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B cell immune response to human bocaviruses

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Abstract

Background: Human bocaviruses (HBoVs) have been demonstrated in respiratory and gastrointestinal infections; however, the immune response to them has not been studied in detail. In this study, we investigated the B cell immune responses to HBoV1 and HBoV2, representing two different species of bocaviruses in humans.

Methods: We analyzed the effects of stimulations with HBoV1 and 2 virus-like particles (VLPs) and of co-stimulation with HBoV1-rhinovirus (RV) on cells of the immune system by flow cytometry, transcriptomics, and luminometric immune assays.

Results: Human B cells, and particularly B regulatory cells (Breg cells), showed an increased immune response to HBoV1-VLPs stimulation. These immune responses were also supported by increased IL-1RA and PDL1 expressions in IL-10$^+$ B cells from peripheral blood mononuclear cells (PBMCs) stimulated with HBoV1-VLPs. In addition, increased levels of IL-10 and IL-1RA were determined in the supernatants of PBMCs following HBoV1-VLPs stimulation. HBoV1-VLPs and RV co-stimulation increased the IL-10$^+$ B cell population. Transcriptome analysis by next-generation RNA sequencing showed an increased expression of IL-10 signalling and Breg cell markers in PBMCs stimulated with HBoV1-VLPs. Furthermore, TGF-β and chemoattractants MIP-1α, MIP-1β and IP10 protein levels were high in the supernatants of PBMCs stimulated with HBoV1-VLPs.

Conclusions: The findings demonstrate that in Breg cells, IL-10 signalling pathways, and anti-inflammatory activity are induced by HBoV1, which can explain the often mild nature of the disease. In addition, the immune regulatory response induced by HBoV1-VLPs may indicate a potential immunomodulatory role of HBoV1 on the immune system and may represent an immune regulatory strategy.

KEYWORDS
B cells, Breg cells, HBoV1, IL-10 signalling, PBMCs

1 | INTRODUCTION

Human bocaviruses (HBoVs) are small single-stranded DNA viruses that are members of the *Parvoviridae* family. This family has two clearly human-pathogenic members – parvovirus B19 and HBoV1.[1] Three other bocaviruses, comprising two different species, have been discovered so far in stools.[2] HBoV1 is found predominantly in the respiratory tract of children and is associated with upper, and mild to life-threatening, lower respiratory tract infections (RTIs).[1–4] On the contrary, HBoV2, HBoV3 and HBoV4 generally infect the gastrointestinal tract; they have been discovered in stool samples, and are recognized as enteric bocavirus types.[2,3] The seroprevalence of HBoVs 1, 2, 3 and 4 have been estimated to be 80%, 50%, 10% and ~0%, in Finland and 67%, 50%, 40% and 1.4%, in China, respectively.[5,6]

HBoV1 respiratory infections occur worldwide and all year round, peaking during late autumn, winter and early spring. HBoV1 infections are clinically characterized by mild to moderately severe acute respiratory symptoms; however, life-threatening RTIs also occur, with chest radiography showing peribronchial or interstitial infiltrates, even necessitating extracorporeal membrane oxygenation (ECMO).[2] HBoV1 was first identified in 2005 in nasopharyngeal samples collected from children with RTIs.[7] HBoV1 infections are most common in children between 6 months and 5 years of age, with a mean age of 2 years.[1] The overall prevalence of HBoV1 in airway samples of children with upper or lower RTIs ranges from 2% to 19%.[1,3,8] HBoV1 has been characterized as a pathogen generally affecting children, but HBoV1 DNA-positivity have also been reported in respiratory samples of adults with RTIs.[2,9,10]

It has been shown that HBoV1 infections may cause rhinorrhoea, cough, wheezing, bronchiolitis, asthma exacerbation and pneumonia.[2,11] Nevertheless, even though HBoV1 is characterized as a respiratory pathogen,[12] its presence at low levels has been additionally demonstrated in the stool samples of patients with gastrointestinal diseases,[13–15] as well as in healthy individuals.[2]

The pathogenesis of HBoV1 has not been fully understood due to the lack of animal models and certain difficulties related to virus replication in cell cultures. HBoV1 does not propagate in traditional monolayer cell culture, but it can replicate in differentiated human airway epithelial cells in an air-liquid interphase (ALI) culture system, where it causes cytopathic effects.[16]

Diagnosis of HBoV1 infections is mainly based on PCR, but due to the prolonged shedding of the virus in the airways, this approach is not recommended.[17,18] Diagnosis of primary acute infection should instead include quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR) for detection of high viral loads and mRNA in respiratory samples; or HBoV1 IgM and IgG, or DNA detection in serum.[19–22]

Due to the prolonged persistence of HBoV1 in the airways, high co-detection rates of HBoV1 with other respiratory viruses such as rhinoviruses (RV), adenovirus, coronaviruses, paramyxoviruses and respiratory syncytial virus (RSV) have been reported in RTIs.[3,23] However, the clinical implications of HBoV1 coinfection in the respiratory tract are poorly understood, due to the conflicting results about the effect of HBoV1 on other respiratory viruses. It has been shown that HBoV coinfection with RSV increased the severity of infection and prolonged the hospitalization period, compared to single acute HBoV or RSV infections.[24] In contrast to RSV-HBoV1 coinfection, RV-HBoV1 coinfection caused non–Th2 type cytokine responses in patients with mild to severe refractory asthma.[25] HBoV1 DNA was detected in germinal centres of adenoid tonsils and was mainly found in B cells and monocytes. In B cell subpopulations, HBoV1 DNA was shown in naïve, activated, and memory B cell populations. Fc-receptor (FcγRII) mediated antibody-dependent enhancement (ADE) of cellular uptake of HBoV1 has been shown in B cells and monocytes.[26]

In this study, we aimed to identify the immune response to HBoV1 in PBMCs. We analysed the effect of HBoV1 VLPs stimulation on the immune system via cell cultures, flow cytometry and RNA-seq transcriptomics; and demonstrated a B and predominantly Breg cell signature with increased IL-1RA and PDL1 expression in IL-10$^+$B cells. The results of RNA-seq transcriptome analysis also showed increased transcription of IL-10 signalling and Breg cell-related immune response genes.

