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TLR 7/8 regulates Type I and Type III Interferon Signaling in RV1b induced Allergic Asthma

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Take Home Message

TLR 7/8 agonist reduced IFNαR1 but induced IFNλRα in PBMCs of preschool children. This induction of IFNλRα offers a new mechanism for therapy of children with RV1b exacerbated asthma.

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Abstract

Question: Interferon responses have been reported to be defective in rhinovirus (RV) induced asthma. The heterodimeric receptor of type I Interferon (IFN) (IFN-α/-β) is composed by IFNαR-1 and IFNαR-2. Ligand binding to the IFN-α/-β receptor complex activates STAT1 and STAT2 intracellularly. Although type III Interferon (IFN-λ) binds to a different receptor containing IFNλRA and IL-10R2, its triggering leads to activation of the same downstream transcription factors. Here we analyzed the effects of Rhinovirus to Interferon type I and III receptors and asked about possible Toll-like receptor 7/8 agonist R848 mediated IFNαR1 and IFNλRα regulation.

Methods: We measured IFN-α, -β, -λ and their receptor levels in PBMCs supernatants and cell pellets stimulated with RV1b and the Toll-like Receptor 7/8 (TLR7/8) agonist (R848), in two cohorts of children with and without asthma recruited at preschool age (PreDicta) and at primary school age (AGENDAS) as well as in cell supernatants from total lung cells isolated from mice.

Results: We observed that R848 induced IFNλR mRNA expression in PBMCs of healthy and asthmatic children, but suppressed the IFNαR mRNA levels. In murine lung cells, RV1b alone and together with R848 suppressed IFNαR protein in T cells compared to controls and in total lung IFNλR mRNA compared to RV1b infection alone.

Answer: In PBMCs from pre-school children, IFNαR mRNA was reduced and IFNλRα mRNA was induced upon treatment with TLR7/8 agonist thus suggesting new avenues for induction of anti-viral immune responses in pediatric asthma.

Key words

IFN-α, IFN-β, IFN-λ, IFNαR, IFNλR, Rhinovirus, TLR7/8, IL-27, IL-10

Introduction

Asthma is a chronic inflammatory disease of the respiratory tract that has variable clinical occurrences. More than 300 million people worldwide are affected and the cumulated incidence is still rising [1]. It has been indicated that T lymphocytes play a prominent role in the pathogenesis of asthma. In particular, T-helper cells type 2 (T_H2 cells) and their cytokines, e.g. Interleukin-5 (IL-5) and IL-13, are overexpressed in allergic asthma [2] starting from childhood [3, 4]. Key problems in asthma are chronic inflammation of the airways and hyper-responsiveness that lead to episodes of coughing, wheezing and shortness of breath [5]. Repeated viral infections may accompany the immune shift towards a T_H2 phenotype and are also considered as crucial factors for asthma exacerbation in children and airway remodeling [4].

Rhinovirus (RV) infections are common [6], but there is evidence that affected patients with asthma may show marked variability in symptoms upon infection [7]. RV is a member of the Picornaviridae family [6]. The major group RV (parts of serotypes of RV-A and all serotypes of RV-B) enters the body through the upper airways [6] and cells via intercellular adhesion molecule 1 (ICAM-1) while the minor group RV (parts of serotypes of RV-A, i.e. RV1b) [6, 8] enters ~~or~~ via members of the low-density lipoprotein receptor family (LDLR) [9, 10]. RV-C enters the cells via Cadherin-related family member 3 (CDHR3) [11]. Toll-like receptor (TLR) 7, TLR8, TLR9 and TLR3 detect virus nucleic acids [12] and lead to the activation of a myeloid differentiation primary response protein (MyD88) dependent pathway [12] resulting in phosphorylation of Interferon regulatory factor (IRF) 7 and IRF3 that lead to the transcription of type I (IFN-alpha and IFN-beta) and type III Interferons (IFN-lambda) [9, 12].

After Interferon alpha (IFN-α) binds to the heterodimeric receptor complex containing the transmembrane glycoproteins Interferon alpha receptor chain 1 (IFNAR1) and IFNAR2, tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) become activated and phosphorylate the transcription factors signal transducer and activator of transcription protein 1 (STAT1) and 2 (STAT2) [13]. Phosphorylated STAT1 and STAT2 form a complex with IRF9, the so-called IFN-stimulated gene factor 3 (ISGF3) complex that translocates to the nucleus and promotes the transcription of IFN-stimulated genes [13]. The signaling pathway of IFN-lambda (IFN-λ, also known as IL-28A, IL-28B and IL-29) is similar to IFN-α, but the IFN-λ receptor has a different structure compared to the receptor of IFN-α and consists of Interferon lambda receptor chain

alpha (IFNλRα) and Interleukin 10 receptor chain 2 (IL-10R2) [14, 15]. The latter receptor chain is also used for some other cytokine signaling pathways, for example the IL-10 or IL-22 signaling pathways [14, 15]. However, the interferon type III-induced immune response is more restricted, because the IFNλRα is expressed on fewer cells as compared to the IFNAR1 [14, 16, 17].

Asthmatic subjects seem to have a defect in their type I IFN levels upon viral infection [18-20], but we recently described that they can produce IFN-α in case of acute exacerbation [9]. Several reports suggested an increased secretion of IFN-λ in asthmatic subjects [14, 21, 22]. Our group also studied IFN-λ secretion after RV1b infection and found an increased production in PBMCs of preschool aged asthmatic children that had a positive RV detection *in vivo* [9, 14].

In this study, we further investigated the production and expression of type I and type III Interferons in asthmatic children. Accordingly, we examined the pathways of those IFNs and their receptors with particular reference to IFNαR1 and IFNλRα. Moreover, we used a TLR7 and TLR8 agonist, denoted R848 that is known to upregulate IFN type I expression [23], to study IFN production in asthma patients upon RV1b infection.

Material and Methods

Human study PreDicta and AGENDAS

In the European Study PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases) - Work-Package 1 (WP1) healthy and asthmatic preschool children between 4-6 years were recruited and examined at recruitment (B0) as well as two years later (F4) in collaboration with the Department of Allergy and Pneumology at the Children Hospital at the FAU University Hospital in Erlangen. The study in Erlangen was approved by the ethics committee of the Friedrich-Alexander University Erlangen-Nürnberg, Germany (Re-No 4435), and is registered in the German Clinical Trials Register (www.germanctr.de: DRKS00004914).

The study AGENDAS (Gender, age and environmental factors that modify immune-response and the development of allergic asthma during the school age in childhood) has been performed in Erlangen with the same team that performed the study PreDicta. In this longitudinal study, primary school children at the age between 6 and 11.9 years were recruited and analysed in collaboration with the Department of Allergy and Pneumology at the Children Hospital at the FAU University Hospital in Erlangen. The study was approved by the local ethics committee of the Friedrich-Alexander University Erlangen-Nürnberg, Germany (Re-No: 212_12 B) and is registered in the German Clinical Trials Register (www.germanctr.de: DRKS00004914).

The recruitment of the subjects, inclusion and exclusion criteria as well as the time scale for clinical visits and data collection were already previously described [9, 24, 25].

In vitro cell culture of human Peripheral Blood Mononuclear cells (PBMC) with Rhinovirus 1b (RV1b) or Resiquimod (R848)

PBMCs were adjusted to a concentration of $1x10^6$ viable cells/ml in complete culture medium. PBMCs were cultured for 48 hours in complete culture medium at 37^oC and 5% CO_2, whereby

parts of them were challenged in vitro with RV1b or 200µl/ml R848 (0.2mg/ml) (InvivoGen, Toulouse, France).

