Supplementary Materials & Methods

Patients

Thirty-one severe asthma patients were recruited from the Division of Respiratory, Immunology and Allergy Medicine at the University Hospital of Lille. The project was declared at the Ministère de l'Enseignement supérieur de la Recherche et de l'Innovation under the number DC 2015-2575 and received the approval from the Comité de Protection des Personnes Nord-Ouest (CP 04/45). All patients signed an informed consent form. Severe asthma was defined according to the ATS/ERS criteria (2). Data collected at enrolment included the number of exacerbations during the last year, respiratory allergies, the rate of peripheral blood eosinophils, the Forced Expiratory volume in one second (FEV1) and the corticosteroid treatments. All patients received inhaled corticosteroids, but none was under biologic therapy. Severe asthma patients were compared to healthy non-atopic non-asthmatic donors (Etablissement Français du Sang). Current smokers and ex-smokers with a cumulative smoking exposure over 10 pack-years were excluded from the study.

PBMC and NK cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood on Ficoll-Paque Plus (Sigma-Aldrich, St Louis, USA). 2.10^6 PBMC/ml were cultured in RPMI containing 2mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, 10% Fetal Bovin Serum (Eurobio, Courtaboeuf, France). NK cells were purified from PBMC by immunomagnetic separation (STEMCELL Technologies, Vancouver, Canada). The purity of NK cells was assessed by flow cytometric analysis using antibody against CD3, CD56 (Biolegend, San Diego, USA), CD14 and CD19 (Beckton Dickinson Biosciences, Franklin Lakes, USA). The purity of isolated NK cells was >95% and viability assessed using Zombie AquaTM Fixable Viability Kit (Biolegend, San Diego, USA) was >90%.

Rhinovirus production

H1-HeLa (ATCC CRL-1958) cell line was grown in Dulbeco's Modified Eagle's Medium (DMEM, Life Technologies, France) containing 10% fetal bovine serum (FBS, Life Technologies, France). Rhinovirus A9 (RV-A9, ATCC VR-489) and rhinovirus 2 (RV-2, ATCC VR-482) were propagated in H1-HeLa cells, in DMEM supplemented with 2% FBS. The infected cells were frozen and thawed three times, then they were centrifuged at 3500 rpm for 10 min, afterwards the supernatant was harvested and used as virus stock stored at -80°C. The viral titre in supernatants of infected cells

was assessed using the end-point dilution assay, and the Spearman-Karber statistical method was used to determine the tissue culture 50% infectious dose (TCID50). The infectious titre of virus stock was 1.44 x 10^7 TCID50/ml.

Cell stimulation

PBMC (2x10^6/ml) or purified NK cells (1x10^6/ml) were stimulated in 24-well plates with Interleukin(IL)-12 (10ng/ml, Peprotech, Rocky Hill, USA) and IL-15 (10ng/ml, Miltenyi, Bergish Gladbach, Germany), or with an agonist for TLR3: Polyinosinic-polycytidylic acid (PIC) (10µg/ml, Invivogen, San Diego, USA), for TLR9: ODN 2395 (10µg/ml, Invivogen, San Diego, USA), for TLR7/8: Resiquimod (R848, 1µg/ml, Invivogen, San Diego, USA), for TLR2/6: FSL1 (1µg/ml, Invivogen, San Diego, USA), for NKp46: Hemagglutinin A of Influenza A (1µg/ml, Interchim, San Diego, USA), or with RV-A9 or RV-2 at Multiplicity Of Infection (MOI) 0.1 and 0.05, respectively, during 24 hours at 37°C in humid atmosphere saturated with 5% CO2. The lowest dose (0.1 MOI), and time point (24 hours) which significantly increased CD69 expression on NK cells were chosen for the rest of the study (data not shown). For the analysis of NK cell degranulation, PBMC were cultured with K562 myeloid tumour cells (ATCC) at a ratio of 100 PBMC for 1 K562 for 3 hours.