Key messages

- IL-10$^+$ B cells and Breg cell populations increase after HBoV1-VLPs and RV stimulation.
- HBoV1-VLPs are actively uptaken by CD19$^+$ B cells.
- The expression of Breg-related and IL-10 signalling genes rises following the stimulation with HBoV1.

2 | METHODS

2.1 | Isolation and virus stimulation of PBMCs, and flow cytometry

PBMCs were isolated from the heparinized peripheral blood of seven adult volunteers (three females and four males) between 25 and 38 years old. The Ethics Committee of Canton of Zurich approved the study protocol and all participants gave informed consent. Briefly, Biocoll solution was added into 50 mL filter tubes and warmed up at RT. After centrifugation, a blood sample diluted with PBS/EDTA (Sigma Aldrich, MO, USA) at 1:2 was gently added to the Biocoll solution. Following the centrifugation, the plasma layer was discarded, and the interphase cell layer was collected in a Falcon tube. After washing three times with PBS/EDTA, cells were resuspended in RPMI 1640 medium supplemented with L-glutamine (2 mmol/L), MEM vitamin, nonessential amino acids, sodium pyruvate, penicillin (100 U/mL), streptomycin (100 µg/mL), kanamycin (Life Technologies, Carlsbad, CA, USA) and 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA).

The HBoV1 and HBoV2-VLPs were produced with the baculovirus expression system in insect cells.[19,27] PBMCs were stimulated with different concentrations (0.3, 0.6, 1.2, and 2.4 μg/mL) of HBoV1-VLPs and HBoV2-VLPs for 24 and 48 h. After incubation, PBMCs were stained with viability dye and surface markers (Table S1). All samples were analysed with a FACSAria III (Beckton Dickinson, Franklin Lakes, NJ, USA).

PBMCs were also induced with HRV-16 (Virapur, San Diego, USA) at 25:1 multiplicity of infection (MOI) and 2.4 μg/mL HBoV1-VLPs for 72 and 120 h. To determine HBoV1 and HRV effect on IgA and IgG4 levels in PBMCs by flow cytometry analysis, IgA and IgG4 antibodies were labelled with APC/Cy7 and PE, respectively, with Lightning-Link® Rapid Conjugation System according to the manufacturer's instruction (Innova Biosciences, Cambridge, UK). Labelled antibodies were stored at 4°C. After incubation, cells were stained with viability dye, intracellular and surface markers. The details of used antibodies in surface and intracellular staining are given in Table S2. The cells were analysed with the FACSAria III (Beckton Dickinson, Franklin Lakes, NJ, USA). Data were evaluated by Kaluza Software (Beckman Coulter, Indianapolis, USA). Detailed methods are given in Data S1.

2.2 | Fluorescent labelling of the HBoV1-VLPs

HBoV1–VLPs were labelled with Lightning-Link® DyLight 650 Rapid Conjugation System according to the manufacturer's instruction (Innova Biosciences, Cambridge, UK). Briefly, 1 μL of LL-Rapid modifier reagent was mixed with 10 μL of VLPs (stock concentration: 0.507 μg/μL). The mixture was added into the Lightning-Link Rapid mix, gently mixed, and incubated for 15 min at RT. Labelled VLPs were stored at 4°C (For detailed methods, see Data S1).

2.3 | Imaging flow cytometry

PBMCs were incubated with DyLight650 labelled HBoV1-VLPs (0.6 μg/mL) for 5 min, 30 min, and 2 h. After washing with PBS, cells were incubated with the viability dye – eF780 for 30 min at 4°C. After that, cells were stained with surface antibodies (Table S3). Measurements were performed by a cell imaging flow cytometer (Image Stream, Seattle, WA, USA). The signal strength was compensated between different channels according to the manufacturer's protocols. The flow speed was stabilized before collecting 30.000 events, and 40× magnification was used for image capturing. Data were analysed using 'Ideas' software (Amnis, Seattle, WA, USA).

2.4 | RNA isolation, next generation RNA sequencing and data analysis

Total RNA was isolated from PBMCs stimulated with HBoV1-VLPs for 6 and 24 h by RNeasy Plus Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions; isolated RNA quality and quantity were assessed by Qubit Fluorometer (Thermo, MA, USA). Library preparation for RNA-seq was carried out with TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA). Sequencing was conducted using Illumina HiSeq 4000 with TruSeq SBS Kit v4-HS reagents (Illumina, San Diego, CA). Raw data are available at the Gene Expression Omnibus (GSE143163, (Jan 06, 2023)). Differential expression analysis between groups was carried out using the edgeR Bioconductor package (Detailed methods are provided in Data S1).

2.5 | Cytokine measurements

PBMCs were stimulated with different concentrations (0.3, 0.6, 1.2 and 2.4 μg/mL) of HBoV1 and HBoV2-VLPs for 24 and 48 h. To determine the long-term virus effect, PBMCs were stimulated with HBoV1-VLPs for 72 and 120 h. After cell culture, supernatants were stored at −80°C. Milliplex human cytokine and chemokine 30 Plex panel were used to determine the following mediators: EGF, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF, RANTES, Eotaxin/CCL11 (Millipore, MA, USA). Minimum detectable concentrations (MinDC) of cytokines are given in Table S4. Fluorescent signals were measured by Bio-Plex 200 System (Bio-Rad, CA, USA).

2.6 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif). The Kruskal–Wallis test with post hoc Dunn's test and two-way ANOVA with Dunnett's multiple comparison test was used to compare differences between groups, as appropriate. A p-value <.05 was considered significant. All results are presented as mean ± standard error of the mean.

3 | RESULTS

3.1 | The stimulation effect of HBoV1 and HBoV2-VLPs on PBMCs

The effect of HBoV1 and HBoV2-VLPs on human PBMCs after 48 h of stimulation was examined by flow cytometry. The percentage of $CD19^+$ and $CD19^+CD25^+CD71^+$ B cells were dose-dependently increased by HBoV1-VLP stimulation compared with the unstimulated condition. Although the percentage of $CD19^+$ B changed significantly after stimulation with the highest dose of HBoV2-VLPs, this effect was not seen in $CD19^+CD25^+CD71^+$ B cells (Figure 1A). The gating strategy for Monocytes, T cells, B cells and NK cells are given in Figure S1.