The growth of RV1b and the description of the RV1b infection itself have been published elsewhere [9, 26, 27]. Afterwards, supernatants were carefully removed and stored at -80°C. For RNA extraction, PBMCs were diluted in QUIazol® Lysis Reagent (Quiagen Sciences, Maryland, USA) and stored at -80°C.

Mice

All mice were maintained under specific pathogen free conditions and had free access to food and water. The government of Mittelfranken, Bavaria approved the experiments (55.2.2,-2532-2-633).

Total Lung cell isolation and *in vitro* cell culture (RV1b and/or R848)

Whole lungs from sacrificed nine weeks old mice were removed and used to isolate total lung cells: lungs were sectioned and mixed with 10 ml PBS, 300 U/ml Collagenase type Ia, 0,015% DNase I and digested for 45 minutes at 37°C. Digested lungs went through a cell strainer of 40µm before they were centrifuged. Pellets were then mixed with Ammonium-Chloride-Potassium Lysing Buffer and incubated 2 minutes at room temperature before they were centrifuged again. Pellets then were washed with PBS. After that, isolated lung cells were centrifuged again and counted. Isolated lung cells were cultured in complete culture medium (RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin and streptomycin) for 24 hours at 5% CO_2 at 37°C. For RV infection, total lung cells were incubated with RV1b for 1 hour at 33°C on a horizontal shaker and washed afterwards with medium and cultured in complete culture medium for 24 hours at 37°C and 5% CO_2. For R848 stimulation cells were cultured in complete culture medium and 200µl/ml R848 for 24 hours at 37°C and 5% CO_2.

Flow cytometric analysis and cellular staining

After 24 hours, cultured total lung cells were harvested, washed and stained with fluochrome conjugated anti-CD4, anti-CD8, anti-CD25, anti-Foxp3 and anti-IFNAR-1 antibodies (see **Table S2**) in staining buffer (PBS/0.1%NaN_3/1.0% fetal bovine serum) at 4°C for 30 min and

afterwards washed in PBS. Fluorescence labelled cells were acquired by using FACS-Canto II (BD Bioscience, Heidelberg) and analyzed with FlowJo (Treestar Inc. Ashland, OR, USA).

Statistical analysis

For statistical analysis, we used Prism version 7 for Windows (GraphPad, La Jolla, CA, USA). Differences were evaluated for significance by using the Kruskal-Wallis test to generate p-value data (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) and corrected for multiple comparisons using statistical hypothesis test Dunn`s now. Except for Figure 2 b, c, d where we used Mann-Whitney-U test. Unless otherwise indicated data are presented as mean values ± SEMs.

Results

RV1b infection suppressed average IFNαR mRNA expression in PBMCs from asthmatic children

All children analyzed in the longitudinal study PreDicta were aged between 4 and 6 years at the time of recruitment (visit B0) and were followed up for two years until the final visit (visit F4) **(Fig 1a)** [9]. Within the PreDicta cohort, asthmatic children with RV in the airways had a defect in Interferon type I levels in their PBMCs and in serum at recruitment (B0), whereas during exacerbation of disease, close to infection, this defect in Interferon type I in serum was not present in asthmatic children [9]. As these findings indicated that IFN-α expression is usually upregulated within hours upon infection, we next looked at later time points by using PBMCs supernatants 48 hours after an infection with RV1b *in vitro* **(Fig 1a)**. Accordingly, cell culture supernatants from PBMCs were collected and analyzed for cytokine content. Comparing the IFN-α production in the supernatants of unstimulated versus RV1b stimulated PBMCs, we did not find any RV1b-dependent IFN-α regulation **(Fig 1b).**

TLR 7/8 agonist R848 induced IFN-α, but suppresses IFNαR mRNA in PBMCs of preschool children with asthma

We next investigated if the TLR 7/8, a single-stranded viral RNA receptor that triggers a rapid innate immune response[28], may restore IFN responses in PBMCs. In these experiments, we incubated PBMCs from asthmatic and control subjects with R848 followed by analysis of IFN-α levels. R848 treatment induced IFN-α levels in healthy control children as well as in asthmatic children **(Fig 1b)**. Furthermore, R848 significantly suppressed the expression of IFNαR mRNA in PBMCs from all children cohorts **(Fig 1c)**.

The TLR7/8 agonist R848 reduced IFN-λ and induces IFNλR mRNA in PBMCs from preschool aged children

The IFN type III pathway is thought to be alternatively activated in the absence of type I Interferons **(Fig S1a)** [15]. Accordingly, we next analyzed IFN-λ in these PBMCs supernatants and found that TLR7/8 agonist R848 treatment reduced IFN-λ levels in the supernatants of asthma and control subjects as compared to untreated cells **(Fig 1d)**. By contrast, R848 induced IFNλR mRNA expression in asthma and control children **(Fig 1e)**. RV1b infection did not lead to any significant regulation of IFNλR mRNA in PBMCs both in control and asthmatic children **(Fig 1e)**. In contrast, Tyk2 mRNA was not regulated after TLR7/8 treatment or RV1b infection *in vitro* **(Fig 1f).**

Immunosuppressive and immunostimulatory role of TLR7/8 R848 agonist in PBMCs of preschool children

As RV-induced lower airway inflammation and impaired lung function in patients with asthma are strongly related to deficient Interferon gamma (IFN-γ) and IL-10 responses [29], we next studied expression of these cytokines as well as the expression of IL-27, a potent IFN-γ inducer[30]. R848 treatment significantly induced the levels of the cytokine IL-10 in the supernatants of PBMCs from control and asthmatic children as compared to untreated control samples **(Fig 1g).** Beside this immunosuppressive effect, R848 treatment induced the immunostimulatory cytokine IL-27 **(Fig 1h)**. However, in the presence of elevated IL-10 and IL-27 levels, the resulting induction of the T helper cell 1 (T_H1) cytokine IFN-γ upon R848 treatment was relatively modest **(Fig 1i).** Nevertheless, a significant induction of IFN-γ levels was detected in R848-treated samples from asthma and control subjects as compared to untreated samples **(Fig 1i)**.

Age dependent decreased IFN-α and IFNαR1 mRNA levels in asthma

Afterwards, we analyzed a new cohort of older control and asthmatic children at primary school age, denoted AGENDAS **(Fig 2a, Table 1**), and asked whether the inhibition of IFN-α and IFNαR1 signaling was also present in this cohort. IFN-α was significantly induced in serum **(Fig 2b)** and tendentially induced at mRNA level in PBMCs from asthmatic children **(Fig S1c)**. Furthermore, we could not detect any difference in IFNαR1 mRNA in these PBMCs **(Fig S1d).**

Table 1: AGENDAS characteristics

AGENDAS	Healthy control children	Asthmatic children
Subjects	6	6
Gender		
Male	33.3 (2/6)	50 (3/6)
Female	66.7 (4/6)	50 (3/6)
Age years	6.68	7.73
FEV_1 <90%	0 (0/6)	0 (0/6)
FEV_1/VC <70%	0 (0/6)	0 (0/6)
Treatment		
No treatment	100 (6/6)	16.7 (1/6)
Steroid	0 (0/0)	33.3 (2/6)
Steroid + nonsteroid	0 (0/0)	50 (3/6)
NPF: RV		
Negative	100 (5/5)	66.7 (4/6)
Positive	0 (0/5)	33.3 (2/6)

Data are presented as n, % (n/N) (n= observed number; N= total number) or mean. FEV_1: forced expiratory volume in 1 s; NPF: nasopharyngeal fluid; RV: rhinovirus

Improved IFN-λ signal transduction in the serum of primary school asthmatic children resulted in reduced number of RV infections in the upper airways

We next found that IFN-λ levels were elevated in the serum of asthmatic children as compared to control children **(Fig 2c)**. Furthermore, the IFNλR mRNA was upregulated in PBMCs of primary school aged children with asthma as compared to control children **(Fig 2d)**. Consistent with an improved IFN type I and type III immune response, the expression of transcription factor STAT2 mRNA **(Fig 2e)** was tendentially upregulated. Moreover, STAT1A mRNA **(Fig 2f)**, the antiviral component of STAT1 [31], but not STAT1B mRNA **(Fig 2g)** was tendentially upregulated in

PBMCs from asthmatic children after RV1b infection *in vitro*. In addition, STAT1B mRNA was elevated in PBMCs from control children after RV1b infection *in vitro* **(Fig 2g)**. It could be possible, that this is the reason why this cohort of children had less RV infections in the airways as compared to the younger PreDicta cohort of the children **(Table I)[9]**. Further researches are needed.

