Comparative Immunology, Microbiology and Infectious Diseases 48 (2016) 54–60

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Bovine natural killer cells are present in *Escherichia coli* infected mammary gland tissue and show antimicrobial activity *in vitro*

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ARTICLE INFO

Article history:
Received 14 November 2015
Received in revised form 29 July 2016
Accepted 3 August 2016

Keywords:
NK cells
Bovine mastitis
E. coli
Innate immunity
Mammary gland epithelial cells

ABSTRACT

Natural killer (NK) cells are early responders in bacterial infections but their role in bovine mastitis has not been characterized. For the first time, we show the presence of NK cells (NKp46$^+$/CD3$^-$) in bovine mammary gland tissue after an intramammary challenge with *Escherichia (E.) coli*. A small number of NK cells was detected in milk from quarters before and during an *E. coli* challenge. *In vitro* cultures of primary bovine mammary gland epithelial cells stimulated with UV irradiated *E. coli* induced significant migration of peripheral blood NK cells (pbNK) within 2 h. Furthermore, pbNK cells significantly reduced counts of live *E. coli in vitro* within 2 h of culture. The results show that bovine NK cells have the capacity to migrate to the site of infection and produce antibacterial mediators. These findings introduce NK cells as a leukocyte population in the mammary gland with potential functions in the innate immune response in bovine mastitis.

1. Introduction

Intramammary infections with *E. coli* are assumed to be the most frequent cause of clinical mastitis in well managed dairy herds and lead to severe economic losses for the dairy industry [1,2]. A large proportion of *E. coli* intramammary infections (IMI) result in mild or moderate clinical signs. However, approximately 10% of *E. coli* IMI lead to severe clinical mastitis and even death of the host [3]. Especially the early immune regulation appears to be crucial in determining the outcome of the infection [4]. A detailed understanding of early immune regulation is therefore important to be able to identify cows at risk and also to develop potential intervention tools. Currently the exact mechanisms of immune regulation during *E. coli* mastitis and the interplay between different leukocyte subsets in the mammary gland are still not completely understood.

Natural killer (NK) cells are known as early responders after pathogen contact and several recent studies point out their potential role in determining polarization and severity of the immune response in bacterial infections [5,6]. Human and mouse NK cells are equipped with toll-like receptors (TLR) 2 and 5 which enable them to directly recognize pathogen-associated molecular patterns (PAMP) [7]. Additionally NK cells can become activated by mediators released from antigen presenting cells after contact with various microorganisms [8–10]. Once activated, NK cells are potent producers of IFN-γ and thereby support the development of a Th1-biased response, placing NK cells at the interface between innate and adaptive immunity [11,12]. Recent studies in mice also demonstrated direct antimicrobial activity of NK cells against *Citrobacter (C.) rodentium* and *Bacillus (B.) anthracis* [13,14]. Depletion of NK cells in mouse infection models resulted in a less severe local inflammation but a higher bacterial load, stronger weight loss and a higher incidence of sepsis among NK cell depleted animals [14,15].

Bovine NK cells are defined as NKp46$^+$/CD3$^-$ lymphocytes [16]. In calves NK cells have been reported to produce IFN-γ in response to *Mycobacterium (M.) bovis* infected dendritic cells (DC) and in infection with *Babesia bovis* [17,18]. In adult cows NK cells acquired cytotoxic effector function *in vitro* after being activated with IL-12 and IL-15 and subsequently reduced the number of *M. bovis*-infected macrophages [19]. A more recent study showed that *M. bovis*-infected bovine DC were able to recruit NK cells and induced IFN-γ production in NK cells. In return, the presence of NK cells increased MHCII expression in the infected DC [20]. There are cur-

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http://dx.doi.org/10.1016/j.cimid.2016.08.001
0147-9571/

rently no peer-reviewed published studies that investigated the presence and potential immune regulatory role of NK cells in the bovine mammary gland or whether there is a direct interaction of NK cells with mastitis pathogens. Gene expression studies, however, revealed an up-regulation of the NK cell mediated cytotoxicity pathway in udder tissue 24 h after intramammary infection with *E. coli*, indicating that NK cells may be involved in the early immune response during *E. coli* mastitis [21,22].

Up to date there is no study on the presence and immune regulatory role of NK cells in the bovine mammary gland in mastitis. The objective of this study was to investigate the presence of NK cells in bovine mammary gland tissue and their potential role in the initial response to an intramammary infection with *E. coli*. We could identify NK cells ($NKp46^+/CD3^-$) in mammary gland tissue and milk of experimentally infected quarters using two color immunohistofluorescence and flow cytometry. Additionally we demonstrated *in vitro* that blood NK cells migrate towards stimulated mammary gland epithelial cells and act bactericidal.

2. Material and methods

2.1. Collection of mammary gland tissue

Mammary gland tissue was collected from 6 mid-lactating cows enrolled in an *E. coli* challenge experiment [23]. The cows were euthanized using a penetrating captive bolt and subsequent exsanguinations at either 24 (n = 3) or 48 (n = 3) h post intramammary challenge with 100 CFU of *E. coli* strain ECZ [24]. Mammary gland tissue was collected from an infected, untreated quarter and an unchallenged control quarter in each cow. Tissue samples were immediately stored in 10% neutral buffered formalin for 24 h at room temperature. All procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number 2010-0078).

