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Justine Devulder, Cécile Chenivesse, Valérie Ledroit, Stéphanie Fry, Pierre-Emmanuel Lobert, Didier Hober, Anne Tsicopoulos, Catherine Duez

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Title: Aberrant anti-viral response of Natural Killer cell in severe asthma

Justine Devulder[1], Cécile Chenivesse[1,2], Valérie Ledroit[1], Stéphanie Fry[1,2], Pierre-Emmanuel Lobert[3], Didier Hober[3], Anne Tsicopoulos[1,2], Catherine Duez[1]

[1]Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France ; [2]CHU Lille, Service de Pneumologie et Immuno-Allergologie, Centre de compétence pour les Maladies Pulmonaires Rares, 59000 Lille, France ; [3]Univ. Lille, CHU Lille, EA 3610 - Pathogenèse virale du diabète de type 1, F-59000 Lille, France

Corresponding author:
Catherine Duez
Center for Infection and Immunity of Lille, Pulmonary Immunity Team
Institut Pasteur de Lille, 1 rue du Pr Calmette, BP 245
59019, Lille Cedex, France
Phone: (33) 3 20 87 71 83
Fax: (33) 3 20 87 73 45
e-mail: catherine.duez@inserm.fr

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Summary

NK cells from severe asthma patients exhibit decreased activation, cytotoxic capacity and IFN-γ production after in vitro stimulation with rhinovirus RV-A9, in comparison to healthy donors. Exhausted phenotype is associated to increased Tim-3 expression.

Abstract

Rhinovirus infections are the main cause of asthma exacerbations. As Natural Killer (NK) cells are important actors of the antiviral innate response, we aimed at evaluating the functions of NK cells from severe asthma patients in response to rhinovirus-like molecules or rhinoviruses. Peripheral blood mononuclear cells from patients with severe asthma and healthy donors were stimulated with pathogen-like molecules or with the rhinoviruses (RV)-A9 and RV-2. NK cell activation, degranulation and IFN-γ expression were analysed. NK cells from severe asthma patients were less cytotoxic than those from healthy donors in response to TLR3, TLR7/8 or RV-A9 but not in response to RV-2 stimulation. Furthermore, when cultured with IL-12+IL-15, cytokines which are produced during viral infections, NK cells from patients with severe asthma were less cytotoxic and expressed less IFN-γ than NK cells from healthy donors. NK cells from severe asthmatics exhibited an exhausted phenotype, with an increased expression of the checkpoint molecule Tim-3. Together, our findings indicate that the activation of NK cells from patients with severe asthma may be insufficient during some but not all respiratory infections. The exhausted phenotype may participate in NK cell impairment and aggravation of viral-induced asthma exacerbation in these patients.

Introduction

Asthma is a chronic inflammatory disease characterized by airway inflammation and hyperresponsiveness (1). Worldwide, 300 million people have asthma, with 5 to 10 percent suffering from severe asthma. According to the European Respiratory Society and American Thoracic Society, severe asthma requires treatment with high doses of inhaled corticosteroids and a second controller for more than 50% of the previous year to prevent it from becoming uncontrolled or which remains uncontrolled despite this therapy (2). Severe asthma is associated with poor quality of life, frequent hospitalizations and mortality due to severe asthma exacerbations (3–5). Asthma exacerbation is defined as the deterioration in the patient's symptoms requiring the use of systemic corticosteroids and/or hospitalization to prevent a serious outcome (6). Approximately 50 to 80% of asthma exacerbations are associated with respiratory viral infections (in adults and children respectively). Among viruses implicated, 50-60% are rhinoviruses (RV) (7,8). Mechanisms underlying virus-induced asthma exacerbations remain poorly understood, even though defective innate immunity in response to respiratory viruses has been highlighted (9). For example, production of IL-15 by alveolar macrophages after *in vitro* RV infection is impaired in asthma patients, and inversely correlates with the severity of lower respiratory symptoms following RV infection *in vivo* (10). RV activates the innate immune system through several pattern recognition receptors (PRR). The capsid is recognized by Toll-Like Receptor (TLR)2, single stranded RNA and double stranded RNA from rhinoviruses are recognized by TLR3, TLR7/8, retinoic acid-inducible gene (RIG)-1 and melanoma differentiation-associated protein (MDA)-5. Engagement of these receptors induces cytokine expression including type I and type III interferons, but also IL-6, IL-12, and IL-15 (11).

Natural Killer (NK) cells are important actors of the antiviral innate immune response (12). Their activation depends on the integration of multiple signals from innate receptors. Some receptors recognize major histocompatibility complex class (MHC)-I and inhibit NK cell activation, whereas others recognize cytokines (like IL-12, IL-15 and IL-18), chemokines, and microbial components leading to the activation of NK cells (13,14). NK cells also express several PRR including TLR2, TLR3, TLR4, TLR7/8, TLR9, NOD2 (nucleotide-binding oligomerization domain 2), NLRP3 and RIG-I. TLR are known to activate NK cell functions either directly or in cooperation with accessory cells in a cytokine or cell-to-cell contact-dependent manner (15). Human NK cells are classically identified by their surface expression of CD56 and lack of CD3, and can be categorized into two subsets, $CD56^{bright}$ and $CD56^{dim}$ NK cells (16).

The involvement of NK cells in the asthmatic disease remains unclear. Modifications in human NK cell phenotype and functional capacities have been observed in asthma, like increased

CD69 and NKG2D expression by peripheral blood NK cells from patients with severe asthma (17,18). In asthmatic children, decreased expression of ICAM-1 and CD62L has been observed on peripheral blood NK cells during acute exacerbation (19). NK cells may regulate eosinophilic inflammation by inducing eosinophil activation and apoptosis (20), but eosinophil apoptosis is reduced in patients with severe asthma (17). Mouse models of allergic asthma highlight a complex role of NK cells, which may participate (21,22) or not (23) in allergic inflammation, or may resolve it (24–26). In a mouse model of asthma exacerbation with a TLR3 agonist, NK cells expressed IL-17A and increased lung inflammation (27).

According to the hypothesis which stipulates that defective innate immunity may lead to virus-induced exacerbation of severe asthma, we aimed to analyse NK cell functions and investigated their activation, cytotoxicity and cytokine production following microbial compound stimulation *in vitro*. We compared the functions of peripheral blood NK cells from patients with severe asthma with healthy donors. We show that NK cells from severe asthma patients are hyporesponsive to *in vitro* stimulation with TLR agonists and human rhinovirus RV-A9. Our exploratory study may help define a novel endotype of severe asthma, which should be confirmed by analysing a larger cohort of patients.

Materials & Methods

Patients

Patient's clinical data are described in table 1. All patients were non-smoker or ex-smoker with a cumulative smoking exposure below 10 pack-years. More detailed information can be found as supplementary material.

PBMC and NK cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood and NK cells were purified by immunomagnetic separation (STEMCELL Technologies, Vancouver, Canada). Further methodological details are described in the supplementary material.

Rhinoviruses production

Rhinovirus A9 (RV-A9, ATCC VR-489) and rhinovirus 2 (RV-2, ATCC VR-482) were propagated in H1-HeLa cells. The infectious titre of virus stock was 1.44×10^7 TCID50/ml, as detailed in the supplementary material.

