

IL-33 augments the effect of rhinovirus HRV16 on inflammatory activity of human lung vascular endothelium – possible implications for rhinoviral asthma exacerbations

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To the Editor,

Epithelium-derived cytokines such as IL-33 and IL-25 are believed to be the key regulatory factors of immune pathogenic mechanisms of bronchial asthma, mainly involving type 2 inflammatory responses.¹ Human rhinovirus (HRV) that belongs to the *Picornaviridae* family and is a non-enveloped virus with a single stranded, positive-sense RNA genome, is responsible for 60 to 80% of virus-associated asthma exacerbations.^{2,3} Vascular endothelium is a semi-permeable barrier that regulates the flow of nutrients, ions, cytokines and immune cells between blood and tissues. As a source of inflammatory mediators, it plays a crucial role in driving local immune responses in physiological and pathological circumstances.^{4,5} Recently, we have found that HRV16 may infect human lung vascular endothelium *via* ICAM-1 entry receptor, activate receptors of innate immunity and subsequent endothelial inflammatory response.⁶ It may also impair its barrier functions.⁷ The answer why rhinovirus is capable of inducing heavy exacerbations in asthmatics, whereas in healthy individuals it usually develops mild, upper respiratory infections, remains unclear. For this reason, we analyzed the effect of IL-33, the cytokine widely distributed in large amounts in airways of asthmatic individuals, on the HRV-induced inflammatory response in the human lung vascular endothelium.

We observed that HRV16 strongly increased the mRNA expression and the release of inflammatory cytokines, including IL-1 β , IL-6 and TNF- α , and chemokines such as RANTES, IP-10, MIP-1 α , MIP-1 β , IL-8 and eotaxin by the Human Microvascular Lung Endothelium (HMVEC-L) (Fig. 1A,B,C). Furthermore, it caused a strong production of growth factors: G-CSF, GM-CSF, FGF and VEGF (Fig. 1D). Expectedly, HRV16 enhanced both mRNA expression and protein release of type I interferons (IFN- β and IFN- λ) (Fig. 2A). Of note, it was able to increase certain T-type immunoregulatory cytokine synthesis: IL-12 and IFN- γ (T1), IL-4, IL-5 and IL-13 (T2), IL-17 (T17), but not IL-10 (Treg) (Fig. 2B). Of other cytokines, HRV16 increased production of IL-2, IL-9, IL-15 (Supp. Fig. 1). This data indicate that HRV is capable of strong inflammatory activation of the lung vascular endothelium.

In order to analyze the effect of IL-33 on the HRV16-induced inflammatory activation of the lung vascular endothelium, HMVEC-L were pre-incubated with IL-33 (1 and 10ng/ml) for 24 hours, and subsequently exposed to HRV16 (MOI3). Of inflammatory cytokines, IL-33 increased the HRV16-induced release of IL-1 β and IL-6. Interestingly, it did not affect TNF- α production. (Fig. 1A) Of

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chemokines, IL-33 enhanced HRV16-induced production of IL-8, eotaxin, MIP-1 α , but not MIP-1 β , MCP-1, RANTES, IP-10 (Fig. 1A,B). Among growth factors, IL-33 caused a robust enhancement of HRV16-induced G-CSF, GM-CSF release, whereas it slightly increased FGF and VEGF production (Fig. 1C). Finally, of T-type immunoregulatory cytokines, IL-33 up-regulated HRV16-induced release of IL-12 (but not IFN- γ) (T1), IL-4, IL-5 and IL-13 (T2), IL-17 (T17), and IL-10 (Treg); however, in small concentrations (Fig. 2B). Importantly, IL-33 enhanced neither IFN- β , nor IFN- λ release (Fig. 2A). IL-33 alone increased the release of the following cytokines: RANTES, IP-10, IL-6, TNF- α , IL-1 β , IL-1R α , G-CSF, GM-CSF, VEGF, IL-12, IL-4, IL-5, IL-13, IL-10, IL-9, but not interferons.

These data made us ask the question about the possible mechanism of the IL-33-driven enhancement of HRV16-induced inflammatory activation of the lung vascular endothelium. We observed that IL-33 increased the capture of HRV16 particles possibly associated with the increase of ICAM-1 entry receptor expression. (Fig. 2C,D,E,F) Furthermore, IL-33 increased the expression of TLR3 and TLR7 (innate immune receptors responsible for sensing viral RNA) as well as IRF3 and IRF7 expression - intracellular signal transmitters from TLRs, leading to the enhanced production of inflammatory cytokines described above (Fig. 2G,H). This may mean that IL-33 might increase the capture of the viral particles by ICAM-1 and enhance the efficacy of sensing the viral RNA by TLRs, thus conditioning endothelial cells for more intense inflammatory, but not interferon, response towards virus.

Considering the fact that asthmatics express IL-33 in larger amounts in airways as compared to non-asthmatic, healthy individuals, we postulate that heavy asthma exacerbations evoked by rhinovirus may be driven by the presence of IL-33 in the airways. IL-33 itself may induce plenty of inflammatory cytokines, chemokines and growth factors that drive asthmatic inflammation by the lung vascular endothelium. Furthermore, our data indicates that during HRV infection, IL-33 may facilitate the higher viral capture (by ICAM-1 increased expression) by the lung vascular endothelium of asthmatics, thus intensifying endothelium-orchestrated inflammation (by IL-1 β , IL-6), promote growth (by G-CAS, GM-CSF) and recruitment of mostly innate immune cells: neutrophils, eosinophils and macrophages (by IL-8, eotaxin, MIP- α). Furthermore, it may enhance mucus production, bronchial hyperresponsiveness, goblet cells hyperplasia (by IL-13), fibrosis and angiogenesis (by FGF, VEGF). Last but not least, IL-33 may enhance HRV-induced T2-type immune responses observed in asthma exacerbations, including IgE production, eosinophil maturation and recruitment (by IL-4, IL-5). At the same time, our data suggests that IL-33 does not enhance parallel anti-viral responses, as it does not increase levels of HRV-induced cytokines that induce intracellular INF-dependent antiviral responses (no IFN- β , IFN- λ , TNF- α increase), NK cell recruitment and activation (no RANTES, IP-10,

MIP-1 β increase) and T cell activity (no IL-2, IL-9, IL-7, RANTES, IP-10 and MCP-1 increase) (comprehensively shown in the Supp. Fig. 2).

Indeed, the insufficient IFN-dependant responses in asthmatics are observed and attributed to heavier course of airway viral infections.⁸ In asthmatics, IL-33 may facilitate higher viral load in the lung vascular endothelium, while IL-33-orchestrated cytokine milieu may enhance innate inflammatory responses without any concomitant increase of antiviral innate and adaptive mechanisms. Overall, our data may open the new field of research on the interactions between airway epithelium and lung vascular endothelium in the pathophysiology of viral airway infections and asthma exacerbations and overall immunopathology of asthma.

