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IL-15 and macrophage secretory factors facilitate immune activation of neonatal natural killer cells by lipoteichoic acid

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ABSTRACT

Neonates possess a relatively "naive", yet inducible immune system. Our hypothesis is that upon strategic antigen exposure, cytokine priming and sensitization by accessory cells, natural killer (NK) cells could be activated to become a functional phenotype. We investigated the *in vitro* stimulation of cord blood (CB) and adult NK cells upon challenge with lipoteichoic acid (LTA), interleukin (IL)-15 and LTA-primed autologous macrophage-conditioned medium, using CD107a and CD69 phenotypes as indicators of activation. We also examined response of CB macrophages to LTA, in terms of P44/42 extracellular signal-regulated kinases (ERK1/2) activation and cytokine secretion. LTA significantly induced secretion of inflammatory cytokines tumor necrotic factor (TNF)-α, IL-6, IL-12 and activated the upstream signal of ERK1/2 phosphorylation in neonatal macrophages. The magnitude of responses to stimulation differed between neonatal and adult NK cells. Co-stimulation with IL-15 was critical for expansion of the CD69 and CD107a NK subpopulations in both neonatal and adult cells, upon a LTA challenge. NK cell activation could be enhanced by LTA-primed autologous macrophages through secretory factors. Our results indicated that neonatal macrophages and NK cells can evoke immunologic responses to a Gram-positive bacterial antigen. The combinatory priming strategy is relevant for development of novel protocols, such as IL-15 treatment, to compensate for the immaturity of the innate immune system in newborns against bacterial infections.

1. Introduction

Gram-positive bacterial infections such as Group B Streptococci in early-onset infection and Coagulase-negative Staphylococci in nosocomial infection are serious complications of newborns. The infection at the initial presentation often occurs with minimal warning signs, but may follow an alarmingly fulminant clinical course, leading to detrimental consequences and death [1,2]. Historically, the immune system of neonates has been considered as compromised and immature, partly due to limited exposure to antigens *in utero* [3,4]. However, recent evidence has suggested that neonates possess a "naive", yet competent immune system with certain degree of functional flexibility and plasticity [5]. Thus, it is possible that upon antigen exposure and cytokine priming, their immune system could be tuned towards a mature functional response.

Natural killer (NK) cells are frontline lymphocytes in the innate immune surveillance against infections. NK cell pattern recognition and cytotoxicity are coordinated by a network of activating and inhibitory receptor signals, responsive to microorganism-infected target cells and soluble ligands in the environment [6]. Functional induction of NK cells by live bacteria and their cell wall components has been reported [7,8]. Bacterial lipopolysaccharide (LPS, Gram-negative organisms) stimulates proliferation of NK cells [9]. The effect and mechanism of lipoteichoic acid (LTA, Gram-positive organisms) on NK cells have not been widely studied. NK cell activation may be mediated by accessory cells. Upon pathogen recognition, immunoregulatory cells such as monocytes, macrophages and dendritic cells transmit signals through either direct contact or soluble factors to induce NK cell responses [10–12]. Recent studies also suggested the existence of specific memory to antigens in subsets of NK cells, rendering them adaptive immune functions [4].

In early life, the innate immune system plays an important role in the rapid evasion of invading pathogens, particularly before building up of the adaptive immune response. However, phenotypic and functional differences exist between neonatal and adult

Abbreviations: APC, allophycocyanin; CB, cord blood; ERK, extracellular signal-regulated kinases; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage-colony stimulating factor; LTA, lipoteichoic acid; MNC, mononuclear cells; NK cells, natural killer cells; PB, peripheral blood; PE, phycoerythrin.

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NK cells, in terms of regulatory subpopulations [13], cytotoxic capacity [14,15], cytokine response [16,17], killer cell immunoglobulin-like receptor (KIR) repertoire [18] and surface expression of adhesins [14,18]. Macrophages from neonates have been suggested as functionally compromised, leading to higher susceptibility to infections [3]. The capacity of producing IL-12 in responding to interferon-γ by neonatal macrophages was found to be lower than that in adults [3].

To date, information is sparse on neonatal NK cells to facilitate insightful assessment of their activation capacity and contribution to the host defense against invading bacteria in newborns. Considering that neonatal NK cells are relatively naive in terms of exposure to immune insults or inflammatory reactions, the important issue regarding boosting the relatively naive NK cell sensitivity in neonates could be addressed by combinatory strategies through bacterial antigen exposure, cytokine priming and stimulation by secretory factors from macrophages. In Neonatal Intensive Care Units, early-onset and nosocomial infections are mainly caused by Group B Streptococci and Gram-positive Coagulase-negative Staphylococci, respectively [19,20]. The functional reactions of neonatal NK cells to Gram-positive bacteria and the feasibility of augmenting the immune response by exposure to inducing factors have not been reported. In this study, we investigated the *in vitro* stimulation of cord blood (CB) and adult NK cells upon challenge with LTA, IL-15 and LTA-primed autologous macrophage-conditioned medium, using cell membrane expressions of CD107a and CD69 as activation markers. We also examined the effects of LTA on P44/42 mitogen-activated protein kinases (MAPKs) ERK1/2 phosphorylation and secretion of tumor necrosis factor (TNF)-α, IL-6 and IL-12 by CB macrophages.

