



Impaired cytolytic activity of asthma-associated natural killer cells is linked to dysregulated transcriptional program in energy metabolism

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ABSTRACT

Natural killer (NK) cells are a cytotoxic subset of the innate lymphoid cells, playing essential roles in host defense against tumors and infections, which, however, are usually functionally compromised in chronic diseases. Atopic diseases, such as allergic asthma, characterized by type 2 immune responses, are usually associated with chronic inflammations. Whether asthma-associated immune environment affects the cytolytic function of NK cells has not been elucidated. Here, YTS, a human NK cell line, was exposed to serum from healthy donors or asthma patients for analysis of its cytolytic function. We found that, serum from asthma patients reduced the cytolytic activity of YTS cells against Raji human B lymphoblasts, in comparison with normal serum. The impairment of cytolytic activity of these YTS cells was accompanied with decreased degranulation potentials, weakened conjugation formation with Raji cells, and premature termination of ERK phosphorylation upon stimulation. Meanwhile, apoptosis or cell death of YTS cells was not increased after exposure to serum from asthma patients. Importantly, such impairment of cytolytic activity of asthma-associated YTS NK cells was accompanied with aberrantly enriched genes involved in oxidative phosphorylation. Taken together, these results demonstrate that the serum of asthma patients directly suppresses the cytolytic function of NK cells, possibly through dysregulation of energy metabolism in NK cells.

1. Introduction

Natural killer (NK) cells are cytotoxic effector cells of the innate immune system, playing critical roles in host anti-viral/anti-tumor immune defense (Vivier et al., 2008). NK cells directly eliminate transformed cells or infected cells through exocytosis of lytic perforin/granzyme granules, or through triggering death receptors on target cells upon target cells recognition.

Allergic disorders, such as allergic asthma, are characterized by type 2 immune responses, and are also usually associated with chronic inflammations (Galli et al., 2008). Although increased activity of pulmonary NK cells was reported in the early phase of allergic airway inflammations (Bi et al., 2017), orthodox functions of NK cells, such as their cytokine production profiles, were shown to be detrimentally

altered in later phases. Compared with healthy individuals, asthma patients had fewer NK cells in the bronchoalveolar lavage (BAL), and displayed decreased cytolytic activity towards tumor cells (Duvall et al., 2017). Importantly, NK cells in the PBMCs of asthma patients with chronic rhinosinusitis exhibited a severe impairment in IFN- γ production (Kim et al., 2013). In addition, NK cells in the peripheral blood of asthma patients were reported to express type II cytokines, and to show constitutive activation of STAT6 (Wei et al., 2005). These studies suggest that allergic asthma might affect not only local, but also systemic immunologic state, as well as the immune cells in the peripheral. Despite the direct analyses of effector functions NK cells in asthma patients, and in mouse models, however, the effects of the asthma-associated immune environment on the cytolytic function of NK cells have not been investigated.

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Metabolism is essential for normal lymphocyte functions. Generally, quiescent lymphocytes engage oxidative phosphorylation (OXPHOS) for bioenergy, which is efficient, yet at a lower rate. On the other hand, aerobic glycolysis is usually required to fuel robust proliferation and biosynthesis of effector molecules upon lymphocyte activation (Pearce et al., 2013). NK cells were reported to require OXPHOS for normal effector functions both in humans (Keating et al., 2016) and in mice (Keppel et al., 2015). Despite these knowledge on NK cell metabolic requirement at normal state, however, the metabolic levels or metabolism-related transcriptional program linked to the aberrant functions of NK cells under disease settings have not been investigated.

In this study, we showed that NK cells exposed to serum from asthma patients displayed impaired cytolytic activity, possibly due to decreased expression of cytotoxic molecules, weakened conjugation formation, as well as prematurely terminated MAPK signaling. Gene sets enrichment analysis further showed that such dysfunction of asthma-associated NK cells was linked to the aberrantly enriched genes involved in the OXPHOS metabolic pathway, which is usually engaged by quiescent, rather than activated, lymphocytes.