Flow cytometry analysis

The following antibodies were used (clone noted in parentheses) : anti-CD3 PECy7 (OKT3), anti-CD56 BV421 (HCD56), anti-CD56 BV510 (HCD56), anti-Tim3 PE (F38-2E2), anti-PD1 PerCP Cy5.5 (NAT105) all from Biolegend (San Diego, USA), anti-CD69 FITC (FN50) and mouse IgG1 κ FITC Isotype control (MOPC-21), anti-IFN-γ PerCP Cy5.5 (B27) and anti-mouse IgG1,κ PerCP Cy5.5 isotype control (MOPC-21) all from Beckton Dickinson Biosciences (Franklin Lakes, USA), anti-CD107a APC ef660 (H4A3) and anti-mouse IgG1,κ APC ef660 isotype control (P3.6.2.8.1), anti-CD62L APC ef780 (DREG56), from Life Technologies-Ebioscience (Carlsbad, USA), The viability of the cells was assessed using Zombie AquaTM Fixable Viability Kit.

After stimulation, PBMC were stained with the viability marker during 20 minutes at room temperature before staining with extracellular antibodies during 30 minutes at 4°C. For intracellular staining, cells were incubated with Cytofix/Cytoperm (Beckton Dickinson Biosciences, Franklin Lakes, USA) during 20 minutes at 4°C before staining with intracellular antibodies during 30 minutes at 4°C. For the analysis of CD107a expression by NK cells, PBMC were cultured with target cells together with antibody against CD107a and monensin (Life Technologies Ebioscience, Carlsbad, USA), and subsequently stained.

Data were acquired on a Canto II flow cytometer (Beckton Dickinson Biosciences, Franklin Lakes, USA) and analysed with FlowJo software. The gating strategy is detailed in Figure S1. Natural Killer cells were identified as a lymphoid population that lacked CD3 expression and expressed CD56. Natural killer cell activation was identified based on the expression of CD69, CD107 and IFN-γ (Figure S1).

Cytokine and Chemokine measurement

25 cytokines (GM-CSF, IFN-α, IFN-γ, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40/p70), IL-13, IL-15, IL-17, TNF-α, eotaxin, CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10) were dosed in culture supernatants by Luminex assay (Life Technologies) according to the manufacturer's instructions.