Decreased percentage of lung IFNαR$^+$CD8$^+$ and IFNαR$^+$CD4$^+$ T-cells after RV1b infection and R848 treatment *in vitro*

We next wanted to directly study the immunomodulatory mechanisms mediated by RV1b and TLR7/8 agonist R848 on IFNαR and IFNλR expression. To this aim, we isolated total lung cells from C57BL/6JRj mice and cultured them either with RV1b or R848 or both **(Fig 3a).** Isolated total lung cells stained with Annexin V and PI (Propidium Iodide) confirmed that 96% of the isolated cells were viable **(Fig S2a).** Moreover, FACS analysis **(Fig S2b)** of these lung cells showed that IFNαR$^+$ lymphocytes **(Fig 3c)** as well as IFNαR$^+$CD8$^+$ **(Fig 3d)** and IFNαR$^+$CD4$^+$ **(Fig 3e)** cells were significantly decreased after RV1b infection together with R848 treatment *in vitro*. However, IFNλRα mRNA expression was downregulated after R848 treatment together with RV1b infection *in vitro* compared to RV1b infection alone **(Fig 3f)**. Successful RV1b infection in the absence and presence of R848 was confirmed by RV1b-PCR **(Fig 3g)**.

TLR7/8 agonist R848 suppresses Tyk2 mRNA expression in murine lung cells

Tyk2 mRNA, in contrast to the results in PBMCs of preschool children, was significantly downregulated by R848 treatment, but not by RV1b infection alone in lung cells **(Fig 3h).** These results are consistent with the different expression of IFNλR mRNA in PBMCs and murine lung cells after R848 treatment *in vitro*. R848 in combination with RV1b also resulted in decreased expression of Tyk2 mRNA **(Fig 3h)**. Moreover, RV1b and R848 in combination inhibited IFN-α production by lung cells **(Fig 3i)**, while TLR7/8 agonist treatment alone had no significant effect. However, IFN-α production induced by RV1b and R848 together is not different from RV1b alone, meaning that R848 does not modify the response to RV1b infection for this parameter.

Discussion

Previous studies suggested that asthmatic children have a defect in IFN type I production [18-20], however, this defect has been shown to be reversible [9]. In this study, we investigated whether this defect in IFN responses could be ameliorated by treatment with R848. As a result, PBMCs treatment with R848 *in vitro* led to an increase of IFN-α in the supernatants of PBMCs, consistent with a previous report [23]. However, we discovered that TLR7/8 agonist has also a limiting effect on IFN type I signaling, because it additionally decreased the expression of IFNαR1 mRNA. The increase of IFN-α in the supernatants of PBMCs after R848 treatment *in vitro* could be the result of a decreased ligand-receptor binding due to the suppression of IFNαR mRNA expression. Due to some limitations of the remaining biological material of the study, we could not use one donor for one stimulation for this evaluation. Nevertheless, similar effects were noted on total murine lung cells upon *in vitro* R848 treatment and such treatment in combination with RV1b infection *in vitro* reduced IFNαR1 expression on both $CD4^+$ and $CD8^+$ lung T cells. However, R848 treatment augmented IFNλR mRNA expression in PBMCs of preschool children suggesting that TLR7/8 activation may favor antiviral immune responses via type III IFN signaling.

RV1b infection *in vitro* led to a decrease of IFNαR1 expression in murine lung cells as well as tendentially in PBMCs of asthmatic preschool children suggesting suppression rather than activation of type I IFN signaling upon RV1b infection. It is possible, that we could not detect a significant difference when analyzing murine CD4+ and CD8+ lymphocytes, because we used RV1b instead of RV-C whose receptor is Cadherin-related family member 3 (CDHR3) [11] or a major group rhinovirus serotype [6]. Alternatively other not yet identified types of lymphocytes were affected. Because of this limitation of our study, further analysis with RV-C or major group rhinovirus serotypes are needed [32]. In fact, RV-C-related wheezing illnesses were found associated with an increased risk of respiratory hospital admission [33] particularly in those children with asthma [34]. Although separated investigations are needed to unreveal the

underlined mechanism of these results, it is possible that this is a mechanism by which the Rhinovirus escapes the immune system of the host either by inhibiting the IFN production or by decreasing its stability and more interestingly by downregulating the IFN-α receptor. Consistent with this type of virus immune escape mechanism, Liu et al. showed that hepatitis C virus (HCV) and vesicular stomatitis virus (VSV) infection resulted in IFNαR degradation leading to weakened IFN type I immune responses [35, 36]. Further researches are needed.

In asthmatic preschool children with RV infection *in vivo*, higher IFN-λ levels were noted in serum [9]. We observed that school aged asthmatic children have significantly higher IFN-λ levels in serum and higher IFNλR mRNA expression in PBMCs as compared to healthy ones, indicating an activation of the IFN-λ pathway.

IFN-λ has already been suggested as alternative antiviral pathway in the presence of suboptimal type I IFN response [17]. In an additional series of studies, we investigated whether R848 is able to induce the IFN-λ signaling pathway. We observed an induction of IFNλR mRNA expression in PBMCs of preschool aged children after R848 treatment *in vitro*, but a decrease of IFN-λ in the supernatants of PBMCs. The lower levels of IFN-λ could be at least partially due to compensatory reduction of cytokine production after induction of receptor expression or high IFN-λ binding to the induced IFN-λ receptor.

It is already known that asthma symptoms may change with age [37]. Consistently, in agreement with the proposed functional relevance of IFN-λ signaling, we found an upregulation of IFNλR expression in the AGENDAS cohort that was associated with a lower percentage of RV infection in the airways compared to the younger PreDicta cohort [9]. This effect was not due to differential use of corticosteroids, as similar percentages of children (preschool children 79.2% [9] and school aged children 83.3%) were treated with this drug.

In addition to IFN type I and III signaling, we found that R848 also regulated IFN-γ and IFN-γ-inducing cytokines such as IL-27 [38]. In fact, R848 caused an induction of IFN-γ and IL-27 cytokine responses that may favor antiviral immune responses. Moreover, R848 augmented IL-10 production. As RV-induced lower airway inflammation and impaired lung function in patients with asthma are strongly related to deficient IFN-γ and IL-10 responses [29] these findings

suggested that R848 may induce several important cytokines that control mucosal immune responses and thus may potentially affect clinical outcome of RV infection.

In conclusion, we showed for the first time that R848 reduce IFNαR mRNA expression. Additionally, our data indicate an important role of the IFN-λ pathway for the clearance of RV1b infection, especially in asthmatic children. Furthermore, we demonstrated that this pathway can be induced via the TLR 7/8 agonist R848 that causes upregulation of IFNλR mRNA and regulates both IFN-γ and IL-10 responses. Thus, triggering of TLR7/8 responses may offer a new therapy approach for asthmatic children. It is currently unclear whether this TLR7/8-dependent IFNλR response is limited to RV1b infection or whether it might be considered for treatment of other severe respiratory tract infections, such as respiratory syncytial virus (RSV) or SARS Coronavirus (SARS-CoV), because Mordstein et al. already showed that IFNλR has a role in the restrictions of RSV and SARS-CoV infections [17]. Collectively, our results indicate an induction of IFN-λ signalling upon treatment with the TLR7/8 agonist R848 thus suggesting new avenues for induction of anti-viral immune responses in pediatric asthma.