2. Materials and methods

2.1. Cell lines and serum exposure

YTS cells (a subclone of the YT NK leukemia cell line) and Raji human B lymphoblast (ATCC® CCL-86™) were maintained in RPMI-1640 medium (Corning) supplemented with 10% (vol/vol) FBS (Hyclone), 2 mM L-Glutamine, 1 mM HEPES, pH 7.4, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Data reported in this study were obtained from 8 healthy donors and 20 asthma patients. The recruited male or female asthmatic patients fulfilled the criteria for asthma, and their allergic status was further confirmed by elevated serum IgE (Fig. 1A). Healthy individuals displayed no evidence of atopy, and showed low levels of serum IgE (Fig. 1A). Information for patients and healthy individuals was listed in Table 1. Serum isolated from healthy individuals and asthma patients was preserved at -80°C before use. Exposure to serum was performed by incubation of YTS cells with the above -mentioned medium mixed with 30% (vol/vol) serum from healthy individuals or asthma patients for 12 h before washed and experiments. For RNA sequencing and transcriptome analysis, pooled serum from healthy individuals or asthma patients was used for serum exposure. Informed consent was obtained from the patients and healthy donors, and the study was approved by the ethical committee of Shenzhen Institutes of Advanced Technology.

2.2. ELISA

Amounts of human IgE in the serum were determined by an ELISA kit from DAKWE (Shenzhen, China) according to the manufacturer's instructions. Briefly, serum from patients or healthy controls was added to ELISA plate pre-coated with anti-human IgE antibody, and the plate was incubated for 2 h at room temperature. After washing, biotinylated anti-human IgE antibody from the kit was applied for 1 h at room temperature, followed by streptavidin-HRP conjugate from the kit for additional 30 min at room temperature after washing. Finally, TMB substrate from the kit was applied after extensive washing, and the 450 nm absorbance was obtained with a Multiskan GO microplate reader (Thermo Scientific, MA, USA).

2.3. Antibodies and reagents

We purchased APC-anti-CD56, purified mouse anti-CD28, APC-anti-mouse IgG, FITC-anti-Granzyme B, PE-anti-Perforin, Foxp3 Fix/Perm Buffer Set from Biolegend (San Diego, CA, USA); rabbit anti-ERK, and mouse anti-p-ERK from Cell Signal Technology (Danvers, MA, USA); goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP from Bosterbio

(Wuhan, China). Flow cytometric analysis of CD28 expression was performed using purified mouse anti-CD28 staining followed by washing and APC-anti-mouse IgG staining. Both Phorbol 12-myristate 13-acetate (PMA) and 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

2.4. Cytolytic assay

Cytolytic activity of YTS cells was assessed by a cytolytic assay using CFSE-7AAD staining. Briefly, YTS cells were incubated with CFSE -labeled Raji cells at the ratio of 10:1 for 4 h. 7AAD was then added into the cell suspensions, and incubated on ice for 10 min, before flow cytometric analysis of the percentages of 7AAD⁺ cells among CFSE⁺ cells.

2.5. Apoptosis detection

Apoptosis of YTS cells was determined by Annexin V - PI staining using an apoptosis detection kit (Transgen, Beijing, China) according to the manufacturer's instructions.

2.6. Conjugation assay

Conjugation formation assay was performed by incubation of CD56-stained YTS cells with CFSE -labeled Raji cells at 37°C for 12 min or for the indicated time, followed by fixation using Foxp3 Fix/Perm Buffer Set (Biolegend, San Diego, CA, USA) and flow cytometric analysis on a cytoflex (Beckman Coulter, Brea, CA, USA). Data were analyzed with FlowJo software (Tree Star). Conjugates formed would display double positive signals for both CD56 and CFSE.

2.7. RNA sequencing and gene set enrichment analysis (GSEA)

Total RNA of YTS NK cells after 12 h of 30% pooled serum -containing complete medium exposure was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). Next generation sequencing library preparations were constructed using Ultra RNA Library Prep Kit for Illumina according to the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA). The libraries were multiplexed and loaded on an Illumina HiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a $2 \times 150\text{bp}$ paired-end configuration. The sequences were processed and analyzed by GENEWIZ (Suzhou, China).

GSEA using Broad Institute software (<http://www.broadinstitute.org/gsea/>) was performed on RNA sequencing data from each samples. GSEA used gene sets from the Molecular Signature Database v.6.1 (Subramanian et al., 2005; Liberzon et al., 2015, 2011) including KEGG pathway gene lists obtained from <http://www.genome.jp/kegg/pathway.html>, as well as BIOCARTA gene sets obtained from <http://software.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=CP:BIOCARTA>. Enrichment scores (ES) obtained by GSEA were used to compare different pathways between exposure with control serum and asthma patients -derived serum.

