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Immune function in arctic mammals: Natural killer (NK) cell-like activity in polar bear, muskox and reindeer

Jean-Pierre Desforges[a,*], Lindsay Jasperse[b], Trine Hammer Jensen[c], Carsten Grøndahl[d], Mads F. Bertelsen[d], Sylvain De Guise[b], Christian Sonne[a], Rune Dietz[a], Milton Levin[b]

[a] Department of Bioscience, Arctic Research Centre, Aarhus University, Frederiksborgvej 399, PO Box 358, DK-4000, Roskilde, Denmark
[b] Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, Storrs, CT, 06269-3089, United States
[c] Aalborg Zoo/Aalborg University, Mølleparkvej 63, 9000, Aalborg, Denmark
[d] Center for Zoo and Wild Animal Health, Copenhagen Zoo, Roskildevej 38, DK-2000, Frederiksberg, Denmark

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ABSTRACT

Natural killer (NK) cells are a vital part of the rapid and non-specific immune defense against invading pathogens and tumor cells. This study evaluated NK cell-like activity by flow cytometry for the first time in three ecologically and culturally important Arctic mammal species: polar bear (*Ursus maritimus*), muskox (*Ovibos moschatus*) and reindeer (*Rangifer tarandus*). NK cell-like activity for all three species was most effective against the mouse lymphoma cell line YAC-1, compared to the human leukemia cell line K562; NK cell response displayed the characteristic increase in cytotoxic activity when the effector:target cell ratio increased. Comparing NK activity between fresh and cryopreserved mouse lymphocytes revealed little to no difference in function, highlighting the applicability of cryopreserving cells in field studies. The evaluation of this important innate immune function in Arctic mammals can contribute to future population health assessments, especially as pollution-induced suppression of immune function may increase infectious disease susceptibility.

1. Introduction

Natural killer (NK) cells are a heterogeneous population of large granular lymphocytes whose primary function is cytotoxic lysis of virus-infected cells and tumor cells. In addition, NK cells prime effective and long lasting adaptive immune responses (Welsh et al., 1991), which highlights the importance of understanding and characterizing these cells and their function. Their cytotoxic activity results from the release of their granule content adjacent to target cells, allowing the released proteins (e.g., perforins and proteases) to induce apoptosis (Abbas et al., 2012). Beyond their "killer" role, NK cells play an important role orchestrating downstream responses via direct interaction with dendritic cells and release of cytokines that help initiate adaptive immunity (Boysen and Storset, 2009). Because of their large heterogeneity and differences in surface markers among species, combined with the lack of available reagents for wildlife species, NK cells are often characterized based on their activity, which has been measured in many non-laboratory, wildlife species (De Guise et al., 1997; Ross et al., 1996; Rousselet et al., 2013; Wirth et al., 2014).

The present study was conducted to measure NK cell-like function in three ecologically and culturally important Arctic mammals – the polar bear (*Ursus maritimus*), muskox (*Ovibos moschatus*), and reindeer (*Rangifer tarandus*) – and help determine the proper target cell for a flow cytometry-based assay. Because studies in Arctic wildlife often require remote field collections and potential long-term sample storage prior to analysis, a secondary objective was to assess the influence of cryopreservation on NK cell activity. A better understanding of NK cell activity in these species will help in future population health assessments, especially in light of climate change and pollutant exposure in the Arctic.

2. Materials and methods

2.1. Animals, blood sampling and isolation of peripheral blood mononuclear cells (PBMCs)

Fresh whole blood was drawn from a polar bear housed at the Copenhagen Zoo (Denmark) in March 2015, as well as from two polar bears at the Aalborg Zoo (Denmark) in June 2014. Briefly, animals were anesthetized using standard protocols as part of veterinary management of the zoological institutions. Blood samples (100–500 ml) were taken from the femoral or jugular vein directly into heparinized blood bags

* Corresponding author.
E-mail address: jpd@bios.au.dk (J.-P. Desforges).

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and immediately placed in a cooler with ice. The cooled blood was transferred to the Bioscience Department at Aarhus University in Roskilde (Denmark) and processed for the isolation of PBMCs within 24 h of collection. Fresh whole blood was drawn from three muskox and three reindeer at the Large Animal Research Station at the University of Alaska Fairbanks. Briefly, animals were trained to stand for blood draws from the jugular vein using an 18 gauge, 1" to 1.5" needle and 20 ml syringe. Blood was then transferred to sodium heparin Vacutainer blood tubes, kept cool, and shipped overnight to the University of Connecticut and processed for the isolation of PBMCs within 24 h of collection. All blood samples were taken under approved by the Institutional Animal Care and Use Committee (IACUC) sampling protocols.

Whole blood was centrifuged at 220*g* for 20 min and buffy coats carefully removed using sterile transfer pipettes and transferred to new 50 ml conical tubes. Buffy coats were washed once with Hanks Balanced Salt Solution (HBSS; Gibco, Grand Island, NY) and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque PLUS 1.077 (GE Healthcare, Pittsburgh, PA) at 400*g* for 30 min. Isolated PBMCs were washed twice with HBSS, assessed for viability and counted on a haemocytometer using trypan blue and visualization with a light microscope. Cells were adjusted to 10×10^6 cells/ml and cryopreserved in a 10% solution of dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MO) in fetal calf serum (FCS; HyClone, GE Healthcare, Logan, UT) as previously described (De Guise et al., 1997) and transferred to a liquid nitrogen container. Cryopreserved polar bear cells were shipped frozen to the laboratory of Dr. Milton Levin at the University of Connecticut, Storrs, Connecticut, using a US Fish and Wildlife Service, Federal Fish and Wildlife permit (#MA48161A-0).