Supplementary figures

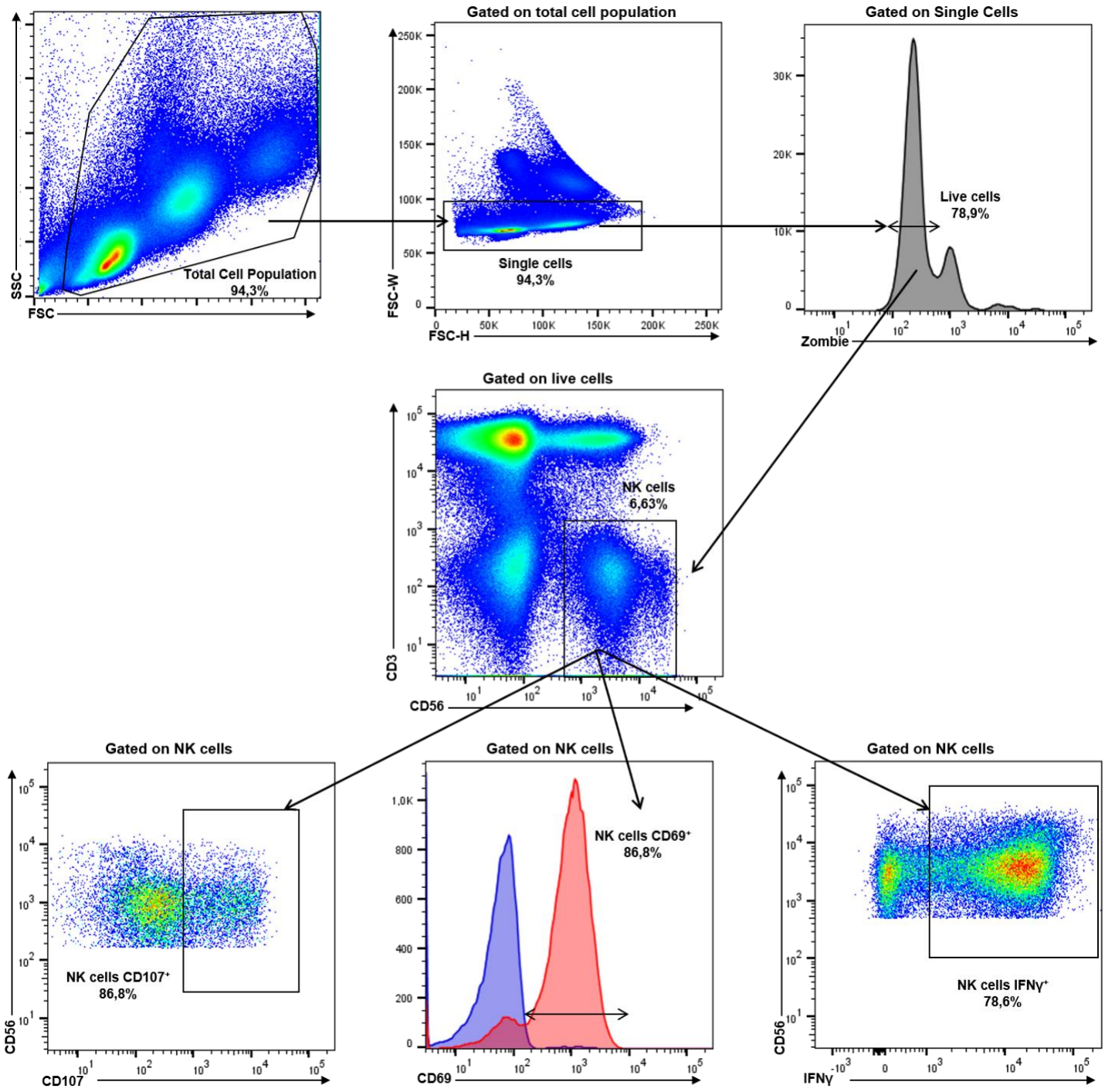

Figure S1: Flow cytometry gating strategies

Single and live cells were gated on PBMC. NK cells were identified as lymphoid cells that lacked CD3 expression and expressed CD56. Surface expression of CD69, CD107a and intracellular expression of IFN-γ were used to define the activation of NK cells.