2.8. Statistics

Statistically significant differences were determined by Student's t-tests. Values of * $P < 0.05$, or ** $P < 0.005$ were considered significant.

3. Results

3.1. Exposure to serum from asthma patients impaired the cytolytic activity of NK cells

To evaluate the effects of asthma-associated immune environment

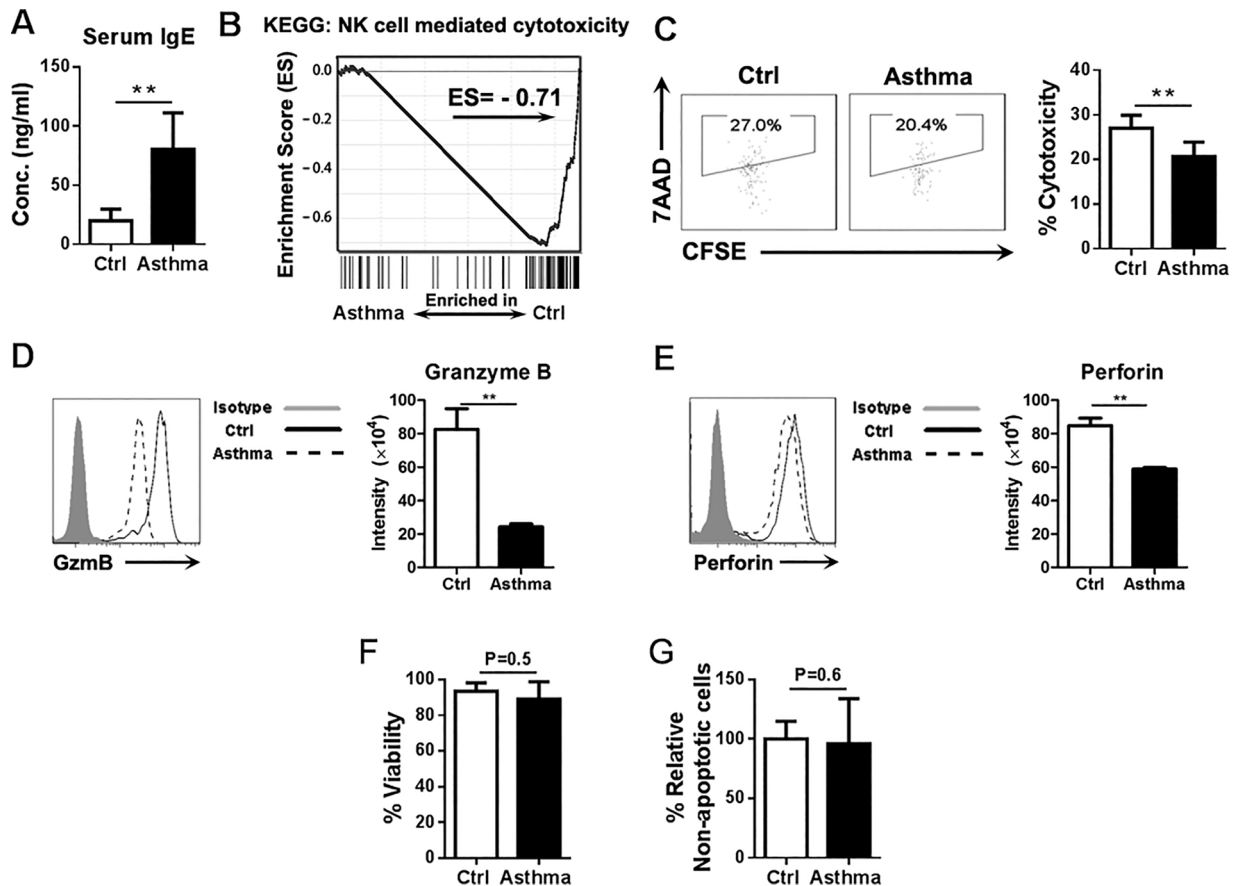


Fig. 1. Exposure to serum from asthma patients impaired the cytolytic activity of NK cells. (A) Concentrations of IgE in the serum from all the asthma patients ($n = 20$) or healthy controls ($n = 8$) included in this study were shown. (B) YTS cells were exposed to pooled serum from asthma patients ($n = 20$) or healthy controls ($n = 8$) for 12 h, before RNA sequencing, and GSEA analysis for KEGG NK cell mediated cytotoxicity pathway. Data was representative of two independent experiments with similar results. (C) YTS cells were exposed to serum from asthma patients or healthy controls, before tested for cytotoxicity against CFSE-labeled Raji cells at the effector : target ratio of 10:1. Raji cells killed in the assay were assessed by the percentages of 7AAD⁺ cells among CFSE⁺ cells. (D) Expression of Granzyme B in serum-exposed YTS cells was determined before cytolytic assay by flow cytometry. (E) Expression of Perforin in serum-exposed YTS cells was determined before cytolytic assay by flow cytometry. (F) Viability of YTS cells after serum exposure in (C) as assessed by trypan blue assay before cytolytic assay was shown. (G) Apoptosis of YTS cells after serum exposure in (C) was determined before cytolytic assay by annexin V and PI staining. Percentages of annexin V⁻ PI⁻ non-apoptotic cells were shown. (C–G) Random samples from healthy controls ($n = 5$) or asthma patients ($n = 7$) were used. (A, C–G) Data are represented as the mean $\pm$ SEM.

Table 1
Characteristics of asthma patients and healthy donors.

	Asthma patients ($n = 20$)	Healthy donors ($n = 8$)
Gender (Male : Female)	09: 11	05: 03
Age (year), mean (range)	48.6 (13–86)	24.5 (22–33)