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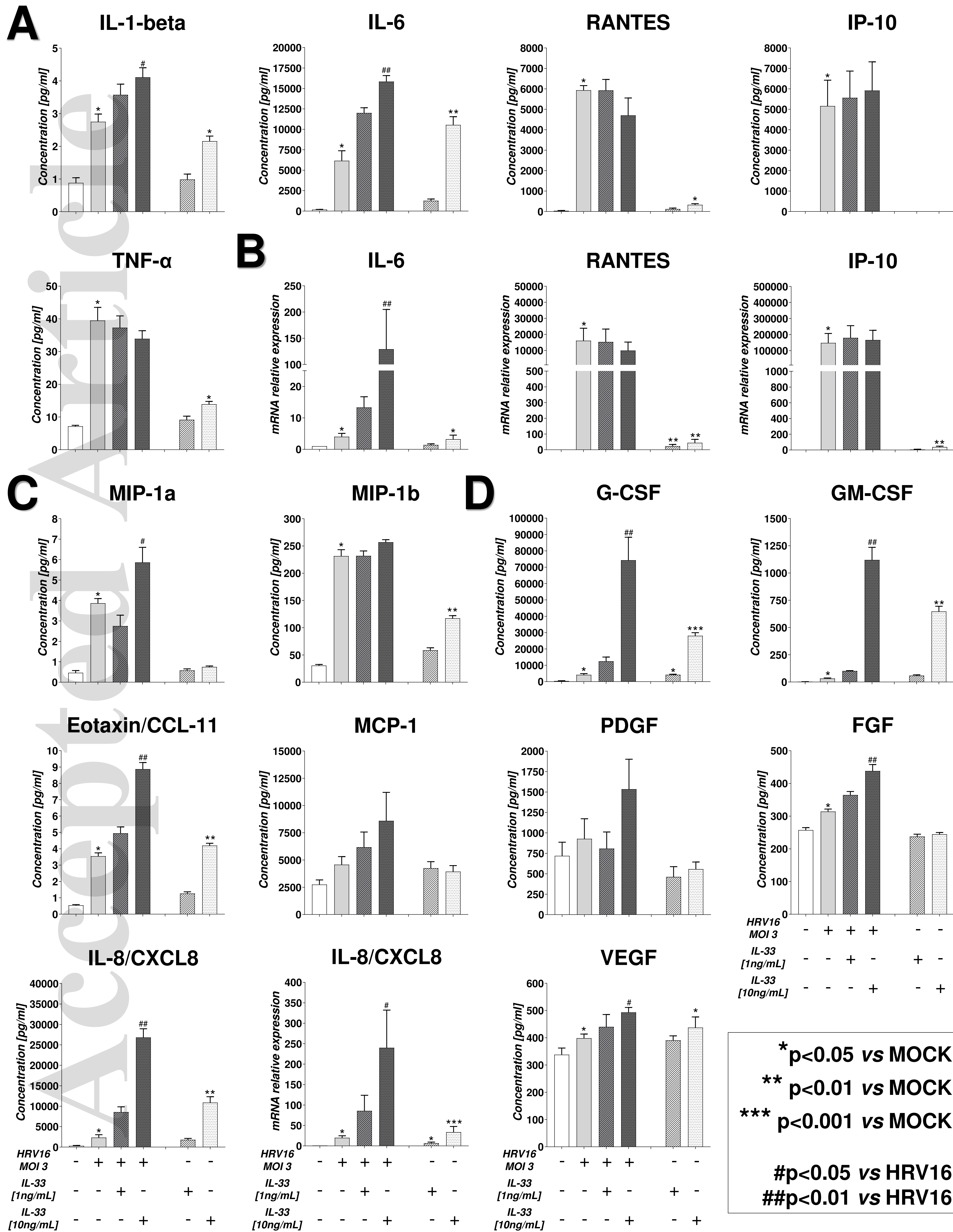
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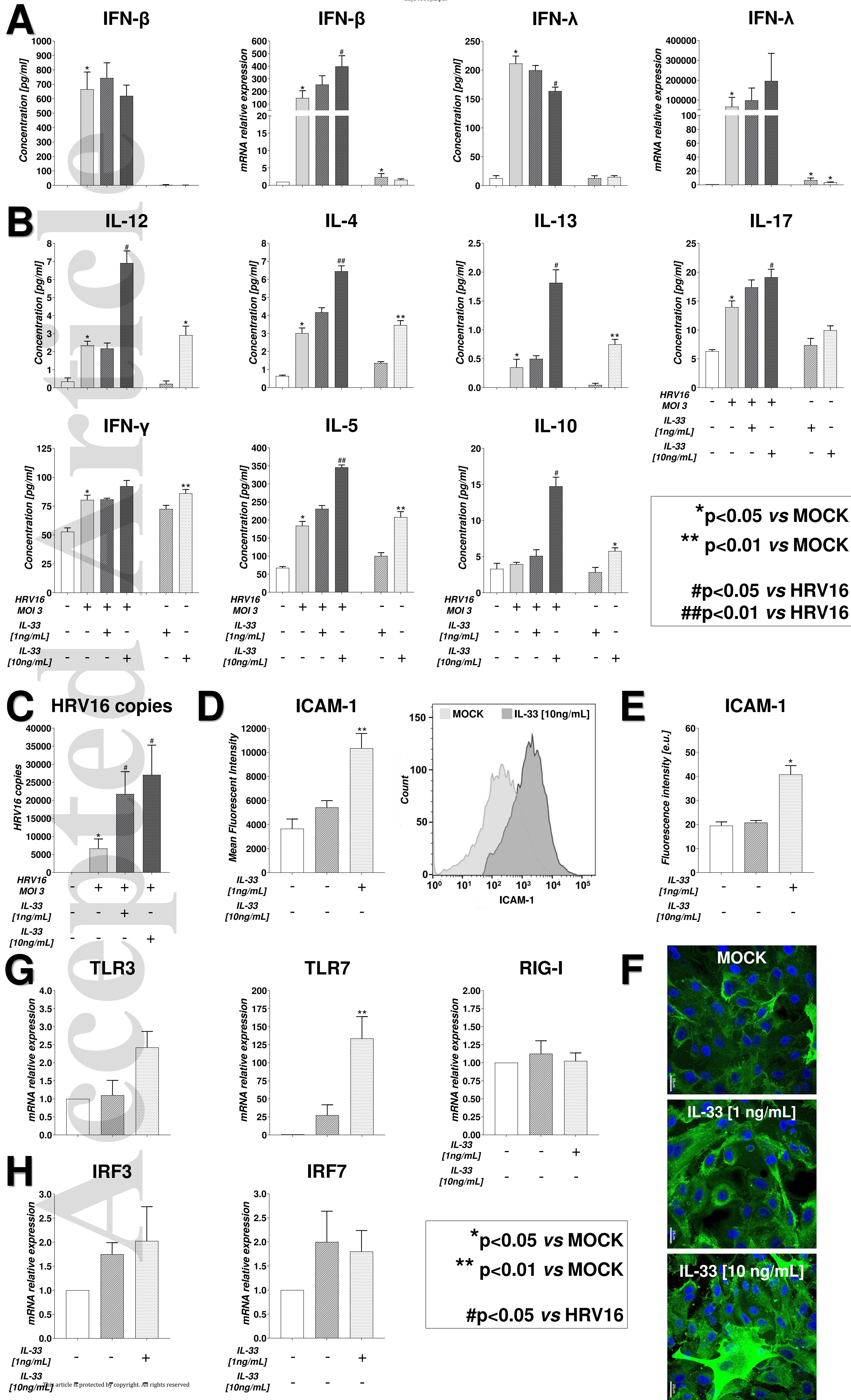
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Figures