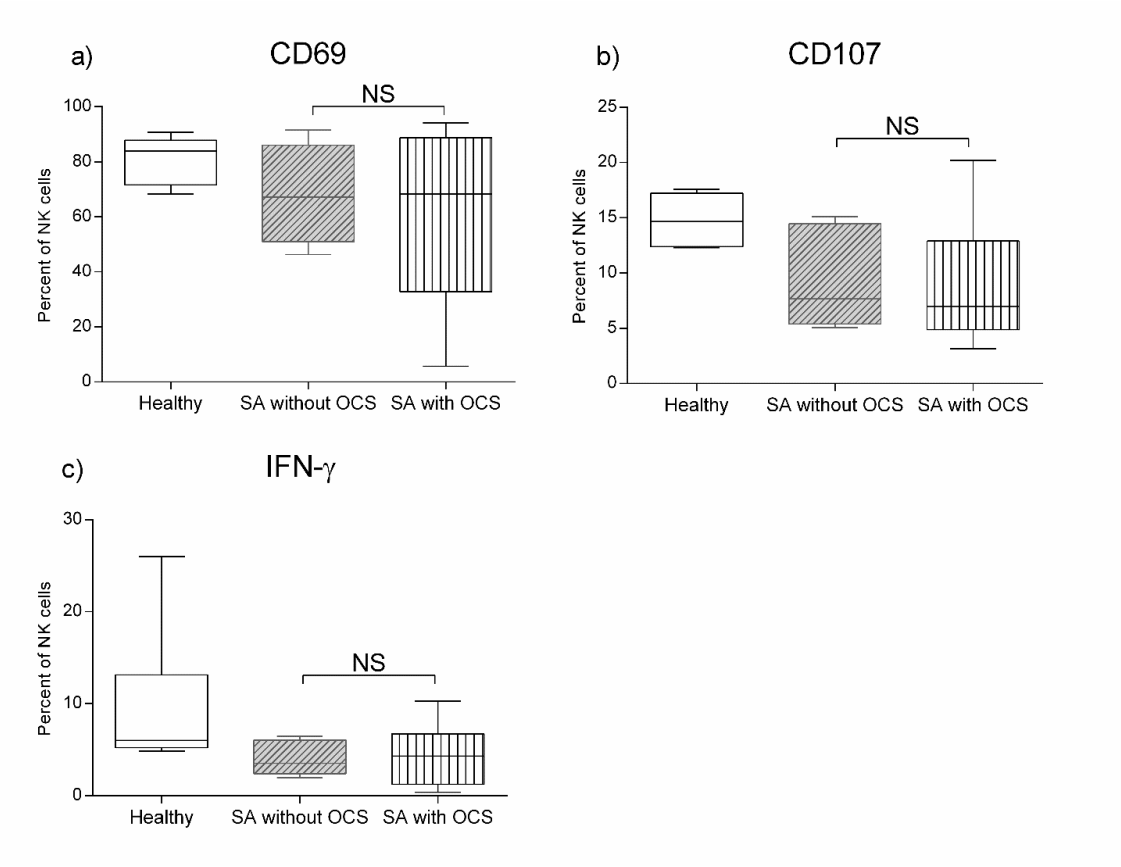

Figure S2: Oral corticosteroid treatment of severe asthma patients affects neither NK cell cytotoxic status, nor IFN-γ expression following PBMC stimulation with RV-A9 rhinovirus.

PBMC from healthy donors and severe asthma patients were stimulated with RV-A9. (a) Percentage of CD69 and (b) CD107a positive NK cells. (c) Percentage of IFN-γ^+ NK cells. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between severe asthma patients (SA) without oral corticotherapy (OCS) and SA with OCS were performed with a Kruskal-Wallis test (NS, non-significant); n=6 healthy donors (white), n=5 SA without OCS (hatched bars), n=8 SA with OCS (vertical bars).

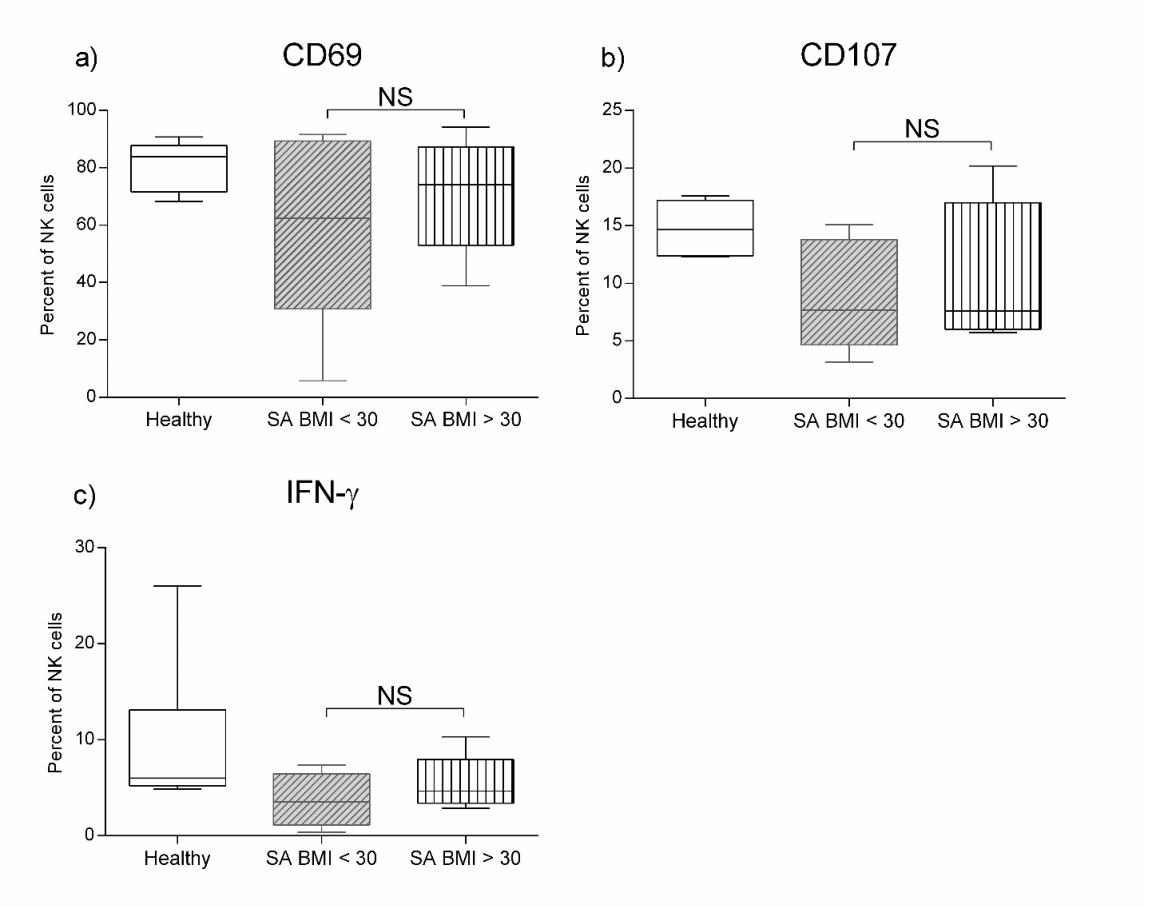

Figure S3: Body Mass Index of severe asthma patients affects neither cytotoxic status nor IFN-γ expression following PBMC stimulation with RV-A9 rhinovirus.