Cell stimulation

PBMC or purified NK cells were stimulated with Interleukin(IL)-12+IL-15, or with an agonist for TLR3: Polyinosinic-polycytidylic acid (PIC), for TLR9: ODN 2395, for TLR7/8: Resiquimod (R848), for TLR2/6: FSL1, for NKp46: Hemagglutinin A of Influenza A (HA), with RV-A9 or RV-2 during 24 hours. Concentrations and manufacturers are provided in supplementary material. 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

After stimulation, PBMC were stained with the viability marker and antibodies for extracellular and intracellular targets. Further methodological details are described in the supplementary material.

Cytokine and Chemokine measurement

CXCL10, CXCL9, granzyme B, and IFN-γ were measured in culture supernatants by Luminex assay (Life Technologies) or ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. More cytokines were measured as shown in the supplementary materials.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All results are expressed as median (min-max). Comparisons between stimuli (figures 1 and 2) were performed using two-way analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons. Comparisons between stimuli (figures 3-5) and between patient's groups were assessed by two-way ANOVA followed by Bonferroni's post-tests. Wilcoxon or Mann-Whitney tests were used for comparison between staining Tim-3 and control antibody, and between patient's groups, respectively (figure 6). The significance level was set at $p<0.05$. Analysis of statistical power was performed using G*Power software (version 3.1.9.4), and α error probability set to 0.05.

Results

Severe asthma is associated with decreased Natural Killer cell activation and cytotoxicity in response to PBMC stimulation with pathogen-like molecules

The effect of PBMC stimulation on NK cell activation was assessed by the expression of CD69 (Figure 1a). Following the stimulation with the cytokine cocktail IL-12+IL-15, or with PIC (TLR3 agonist), R848 (TLR7/8 agonist) and CpG (TLR9 agonist), the proportion of $CD69^+$ NK cells from healthy donors significantly increased compared to the medium condition, suggesting that NK cells were activated. Similarly, NK cells from patients with severe asthma were activated by IL-12+IL-15, R848 and CpG stimulation. However, in response to PIC, the percentage of $CD69^+$ NK cells was significantly lower in patients with severe asthma than in healthy donors.

The cytotoxicity of NK cells was evaluated with the expression of CD107a and the release of granzyme B in PBMC supernatants after culture with K562 target cells (Figure 1b, 1c). After PBMC stimulation with IL-12+IL-15, PIC, R848 and CpG, NK cells from healthy donors showed signs of degranulation as the proportion of $CD107a^+$ NK cells significantly increased compared to the medium condition. The concentration of granzyme B in supernatants was significantly increased in response to IL-12+IL-15, and not significantly in response to PIC and R848. Similarly, NK cells from patients with severe asthma exhibited signs of degranulation in response to IL-12+IL-15, R848 and CpG. In contrast, the percentage of $CD107a^+$ NK cells significantly decreased in response to R848 and non-significantly to PIC and CpG in patients with severe asthma compared to healthy donors. The concentration of granzyme B seemed to be diminished in the supernatants of PBMC from patients with severe asthma after PIC but no other stimulation. No differences were observed between different phenotypes of severe asthma, such as allergic versus non-allergic asthma, eosinophilic versus non-eosinophilic asthma, exacerbation prone or not. No differences were seen either when comparing severe asthma patients treated or not with oral corticotherapy (Figure S2) or with body mass index (BMI) inferior or superior to 30 kg/m^2 (Figure S3). However, due to a lack of statistical power (0.075 and 0.099 for CD69, 0.052 and 0.091 for CD107, and 0.051 and 0.171 for IFN-γ, for separation based on corticotherapy or BMI respectively), we cannot conclude to a true absence of difference. Altogether, these results indicate that NK cells from severe asthma patients have impaired activation and cytotoxicity in response to PIC and R848.

Severe asthma is associated with decreased IFN-γ production by NK cells in response to stimulation with IL-12+IL-15

IFN-γ is the main cytokine expressed and rapidly produced by NK cells following their activation. PBMC stimulation with IL-12+IL-15 significantly increased the release of IFN-γ (Figure 2a), as well as interferon-inducible chemokines, CXCL9 and CXCL10 (Figure 2c and 2d). However, although IFN-γ release by PBMC from severe asthma patients was significantly lower compared

to healthy donors, levels of CXCL9 and CXCL10 were increased or unchanged, respectively. While no other stimulation induced CXCL9, CXCL10 was induced by CpG stimulation, similarly in both patients, suggesting differential regulation of these two interferon-inducible chemokines. NK cells were one of the cellular sources of IFN-γ, as IL-12+IL-15 stimulation significantly increased the percentage of IFN-γ^+ NK cells both in healthy and severe asthma patients (Figure 2b), although the percentage of IFN-γ^+ NK cells was significantly lower in severe asthma patients than in controls. However, while R848 significantly increased IFN-γ secretion by PBMC only in patients with severe asthma, IFN-γ^+ NK cells were increased in both type of donors, suggesting that other cells produced IFN-γ after PBMC stimulation with R848. The increased expression and production of IFN-γ after R848 stimulation, whereas CXCL9 and CXCL10 were not increased, strengthen the idea that the latter were independent of IFN-γ. Overall, these results show that NK cells from patients with severe asthma may also be deficient in their ability to produce the anti-viral cytokine IFN-γ.

Severe asthma is associated with deficient Natural Killer cells response to *in vitro* RV-A9 stimulation

To closely mimic the pathophysiological conditions, PBMC were stimulated with live rhinovirus A9 (RV-A9), which belongs to the major group of rhinovirus. RV-A9 significantly increased the proportion of CD69$^+$ NK cells both in healthy donors and patients with severe asthma (Figure 3a), however the percentage of CD69$^+$ NK cells in severe asthma patients was slightly lower compared to healthy donors. Purified NK cells were not activated by RV-A9, suggesting that other cells activated by RV-A9 are necessary intermediates for NK cell stimulation (Figure S4). The proportion of CD107$^+$ NK cells significantly increased following the stimulation of PBMC with RV-A9 in both groups, indicating that NK cells were sufficiently activated to degranulate after target cell encounter. In parallel, granzyme B release increased in PBMC supernatants (Figure 3c). In severe asthma patients, the proportion of CD107$^+$ NK cells and the concentration of granzyme B were significantly lower compared to healthy donors (Figure 3b, 3c). Similarly, RV-A9 increased IFN-γ expression by NK cells and IFN-γ production in both groups, but significantly less in severe asthma patients (Figure 3d, 3e). Altogether, these results suggest that NK cells from severe asthma patients are deficient in their activation, cytotoxicity, and IFN-γ production in response to RV-A9. However, when PBMC from severe asthma patients were activated with another rhinovirus (RV-2), which belong to minor-group of rhinovirus, NK cells exhibited activation, cytotoxicity and IFN-γ production similarly to NK cells from healthy donors (Figure 4). This latter result suggests that RV-A9 and RV-2 induced differential response in NK cells from

severe asthma patients and that hyporesponsiveness to viruses is a specific but not universal feature of NK cells in severe asthma.