on the cytolytic function of NK cells, we exposed YTS NK cells to serum from asthma patients or healthy controls. High levels of serum IgE in serum from asthma patients included in this study confirmed this characteristic of the disease in these individuals (Fig. 1A) (Galli et al., 2008). Gene sets enrichment analysis (GSEA) showed that after control serum exposure, YTS NK cells displayed an enrichment of genes involved in NK cell mediated cytotoxicity (Fig. 1B) over the asthma group, indicating that YTS NK cells exposed to serum from asthma patients might be compromised in cytolytic activity. In vitro cytolytic assay indeed showed decreased cytolytic activity against Raji cells by these asthma-associated NK cells (Fig. 1C). Cytolysis of target cells by NK cells requires the release of preformed lytic granules in NK cells (Smyth et al., 2005). We next assessed the expression of Granzyme B and Perforin in serum-exposed YTS NK cells without any stimulations, and found that both Granzyme B and Perforin were lower in YTS cells in

the asthma group (Fig. 1D and E). These results demonstrate that asthma-associated YTS NK cells possessed decreased cytolytic activity, as well as reduced cytolytic potentials, as evidenced by fewer pre-formed lytic granules. Meanwhile, exposure to serum from asthma patients didn't increase cell death or apoptosis of YTS cells, compared to control serum exposure (Fig. 1F and G), indicating that the decreased cytotoxicity was a result of dysfunction in YTS cells.

3.2. Exposure to serum from asthma patients impaired conjugation formation of NK cells

NK cell cytotoxicity requires conjugation formation of NK cells with target cells, which involves the regulation of actin cytoskeleton, and adhering to target cells through adhesion molecules (Mace et al., 2014; Orange, 2008). GSEA showed an enrichment of these related pathways in control YTS NK cells, suggesting that the ability of asthma-associated YTS NK cells to form conjugation with Raji target cells might be compromised (Fig. 2A). Indeed, we observed fewer conjugates formed between Raji cells and YTS cells of the asthma group, than between Raji cells and control YTS cells (Fig. 2B–D), indicating that YTS cells decreased the ability of forming conjugation with target cells, after exposure to serum from asthma patients. Since conjugation formation of NK cells with target cells requires NK cell activation upon target cell