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Author contributions

Jasmin Krug generated the laboratory results at the Molecular Department in Erlangen. She correlated, analysed and interpreted all the data and generated all the Figures, Tables and supplementary material associated to this manuscript. She wrote the introduction, methods, result section and the discussion. She was involved in drafting the manuscript and edited it and contributed to the revision. A.K. recruited, analysed and diagnosed the children from the WP1 PreDicta Cohort in Erlangen and the children from the AGENDAS cohort. J.K. helped with the

murine data recruitment. T.V. provided the respiratory viruses' analysis for PreDicta and AGENDAS. M.A. and B.S. provided the Multiplex cytokines data for the Erlangen WP1 Cohort PreDicta reported and correlated in Figures 1C, 2B, 2C, 2D. N.P. was the coordinator of the PreDicta studies and P.X. helped N.P. to establish the different European cohorts in PreDicta. T.Z. was involved in the recruitment and analysis of the PreDicta studies. S.F. contributed to the design of the study, writing the first draft of the manuscript and to edit it, analysed and mentored all the studies for the PreDicta and AGENDAS part in Erlangen and contributed to the revision of the manuscript.

Figure legends

Figure 1: Immunoregulatory and immunosuppressive function of TLR7/8 agonist (R848) in PBMCs of healthy and asthmatic preschool children.

a. Design of the two human studies PreDicta and AGENDAS and the experimental design of the study PreDicta. **b.** IFN-α levels in the supernatants (SN) of PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* cell culture with RV1b or R848 (CN: UNST n=13, RV n=16, R848 n=19, A: UNST n=20, RV n=19, R848 n=21, $p_{(CN\ UNST - CN\ R848)} = 0.0289$; $p_{(CN\ RV - CN\ R848)} < 0.0001$; $p_{(A\ UNST - A\ R848)} = 0.0011$; $p_{(A\ RV - A\ R848)} < 0.0001$). **c.** IFNaR1 mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* cell culture with RV1b or R848 (CN: UNST n=5, RV n=8, R848 n=17; A: UNST n=5, RV n=5, R848 n=16; $p_{(CN\ UNST - CN\ R848)} = 0.0045$; $p_{(CN\ RV - CN\ R848)} = 0.0001$; $p_{(A\ UNST - A\ R848)} = 0.0005$; $p_{(A\ RV - A\ R848)} = 0.0115$). **d.** IFN-λ levels in the supernatants (SN) of PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* culture with RV1b or R848 (CN: UNST n=14, RV n=14, R848 n=14, A: UNST n=11, RV n=11, R848 n=11; $p_{(CN\ UNST - CN\ R848)} = 0.0038$; $p_{(A\ UNST - A\ R848)} = 0.0008$). **e.** IFNLR mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children (F4) after *in vitro* culture with RV1b or R848 (CN: UNST n=12, RV n=9, R848 n=13, A: UNST n=8, RV n=9, R848 n=8; $p_{(CN\ UNST - CN\ R848)} = 0.0005$; $p_{(CN\ RV - CN\ R848)} = 0.0051$; $p_{(A\ UNST - A\ R848)} = 0.0084$; $p_{(A\ RV - A\ R848)} = 0.001$). **f.** Tyk2 mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children (F4) after *in vitro* culture with RV1b or R848 (CN: UNST n=5, RV n=3, R848 n=13, A: UNST n=3, RV n=7, R848 n=8). **g.** IL-10 levels in the supernatants (SN) of PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* culture with RV1b or R848 (CN: UNST n=17, RV n=20, R848 n=20, A: UNST n=22, RV n=23, R848 n=21; $p_{(CN\ UNST - CN}$

$_{R848)}$ < 0.0001; p $_{(CN\ RV - CN\ R848)}$ < 0.0001; p $_{(A\ UNST - A\ R848)}$ < 0.0001; p $_{(A\ RV - A\ R848)}$ < 0.0001). **h.** IL-27 levels in the supernatants (SN) of PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* culture with RV1b or R848 (CN: UNST n=8, RV n=11, R848 n=20, A: UNST n=12, RV n=12, R848 n=21; p $_{(CN\ UNST - CN\ R848)}$ < 0.0001; p $_{(CN\ RV - CN\ R848)}$ < 0.0001; p $_{(A\ UNST - A\ R848)}$ < 0.0001; p $_{(A\ RV - A\ R848)}$ < 0.0001). **i.** IFN-γ levels in the supernatants of PBMCs of healthy (CN) and asthmatic (A) children after *in vitro* culture with RV1b or R848 (CN: UNST n=6, RV n=18, R848 n=17, A: UNST n=12, RV n=15, R848 n=18; p $_{(CN\ UNST - CN\ RV)}$ = 0.0201; p $_{(CN\ UNST - CN\ R848)}$ = 0.0003; p $_{(A\ UNST - A\ RV)}$ = 0.0251; p $_{(A\ UNST - A\ R848)}$ < 0.0001; p $_{(A\ RV - A\ R848)}$ = 0.0237).

B0 = Baseline visit, F4 = Follow-Up visit after 24 months; PBMC = peripheral blood mononuclear cell; CN = control children; A = asthmatic children; UNST = Unstimulated; RV = Rhinovirus; R848 = TLR7/8 agonist.

All data are presented as individual points and mean values ± SEM. Differences between the cultures were evaluated for significance by using the Kruskal-Wallis test to generate p-value data (* p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001) and corrected for multiple comparisons using statistical hypothesis test Dunn`s.

Figure 2. Asthmatic primary school age children have more IFNλ and STAT2 compared to control children and improved in vivo RV clearance.

a. Experimental design of the study AGENDAS **b.** IFNλ (IL28B/29) levels in serum of healthy (CN) and asthmatic (A) children (CN n=7, A n=4; p $_{(CN\ -\ A)}$ = 0.0095). **c.** IFNLR mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children (CN n=6, A n=5; p $_{(CN - A)}$ = 0.0303). **d.** STAT2 mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children (CN n=6, A n=5; p $_{(CN - A)}$ = 0.0823). **e.** STAT1A mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children without and with RV1b infection *in vitro*. (CN: UNST n=5, RV n=5, A: UNST n=3, RV n=3). **f.** STAT1B mRNA expression in PBMCs of healthy (CN) and asthmatic (A) children without and with RV1b infection *in vitro*. (CN: UNST n=4, RV n=5, A: UNST n=3, RV n=1; p $_{(CN - A)}$ = 0.0159).

PBMC = peripheral blood mononuclear cell; CN = control children; A = asthmatic children; UNST = Unstimulated; RV = Rhinovirus; R848 = TLR7/8 agonist.

All data are presented as individual points and mean values ± SEM. Differences between healthy and asthmatics were evaluated for significance by using the Mann-Whitney-U test (b, c, d) and differences between the cultures were evaluated for significance by using the Kruskal-Wallis test (e, f) to generate p-value data (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) and corrected for multiple comparisons using statistical hypothesis test Dunn`s (e,f).