We did not find any differences in the percentage of T cell subpopulation, monocytes, and NK cells after incubation with VLPs of either HBoV1 or HBoV2 (Figure S2).

3.2 | Increased IL-1RA and PDL1 levels in IL-10$^+$ B cells after HBoV1 and RV stimulation

In order to determine the single and coinfection effects of the viruses on the Breg cells, PBMCs were stimulated with 2.4 μg/mL of HBoV1 and/or RV-16 at 25 multiplicity of infection (MOI) for 72 h. The gating strategy is given in Figure S3. After virus stimulation, IL-10$^+$ B cells were increased in the CD19$^+$ B cell population. The highest increase was determined in the co-stimulated conditions (Figure 1B).

IL-1RA protein level in IL-10$^+$ B cells increased under all stimulation conditions in 72 h, but not in IL-10$^-$ B cells (Figure 1B). Compared to the control, IL-1RA synthesis in IL-10$^+$ B cells increased following the HBoV1-VLPs, RV, and co-stimulation for 120 h, while IL-1RA synthesis did not change in IL-10$^-$ B cells after virus-containing conditions (Figure 1B). PDL1 synthesis increased in IL-10$^+$ B cells after stimulation with viruses for 72 h, but no difference was determined in IL-10$^-$ B cells (Figure 1B).

The effect of respiratory virus stimulation on the two different Breg populations was investigated after 72 h of stimulation. Following the HBoV1-VLPs, RV and co-stimulation, compared to the control, CD19$^+$CD25$^+$CD71$^+$CD73$^-$IL-10$^+$ Breg cells showed a tendency to increase. However, the results did not reach statistical significance (Figure 1C). The percentage of CD19$^+$CD24^{high}CD38^{high}IL-10$^+$ Breg cells was higher than the control under all virus-stimulated conditions (Figure 1C).

3.3 | HBoV1-VLPs interaction with B cells

To figure out whether HBoV1-VLPs attached or entered B cells, fluorescent dye-labelled (DyLight 650) HBoV1-VLPs were used to stimulate PBMCs. Dye-labelled HBoV1 and stained B cells were investigated by flow cytometry analysis following 5 min, 30 min and 2 h of stimulation. Lymphocytes and monocytes gating strategy are given in Figure S4. Unlabelled VLPs and unstimulated cells were used as controls. Even though a low concentration of VLPs was chosen for stimulation, 3.3% of B cells were positively labelled with VLPs in the first 5 min of stimulation. In 30 min, the double-positive B cell population increased over two-fold (7.4%), and this increase reached 21.9% after 2 h. The flow cytometry results of unlabelled HBoV1-VLP and unstimulated cells were negative for CD19$^+$VLPs$^+$ B cells (Figure 2A).

According to the imaging flow cytometry results (image stream), the dye-labelled HBoV1-VLPs (red colour) seem to be located inside the B cells (green colour). Unstimulated and unlabelled samples were negative for Dylight 650 (Figure 2B). VLPs were determined in the monocytes first 30 min (Figure S5A–D), while CD4$^+$ and CD8$^+$ cells were found to be positive for labelled VLPs after 2 h of stimulation (Figure S5).

3.4 | HBoV1-VLPs stimulation upregulates Breg markers and virus response genes

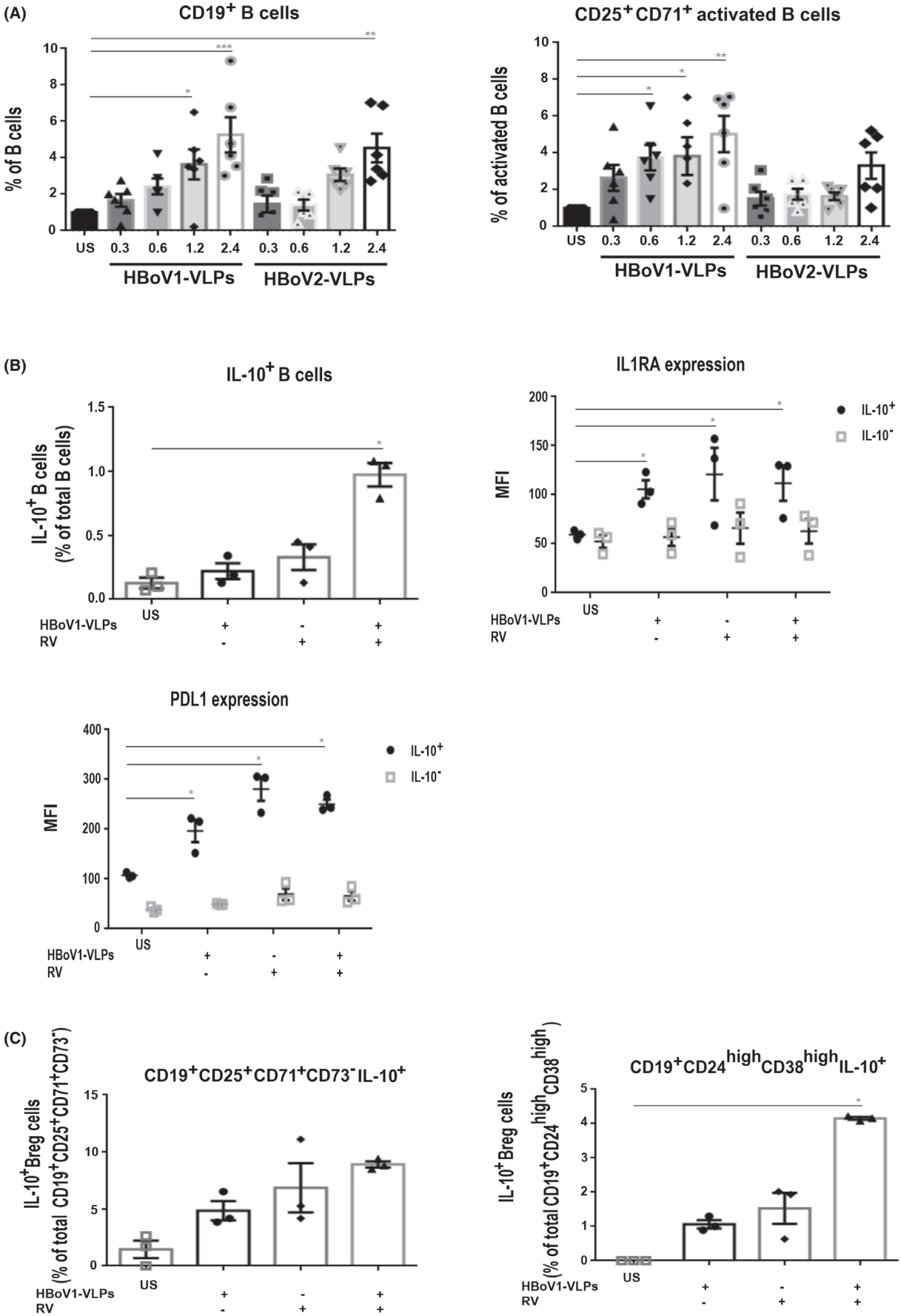
To determine the effect of HBoV1 infection on the transcriptome, a relatively high concentration of viral particles (2.4 μg/mL) was used for PBMC stimulation during two different time points (6 and 24 h). β-diversity analysis (principal component analysis [PCA]) of the PBMCs transcriptomes after HBoV1-VLPs stimulation for 6 and 24 h, showed differential gene expression profiles compared to the unstimulated samples. The variance between virus stimulated and unstimulated conditions were higher in 24 h when compared to 6 h (Figure S6A,B).