Natural Killer cells from severe asthma patients exhibit an exhausted phenotype

Because RV-A9 stimulation of NK cells is indirect, we wondered if NK cells from severe asthma patients were deficient or responded to a deficient activation. IL-12+IL-15 can directly stimulate purified NK cells, and indeed increased the proportion of IFN-γ$^+$ NK cells (Figure 5a) and IFN-γ secretion by purified NK cells from both donor types (Figure 5b). However, both IFN-γ$^+$ NK cells and IFN-γ concentration were significantly decreased in severe asthma patients compared to healthy subjects, suggesting that NK cells from severe asthma patients present an intrinsic defect leading to a decreased expression and production of IFN-γ. Therefore, although several other cells may behave abnormally in severe asthma patients in response to RV-A9, NK cells per se exhibit aberrant functions.

An increasing number of studies suggest that NK cells can become functionally exhausted during chronic infections. The checkpoint molecules or exhaustion markers NKG2A and programmed cell death protein 1 (PD-1) were detected on NK cells but their expression was not modified between healthy donors and severe asthma patients (Figure S5). In contrast, Tim3 expression by NK cells was significantly increased in severe asthma patients compared to healthy donors (Figure 6a-e). This was restricted to NK cells as Tim3 expression by CD3$^+$T cells was identical in healthy donors and severe asthma patients (Figure 6f-h). Tim3 expression on NK cells did not increase after *in vitro* treatment of NK cells with dexamethasone (Figure S6), suggesting that the observed increased expression of Tim3 on NK cells from severe asthma patients may be independent of inhaled corticosteroid treatment. Therefore, increased Tim3 expression suggests that NK cells from severe asthma patients may be exhausted, which may partially account for their impaired functions in response to TLR agonists and RV-A9 stimulation.

Discussion

In this study, we explored NK cell functions in severe asthma patients and healthy donors in response to stimulation either with molecules mimicking viruses or with a live virus. We chose to stimulate mononuclear cells in order to allow accessory cells to interact with NK cells.

In order to mimic RV stimulation of human PBMC, we first used synthetic agonists for TLR and cytokines produced during viral infection. Stimulation with IL-12+IL-15 or through TLR involved in nucleic acid recognition led to significant NK cell activation and/or degranulation in both

donors. CD69 expression on NK cells was significantly decreased in severe asthma patients only after TLR3 stimulation, whereas CD107 expression was significantly lower after TLR7/8 stimulation, and non-significantly lower after TLR3 and TLR9 stimulation. CD69 was originally described as an early activation marker but may potentially regulate other functions like tissue retention and metabolism of expressing cells (28). Moreover, non-conditioned tumour associated-NK cells were shown to express high percentage of the CD69 activation marker but weak cytolytic activities (29), showing that CD69 expression may be dissociated from NK cell degranulation. Finally, poly(I:C) administered in mice was found to activate mouse NK cells and to upregulate CD69 expression indirectly through accessory cells. NK cells from mice deficient for MDA5 but not TLR3, two receptors for poly(I:C), exhibited decreased CD69 expression, suggesting that MDA-5 had a predominant role in NK cell CD69 upregulation induced by poly(I:C) (30). We can therefore hypothesize that MDA-5 signaling might be deficient in accessory cells from severe asthma patients leading to decreased NK cell CD69 expression, in contrast to TLR3, 7/8 and 9 signaling. Impaired NK cell cytotoxic function after PBMC stimulation with IL-12+IL-15 or TLR involved in RV immune response (TLR3 and TLR7/8) in severe asthma patients, are consistent with studies previously published showing that peripheral blood NK cells from severe asthma patients have impaired killing of K562 myeloid target cells after IL-2 stimulation (18). We measured granzyme B secretion in PBMC supernatant and found that this molecule, which is released from cytotoxic granules and particularly involved in target cell lysis was less secreted, similarly to decreased CD107 expression (witness of degranulation) in severe asthma patients, but only after TLR3 stimulation. However, discrepancy between CD107 expression and granzyme B secretion was observed for R848 and CpG stimulation, suggesting that other cells among PBMC may release granzyme B as previously published (31), and that this granzyme B secretion is not affected in PBMC from severe asthma patients. Defective cytotoxicity of NK cells from severe asthma patients after stimulation through TLR3 and TLR7/8, both involved in RV immune response, are in agreement with the decreased cytotoxicity (CD107a and granzyme B) observed after activation with RV-A9. The defective cytotoxicity of NK cells is also consistent with the increased viral load observed in asthma patients during infection (32), although the severity of lower respiratory tract symptoms is not related to viral load (33)

Another important function of NK cell consists of cytokine production, and more particularly IFN-γ production. Lower IFN-γ expression in NK cells and secretion in PBMC were measured in severe asthma patients compared to healthy donors. Similarly, NK cells from patients suffering from atopic dermatitis were shown to express less IFN-γ following PBMC stimulation with PMA

and ionomycin (34). Although IFN-γ production by NK cells is dependent on accessory cells for TLR or RV stimulation, our results on purified NK cells stimulated with IL12+IL-15 also indicate that the defect in its production is, at least partly, intrinsic i.e. independent of other cells.

As previously shown *in vivo* (35) or *in vitro* using another cell type (36), CXCL9 was found increased in the supernatants of PBMC from severe asthma patients after IL-12+IL-15 stimulation. Monocytes may be the source of CXCL9, as previously shown after IL-12+IL-18 stimulation, independently of IFN-γ (37). We could not detect any soluble cytokines known to be involved in NK cell stimulation, like type I IFN, IL-12, IL-15 or IL-18, in PBMC supernatants after TLR or RV-A9 stimulation (data not shown). These cytokines may be already used by cells, not involved, or may stimulate NK cells in their membrane form as shown for IL-15 (38) or for IL-18 which can be released in close vicinity of NK cells (39).

We could not show obvious decreased expression of stimulating receptors on NK cells from severe asthma patients (data not shown). Therefore, we focused on NK cell exhaustion signs. Immune cell exhaustion describes the status of dysfunction of immune cells, usually under the settings of tumours or chronic infections, characterized by decreased effector functions and associated with increased checkpoint molecules (40). For example, in HIV-infected patients, polyfunctional $CD62L^{+}$ NK cells express high levels of Program Cell Death (PD)-1, NKG2A, and T-cell immunoglobulin and mucin-domain containing (Tim)-3. The expression of these markers on NK cell surface is associated with a decreased cytotoxicity and IFN-γ expression in response to TLR3 stimulation (40). Here we showed that the expression of Tim-3 by NK cells was higher in severe asthma patients compared to healthy donors. The expression levels of PD-1 and NKG2A on NK cells were similar between severe asthma patients and healthy donors. Overexpression of these three checkpoint molecules has been associated with NK cell exhaustion and dysfunction in chronic hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infection. However, although impaired TLR3 response of NK cells was shown to be associated with increased expression of NKG2A, PD-1 and Tim-3 in chronic exposure to HIV (40), in HBV chronic infection only Tim-3 over-expression was associated with reduced function of NK cells (41). Together with our results, these data suggest that NK cell dysfunction may be associated with differential sustained over-expression of checkpoint molecules depending on the pathology. In severe asthma, we can hypothesize that chronic inflammation may induce NK cell dysfunction in particular through Tim-3 signaling, leading to inefficient activation of NK cells in response to RV-A9. The effect of Tim-3 blockade on restoring NK cell activity needs to be further explored as previously shown in chronic virus infection (42) or cancer (43) setting.