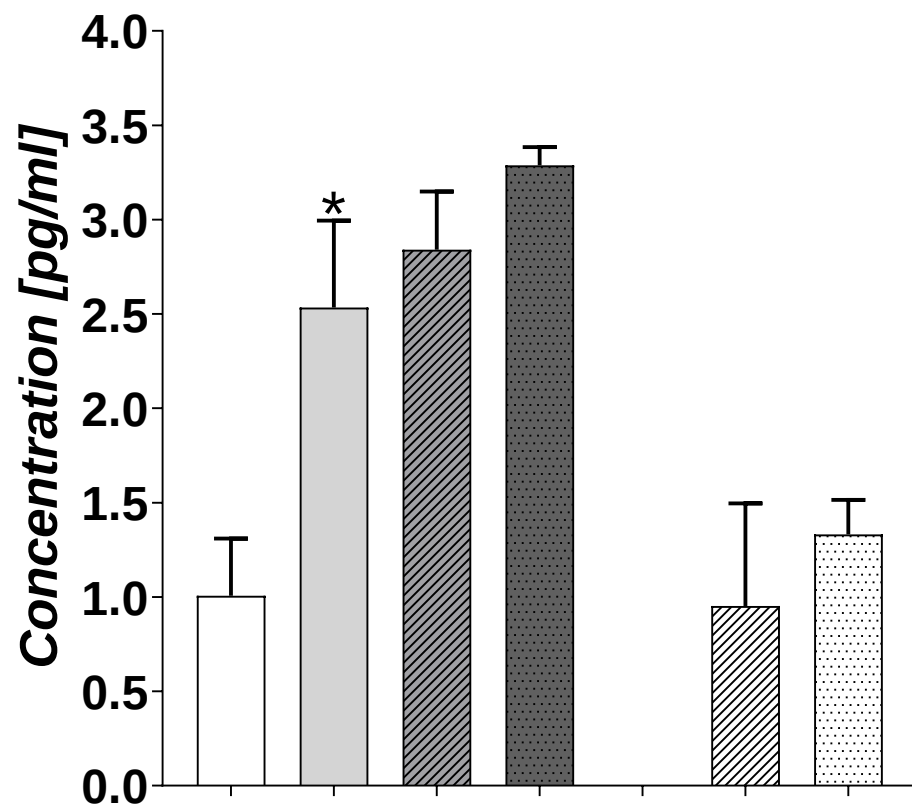
Figure 1. Cytokine mRNA expression in cells and cytokine concentrations in supernatants from HMVEC-L incubated with HRV16 (MOI3) alone or upon cell 24-hour pre-stimulation with IL-33 (1 or 10ng/ml) or stimulated only with IL-33 (1 or 10ng/ml). Inflammatory cytokines concentrations (A) and mRNA expression (B), chemokines (A), and growth factors (D) concentrations; The Kruskal-Wallis test with Dunn's multiple comparison test were used to analyze differences between groups. Data are representatives of four independent experiments as means $\pm$ SEM; (n=4-6); (BioPlex), *p<0.05, **p<0.01, and ***p<0.001 as compared to Mock cells, #p<0.05 and ##p<0.01 as compared to HRV16-induced cells;

Figure 2. Cytokine mRNA expression in cells and cytokine concentrations in supernatants from HMVEC-L incubated with HRV16 (MOI3) alone or upon cell 24-hour pre-stimulation with IL-33 (1 or 10ng/ml) or stimulated only with IL-33 (1 or 10ng/ml). Interferons (A) and immunoregulatory cytokines (B). The effect of HMVEC-L pre-stimulation with IL-33 (1 and 10ng/ml) on HRV16 copies (C), ICAM-1 surface expression (FACS: D), (confocal microscope: E,F). The effect of IL-33 (1 and 10ng/ml) on TLR3, TLR7, RIG-I (G) and IRF3, IRF7 (H) mRNA expression in HMVEC-L. The Kruskal-Wallis test with Dunn's multiple comparison test were used to analyze differences between groups. (real time PCR, (BioPlex), flow cytometry [ICAM-1-FITC Abs], confocal microscope [green – ICAM-1-FITC, blue nuclei– DAPI]). Data are representatives of four independent experiments as means $\pm$ SEM; (n=4-6); (BioPlex), *p<0.05, **p<0.01, and ***p<0.001 as compared to Mock cells, #p<0.05 and ##p<0.01.