Co-expression modular analysis identified 10 co-expressed modules. The largest module – M1, contained 267 co-expressed genes, while the smallest module – M10, contained 40 genes (Figure 3A). All modules showed significant module activity (Figure 3B). A gene set enrichment analysis of HBoV1-VLPs stimulated PBMCs revealed statistically significant over representation of inflammatory and immune response (M2), inflammatory response and chemotaxis (M3), defence response to virus and type I interferon signalling pathways (M4), neutrophil degranulation and inflammatory response (M6) (Figure 3B).

According to the flow cytometry analysis, we determined that the frequency of IL-10$^+$ Breg cell population increased in HBoV1-VLPs stimulated conditions. To assess the effect of HBoV1-VLPs on Breg cell differentiation based on the transcriptional level, IL-10$^+$ Breg cell markers were selectively analysed. In the 6-h stimulation with HBoV1-VLPs, the expression level of GLRX, IRF4, LTA, IL-1RN, CD38, FASLG, EGR3, LRRC32, CD274, IL2RA, AREG, IL-6 and SOCS3 genes were upregulated (Figure 4A). After 24-h stimulation, the expression of AREG, FASLG, EGR3, GLRX, IL-2RA, IRF4, LTA and SOCS3 genes was still high compared to the unstimulated condition. CD274, IL-1RN, CD38 and IL-6 transcription were upregulated in 24 h conditions compared to 6 h (Figure 4B).

We analysed the effect of HBoV1-VLPs on the expression of chemokines in PBMCs. The expression level of CCL3, CCL4, CCL7, CCL20, CCL3L1 and CCL4L2 slightly increased after 6 h of stimulation with HBoV1-VLPs. CCL2, CXCL10 and CXCL11 transcription was strongly upregulated in both 6 and 24 h of stimulations with VLPs. In a 24-h stimulated condition, CCL13, CCL17, CCL18, CCL19, CXCL9, CXCL12, CXCL13 mRNA levels were higher than in an unstimulated condition (Figure 4C).

FIGURE 1 HBoV1-VLPs stimulation increased B and Breg cell population. (A) Fold change percentage of CD19$^+$ and CD25$^+$ CD71$^+$ B cells following the stimulation of PBMCs with HBoV1 and HBoV2-VLPs for 48 h ($n=6$). (B) IL-10$^+$ B cell population after stimulation with HBoV1-VLPs (2.4 μg/mL) and RV (25 MOI) for 72 h. PDL1 and IL1RA expression levels in IL10$^+$ and IL10$^-$ B cells following HBoV1-VLPs and RV incubation ($n=3$). (C) IL10$^+$ Breg cell populations in PBMCs stimulated with HBoV1-VLPs (2.4 μg/mL) and RV (25 MOI) ($n=3$). RV, Rhinovirus-16. Values are mean ± SEM. Statistical significance was determined using one-way or two-way ANOVA, $^*p<.05$, $^{**}p<.01$, $^{***}p<.001$.



