. M., Scott, P. (1993) Natural killer cells are a source of interferon γ that drives differentiation of CD4+ T cell subsets and induces early resistance to *Leishmania major* in mice. *J. Exp. Med.* **178,** 567–577.
29. Zingoni, A., Sornasse, T., Cocks, B. G., Tanaka, Y., Santoni, A., Lanier, L. L. (2005) NK cell regulation of T cell-mediated responses. *Mol. Immunol.* **42,** 451–454.
30. Esin, S., Batoni, G., Pardini, M., Favilli, F., Bottai, D., Maisetta, G., Florio, W., Vanacore, R., Wigzell, H., Campa, M. (2004) Functional characterization of human natural killer cells responding to *Mycobacterium bovis* bacille Calmette-Guerin. *Immunology* **112,** 143–152.
31. Junqueira-Kipnis, A. P., Kipnis, A., Jamieson, A., Juarrero, M. G., Diefenbach, A., Raulet, D. H., Turner, J., Orme, I. M. (2003) NK cells respond to pulmonary infection with *Mycobacterium tuberculosis*, but play a minimal role in protection. *J. Immunol.* **171,** 6039–6045.
32. van Crevel, R., Ottenhoff, T. H., van der Meer, J. W. (2002) Innate immunity to *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* **15,** 294–309.
33. Clayberger, C., Krensky, A. M. (2003) Granulysin. *Curr. Opin. Immunol.* **15,** 560–565.
34. Frey, M., Packianathan, N. B., Fehniger, T. A., Ross, M. E., Wang, W. C., Stewart, C. C., Caligiuri, M. A., Evans, S. S. (1998) Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J. Immunol.* **161,** 400–408.
35. Cooper, M. A., Fehniger, T. A., Caligiuri, M. A. (2001) The biology of human natural killer-cell subsets. *Trends Immunol.* **22,** 633–640.
36. Moretta, L., Bottino, C., Pende, D., Vitale, M., Mingari, M. C., Moretta, A. (2004) Different checkpoints in human NK-cell activation. *Trends Immunol.* **25,** 670–676.
37. Sivori, S., Vitale, M., Morelli, L., Sanseverino, L., Augugliaro, R., Bottino, C., Moretta, L., Moretta, A. (1997) p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J. Exp. Med.* **186,** 1129–1136.
38. Nakajima, H., Cella, M., Langen, H., Friedlein, A., Colonna, M. (1999) Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur. J. Immunol.* **29,** 1676–1683.
39. Garni-Wagner, B. A., Purohit, A., Mathew, P. A., Bennett, M., Kumar, V. (1993) A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J. Immunol.* **151,** 60–70.
40. Gunturi, A., Berg, R. E., Forman, J. (2003) Preferential survival of CD8 T and NK cells expressing high levels of CD94. *J. Immunol.* **170,** 1737–1745.