Figure 3: Downregulation of IFNαR in lung cells after culture with RV1b and TLR7/8 agonist (R848)

a. Experimental design of the mouse experiment. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com. **b.** Flow cytometry analysis of Lymphocytes gated on all cells after in vitro culture of total lung cells with RV1b or R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - RV)} = 0.0254$). **c.** Flow cytometry analysis of IFNAR1+ Lymphocytes gated on lymphocytes after *in vitro* culture of total lung cells with RV1b or R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - RV)} = 0.0383$; $p_{(UNST - R848)} = 0.0233$). **d.** Flow cytometry analysis of CD8+ IFNaR1+ cells gated on CD8+ lymphocytes after *in vitro* culture of total lung cells with RV1b or R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - RV+R848)} = 0.0452$). **e.** Flow cytometry analysis of CD4+ IFNaR1+ cells gated on CD4+ lymphocytes after *in vitro* culture of total lung cells with RV1b or R848 or both (UNST n=5, RV n=10, R848 n=5, RV+R848 n=5; $p_{(UNST - RV+R848)} = 0.0275$). **f.** IFNLRA mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b or R848 or both (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5; $p_{(RV - RV+R848)} = 0.0119$). **g.** RV1b mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b or R848 or both and a representative picture of the corresponding electrophoresis. (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - RV)} = 0.0228$; $p_{(UNST - RV+R848)} = 0.0043$). **h.** Tyk2 mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b or R848 or both (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - R848)} = 0.0394$; $p_{(RV - R848)} = 0.0189$). **i.** IFN-alpha levels in the supernatant (SN) of total lung cells after *in vitro* culture of total lung cells with RV1b or R848 or

both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5; $p_{(UNST - RV+R848)} = 0.0021$; $p_{(R848 - RV+R848)} = 0.0234$).

UNST = Unstimulated; RV = Rhinovirus; R848 = TLR7/8 agonist; FACS = Fluorescence-activated cell sorting.

All data are presented as mean values + SEM. Differences were evaluated for significance by using the Kruskal-Wallis test to generate p-value data (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$) and are corrected for multiple comparisons using statistical hypothesis test Dunn`s.

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Supplementary Material

TLR 7/8 regulates Type I and Type III Interferon Signaling in RV1b induced Allergic Asthma

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Take Home Message

TLR 7/8 agonist reduces IFNαR1 but induces IFNλRα in PBMCs of preschool children. This induction of IFNλRα offers a new mechanism for therapy of children with RV1b induced asthma.

Supplementary Methods

Isolation of human Peripheral Blood Mononuclear cells (PBMC)

Heparinized blood was transferred to a sterile 15ml tube and diluted with an equal volume of room temperature PBS and inverted. The diluted blood was overlayed on top of Ficoll-Hypaque. After density gradient centrifugation, peripheral blood mononuclear cells (PBMC) were collected between plasma and Ficoll. PBMCs were washed twice with RPMI 1640 medium and then cultured as described below.

Quantitative real-time PCR

To amplify the resulting template cDNA we used quantitative real-time PCR (qPCR) with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with a cycle of 2 min 98°C, 50 cycles at 5s 95°C, 10s 60°C, followed by 5s 65°C and 5s 95°C and a cycle of 2 min 95°C, 42 cycles at 15s 95°C, 15s 56°C, 15s 72°C, followed by 30s 95°C, 5s 60°C and 5s 95°C. The Primer sequences we used are listed in Table S1. To normalize the mRNA of genes of interest, we used the housekeeping gene HPRT (Hypoxanthine Guanine Phosphoribosyl Transferase). To calculate the mRNA expression levels we used ΔΔCt-method: First, we determined the difference of Ct-value of the analyzed gene and the reference gene Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) (ΔCt = Ct (target gene) – Ct (reference gene)). Next, we determined the difference of the ΔCt-value of the analyzed gene and the mean of ΔCt-values of the control group (either unstimulated PBMCs from control children or PBMCs of control children or unstimulated murine lung cells) to determine ΔΔCt (ΔΔCt = ΔCt (treatment) – Mean ΔCt (control)). Gene expression then is showed as n-fold expression ($E = 2^{-\Delta\Delta Ct}$) (Control group = 1, therefore for the treatment value < 1 means decreased and value > 1 means increased).

FEV1

FEV1 and FVC were measured at Baseline visit (B0) by using spirometry. After a period of normal breathing the participant should inhale maximally, directly followed by maximal and fast exhalation. The volume exhaled in one second is FEV1. The total exhaled volume is FVC. The ratio FEV1/FVC is known as Tiffenau Index.

Rhinovirus detection in nasopharyngeal fluid (NPF)

The Department of Virology, University of Turku (Finland) performed the detection of rhinovirus (RV-A, RV-B, RV-C) in nasopharyngeal swab obtained from the children as described in detail previously [1].

ELISA

To detect human IFN-alpha levels we used VeriKine^{TM} Human IFN Alpha ELISA Kit and VeriKine-HS^{TM} Human Interferon Alpha All Subtype TCM ELISA Kit (Pestka Biomedical Laboratories, New Jersey, USA). For human IL-28B/29 levels we used DuoSet ELISA Development System (R&D, Minneapolis, USA). To detect murine IFN-alpha level in cell culture supernatants we used mouse IFN alpha Platinum ELISA Kit (Affymetrix eBioscience, Vienna, Austria). Each Elisa was performed according to the manufacturer´s protocol.

IFN-α, IL-27, IFN-γ Milliplex analysis in PBMCs supernatants

IFN-α, IFN-γ, IL-27 analysis was performed by using Milliplex MAP kits: >22-plex human cyto/Th17 Magnetic Bead Panel (EMD, Millipore). This is a Luminex x-MAP technology (x = analyte, MAP = multi-analyte profiling) - based 22-plex human cyto/ Th17 Magnetic Bead Panel (from EMD, Millipore). The samples were thawed and the procedure followed the manufacturer's instructions for the quantitative assessment of the cell culture supernatants after overnight incubation period (12h). Analysis was done using Luminex 200 machine. Concentration of the proteins in samples was expressed as pg/ml.

RV detection

To verify the RV1b infection we performed a PCR with primers designed to amplify the RV1b RNA, as previously described [2]. The Primers OL26 and OL27 (see **Table S1**) were used to

detect RV1b and mβ-Actin as housekeeping gene. For further analyses and quantification, we used QIAxcel Advanced System (Quiagen, Hilden, Germany).

R848

R848 (InvivoGen, Toulouse, France) is very specific for TLR7 and TLR8, CAS number: 144875-48-9 (free base). The company: 'R848 (Resiquimod) is an imidazoquinoline compound with potent anti-viral activity. This low molecular weight synthetic molecule activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway [3, 4].

Recently, R848 was shown to trigger NF-κB activation in cells expressing murine TLR8 when combined with poly(dT) [5].

Unlike other commercially available R848 preparations, InvivoGen's R848 is water soluble, validated for TLR7/8 potency and tested to ensure the absence of TLR2 or TLR4 contamination. It has a vaccine grade.

Supplementary Figure Legend

Figure S1. IFN-α and IFNAR1 regulation in asthmatic children

A. IFN signaling pathways. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com. **B.** IFN-alpha levels in the supernatant of PBMCs of healthy (CN) and asthmatic (A) PREDICTA children after *in vitro* cell culture with RV or R848 at F4 (CN: UNST n=14, RV n=14, R848 n=14; A: UNST n=11, RV n=11, R848 n=11; $p_{(CN\ UNST - CN\ R848)} = 0.0004$; $p_{(CN\ RV - CN\ R848)} = 0.0061$; $p_{(A\ UNST - A\ R848)} = 0.0107$; $p_{(A\ RV - A\ R848)} = 0.0107$). **C.** IFN–α levels in serum of healthy (CN) and asthmatic (A) AGENDAS children (CN n=6, A n=4, $p_{(CN - A)} = 0.0143$). **D.** IFN-α mRNA expression in PBMCs of healthy (CN) and asthmatic (A) AGENDAS children (CN n=5, A n=5). E. IFNAR1 mRNA expression in PBMCs of healthy (CN) and asthmatic (A) AGENDAS children (CN n=6, A n=5).