The involvement of corticosteroids in NK cell defect in severe asthma needs to be questioned. Indeed, treatments for severe asthma are mainly constituted with high doses of inhaled and/or systemic corticosteroids as anti-inflammatory agents. It was shown that intramuscular injection of triamcinolone modifies BAL NK cell phenotype, but not BAL IFN-γ level. Furthermore, *in vitro* treatment of PBMC with dexamethasone significantly inhibits the capacity of NK cells to lyse K562 target cells, both in healthy donors and severe asthma patients (18). In our study, all severe asthma patients were treated with inhaled corticosteroids, and 45.2% of them received systemic corticosteroids. The cytotoxicity and IFN-γ expression were not different between patients with and without systemic corticosteroids. However, statistical power was insufficient to conclude to a true absence of difference. We analysed Tim-3 expression on NK cells after *in vitro* treatment of PBMC isolated from 3 different healthy donors with increasing doses of dexamethasone. Dexamethasone did not increase Tim-3 expression for the 10 and 100 ng/ml doses. Moreover, PBMC *in vitro* stimulation with a different RV, this one belonging to the minor-group (i.e. RV-2), induced NK cell activation levels (increased CD69, CD107 and IFN-γ expression) similar between severe asthma patients and healthy subjects. This result may seem puzzling. However, exposure of primary monocytic cells to two genetically similar serotypes of RV, one major- and one minor- group, was shown to elicit differential activation of signaling molecules and transcription factors leading to different responses (44). Our observation suggests that RV serotypes may lead to differential NK cell activation depending on the patient status, but further studies comparing several strains of each group are needed to conclude. Moreover, our results suggest that there is not a general viral deficient response to rhinoviruses but rather that NK cells from severe asthma patients responds with a virus-specific signaling response. Moreover, this result further emphasizes that a defect in NK cells may be related to the severe asthmatic status rather than to the treatment, although corticosteroids may certainly modify NK cell-induced immune response in severe asthma patients, and therefore have adverse effects in RV-induced exacerbations.

In conclusion, we found that NK cells from severe asthma patients have impaired *in vitro* functions in response to one serotype of rhinovirus, and some virus-mimicking molecules (TLR3, TLR7/8 agonists). This defect may be linked to NK cell exhaustion and may contribute to the severity of virus-induced asthma exacerbations in severe asthma patients. NK cell exhaustion may also be responsible for decreased cytotoxicity of NK cells from severe asthma patients towards eosinophils (18). Therefore, our study emphasizes that aberrant NK cell functions may participate to the severity and virus-induced exacerbation of asthma.

Acknowledgments

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Figure legends

Figure 1: NK cells from severe asthma patients exhibit a defective activation following PBMC stimulation with molecules mimicking microbial compounds.

PBMC from severe asthma patients and healthy donors were stimulated with IL-12+IL-15, FSL1 (TLR2/6 agonist), polyinosinic-polycytidylic acid (PIC, TLR3 agonist), resiquimod (R848, TLR7/8 agonist), CpG oligonucleotides (CpG, TLR9 agonist), or hemagglutinin A of influenza A (HA, recognized by NKp46) for 24 hours. (a) Percentage of CD69 and (b) CD107a positive NK cells. (c) Concentration of Granzyme B in PBMC supernatants. Horizontal lines represent the median, boxes the interquartile range and whiskers the range. Statistical comparisons between medium condition and stimulated conditions were performed with two-way ANOVA followed by Dunnett's post-test ($^{£}p<0.0001$, $^{\lambda}p<0.01$, $^{\omega}p<0.05$). Healthy donors (n=8, white) and severe asthma patients (n=14, grey) were compared with two-way ANOVA followed by Bonferroni's post-test (**p<0.01, ****p<0.0001).

Figure 2: Decreased production of IFN-γ following PBMC stimulation with IL-12+IL-15 but increased production of CXCL9 in severe asthma patients.

PBMC from severe asthma patients and healthy donors were stimulated with IL-12+IL-15, FSL1 (TLR2/6 agonist), polyinosinic-polycytidylic acid (PIC, TLR3 agonist), resiquimod (R848, TLR7/8 agonist), CpG oligonucleotides (CpG, TLR9 agonist), or hemagglutinin A of influenza A (HA, recognized by NKp46) for 24 hours. (a) Concentrations of IFN-γ (pg/ml) in supernatant. (b) Intracellular expression of IFN-γ in NK cells expressed as percentage of positive NK cells. (c) Concentration of CXCL9 and (d) CXCL10 (pg/ml). Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between medium condition and stimulated conditions were performed with two-way ANOVA followed by Dunnett's post-test ($^{£}p<0.0001$, $^{\$}p<0.001$, $^{\omega}p<0.05$). Healthy donors (n=8, white) and

severe asthma patients (n=14, grey) were compared with two-way ANOVA followed by Bonferroni's post-test (**p<0.01, ****p<0.0001).

Figure 3: NK cells from severe asthma patients exhibit decreased cytotoxic status and IFN-γ expression following PBMC stimulation with rhinovirus RV-A9.

PBMC from healthy donors (n=6, white) and severe asthma patients (n=9, grey) were stimulated with RV-A9. (a) percentage of CD69 and (b) CD107a positive NK cells. (c) Granzyme B secretion in PBMC supernatants. (d) Percentage of IFN-γ+ NK cells and (e) concentration of IFN-γ in PBMC supernatant. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between medium and RV-A9 condition were performed with two-way ANOVA followed by Bonferroni's post-test ($^{£}$p<0.0001, $^{\$}$p<0.001, $^{\lambda}$p<0.01). Healthy donors and severe asthma patients were compared with two-way ANOVA followed by Bonferroni's post-test (****p<0.0001, **p<0.01, *p<0.05).

Figure 4: NK cells from severe asthma patients does not exhibit decreased cytotoxic status and IFN-γ expression following PBMC stimulation with RV-2.

PBMC from healthy donors (n=7, white) and severe asthma patients (n=5, grey) were stimulated with RV-2. (a) percentage of CD69, (b) CD107a and (c) IFN-γ positive NK cells. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between medium and RV-2 condition were performed with two-way ANOVA followed by Bonferroni's post-test ($^{£}$p<0.0001, $^{\lambda}$p<0.01, $^{\omega}$p<0.05). Healthy donors and severe asthma patients were compared with two-way ANOVA followed by Bonferroni's post-test (NS, non-significant).

Figure 5: Defect of IFN-γ expression and secretion by NK cells from severe asthma patients after direct stimulation with IL-12+IL-15.