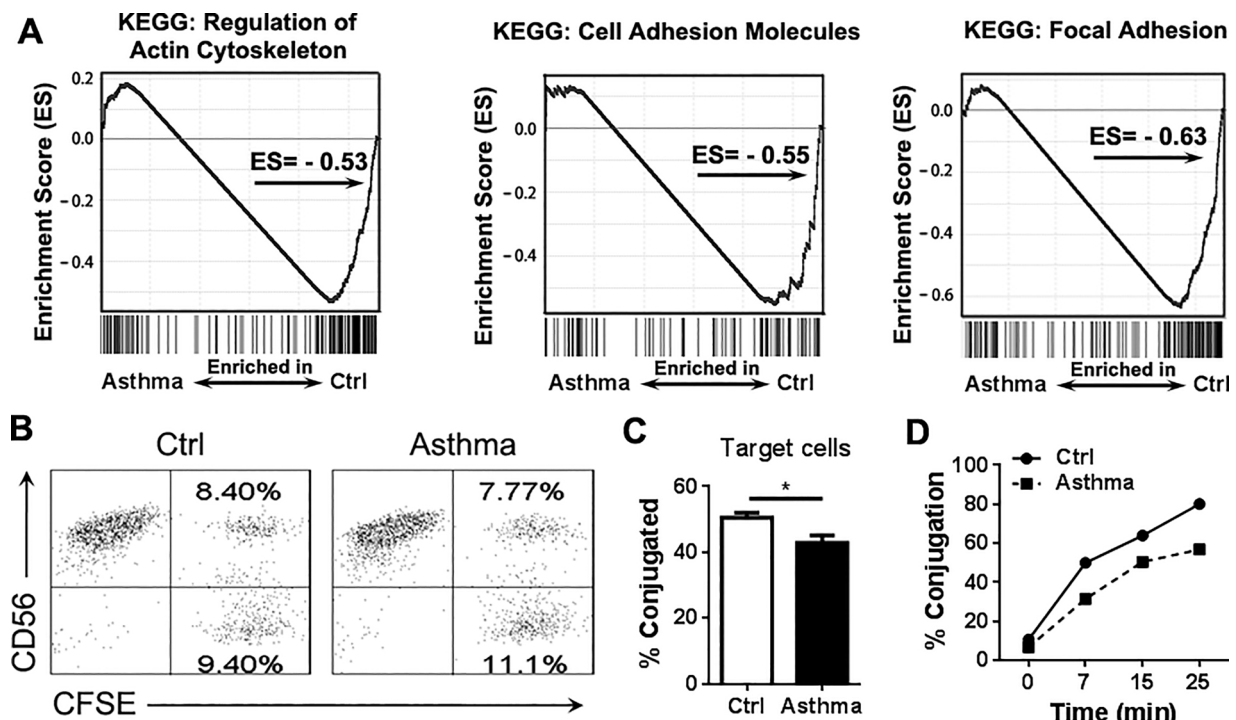


Fig. 2. Exposure to serum from asthma patients impaired conjugation formation of NK cells with Raji cells. (A) YTS cells were exposed to pooled serum from asthma patients ($n = 20$) or healthy controls ($n = 8$) for 12 h, before RNA sequencing, and GSEA analysis for the indicated KEGG pathways. Data was representative of two independent experiments with similar results. (B–D) CD56⁺ YTS cells exposed to serum from asthma patients or healthy controls were tested for conjugation formation with CFSE-labeled Raji cells. (B) Representative results at 12 min were shown. (C) Statistical analysis of the percentages of conjugated cells among Raji cells at 12 min was shown, and represented as the mean $\pm$ SEM. Healthy controls ($n = 3$). Asthma patients ($n = 5$). (D) The percentages of conjugated cells among Raji cells at different time points were shown. This experiment was repeated twice.

activation (Mace et al., 2014; Orange, 2008), this result suggests that the activation upon Raji target cell recognition by YTS cells might be impaired after exposure to serum from asthma patients.

3.3. Exposure to serum from asthma patients prematurely terminates ERK phosphorylation in NK cells

NK cell activation upon target cell recognition depends on the integrated signals through both activating and inhibitory surface receptors (Watzl and Long, 2010). While YTS NK cells lack most inhibitory surface receptors, the activating receptor, CD28, is a major activating receptor for YTS NK cells (Chen et al., 2006). Here, CD28 expression on asthma patient serum-exposed YTS cells showed only modest decrease, as compared with control YTS cells (Fig. 3A and B). On the other hand, the signaling pathway from PI3K via Rac to ERK (a MAPK) has been shown to be essential for NK cell cytotoxicity (Billadeau et al., 1998; Djeu et al., 2002; Wei et al., 1998). Here, we identified the gene sets enrichment of both MAPK and RAC1 pathways in control YTS NK cells, as compared with asthma-associated NK cells (Fig. 3C), suggesting that these pathways might be downregulated in asthma-associated NK cells. For confirmation, we compared the phosphorylation of ERK in YTS NK cells following PMA stimulation (Fig. 3D). We found that YTS cells exposed to serum from asthma patients prematurely terminated ERK phosphorylation following PMA stimulation, compared to YTS cells exposed to control serum (Fig. 3D). These results demonstrated that YTS NK cells were hyporesponsive after exposure to serum from asthma patients.