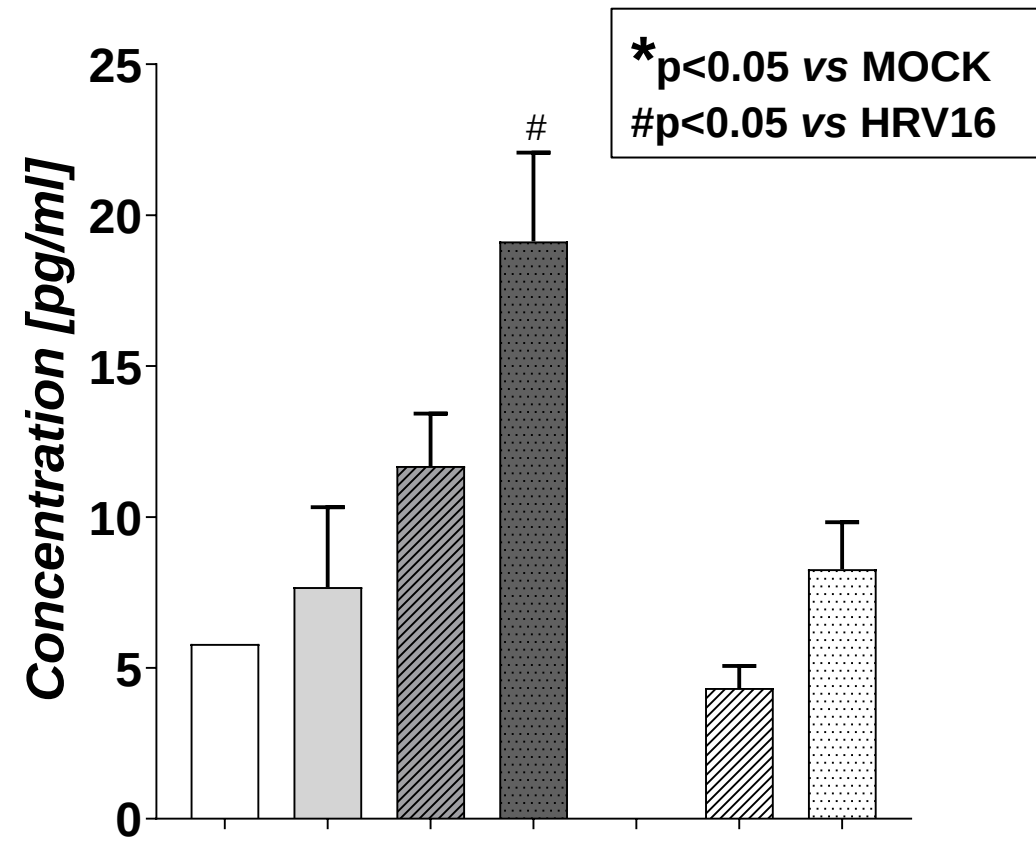




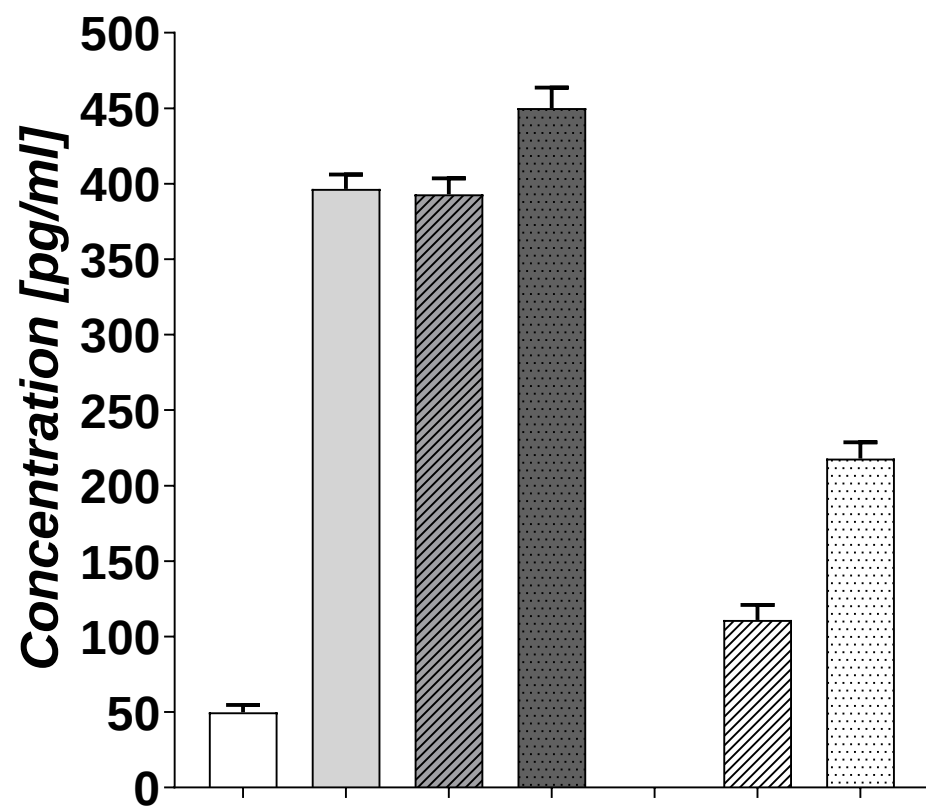
IL-2



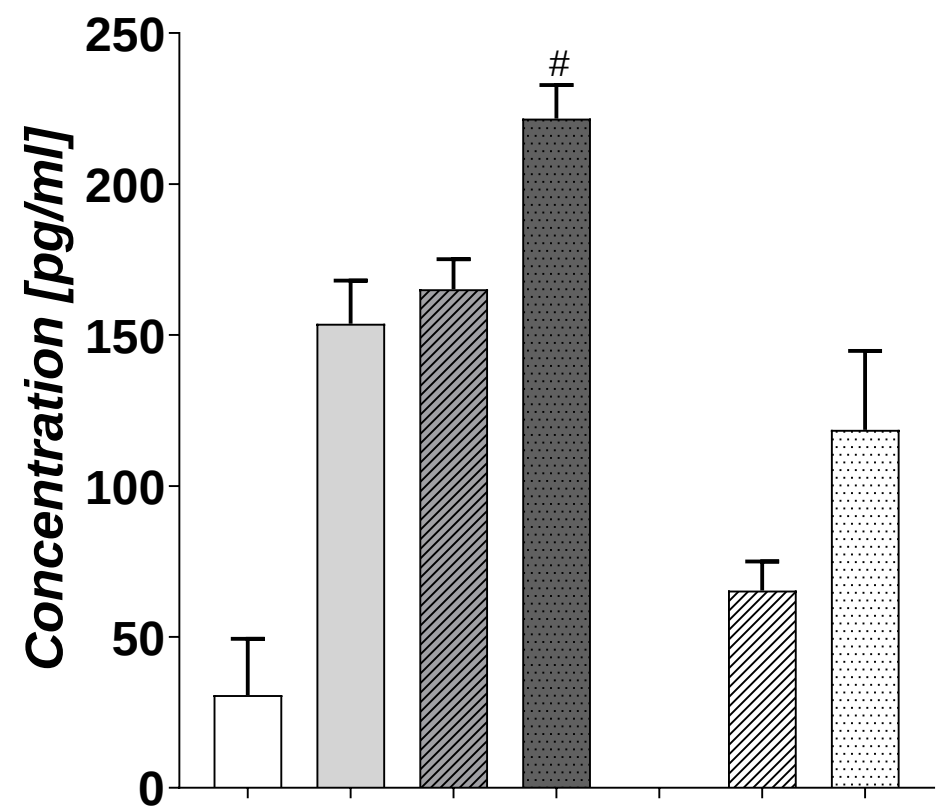
IL-7



IL-9