Figure S2. FACS analysis of dissociated murine lung cells

A. Annexin V and PI (Propidium Iodide) FACS staining of isolated total lung cells. **B**. Gating strategy for FACS analysis of total lung cells

Supplementary Tables

Table S1: Primers used for quantitative real-time PCR

Gene	Primer sequences
hHPRT	Fwd: 5´TGA CAC TGG CAA AAC AAT GCA 3´
	Rev: 5´GGT CCT TTT CAC CAG CAA GCT 3´
hIFNAR1	Fwd: 5´ACA CCA TTT CGA AAA GCT CA 3´
	Rev: 5´CCA TCC AAA GCC CAC ATA ACA C 3´
hIFNalpha	Fwd: 5´AGG AGT TTG ATG GCA ACC AG 3´
	Rev: 5´CTC TCC TCC TGC ATC ACA CA 3´
hTYK2	Fwd: 5´CC CCT GGA GAT CTG CTT TG 3´
	Rev: 5´TCT GGG TTG GCT CAT AGG TC 3´
hIFNLR	Fwd: 5´ACA ACA AGT TCA AGG GAC GC 3´
	Rev: 5´GCC GGC TCC ACT TCA AAA AG 3´
hSTAT2	Fwd: 5´TCC CAG GAT CCT ACC CAG TT 3´
	Rev: 5´TGG CTC TCC ACA GGT GTT TC 3´
hSTAT1A	Fwd: 5´GTG TCT GAA GTT CAC CCT TCT 3´
	Rev: 5´AAG GAA AAC TGT CGC CAG AGA 3´
hSTAT1B	Fwd: 5´CGG AGG CGA ACC TGA CTT 3´
	Rev: 5´TGT CAC TCT TCT GTG TTC ACT T 3´
RV1b (OL26/27)	Fwd: 5´GCA CTT CTG TTT CCC C 3´
	Rev: 5´CGG ACA CCC AAA GTA G 3´
mHPRT	Fwd: 5´GCC CCA AAA TGG TTA AGG TT 3´
	Rev: 5´TTG CGC TCA TCT TAG GCT TT 3´
mß-Actin	Fwd: 5´TGT TAC CAA CTG GGA CGA CA 3´
	Rev: 5´GGG GTG TTG AAG GTC TCA AA 3´
mTYK2	Fwd: 5´TCT TGG ACT CTG CCT CGT TT 3´
	Rev: 5´TGA GAG CAA GGT GAC ACA GG 3´
mIFNLRA	mIFNλRα QuantiTect Primer Assay (Cat. No: QT00494137) (Qiagen, Venlo, Netherlands)

Table S2: Antibodies used for Flow cytometric analysis and cellular staining

Antibody	Clone	Company	Order No.
Anti-CD4 BV421	RM 4-5	BD	740007
Anti-CD4 PerCP-Cy5.5	RM 4-5	BD	550954
Anti-CD8a V500	53-6.7	BD	560776
Anti-CD8a PE Vio770	REA601	Miltenyi	130-119-123
Anti-IFNAR1 APC	MAR1-5A3	Biolegend	127313
Anti-CD25 BV421	PC61	Biolegend	102033
Anti-Foxp3 APC	3G3	Miltenyi	130-113-470

Reagent	Company	Order No.
Propidium Iodide Staining Solution	BD	556463
APC Annexin V	BD	550474

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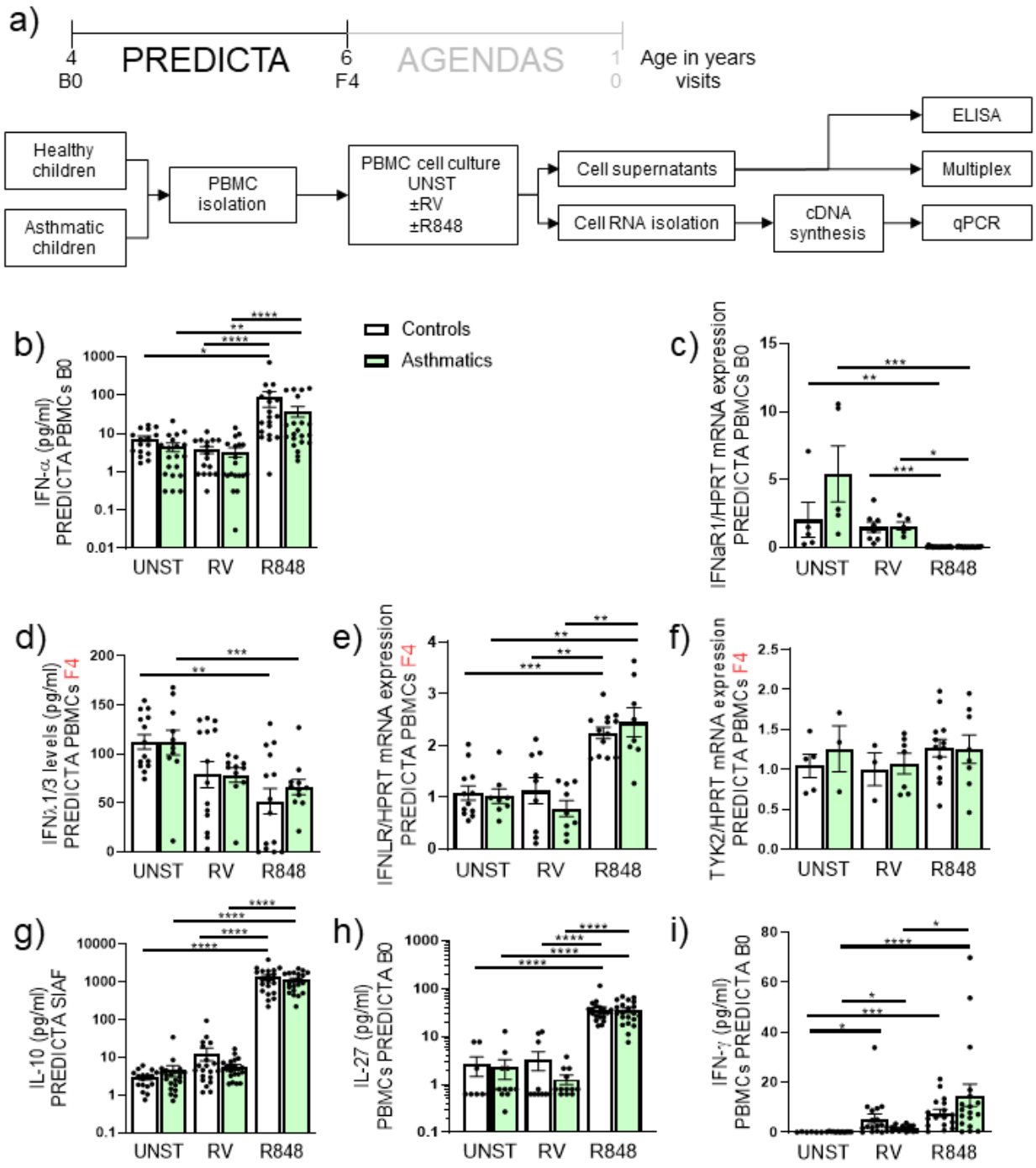

a)
4
B0
PREDICTA
6
F4
AGENDAS
1
0
Age in years
visits
Healthy children
Asthmatic children
PBMC isolation
PBMC cell culture
UNST
±RV
±R848
Cell supernatants
Cell RNA isolation
cDNA synthesis
ELISA
Multiplex
qPCR
b)
IFN-α (pg/ml)
PREDICTA PBMCs B0
Controls
Asthmatics
UNST
RV
R848
c)
IFNaR1/HPRT mRNA expression
PREDICTA PBMCs B0
d)
IFNλ1/3 levels (pg/ml)
PREDICTA PBMCs F4
e)
IFNLR/HPRT mRNA expression
PREDICTA PBMCs F4
f)
TYK2/HPRT mRNA expression
PREDICTA PBMCs F4
g)
IL-10 (pg/ml)
PREDICTA SIAF
h)
IL-27 (pg/ml)
PBMCs PREDICTA B0
i)
IFN-γ (pg/ml)
PBMCs PREDICTA B0