3.4. Exposure to serum from asthma patients enriched genes involved in oxidative phosphorylation in NK cells

The above results demonstrated the impaired cytolytic functions of asthma-associated NK cells with compromised critical steps involved in

NK cell cytotoxicity. Since T cell dysfunction has been suggested to be driven by metabolic dysregulation, we set out to search for enriched metabolic pathways in asthma-associated YTS NK cells. We observed genes for the OXPHOS pathway were enriched in YTS NK cells exposed to asthma serum, with an enrichment score of 0.65 (Fig. 4A). Leading edge genes driving gene set enrichment of KEGG Oxidative Phosphorylation pathway in asthma-associated YTS NK cells were mostly genes involved in mitochondrial respiration, such as mitochondrial H⁺ ATP synthase subunits, NADH:ubiquinone oxidoreductase subunits, as well as cytochrome c oxidase subunits (Fig. 4B). These results indicated the aberrant cellular requirement for OXPHOS metabolic program in asthma-associated NK cells, and suggest that dysregulated metabolism might underlie the impaired effector functions of these NK cells.

4. Discussions

In this study, we demonstrate that NK cells exposed to serum from asthma patients showed impaired cytolytic activity, as evidenced by decreased target cell killing, weakened conjugation formation, and hyporesponsive intracellular signaling upon target cell recognition. Given that NK cells from asthma patients also showed impaired production of IFN- γ and TNF- α (Kim et al., 2013; Wei et al., 2005), together these data indicate that the anti-tumors/infections function of asthma-associated NK cells were functionally impaired. Importantly, we discovered a link between the dysfunction of asthma-associated NK cells and a dysregulated metabolic requirement by these NK cells.

Metabolism holds great potential to therapeutically recover/enhance NK cell function (Gardiner and Finlay, 2017). OXPHOS is usually engaged by quiescent lymphocytes for generation of energy at a low rate, rather than by activated lymphocytes that need robust biosynthesis for proliferation and production of effector molecules. The relatively increased cellular requirement for OXPHOS transcriptional program in asthma-associated dysfunctional NK cells suggest that the