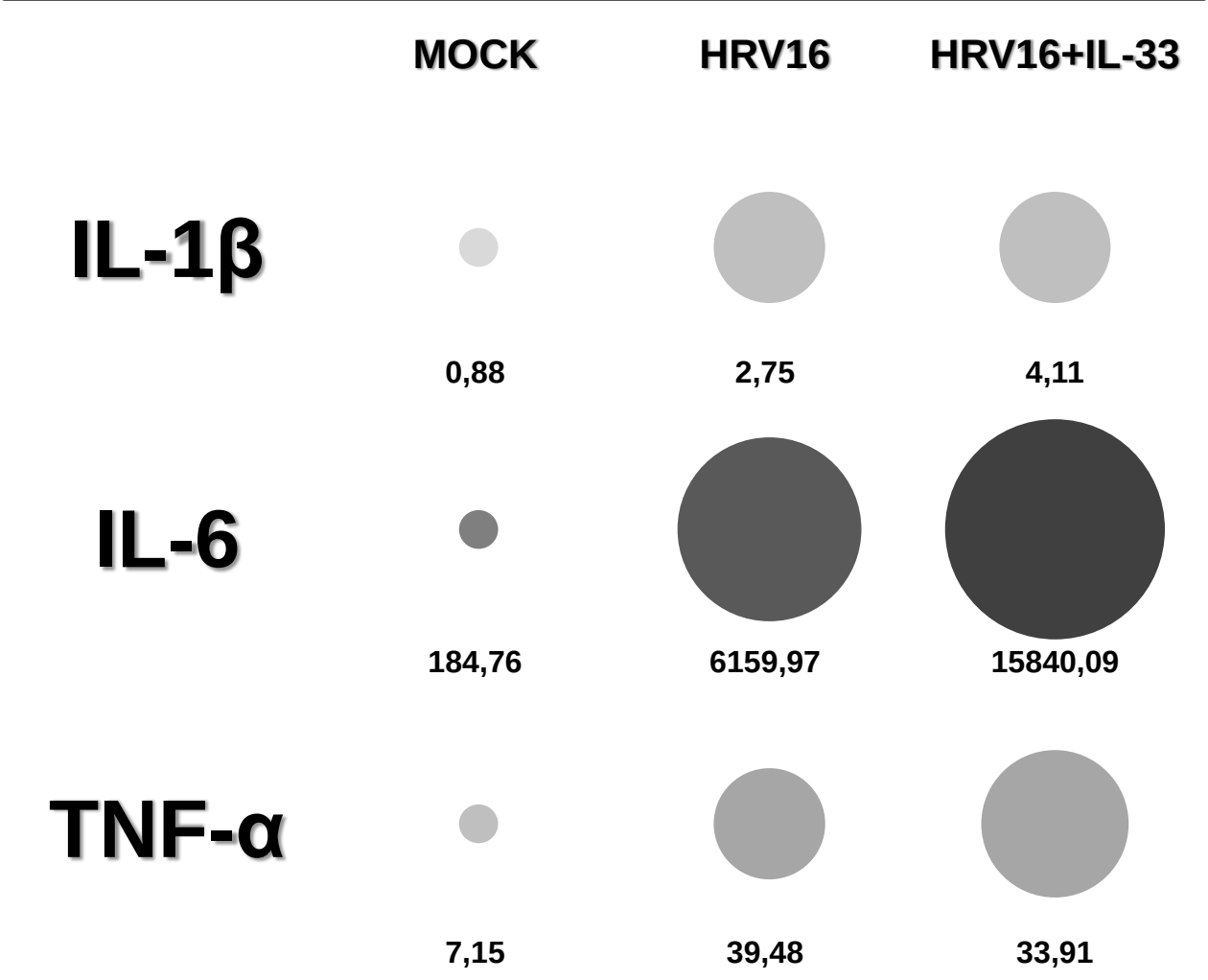
IL-15



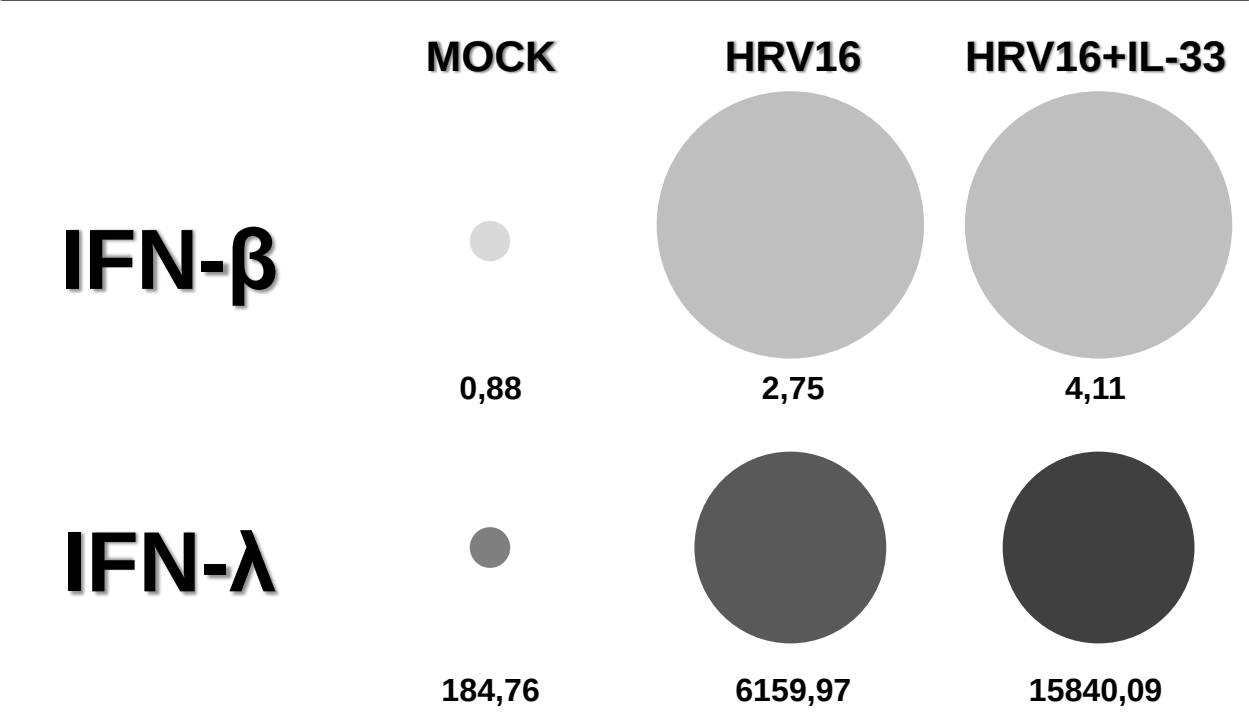
HRV16 MOI 3	-	+	+	+	-	-
IL-33 [1ng/mL]	-	-	+	-	+	-
IL-33 [10ng/mL]	-	-	-	+	-	+