Figure 1

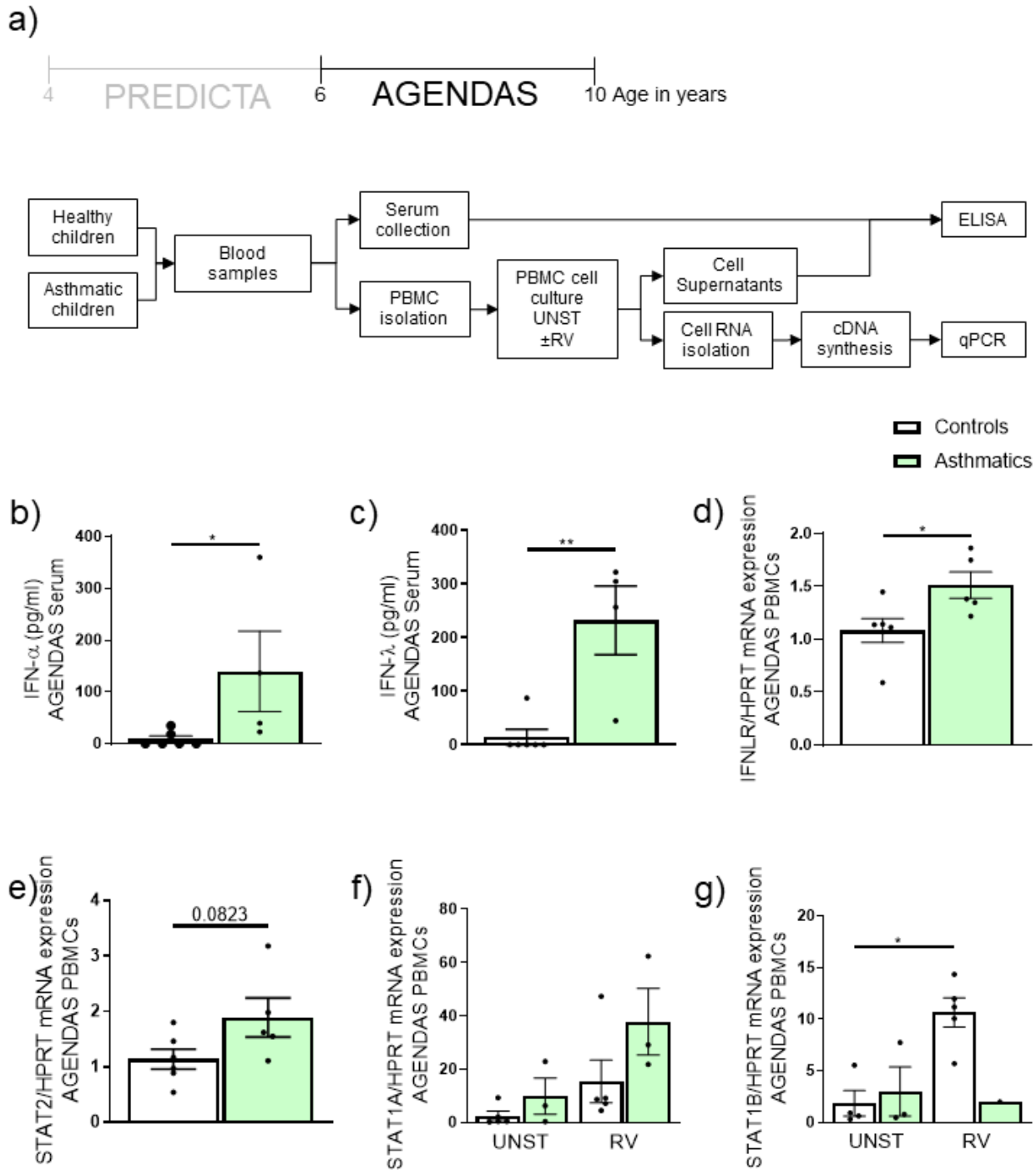

a)
4
PREDICTA
6
AGENDAS
10 Age in years
Healthy children
Asthmatic children
Blood samples
Serum collection
PBMC isolation
PBMC cell culture UNST ±RV
Cell Supernatants
Cell RNA isolation
cDNA synthesis
ELISA
qPCR
Controls
Asthmatics
b)
IFN-α (pg/ml) AGENDAS Serum
c)
IFN-λ (pg/ml) AGENDAS Serum
d)
IFNLR/HPRT mRNA expression AGENDAS PBMCs
e)
STAT2/HPRT mRNA expression AGENDAS PBMCs
0.0823
f)
STAT1A/HPRT mRNA expression AGENDAS PBMCs
UNST
RV
g)
STAT1B/HPRT mRNA expression AGENDAS PBMCs
UNST
RV