PBMC from healthy donors and severe asthma patients were stimulated with RV-A9. (a) Percentage of CD69 and (b) CD107a positive NK cells. (c) Percentage of IFN-γ^+ NK cells. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between severe asthma patients (SA) with Body Mass Index (BMI) < 30 kg/m^2 and SA with BMI > 30 kg/m^2 were performed with a Kruskal-Wallis test (NS, non-significant); n=6 healthy donors (white), n=7 SA with BMI < 30 kg/m^2 (hatched bars), n=5 SA with BMI > 30 kg/m^2 (vertical bars).

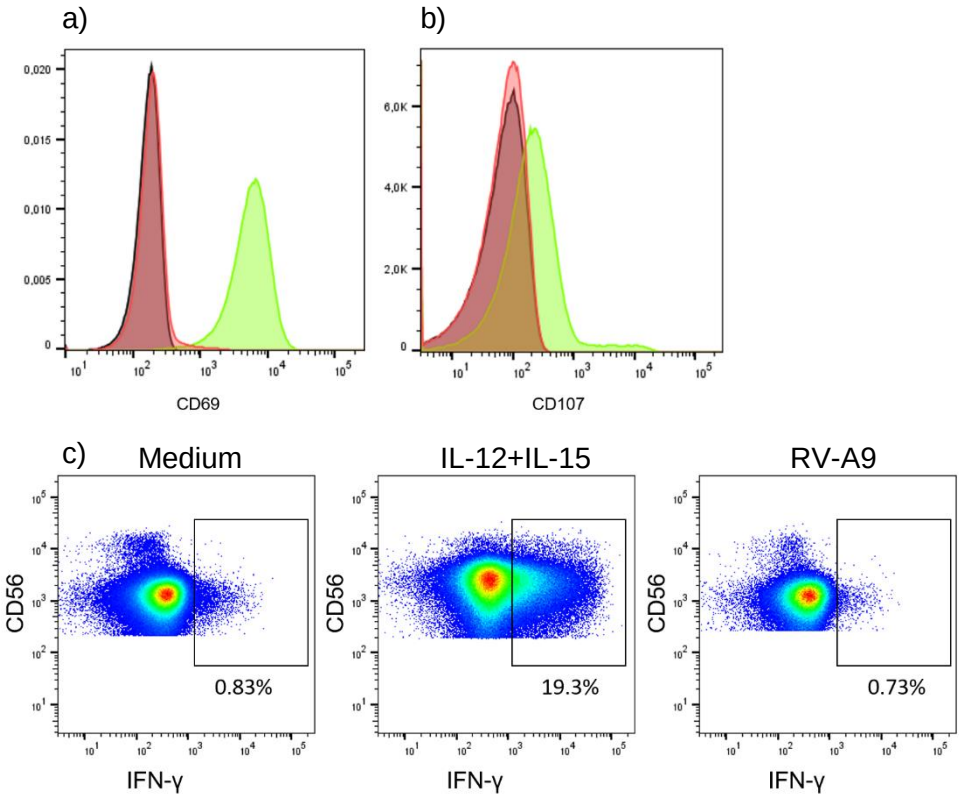

Figure S4: Purified NK cells are not activated by rhinovirus.

NK cells from a healthy donor were purified and stimulated with medium (in black), with IL-12+IL-15 (in green) or with RV (in red). Purity of NK cells was assessed by flow cytometry and was over 95%. **a)** $CD69^+$, **b)** $CD107^+$ and **c)** IFN-γ^+ NK cells were identified by flow cytometry. Percentage of NK cells positive for IFN-γ are shown.