NK cells from healthy donors (n=8, white) and severe asthma patients (n=9, grey) were purified and stimulated with IL-12+IL-15. (a) Percentage of IFN-γ+ NK cells and (b) concentration of IFN-γ in NK cell supernatant. Horizontal lines represent the median, boxes represent the interquartile range and whiskers represent the range. Statistical comparisons between medium and IL12/15 condition were performed with two-way ANOVA followed by Dunnett's post-test ($^{£}$p<0.0001). Healthy donors and severe asthma patients were compared with two-way ANOVA followed by Bonferroni's post-test (****p<0.0001, **p<0.01).

Figure 6: Increased Tim3 expression restricted to NK cells from severe asthma patients.

PBMC were obtained from healthy donors (n=7) and severe asthma patients (n=15), and stained with Tim3 specific monoclonal antibody or control isotype antibody. One representative histogram is shown for NK cell staining in (a) for healthy donors and (b) for severe asthma patients, with Tim3 staining in grey and control isotype staining in white. Mean Fluorescence Intensity (MFI) values are shown for Tim3 and isotype antibodies for NK cells from healthy donors (c) and severe asthma patients (d). Ratio MFI Tim3 / MFI Isotype was compared for NK cells between healthy subjects (H, white) and severe asthma patients (SA, grey) (e). In parallel, MFI values are shown for Tim3 and isotype antibodies for T cells from healthy donors (f) and severe asthma patients (g). Ratio MFI Tim3 / MFI Isotype was compared for T cells between healthy subjects (H, white) and severe asthma patients (SA, grey) (h). 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 Tim3, or Mann-Whitney test for difference between healthy and severe asthma subjects).

Table 1: Patient characteristics. Data are presented as median (range) unless otherwise stated. ACT: asthma control test, FEV1: forced expiratory volume in 1 second, GORD: gastro-oesophageal reflux disease, OCS: oral corticosteroids, NA: nonapplicable, # based on positive skin test.

	Healthy donors	Severe asthma patients
Subjects n	11	31
Age years	38 (24 – 61)	57 (19 – 78)
Females (%)	64%	58%
BMI >30 kg/m^2 (%)	0%	47.2%
ACT >20 (%)	NA	17.7%
Exacerbations in the last year	NA	4 (0 – 12)
FEV1% predicted	100 (95 – 105)	68 (34 – 112)
Blood eosinophils per mm^3	0 (0 – 168)	350 (0 – 1320)
Atopy# (%)	0%	35.4%
Sinusitis (%)	0%	9.5%
GORD (%)	0%	26.8%
Long-term OCS (%)	0%	45.2%

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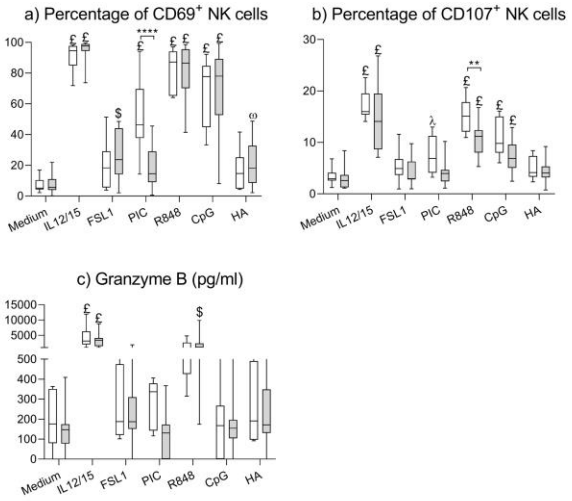
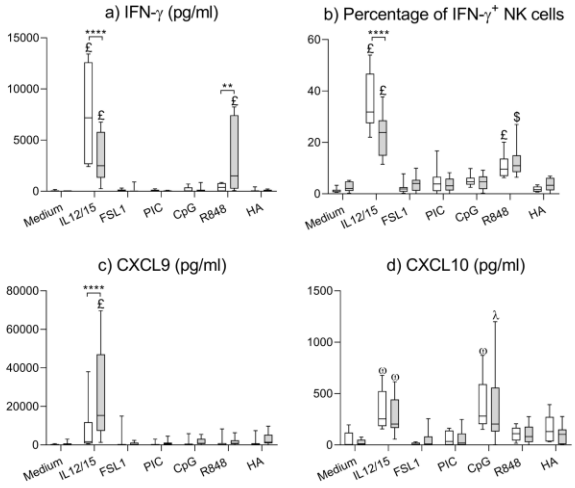
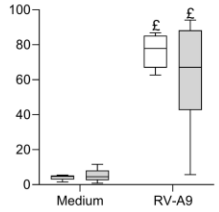
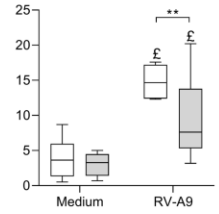

a) Percentage of CD69+ NK cells
b) Percentage of CD107+ NK cells
c) Granzyme B (pg/ml)
Medium
IL12/15
FSL1
PIC
R848
CpG
HA


a) IFN-γ (pg/ml)
b) Percentage of IFN-γ+ NK cells
c) CXCL9 (pg/ml)
d) CXCL10 (pg/ml)
Medium
IL12/15
FSL1
PIC
CpG
R848
HA

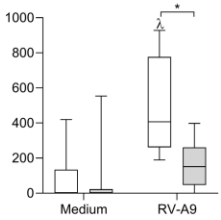
a) Percentage of CD69^{+} NK cells



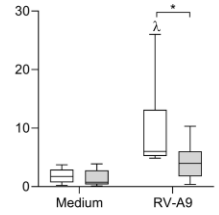
b) Percentage of CD107^{+} NK cells



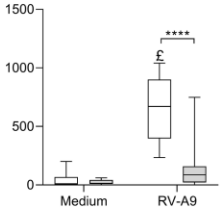
c) Granzyme B (pg/ml)



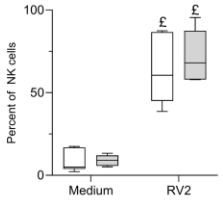
d) Percentage of IFN-γ^{+} NK cells



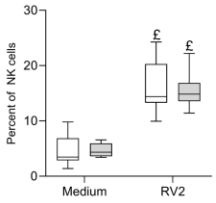
e) IFN-γ (pg/ml)