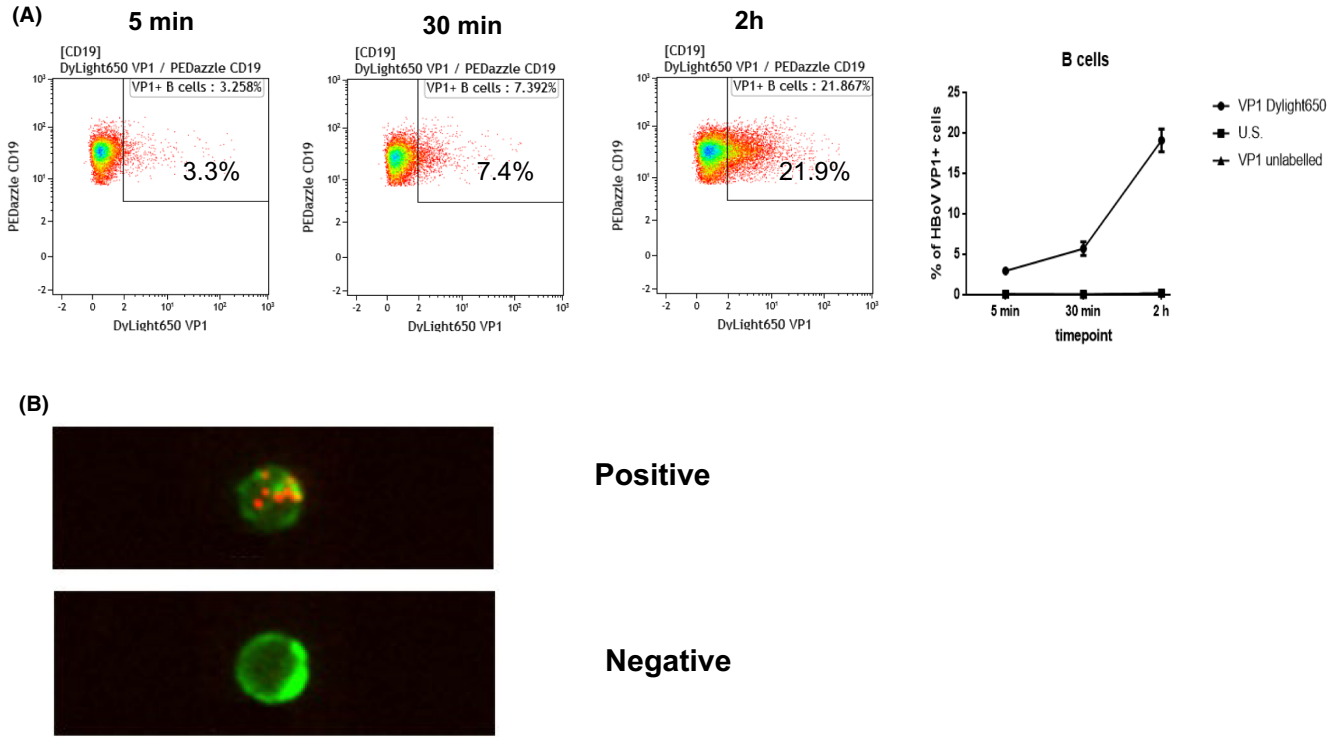


FIGURE 2 The uptake of HBoV1-VLPs into CD19$^+$ B cells increases for 2 h. PBMCs were incubated with DyLight650-labelled HBoV1-VLPs (VP1) and analysed at the designated time points by flow cytometry. (A) A representative of cell flow cytometry of CD19$^+$ VP1+ cells. Data are mean with CI of 95% of 3 donors. (B) Imaging of HBoV1-VLPs with B cells after incubation with VP1. B cells were analysed with anti-CD19 PE/Dazzle (green) and HBoV1-VLPs (red).

We examined the cytokine profile differences in PBMCs stimulated by HBoV1-VLPs. The transcription of interferon genes including IFNB1, IFNG, IFNL2, and IFNL1 increased in both stimulated conditions, but IFNW1, IFNA14, IFNA2, IFNA8, IFNB1, IFNA, and IFNL expression was upregulated mainly following 24 h of VLPs stimulations. The expression of EBI3, IL-15, IL-27, IL-7 and IL-12A genes playing a role in the proliferation, differentiation, and activation of T and B cells, was highly increased in conditions induced by HBoV1-VLPs for 24 h. Meanwhile, the expression of certain gene groups, including TGF-related genes (such as TGFB2, TGFB3), TGFA, and proinflammatory cytokines (such as IL16 and IL23A) decreased only in 24 h VLPs-stimulated conditions (Figure 4D).

We also investigated the interaction between the expression of the immune receptor genes and HBoV1-VLPs stimulation. Two main groups (containing numerous subgroups) are distinguished based on their expression profiles. In the first group, IL-10 and IFN receptor gene mRNA increased in the 24 h VLPs-stimulation conditions. The second group of gene expression, IL-4R, CD4, IL-13RA1 and TGF receptors, decreased after HBoV1-VLPs induced condition for 24 h (Figure 4E).

3.5 | Pathway analysis of top 200 differentially expressed genes indicate IL-10 signalling pathway

Reactome analysis results of top 200 differentially expressed genes (DEGs) demonstrated overexpression of IL-10 signalling pathway genes after HBoV1-VLPs stimulation for 6 and 24 h. Pathway analysis showed that antiviral response, interferon signalling and cytokine signalling genes were overexpressed as early response genes after 6 h of HBoV1 stimulation (Figure S7A). After 24 h of HBoV1 stimulation, the expression of the immune system, inflammasome and IL-27 signalling pathway genes increased as late response genes (Figure S7B). Protein–protein interaction of these genes were demonstrated by STRING analysis; the most significant pathway results and IL-10 signalling pathway genes are shown in the circle with protein–protein interactions (Figure S8A,B).

3.6 | Cytokine profile change in HBoV1 and HBoV2-VLPs induced PBMCs

The effect of HBoV1 and HBoV2-VLPs on cytokine, chemokine and growth factor profiles was investigated by PBMC stimulation with increasing concentrations of HBoV1 and HBoV2-VLPs for 24 and 48 h.

IL-10, GM-CSF, MIP-1α and MIP-1β protein levels were significantly increased by stimulation with the highest concentration (2.4 μg/mL) of VLPs after 24 h compared to the unstimulated conditions. IL-1RA protein levels were significantly raised in 1.2 and 2.4 μg/mL, while IP10 was increased in 0.3, 0.6 and 2.4 μg/mL concentrations. A significant increase in TNF-β and MCP-1 levels were seen only in samples stimulated with 1.2 μg/mL of HBoV1-VLPs (Figure 5A).