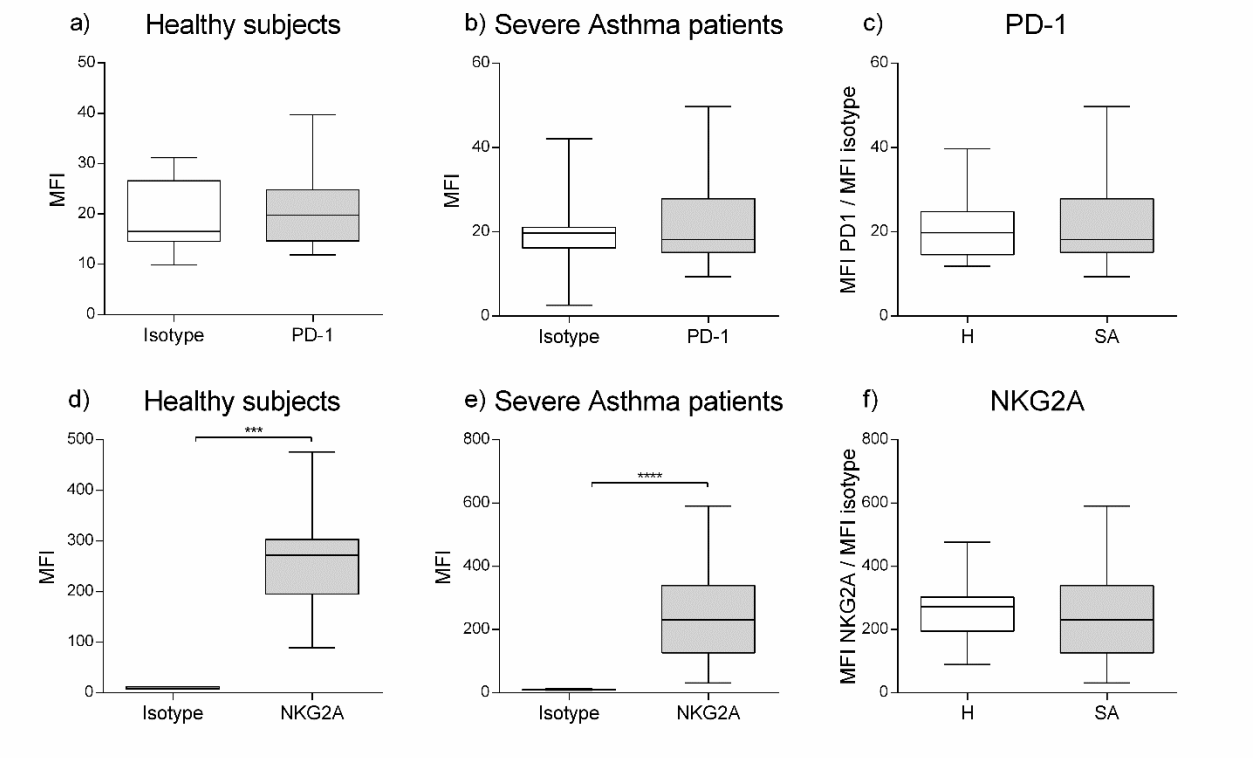

Figure S5: Expression of PD-1 and NKG2A on NK cells.

PBMC were obtained from healthy donors (n=7) and severe asthma patients (n=15), and stained with PD-1 or NKG2A specific monoclonal antibody or control isotype antibody for flow cytometry analysis. Mean Fluorescence Intensity (MFI) values are shown for PD-1 and isotype antibodies for healthy donors (a) and severe asthma patients (b). Ratio MFI PD-1 / MFI Isotype was compared between healthy subjects (H, white) and severe asthma patients (SA, grey) (c). Mean Fluorescence Intensity (MFI) values are shown for NKG2A and isotype antibodies for healthy donors (d) and severe asthma patients (e). Ratio MFI NKG2A / MFI Isotype was compared between healthy subjects (H, white) and severe asthma patients (SA, grey) (f). Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. *p<0.05, ****p<0.0001 (Wilcoxon test for paired isotype versus PD-1 or NKG2A, or Mann-Whitney test for difference between healthy and severe asthma subjects).