2. Materials and methods

2.1. Sample collection

All human blood samples were collected and used in accordance with procedures approved by the Ethics Committee for Clinical Research of the Chinese University of Hong Kong. CB samples were collected in preservative-free heparin (10 IU/ml) during normal, full-term vaginal delivery of cases without blood precaution, fever, infection, smoking history or genetic disorders. Written informed consent was obtained from all the mothers. Buffy coat samples were obtained from peripheral blood (PB) of adult male donors (aged 20–25 years).

2.2. Preparation of macrophages and NK cells

Primary mononuclear cells (MNC) from CB and adult PB were obtained by density-gradient separation on Ficoll-Paque (Amersham Biosicences, Uppsala, Sweden). For enrichment of macrophages, MNC (5×10^6/ml) were cultured for 2 h in 5% CO_2. Non-adherent cells were removed and remaining cells were cultured in 1 ml of RPMI containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and granulocyte macrophage-colony stimulating factor (GM-CSF, 4 ng/ml) (Peprotech, Rocky Hill, NJ, USA). The culture was maintained for 11–12 days, with non-adherent cells removed and medium changed every 2 days. NK cells were isolated from MNC by negative selection using the human NK cell isolation kit (MiniMACS; Miltenyi Biotech, Bergisch Gladback, Germany). The purity of enriched NK cells was determined by flow cytometry using the BD Multitest™ antibody mixture (BD Pharmingen, San Jose, CA, USA) containing anti CD3-fluorescein isothiocyanate (FITC)/CD16CD56-phycoerythrin (PE)/CD45-perodonon chlorophyll protein (PerCP) /CD19-allophycocyanin (APC). Cells were acquired by the FACSCalibur cytometer (BD Pharmingen). Enriched NK cells (CD3-CD16+56+) had purity >95% (97.5 ± 0.9%) as measured by the FlowJo software (Tree Star, Ashland, Oregon, USA). The viability of macrophages or NK cells was >95% as determined by trypan blue exclusion staining.

2.3. ERK phosphorylation and secretory cytokines in macrophage culture

Macrophages cultured in 6-well plates were treated with LTA (20 μg/ml; Invivogen, San Diego, CA, USA) for different time durations. The plates were centrifuged at 300*g* for 10 min at 4 °C and the supernatant was collected for quantifying IL-6, TNF-α and IL-12 by ELISA assay (Peprotech). The cell pellet was lysed and the protein concentration was determined by the DC Protein Assay Kit II using bovine serum albumin as the standard (BioRad; Hercules, CA, USA). Analysis of ERK phosphorylation was performed by Western blot analysis and electrochemiluminescence detection using specific antibodies against phospho-ERK1/2 and ERK1/2 (Cell Signaling; Beverly, MA, USA) as previously described [21].

2.4. NK cell activation in response to LTA, IL-15 and LTA-stimulated autologous macrophage-conditioned medium

Enriched NK cells from CB or adult PB were treated for 48 h in culture under different conditions (with or without 20 μg/ml LTA or 5 ng/ml IL-15). For expression of CD69, NK cells were stained with CD69-PE antibody (BD Pharmingen), and analyzed by flow cytometry. For assessment of CD107a expression, NK cells were further challenged with K562 cells (2×10^6/ml) for 4 h. To detect spontaneous degranulation, a control culture without K562 cells was included in every experiment [22]. The detection of CD107a expression on NK cells was performed by flow cytometry after staining with antibodies against CD107a-PE and CD56-APC (BD Pharmingen).

For collection of macrophage-conditioned medium, macrophages were cultured for 9 days in RPMI containing 10% FBS and resuspended at a density of 2×10^5 cells/ml. These cells were further cultured for 72 h. Total medium change was performed and the cells were treated with LTA (0 or 20 μg/ml), without GM-CSF (Supplementary Fig. 1). After 48 h, the culture supernatant was collected and used for treatment of autologous NK cells at a cell density of 1×10^6/ml, with or without 5 ng/ml of IL-15. After 48 h, NK cells were analyzed for membrane expressions of CD107a and CD69. Basal levels of IL-15 in the macrophage-conditioned media, with or without LTA treatment, were quantified by ELISA assay (R&D Systems, Minneapolis, MN, USA).

2.5. Statistical analysis

Statistical analyses were determined using the Student's paired *t*-test for comparing paired data, or *t*-test for non-paired experiments comparing data between adult and CB NK cells using the software SPSS version 16.0 (IBM, Armonk, NY, USA). *P*-values were adjusted by Bonferroni correction wherever multiple comparisons were applied. Western blot data were analyzed by the Friedman test followed by Dunn's Multiple Comparison test and a *P*-value of $\leqslant 0.05$ was considered significant. All results were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. LTA induced ERK1/2 phosphorylation in CB macrophages

Western blotting analysis ($n = 3$) demonstrated very low levels of phospho-ERK in the control culture (without LTA) (Fig. 1a). In