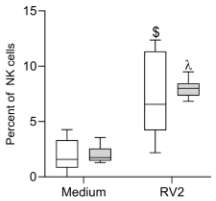
a) Percentage of CD69^{+} NK cells

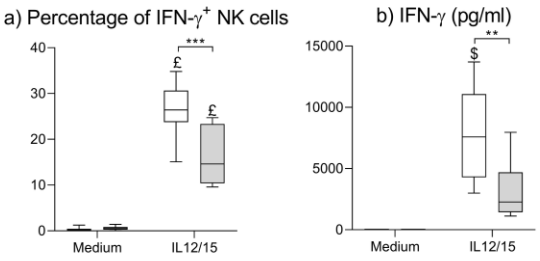


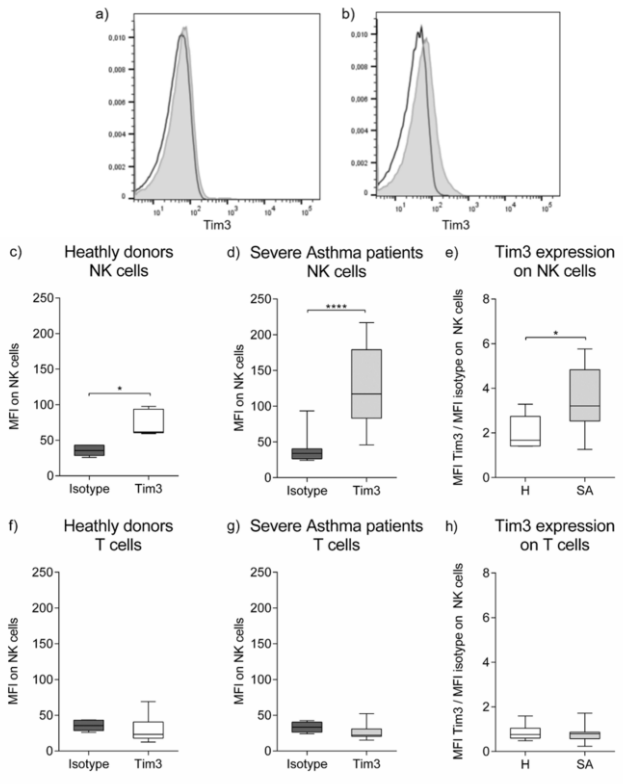
b) Percentage of CD107^{+} NK cells



c) Percentage of IFN-γ^{+} NK cells




a) Percentage of IFN-γ+ NK cells
40
30
20
10
0
***
£
£
Medium
IL12/15
b) IFN-γ (pg/ml)
15000
10000
5000
0
**
$
Medium
IL12/15


a)
b)
Tim3
Tim3
c) Heathly donors NK cells
d) Severe Asthma patients NK cells
e) Tim3 expression on NK cells
MFI on NK cells
MFI on NK cells
MFI Tim3 / MFI isotype on NK cells
Isotype
Tim3
H
SA
f) Heathly donors T cells
g) Severe Asthma patients T cells
h) Tim3 expression on T cells

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

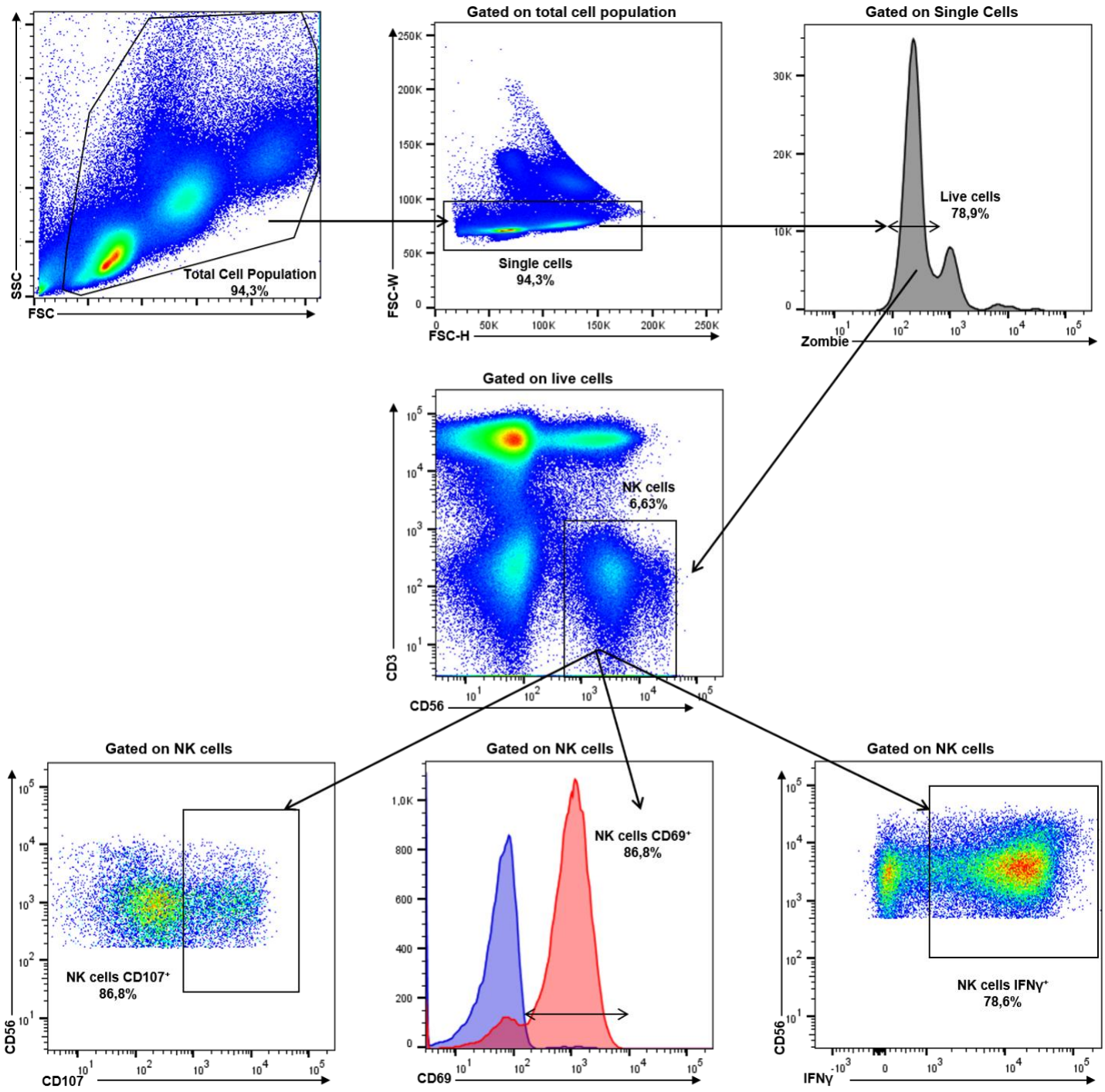


Figure S1: Flow cytometry gating strategies

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.