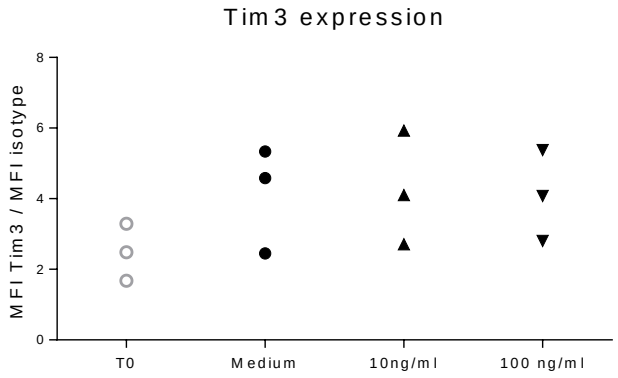

Figure S6: In vitro stimulation of PBMC with increasing doses of dexamethasone does not modify the expression of Tim3 on NK cells.

PBMC from three healthy donors were stimulated with Dexamethasone at 10ng/ml or 100ng/ml for 24 hours and stained with Tim3 specific monoclonal antibody or control isotype antibody for flow cytometry analysis. Values are expressed as Ratio MFI (Mean Fluorescence Intensity) Tim3 / MFI Isotype.

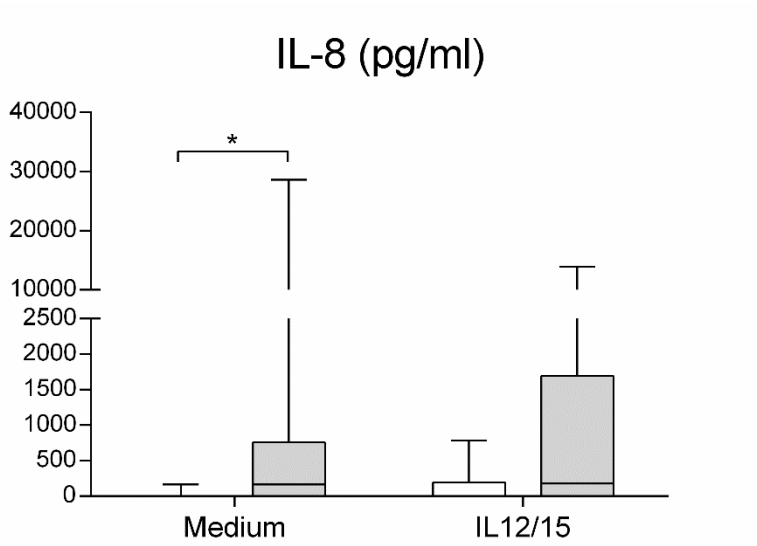

Figure S7: IL-8 production by purified NK cells from severe asthma patients.

NK cells from severe asthma patients (n=11, grey) and healthy donors (n=7, white) were purified and stimulated with IL-12+IL-15. Purity of NK cells assessed by flow cytometry was over 95%. Values are expressed in pg/ml. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between healthy donors and severe asthma patients were made with a two-way ANOVA followed by Bonferroni's post-test (*$p<0.05$).

Supplementary results

Cytokine production by PBMC in response to stimulation with molecules mimicking microbes

We then measured the release of cytokines and chemokines known to be produced by or act on NK cells, or involved in asthma. PBMC supernatants were recovered 24 hours after activation with the three TLR agonists seen to increase NK cell activation and cytotoxicity, and the IL-12+IL-15 stimulation. IL-1β, IL-1RA, IL-6, IL-7, IL-8, IL-10, IL-12, CCL2, CCL3, CCL4, CCL5 and TNF-α were significantly increased only after R848 compared to medium condition similarly in healthy donors and severe asthma patients, except for IL-10 and CCL5 levels which were significantly higher in severe asthma patients compared to healthy donors (data not shown). IL-7 and IL-8 levels were significantly increased after IL-12+IL-15 stimulation, but only IL-8 was significantly increased in severe asthma patients compared to healthy donors. Purified NK cells from severe asthma patients produced IL-8 without any further stimulation than being in culture, suggesting that NK cells may be partly responsible for increased IL-8 production in severe asthma patients. However, IL-12+IL-15 did not enhance this production (Figure S7). IFN-α was significantly increased only after CpG compared to medium condition, similarly in healthy donors and severe asthma patients (data not shown). Th2 (IL-4, IL-5 and IL-13) and Th17 (IL-17A and IL-17F) cytokines were not detected in PBMC supernatants. Therefore, after 24 hours of stimulation, cytokines and chemokines were mainly induced by R848 and only rare differences between severe asthma patients and healthy donors were seen.