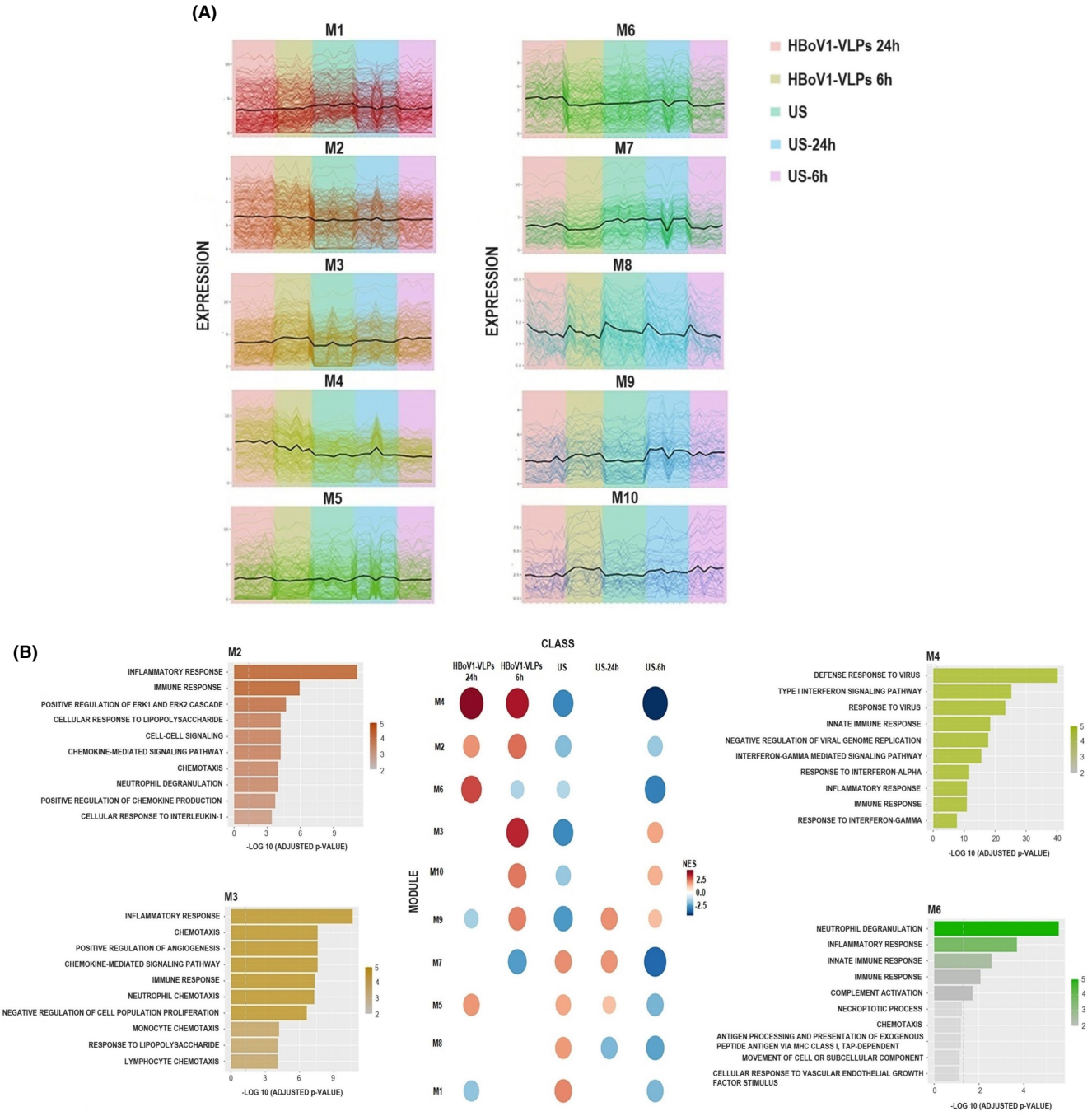


FIGURE 3 PBMCs induced with HBoV1-VLPs for 6 and 24 h form 10 co-expressed modules. (A) The largest co-expressed genes module is M1, and the smallest module is M10 ($n = 6$). Five different colours symbolize stimulated, and unstimulated conditions. (B) Gene set enrichment and over-representation analysis results of the investigated groups.

In 48-h stimulations, dose dependent responses were seen in IL-10, IL-1RA, TNF-β, MIP-1α, MIP-1β, MCP1, IFN-γ and IP10. Compared with the unstimulated condition results, protein levels changed in IL-10, MIP-1α and IFN-γ, reaching significance in stimulations with the highest concentration of VLPs. Similarly, 24-h stimulations showed that the IP10 level significantly changed in 0.6 and 2.4 μg/mL VLPs-induced conditions, but not with the lowest concentration. MIP-1α synthesis significantly changed in samples stimulated with 1.2 and 2.4 μg/mL of HBoV1-VLPs. Additionally, when compared to the unstimulated samples, IL-1RA, TNF-β and MCP-1 synthesis increased under all VLPs concentrations, with the exception of the lowest concentration (0.3 μg/mL) (Figure 5B). Cytokines, whose protein levels were measured, but were not statistically different, are given in Figure S9A,B.

For determination of the long-term effect of HBoV1, we stimulated PBMCs with the highest concentration (2.4 μg/mL) of HBoV1-VLPs for 3 (72 h) and 5 days (120 h). Protein synthesis of IL-10, IL-4, IL-6, GM-CSF, IL-7, IL-1α, IL-12p70, IL-2, EGF, IFN-γ and IFN-α2