Splenocytes from female B6C3F1 mice (*Mus musculus,* n = 8; Charles River Laboratory, MA), were used to assess the effects of liquid nitrogen cryopreservation on NK cell-like activity. Mice were housed at the University of Connecticut's laboratory animal facility and sacrificed using procedures approved by the IACUC. Lymphocytes were isolated from the spleen as previously described (Levin et al., 2008). From the same mice, NK cell-like activity was assessed immediately after processing (fresh), as well as after storage in liquid nitrogen for approximately 3 weeks (cryopreserved).

2.2. NK cytotoxicity assay

Two different target cells, both recognized as generally sensitive to NK cytotoxic activity, were assessed for the species used in this study: YAC-1, a murine lymphoma cell line and K-562, a human erythroleukemic cell line. Target cells were obtained from ATCC (Rockville, MD) and were cultured in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with (all from Gibco, Grand Island, NY) 1 mM sodium pyruvate, 100 μM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, along with 10% FCS), at 37 °C with 5% CO_2. Target cells were passed 1:10 and 1:2 in complete media 48 and 24 h, respectively, prior to each assay.

NK-like cytotoxic activity was measured as the mortality of target cells at different PBMC (effector) to target cell ratios using flow cytometry as previously described (De Guise et al., 1997). Since NK cells were not isolated here, the enumerated effector cell population included all PBMCs. Nonetheless the proportion of NK cells was constant for each target cell used and in each effector:target (E:T) ratio tested. Briefly, cyrovials containing PBMCs were removed from liquid nitrogen storage and quickly thawed in a 37 °C water bath, washed twice in complete DMEM and assessed for viability and counted on a haemocytometer using trypan blue and visualization with a light microscope. Viability was generally > 90% (data not shown).

Target cells were incubated with the lipophilic membrane dye 3,3′-dioctadecyloxarbocyanine perchlorate (DiO, Molecular Probes, Eugene, OR) prior to being mixed with the appropriate concentration of PBMCs or mouse cells to achieve effector:target (E:T) ratios of 100:1, 50:1, 25:1 and 12.5:1, in duplicates. The cell mixtures (and target cells alone to assess spontaneous target cell mortality, four replicates per individual) were centrifuged for 30 s and then incubated for 150 min at 37 °C in 5% CO_2. Following centrifugation at 220*g* for 10 min, the supernatant was discarded and cells were re-suspended in complete DMEM and placed on ice before immediate analysis. The mortality of the target cells was evaluated by the addition of 50 μl/ml propidium iodide (PI, Molecular Probes, Eugene, OR) immediately before acquisition using two color (DiO vs PI) flow cytometry. The fluorescence of approximately 1000 target cells was read using a FACScan (Becton Dickinson, Rutherford, NJ) flow cytometer and the CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA). Labeled target cells were distinguished from effector cells due to their increased fluorescence in FL-1 (DiO). Dead (or dying) target cells that incorporated propidium iodide had a higher fluorescence in FL-3 (PI). The percent target cell mortality was calculated as: [# dead target cells/(# dead target cells + # live target cells)] x 100. The percent of spontaneous target cell mortality was subtracted from each target cell mortality at each E:T ratio to calculate NK cell-like specific cell mortality. The percent of spontaneous target cell mortality was typically between 2 and 8% and replicate (four replicates per individual) variation was small as indicated by standard errors all below 1 and representing a small percentage of the averages (< 10–20%).

2.3. Statistical analysis

The results are expressed as mean ± standard error and comparisons between experimental groups (target cell type and E:T ratio or cryopreservation and E:T ratio) were evaluated using two-way ANOVA. All statistics were done using the open source software R (R Core Team, 2016) and graphics done using R package "ggplot2" (Wickham, 2009).

3. Results and discussion

Given the important role of NK cells in the initial response to viral infection and aberrant tumor cells, as well as their increasingly clear role in promoting adaptive responses (Boysen and Storset, 2009), describing and evaluating NK function in never before evaluated wildlife species is critical for future immunological screening in these species. NK activity is also recognized as a sensitive biomarker to assess the immunotoxic potential of a chemical. The US National Toxicology Program has demonstrated that changes in NK cell function could help predict changes in host resistance to infectious pathogens using the rodent model (Luster et al., 1993, 1992). These factors support the need to understand this important immune function in species of interest.

The results of the NK-like cytotoxic activity against YAC-1 and K562 target cells for polar bears are shown in Fig. 1. Polar bear NK activity showed the characteristic pattern of activity against both target cells, whereby specific mortality increased with increasing E:T ratio ($p = 0.030$). Although there were no significant differences between NK activity against YAC-1 or K562 across E:T ratios ($p = 0.85$), polar bear NK activity displayed relatively less variation against YAC-1 compared to K562 (Fig. 1) and as such YAC-1 appeared to be the more appropriate target cell for use in polar bear studies. To our knowledge, only one other reference to polar bear NK function has been published to date (Brousseau et al., 1999) and this report also used YAC-1 cells to assess polar bear NK cell activity. YAC-1 has been shown to be most sensitive to NK function in other aquatic animals, including seals (Ross et al., 1996) and sea turtles (Rousselet et al., 2013). In contrast, K562 cells appear more sensitive than YAC-1 cells to NK cell activity in cetaceans, including bottlenose dolphin (*Tursiops truncatus*; Gebhard et al., 2015) and beluga whales (De Guise et al., 1997).

Muskox and reindeer NK cell-like activity against YAC-1 and K562 cells is shown in Fig. 1. While NK activity showed the characteristic