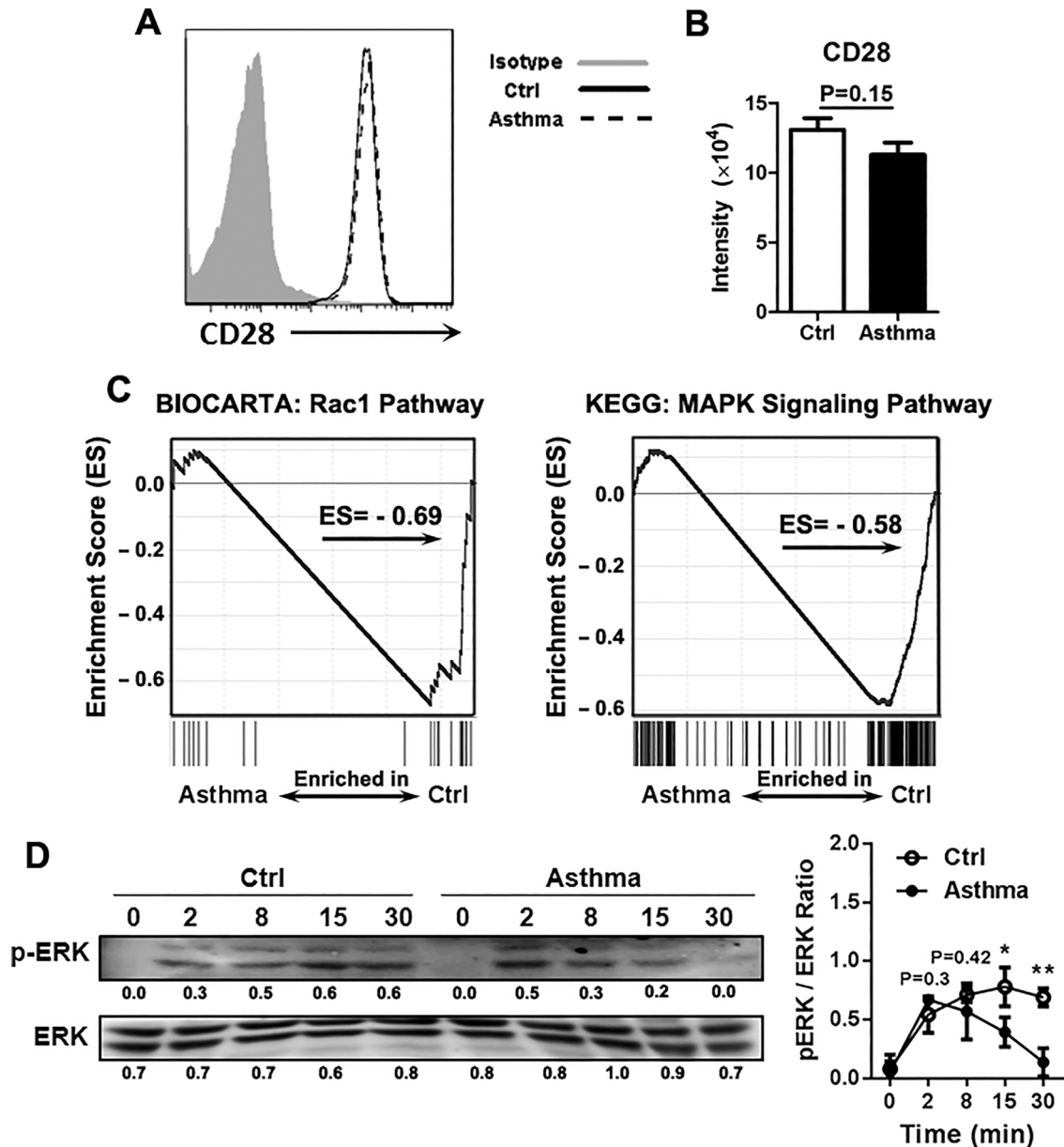


Fig. 3. Exposure to serum from asthma patients prematurely terminates ERK activation in NK cells. (A) CD28 expression on YTS cells was determined by flow cytometry after exposure to serum from asthma patients or healthy controls. (B) Statistical analysis of the intensity of CD28 expression on YTS cells in (A) was shown. Healthy controls (n = 5). Asthma patients (n = 7). (C) YTS cells were exposed to pooled serum from asthma patients (n = 20) or healthy controls (n = 8) for 12 h, before RNA sequencing, and GSEA analysis for BIOCARTA Rac1 pathway and KEGG MAPK signaling pathway. Data was representative of two independent experiments with similar results. (D) After exposure to serum from asthma patients or healthy controls, YTS cells were stimulated with PMA for the indicated time (0, 2, 8, 15 or 30 min), and were examined for the phosphorylation of ERK by western blot. The representative results were shown on the left. The intensity of each band was shown below the indicated bands. Statistical analysis of the ratio of phosphorylated ERK to total ERK was shown on the right. Data are pooled from 3 independent experiments. (B&D) Data are represented as the mean $\pm$ SEM.