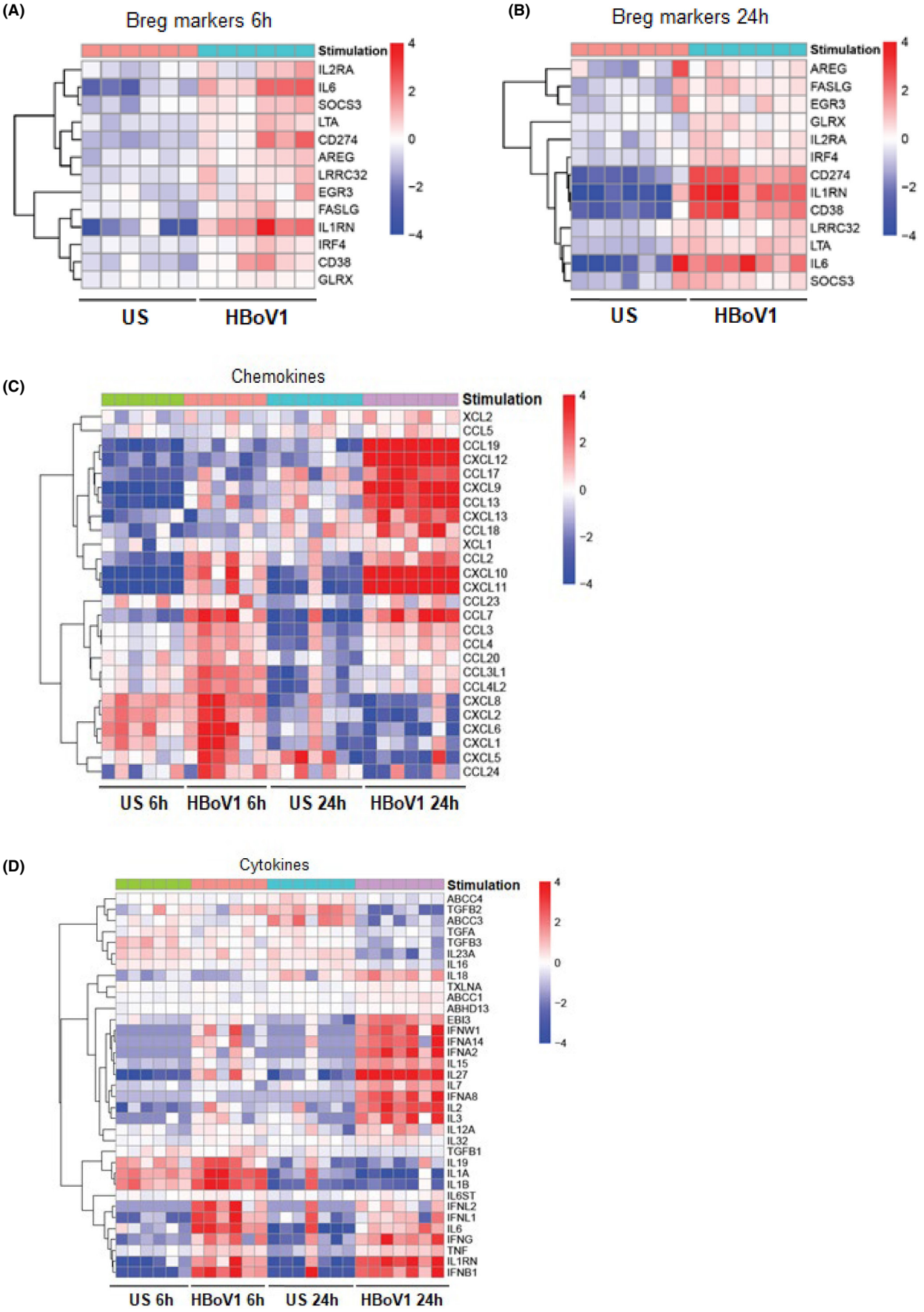


FIGURE 4 HBoV1-VLPs stimulation alters the profile of Breg, chemokines, cytokines and immune receptor genes. The expression of Breg marker genes were significantly changed in PBMCs after 6 h (A) and 24 h (B) of stimulation with HBoV1-VLPs (2.4 μg/mL); (C) The expression of chemokines, (D) cytokines and (E) immune receptors genes were significantly changed in PBMCs after 6 and 24 h stimulation with HBoV1-VLP (2.4 μg/mL).

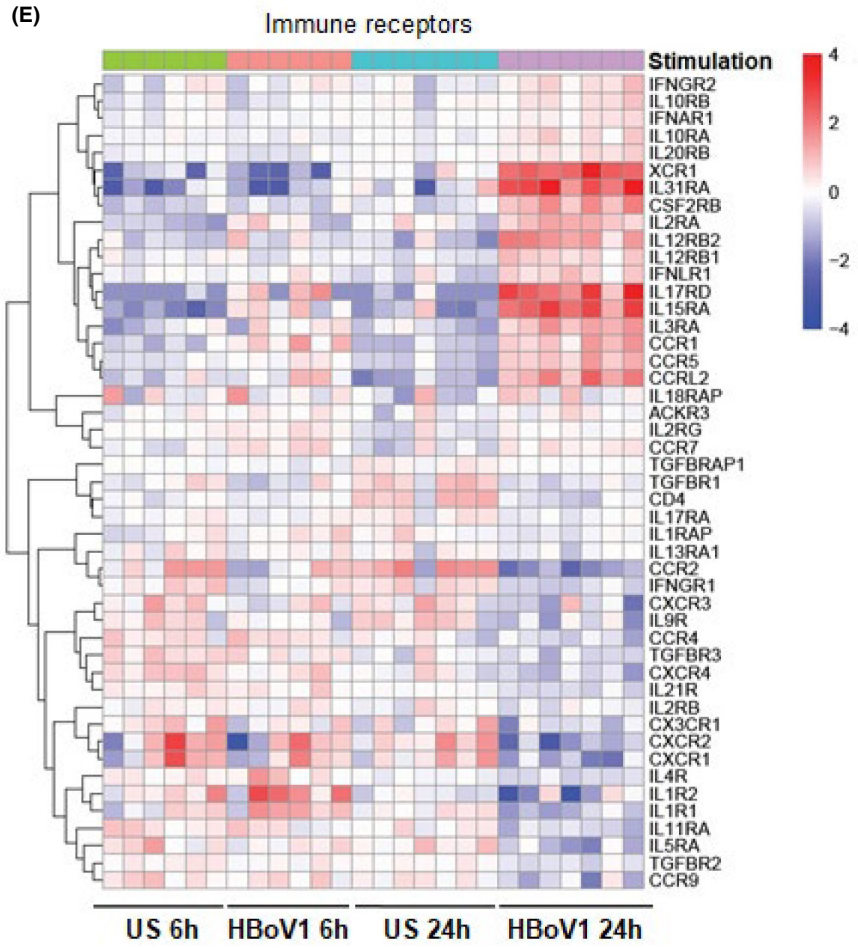


FIGURE 4 (Continued)

increased after HBoV1 administration in both time points. IL-1RA, G-CSF, IL-8, MIP-1β and VEGF protein levels increased in 3 days, but decreased in 5 days. MIP-1α and TNF-α synthesis changed only in 5 days (Figure S10).

4 | DISCUSSION

HBoV1 is a common agent of mild to fatal respiratory tract infections in childhood, with still unclear effects on the immune system. In this study, we showed that stimulation of PBMCs with HBoV1-VLPs increased the percentage of B cells, activated B cells and Breg cells. Stimulation with HBoV1 caused transcriptome profile differences in PBMCs. Additionally, HBoV1-VLPs induced the production of inflammatory mediators, including proinflammatory and anti-inflammatory cytokines and chemokines in PBMC cultures.