HRV16 MOI 3	-	+	+	+	-	-
IL-33 [1ng/mL]	-	-	+	-	+	-
IL-33 [10ng/mL]	-	-	-	+	-	+

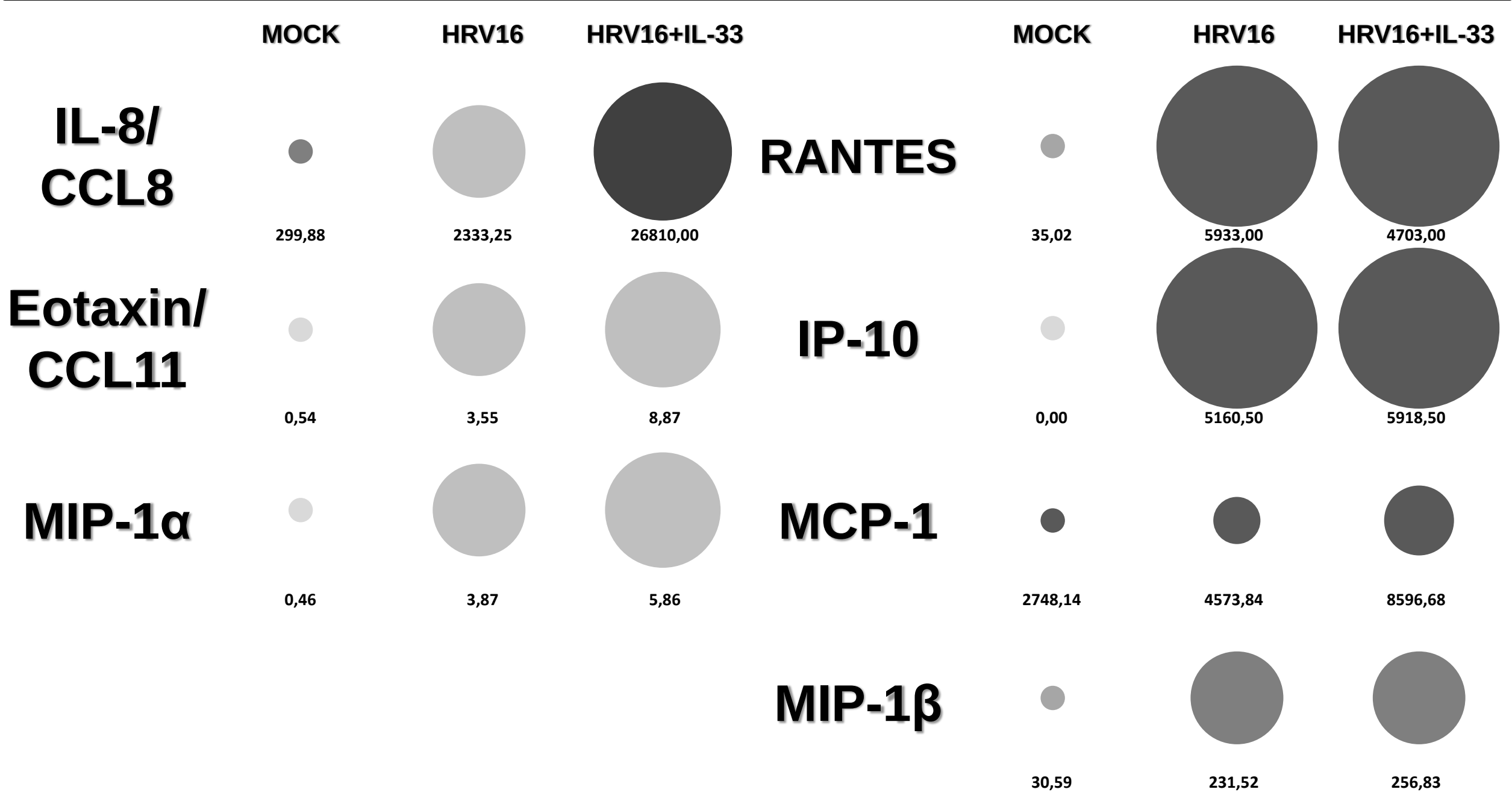
Pro-inflammatory



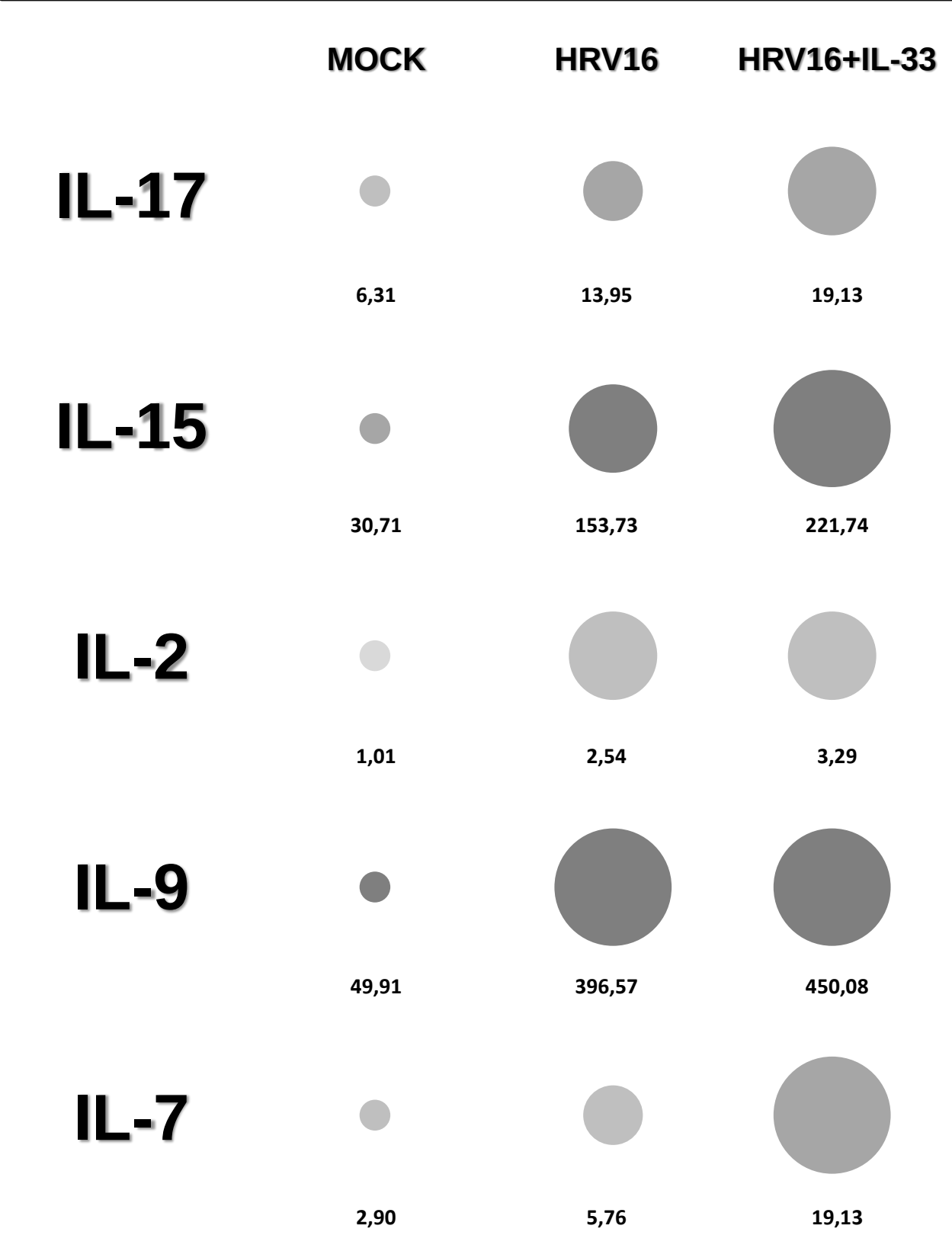
Antiviral



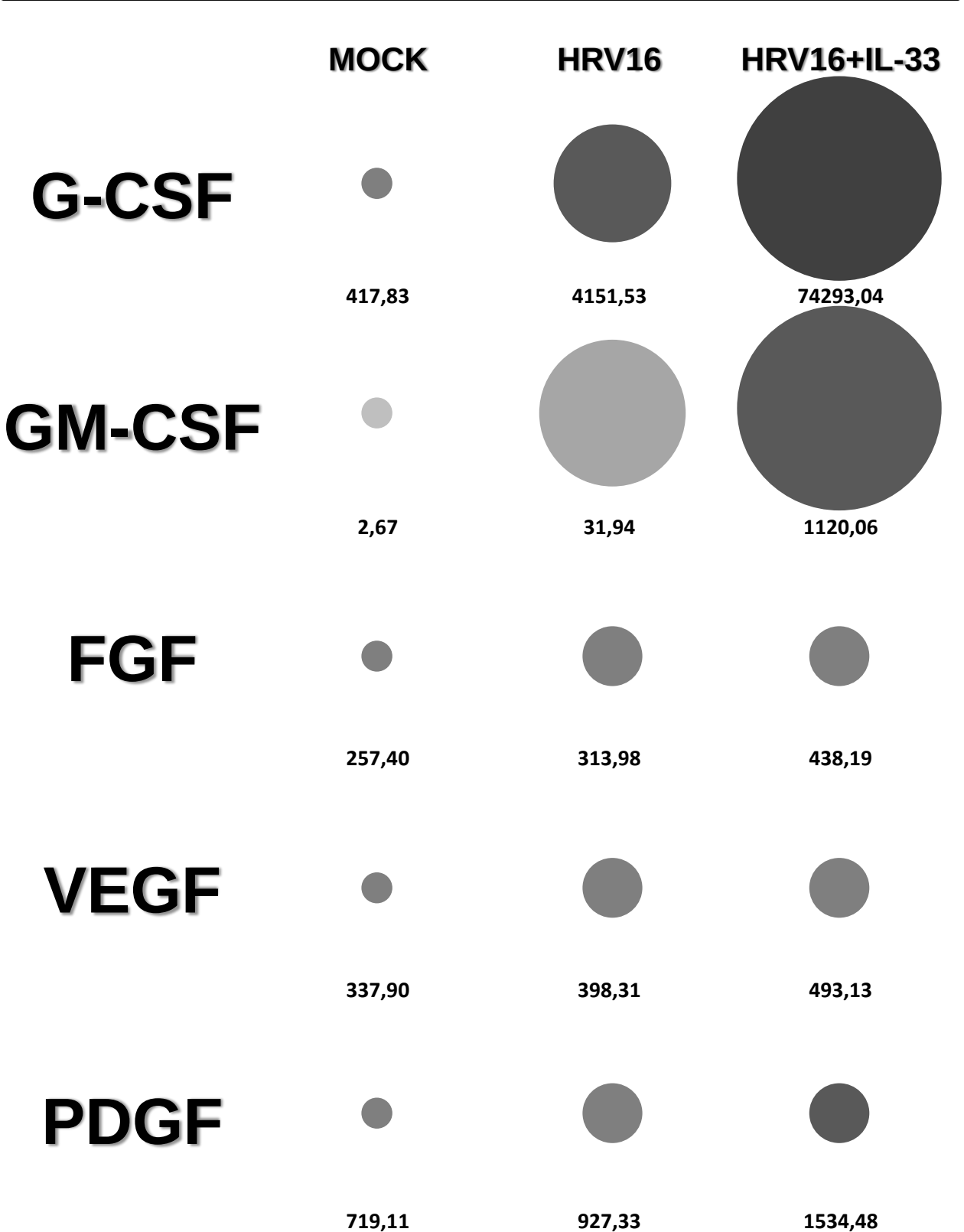
Chemokines



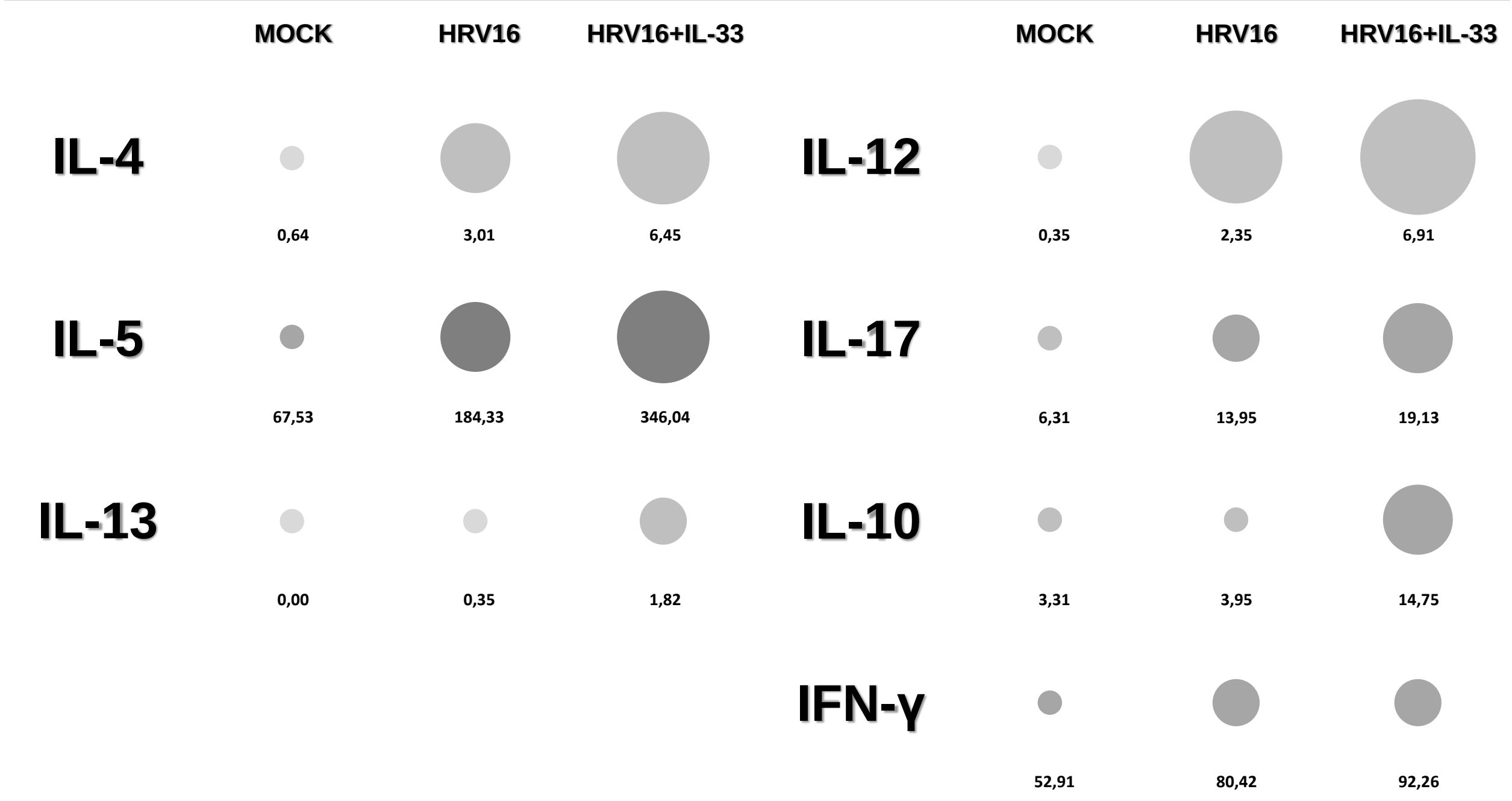
Other Cytokines



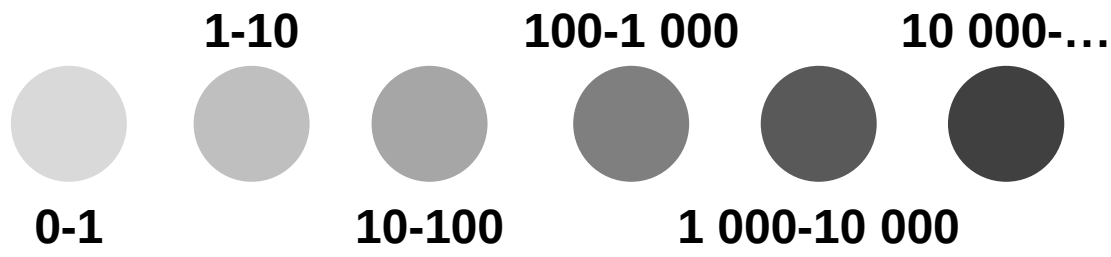
Growth Factors



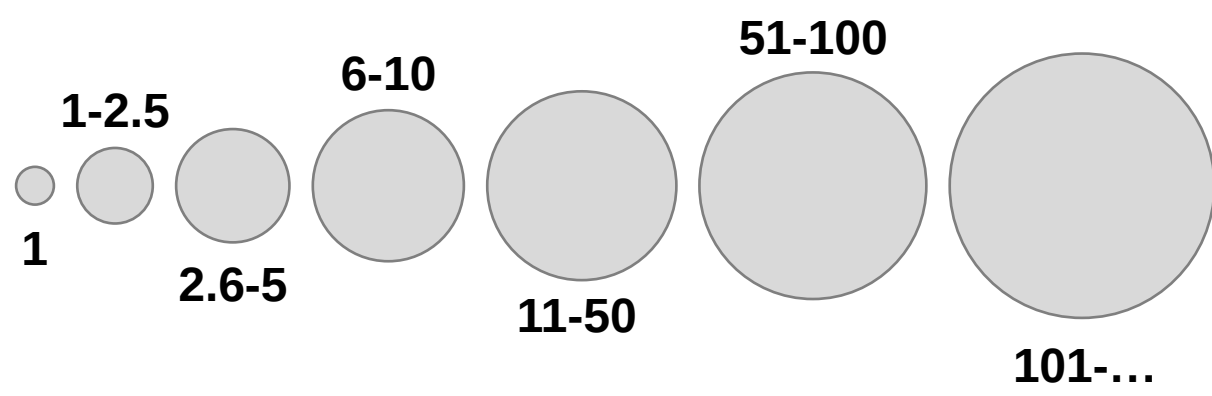
Immunomodulatory



Mean Value
Range



The scale of
multiples of
mock value



SUPPLEMENTARY

Material and methods

Cells

Human lung microvascular endothelial cells (HMVEC-L) (CC-2527, Lonza, Walkersville, MD, USA) were cultured in Endothelial Basal Medium-2 (EGM-2) (CC-3156, Lonza, Walkersville, MD, USA), supplemented with EGM-2MV Endothelial Growth Medium-2 MV SingleQuots (CC-4147, Lonza, Walkersville, MD, USA). HMVEC-L were cultured in an incubator at 37 °C, 5% CO₂ concentration (culture conditions). After obtaining 80-90% confluence cells were trypsinized with 0.05% trypsin and 0.02% EDTA (59417C, Sigma Aldrich, St. Louis, MO, USA) and after that neutralized by Trypsin Neutralizing Solution (CC-5002, Lonza, Walkersville, MD, USA). The cells were ready to perform experiments.