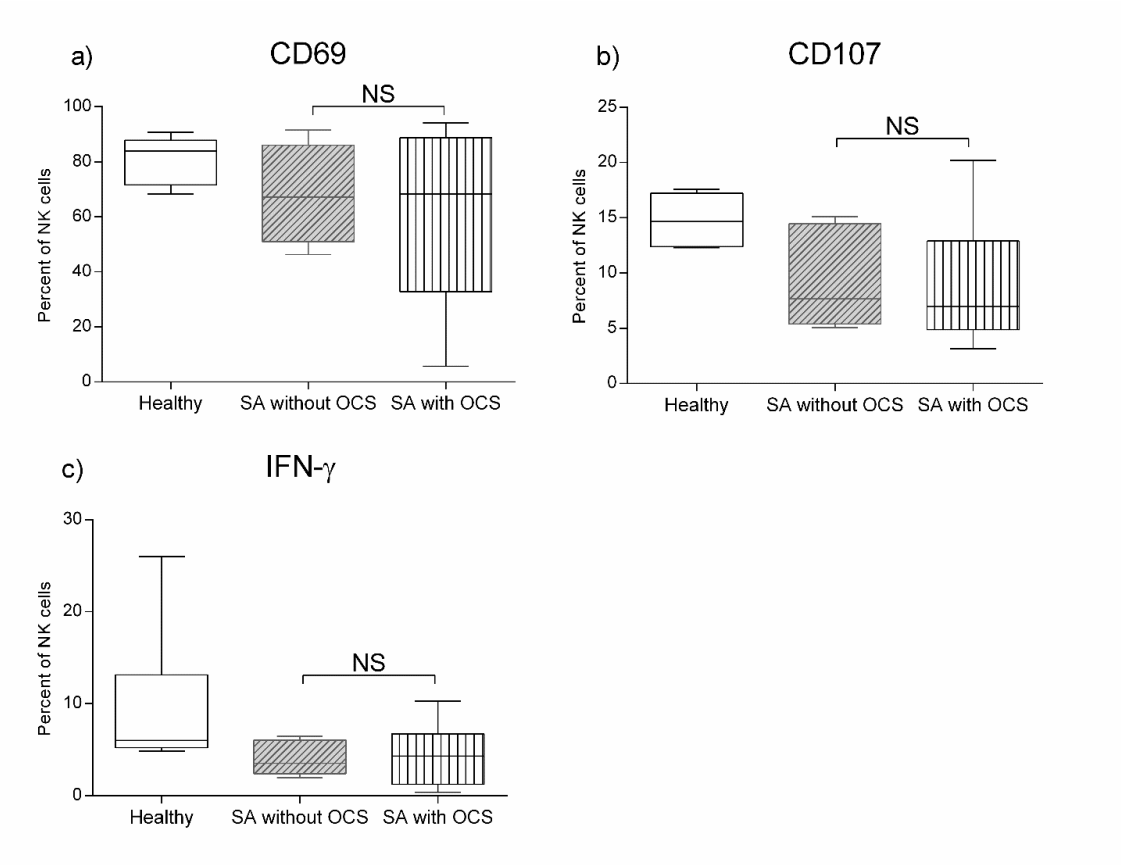


Figure S2: Oral corticosteroid treatment of severe asthma patients affects neither NK cell cytotoxic status, nor IFN-γ expression following PBMC stimulation with RV-A9 rhinovirus.

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).

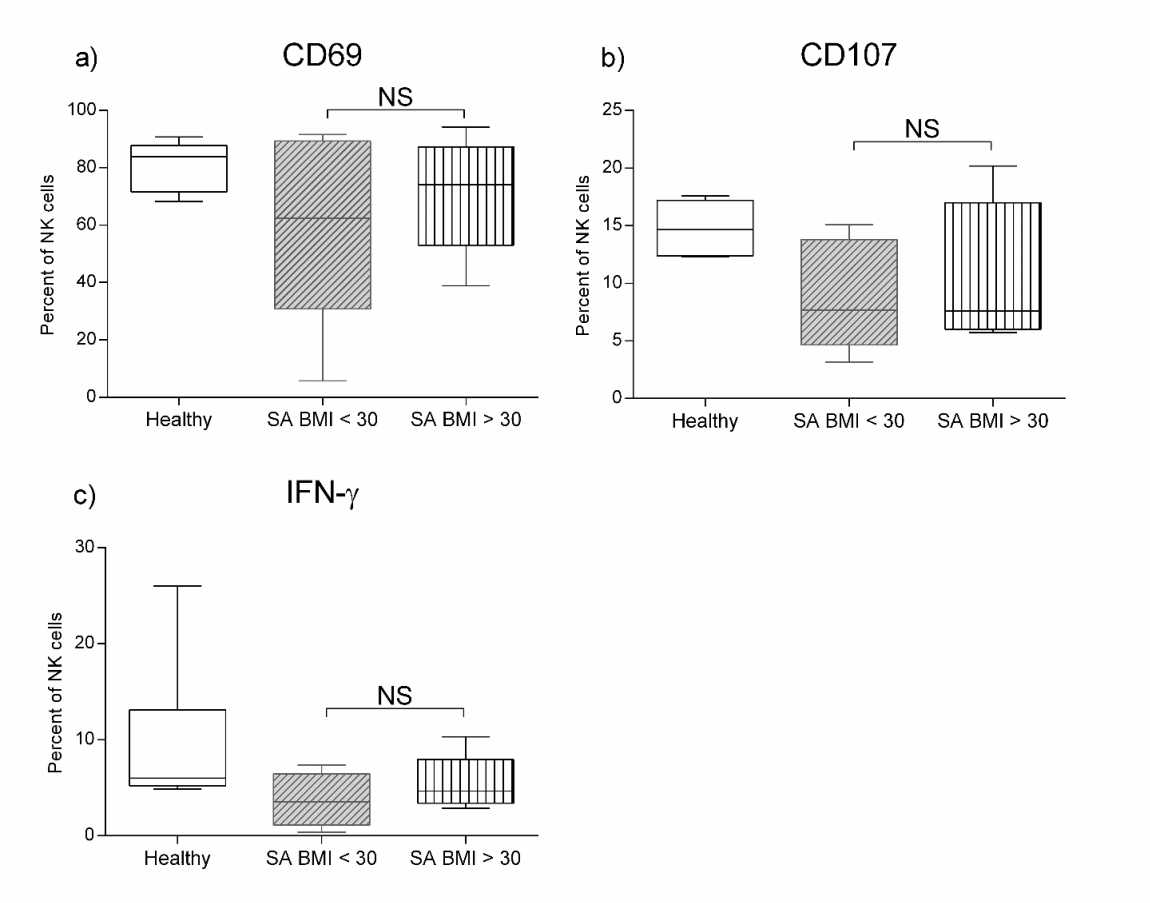


Figure S3: Body Mass Index of severe asthma patients affects neither cytotoxic status nor IFN-γ expression following PBMC stimulation with RV-A9 rhinovirus.

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).