2.2. Immunohistofluorescence

Formalin fixed tissue samples were embedded in paraffin and sectioned at 5 μm thickness. For immunofluorescence staining sections were deparaffinized 3 times for 10 min in 100% xylene (Sigma-Aldrich, St. Louis, MO) followed by 100%, 95% and 70% ethanol for 5 min each and washed with DI water. For antigen retrieval slides were submerged with pre-warmed Trypsin solution (0.1% trypsin, 0.1% calcium chloride in 20 mM TBS, pH 7.6, all Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C for 5 min. The reaction was stopped with cold tap water. Slides were washed 3 times in PBS-tween (PBS containing 0.5% Tween®20, Sigma-Aldrich, St. Louis, MO) for 5 min each. Unspecific binding was blocked by submerging slides with 10% normal goat serum (Life Technologies, Carlsbad, CA) for 30 min at room temperature on an orbital shaker. Primary antibodies were diluted in PBS containing 0.5% BSA and 0.05% sodium azide. The sections were submerged with the respective primary antibody mix of mouse anti bovine NKp46 and rabbit anti human CD3 cross reactive for bovine CD3 (manufacturer and concentration Table 1) and incubated overnight at 4 °C. Subsequently sections were washed 3 times in PBS-tween for 5 min on an orbital shaker. Secondary antibodies were diluted respectively (Table 1) in PBS containing 0.5% BSA and 0.05% sodium azide. Controls were incubated with secondary antibody only and showed no unspecific binding of either antibody. Slides were submerged with the respective secondary antibody mix and incubated for 20 min at room temperature protected from light on an orbital shaker. Subsequently slides were washed 3 times with PBS-tween and mounted with Vectashield mounting medium containing DAPI (Vector labs, Burlingame, CA). The sections were visualized with an Olympus BX-50 microscope (Olympus, Center Valley, PA) using MetaMorph acquisition and image analysis software (Molecular Devices, Sunnyvale, CA). In total sections from 3 infected, untreated quarters and 3 unchallenged control quarters at 24 h post challenge and 3 infected, untreated quarters and 3 unchallenged control quarters at 48 h post challenge were analyzed. In each section 5 vision fields were analyzed and presence or absence of NK cells was recorded.

2.3. Blood and milk samples

Blood and milk samples were obtained from mid-lactating cows housed at the Cornell Teaching Dairy Barn or the Cornell Large Animal Teaching and Research Facility. Cows (n = 6) selected for blood sampling were determined healthy if their cow file did not show any diseases within the last month and after brief general examination. A volume of 200 ml blood was obtained by puncture of the jugular vein into an autoclaved evacuated glass bottle containing 40 mM EDTA, pH 7.4 (Sigma-Aldrich, St. Louis, MO). Cows selected for milk samples (n = 8) had SCC < 200,000 and were tested culture negative twice before sampling. Fifty ml of milk were collected under aseptic conditions before and after intramammary challenge with 100 CFU *E. coli* ECC-Z and kept on ice until further processed. All sampling procedures were approved by the Cornell Institutional Animal Care and Use Committee (project number 2007-0110).

2.4. NK cell separation from blood mononuclear cells

A volume of 200 ml blood was diluted 1:1 with PBS and layered on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 18 °C for 30 min at 1000 × *g*. The interphase containing mononuclear cells (MNC) was collected and washed three times in PBS (500 × g, 250 × g and twice at 100 × g, 4 °C). Contaminating red blood cells were lysed during the first wash step by exposing them to sterile, hypotonic saline solution for 10 s. Mononuclear cells were re-suspended in 5 ml PBS containing 2 mmol/l EDTA and 0.5% BSA and filtered through a cell strainer (mesh size: 40 μm, Corning, Tewksbury, MA) to remove cell clumps. Separation of 200 ml blood resulted in a total of 5×10^8 to 1×10^9 MNC which were centrifuged at 300 × *g* (10 min, 4 °C) and labeled with a monoclonal antibody mix containing antibodies to bovine CD3, CD4, CD14, CD21, γδ-T cell receptor (WC1) and MHC II at 0.005 mg/ml each (all Kingfisher Biotech, St Paul, MN) for 30 min at 4 °C. Subsequently cell suspension was washed at 300 × g, 4 °C for 10 min and incubated with goat anti mouse IgG coupled with paramagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C. After labeling, the cells were washed with PBS-EDTA (300 × g, 10 min, 4 °C). Subsequent magnetic separation with the MidiMACS separation unit (MiltenyiBiotec, Bergisch Gladbach, Germany) was conducted according to the manufacturer's instructions to deplete the cell suspension of T-cells, B-cells, monocytes and MHCII+ cells. This process resulted in a cell suspension with 20–40% NKp46+ cells (pbNK cells). Pre-trials have shown that the proportion of NKp46+/CD3+ cells was below 1% in peripheral blood leukocytes (data not shown). To increase the purity of pbNK cells in the pre-enriched cell suspension the cells were labeled with monoclonal mouse anti bovine NKp46 conjugated to Alexa Fluor® 488 and NKp46+ cells were sorted using a FACSaria cell sorter (Becton Dickinson, Franklin Lakes, NJ). Purity of the pbNK cell suspension after magnetic depletion and cell sorting was ≥98%.

2.5. Preparation of milk leukocytes and flow cytometric analysis

Aseptic milk samples were diluted 1:1 in PBS and centrifuged at 550 × *g* for 15 min at 4 °C. After removing the cream layer and discarding of the supernatant the cell pellet was resuspended in PBS and washed twice (550 × g, 15 min at 4 °C). Subsequently cells were