Infection of cells with HRV16 and HRV16 inactivation

Microvascular endothelial cells were incubated with human rhinovirus HRV16 (viral stocks were used at 1.26×10^7 TCID₅₀/mL), which was received in cooperation with the Department of Respiratory Medicine, Imperial College London, UK, courtesy of Professor Sebastian Johnston. (n=4-6) HMVEC-L monolayers were treated with HRV16 at a multiplicity of infection (MOI) of 3.0 or exposed to medium alone (negative control). The cells with the virus were then incubated for 3 hours at 37°C, 5% CO₂ concentration. After incubation, viral suspension was removed and the cells were washed three times with HEPES buffered saline solution (CC-5022, Lonza, Walkersville, MD, USA), fresh medium was added and cells were further cultured.

Isolation of mRNA and real-time polymerase chain reaction (RT-PCR)

mRNA was extracted from HMVEC-L using the RNAeasy Mini Kit (74106, Qiagen, Hilden, Germany) following the manufacturer's instruction. Subsequently, cDNA was synthesized by using the RevertAid H Minus first strand cDNA synthesis kit (K1632, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primers were used for IFN- β , IFN- λ , RANTES (CCL5), IP-10, IL-6, IL-8, TLR3, TLR7, RIG-I, IRF3 and IRF7 (Thermo Fisher Scientific, Waltham, MA, USA). mRNA expression was made using Step One Plus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) and results were normalized to GAPDH (Thermo Fisher Scientific, Waltham, MA, USA) as the

housekeeping gene. Quantitative reverse transcriptase real-time PCR (qRT-PCR), using the Genesig Kit (PrimerDesign, Chandler's Ford, UK) for detection and quantification of HRV16.

Protein concentration

Protein concentrations were measured using enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instruction. IFN- β (Human IFN-beta DuoSetELISA, Cat. no DY814, Assay range – 7.8 – 1000 pg/mL, R&D Systems, Minneapolis, MN, USA), RANTES (Human CCL5/RANTES DuoSet ELISA, Cat. no DY278, Assay range 7.8 – 1000 pg/mL, R&D Systems, Minneapolis, MN, USA) and IL-8/CXCL8 (Human IL-8/CXCL8 DuoSetELISA, Cat. no DY208, Assay range – 31.3 – 2 000 pg/mL, R&D Systems, Minneapolis, MN, USA). Protein concentrations were measured using Bio-Plex Pro Human Cytokine Assays (Cat. no M50-0KCAF0Y, Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction.

Targets	Assay working ranges [pg/mL]	Assay sensitivity [pg/mL]
IL-1 β	3.2 – 3261	0.6
IL-1R α	81.1 – 70487	8.5
IL-2	2.1 – 17772	1.6
IL-4	2.2 – 3467	0.7
IL-5	3.1 – 7380	0.6
IL-6	2.3 – 18880	2.6
IL-7	3.1 – 6001	1.1
IL-8	1.9 – 26403	1.0
IL-9	2.1 – 7989	2.5
IL-10	2.2 – 8840	0.3
IL-12 (p70)	3.3 – 13099	3.5
IL-13	3.7 – 3137	0.7
IL-15	2.1 – 2799	2.4
IL-17	4.9 – 12235	3.3
Eotaxin	40.09 – 5824	2.5
Basic FGF	27.2 – 7 581	1.9
G-CSF	2.4 – 11 565	1.7
GM-CSF	63.3 – 6 039	2.2
IFN- γ	92.6 – 52 719	6.4

IP-10	18.8 – 26 867	6.1
MCP-1	2.1 – 1 820	1.1
MIP-1 α	1.4 – 836	1.6
MIP-1 β	2.0 – 1 726	2.4
PDGF-BB	7.0 – 51 933	2.9
RANTES	2.2 – 8 67	1.8
TNF- α	5.8 – 95 484	6.0
VEGF	5.5 – 56 237	3.1

Confocal microscopy

HMVEC-L monolayers were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and then incubated with anti-ICAM-1 antibodies (1:200) (PE anti-human CD54, 322708, Biolegend, San Diego, CA, USA) over night at 4°C. Subsequently, the cells were washed three times in PBS and the cell nuclei was stained with DAPI (4',6-diamidino-2-phenylindole) (1:2000, 15 minutes at room temperature) (D1306, Invitrogen, Carlsbad, CA, USA). Leica TCS SP8 confocal microscope (Leica-Microsystems, Wetzlar, Germany) featuring 63x/1.40 lens (HC PL APO CS2 Oil Immersion) was used to evaluate the state of intercellular junctional proteins. For visualization of cells supercontinuum laser with 532 nm excitations and emission at 562-597 nm was used. For nuclei stained with DAPI, excitation and emission parameters were 405 nm and 460-480 nm, respectively. Leica software (Wetzlar, Germany) was used to analyze the data.

Analysis of ICAM-1 surface expression on HMVEC-Ls by flow cytometry

ICAM-1 surface expression in HMVEC-L was assessed in a Becton-Dickinson flow cytometer. For ICAM-1 analysis, anti-CD54-PE antibodies (PE antihuman CD54, 322708, Biolegend, San Diego, CA, USA) were used according to the manufacturer's protocol. PE IgG1, κ isotype (400114, Biolegend, San Diego, CA, USA) was used as an isotype control. All assessments were performed on BD LSRFortessa flow cytometer and data were analyzed using BD FACSDiva Software.

Statistical analysis

The results are presented as mean $\pm$ SD for variables with a normal distribution of values. The distribution of particular variables was verified by the Shapiro–Wilk W test, whereas Levene's was performed to test homogeneity of variances. **The Kruskal-Wallis test with**

74 Dunn's multiple comparison test were used to analyze differences between groups. Statistical
75 evaluations were performed using GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla,
76 CA, USA) software. Differences less than $p=0.05$ were regarded as significant.

Supplementary figures.

Supplementary figure 1. Immune cytokine concentrations in supernatants from HMVEC-L incubated with HRV16 (MOI3) alone or upon cell 24-hour pre-stimulation with IL-33 (1 or 10ng/ml) or stimulated only with IL-33 (1 or 10ng/ml). The Kruskal-Wallis test with Dunn's multiple comparison test were used to analyze differences between groups. Data are representatives of four independent experiments as means $\pm$ SEM; (n=4-6); (BioPlex) $p < 0.05$ as compared to Mock cells, # $p < 0.05$ as compared to HRV16-induced cells;

Supplementary figure 2. Comprehensive overview of concentration values and folds of increases of cytokines in supernatants from HMVEC-L incubated with HRV16 (MOI3) alone or upon cell 24-hour pre-stimulation with IL-33 (10ng/ml) reflecting the magnitude of the cytokine presence in the lung vascular endothelium-derived milieu. Data are representatives of four independent experiments as means $\pm$ SEM; (n=4-6), (BioPlex);