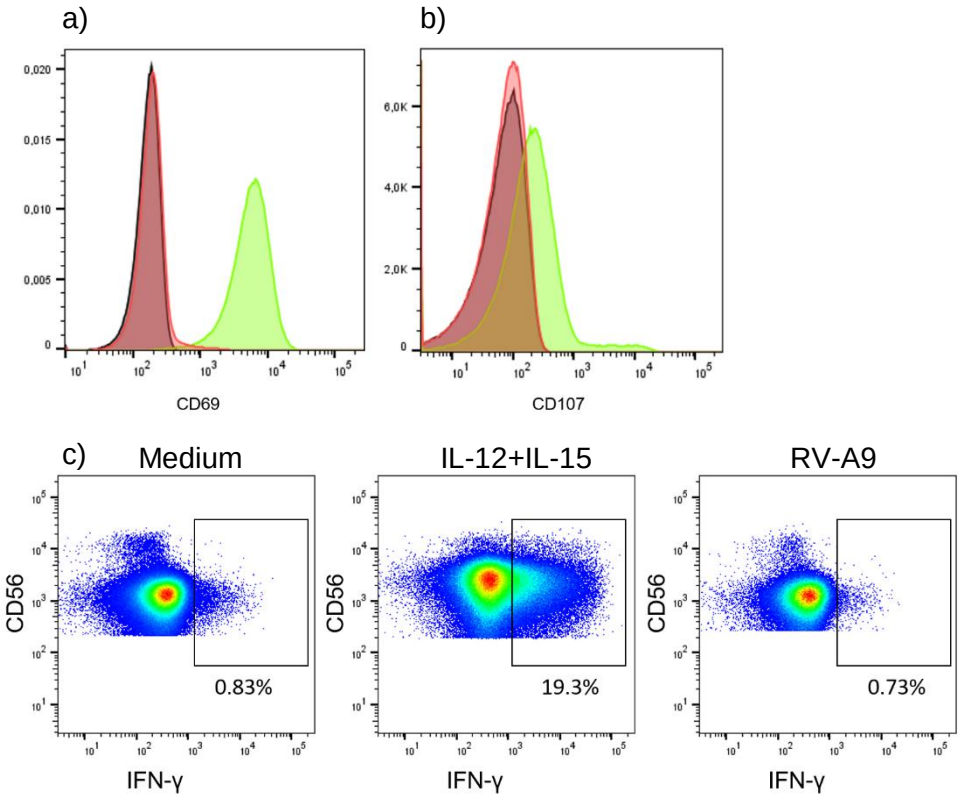


Figure S4: Purified NK cells are not activated by rhinovirus.

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.

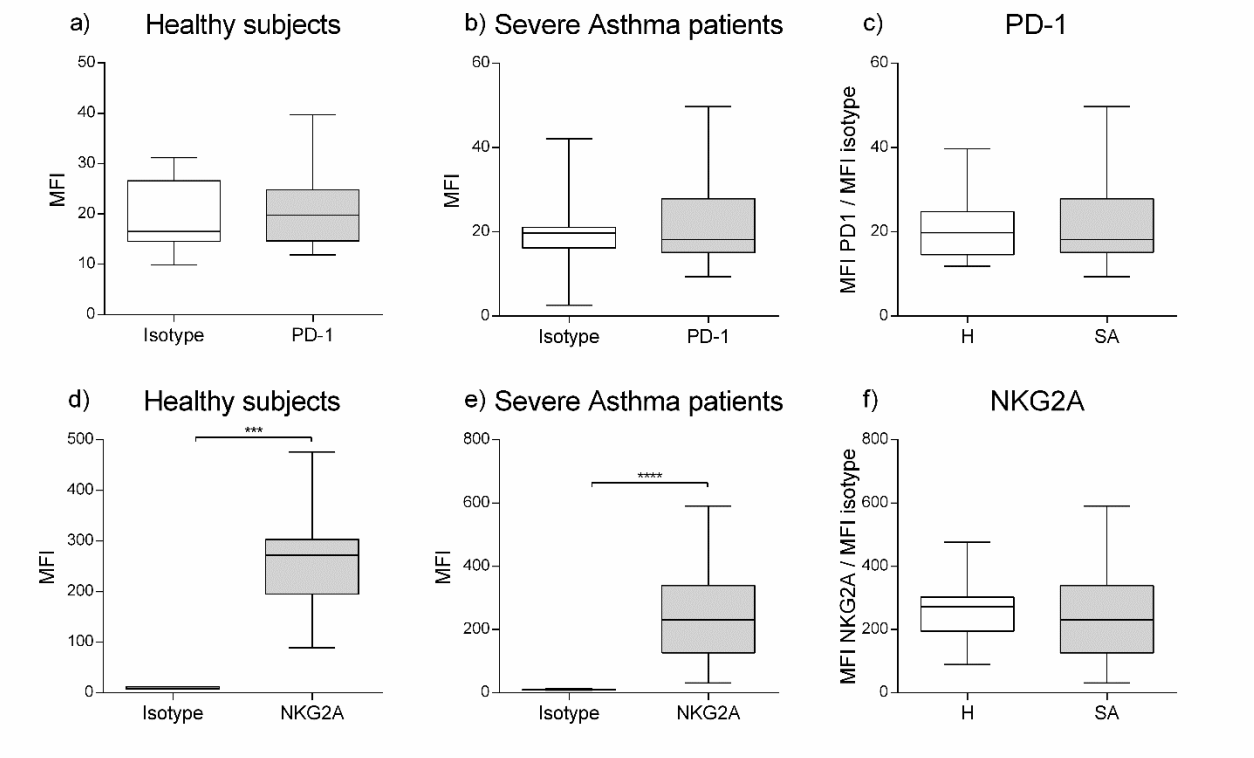


Figure S5: Expression of PD-1 and NKG2A on NK cells.

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).

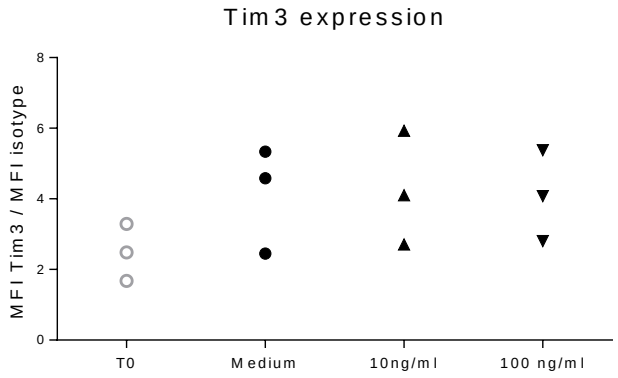


Figure S6: In vitro stimulation of PBMC with increasing doses of dexamethasone does not modify the expression of Tim3 on NK cells.

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.

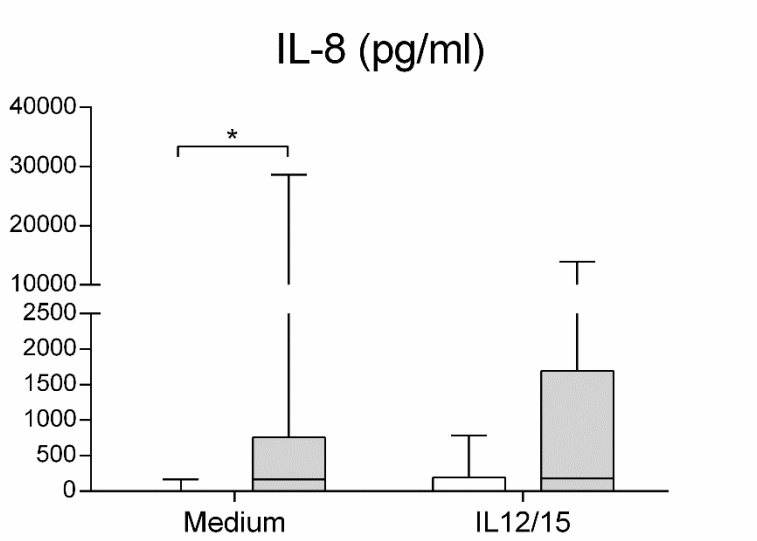


Figure S7: IL-8 production by purified NK cells from severe asthma patients.

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.