Figure 2

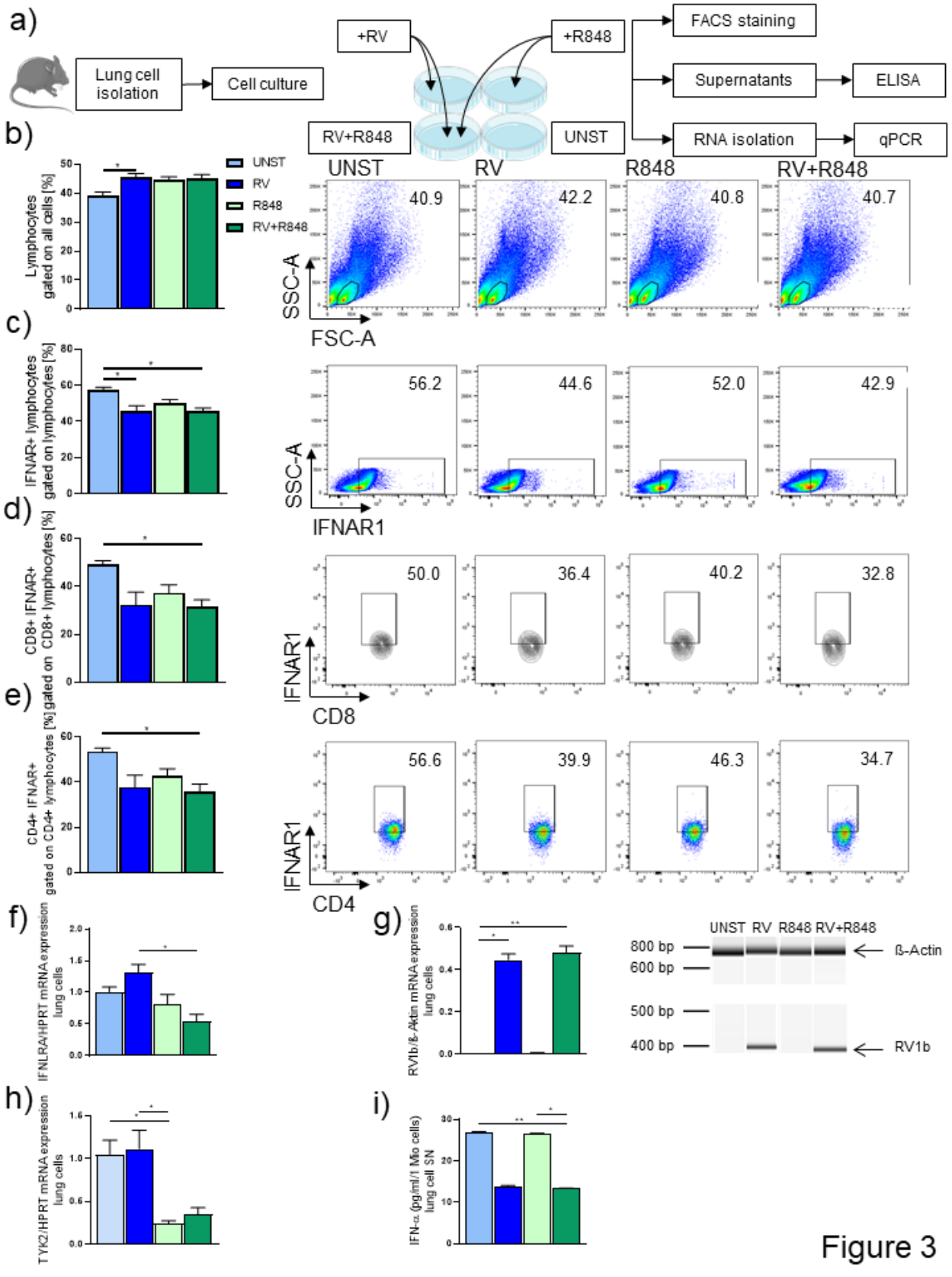
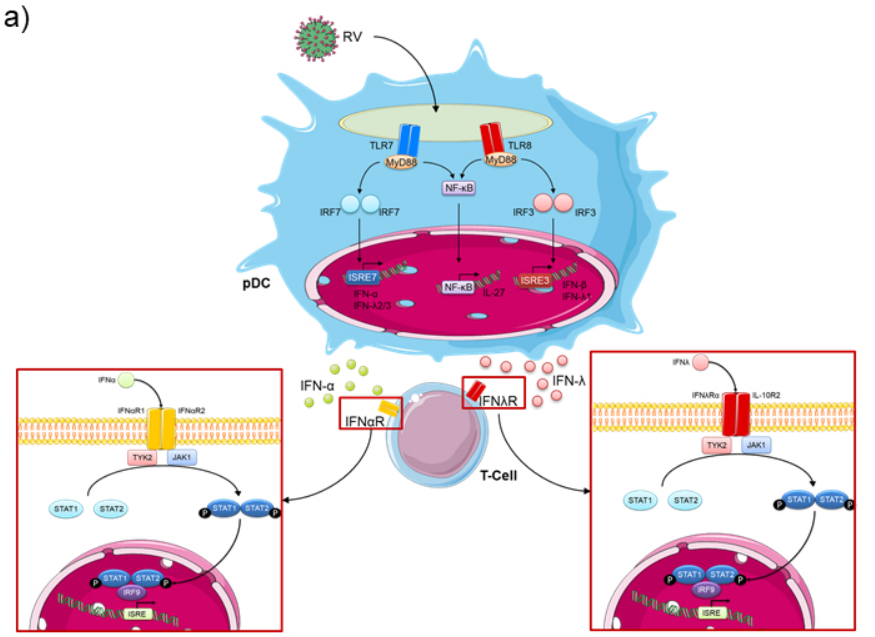
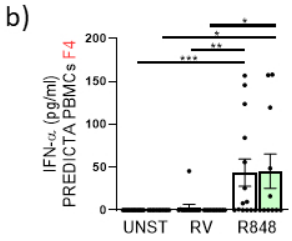
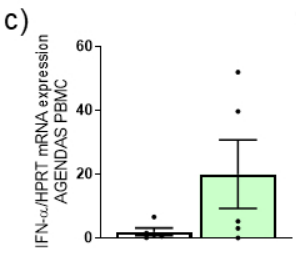
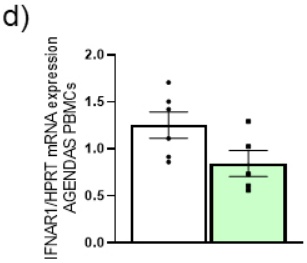

a)
Lung cell isolation
Cell culture
+RV
+R848
RV+R848
UNST
FACS staining
Supernatants
ELISA
RNA isolation
qPCR
b)
Lymphocytes gated on all cells [%]
UNST
RV
R848
RV+R848
UNST
RV
R848
RV+R848
40.9
42.2
40.8
40.7
SSC-A
FSC-A
c)
IFNAR+ lymphocytes gated on lymphocytes [%]
56.2
44.6
52.0
42.9
SSC-A
IFNAR1
d)
CD8+ IFNAR+ gated on CD8+ lymphocytes [%]
50.0
36.4
40.2
32.8
IFNAR1
CD8
e)
CD4+ IFNAR+ gated on CD4+ lymphocytes [%]
56.6
39.9
46.3
34.7
IFNAR1
CD4
f)
IFNLRA/HPRT mRNA expression lung cells
g)
RV1b/ß-Aktin mRNA expression lung cells
UNST RV R848 RV+R848
800 bp
600 bp
500 bp
400 bp
ß-Actin
RV1b
h)
TYK2/HPRT mRNA expression lung cells
i)
IFN-α (pg/ml/1 Mio cells) lung cell SN

Figure 3

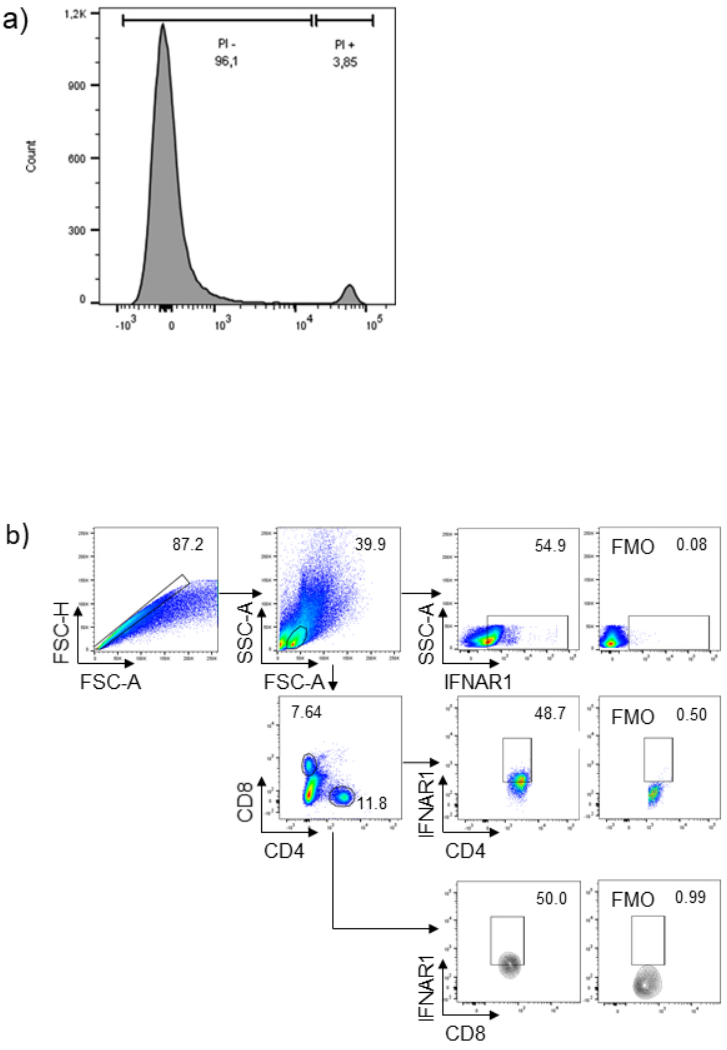








Suppl. Figure S1



Suppl. Figure S2