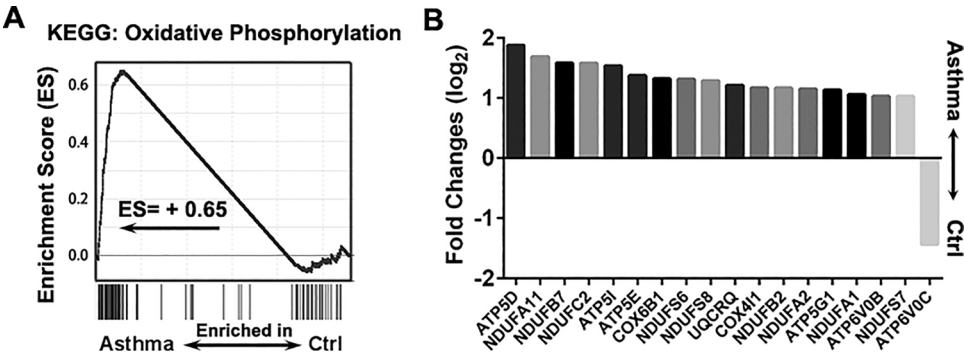


Fig. 4. Exposure to serum from asthma patients increased genes for the OXPHOS pathways in NK cells. (A) YTS cells were exposed to pooled serum from asthma patients (n = 20) or healthy controls (n = 8) for 12 h, before RNA sequencing, and GSEA analysis for KEGG Oxidative Phosphorylation pathway. Data was representative of two independent experiments with similar results. (B) Leading edge genes driving gene set enrichment of KEGG Oxidative Phosphorylation pathway. Genes with both fold changes over 2 and RPKM > 10 were shown as log₂ values. The darker of the column indicates the higher RPKM value of the gene.

metabolic status of these cells might be shifted away from robust biosynthesis characteristic of effector lymphocytes. This aberrant shift in energy metabolism might underlie the impaired effector function of asthma-associated YTS NK cells we observed. On the other hand, up-regulation of genes involved in OXPHOS, with OXPHOS levels actually suppressed, were reported for T cells during the process of establishing exhaustion, which reflects the cellular need, rather than the actual levels of cellular metabolism. Despite the transcriptional up-regulation of genes involved in OXPHOS, it would be interesting for further studies to investigate the actual levels of OXPHOS, as well as other metabolic pathways, in asthma-associated NK cells.

Also, our observations that serum from asthma patients directly affects NK cell cytolytic activity, as well as genes involved in OXPHOS, suggest that immuno-regulatory factors might exist in the serum of asthma patients which could act on NK cells, possibly via metabolic pathways.

One such potential soluble factor might be TGF- β , the immuno-suppressive cytokine frequently detected in chronic diseases. Indeed, increased levels of TGF- β was detected in asthma patients (Wong et al., 2001; Balzar et al., 2005). Also, TGF- β signaling was active in asthmatic airways (Sagara et al., 2002). TGF- β might contribute to the functional exhaustion of asthma-associated NK cells (Bi and Tian, 2017), in a way similar with that observed in tumors or chronic infections (Castriconi et al., 2003; Donatelli et al., 2014; Sun et al., 2012). Importantly, TGF- β signaling pathway was reported to repress NK cell metabolism and function (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018), and inhibition of the canonical TGF- β signaling pathway could provide a metabolic advantage to NK cells and could improve functional responses (Zaiatz-Bittencourt et al., 2018). However, in the settings of asthma, further studies are required to reveal the dynamic levels of TGF- β in patients. Also, it would be interesting to neutralize TGF- β to see whether this could recover both the impaired function and dysregulated metabolic transcriptional program of NK cells after asthma patient-derived serum exposure.

Alternatively, type-2 cytokines (e.g. IL-4 and IL-13) were usually present in asthma patients, which could induce type-2 biased status of NK cells (Wei et al., 2005) in these context, and might result in the impairment of the orthodox cytolytic function of NK cells. IL-4 and IL-13 activate an STAT6-dependent switch of metabolic program in macrophages (Odegaard and Chawla, 2011), suggesting that type-2 cytokines like IL-4 and/or IL-13 might also induce a metabolic reprogramming in asthma-associated NK cells, resulting in the dysfunctional status. Further studies are required to determine the role of these cytokines and the downstream STAT6 in the detrimental alterations in the cytolytic function of NK cells in the current setting.

In conclusion, our study demonstrates the compromised immunosurveillance functions of asthma-associated NK cells, as well as the association with dysregulated transcriptional program in energy metabolism, and sheds light on the abnormal immune status of asthma patients. According to our data, concerns for patients with atopic disorders should not only be paid to the control of the diseases, but also be paid to the host immunosurveillance status, which is normally fueled by properly regulated metabolism. Interventions might be required to restore the dysfunctional effector functions of NK cells in these patients, possibly by developing techniques that target metabolism-interfering factors present in these patients, such as TGF- β or type-2 cytokines. Also, therapeutics with dual functions of both disease control and NK cell invigoration might be appreciated.

Author contributions

H.W. performed the great majority of experiments and analyzed the data. J.B. conceived the research, designed experiments, performed the transcriptome analysis, analyzed the data, and wrote the manuscript. G.W. collected serum from healthy donors and asthma patients. C.Z., Z.L. and L.C. provided technical assistance. X.W. conceived and

supervised the research.

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Conflict of interests

The authors declare no competing financial interests.

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