HBoV1 studies have previously been mainly focused on epidemiological investigations and/or virus effects and propagation in airway epithelium.[2,27,28] Only a limited number of studies investigated the role of the adaptive cellular immune response after HBoV1 infection. One of these studies demonstrated that IFN-γ secretion was significantly increased in PBMCs obtained from HBoV-seropositive healthy individuals when compared to the control group. Depletion of $CD4^+$ or $CD8^+$ T cells from PBMC cultures showed that IFN-γ secretion was dependent on $CD4^+$ T cells, but not on $CD8^+$ T cells.[29]

Another study demonstrated that in nasopharyngeal aspirates from children, Th1 and Th2 type cytokine secretion depends on HBoV.[30] The proliferation of $CD4^+$ T cells, but not $CD8^+$ T cells stimulated with HBoV VP2-VLP were reported in HBoV seropositive individuals. These $CD4^+$ T cells also produced a higher amount of IFN-γ, IL-10 and IL-13 than $CD8^+$ T cells.[31] Viral DNA

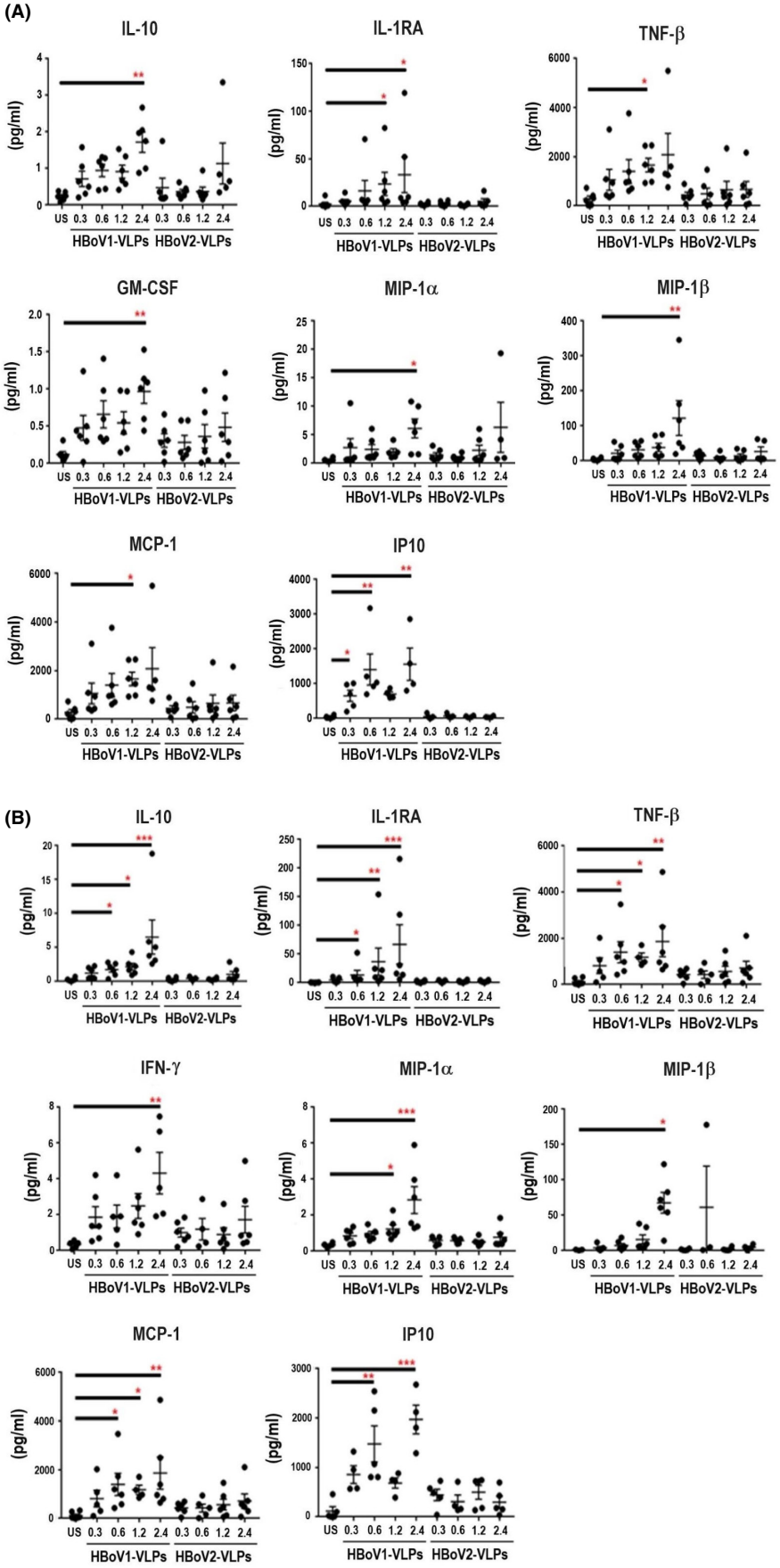


FIGURE 5 The stimulation of PBMCs with HBoV1-VLPs altered the inflammatory cytokine profile. PBMCs were stimulated with VLPs of HBoV1 and HBoV2 for 24 and 48 h to profile cytokines. (A) The protein level of proinflammatory, anti-inflammatory, chemokine and cytokine inhibitory molecules after 24 h of stimulation with HBoV1-VLPs or HBoV2-VLPs. (B) The protein level of proinflammatory, anti-inflammatory, chemokine and cytokine inhibitory molecules after 48 h of stimulation with HBoV1-VLPs or HBoV2-VLPs. Values are mean ± SEM. Statistical significance was determined using Kruskal-Wallis test, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

of HBoV1 was detected in B cells, monocyte and proportion of T cells in adenoid germinal centre. In B cell population HBoV1 DNA was determined mainly in naïve, activated and memory B cells, and monocytes.[26]

The role of B cell subtypes as an immune response against HBoV1 infection has not been previously studied in detail. Humoral B-cell immunity to HBoVs has been investigated in only a few studies. Systemic B-cell responses consisting of IgM and IgG have been studied in children with respiratory disease.[19,32] After HBoV1 infection, a low amount, or even lack of specific IgG responses was observed in children with pre-existing HBoV2 or HBoV3. It has been suggested that this result can be explained by the original antigenic sin phenomenon (OAS).[6,33] OAS may mask the current immune response and show a boost of the IgG to the prior HBoV2 or HBoV3 infections.

In the present study, we determined that the percentage of $CD19^+$ and $CD19^+CD25^+$ $CD71^+$ B cells was increased by HBoV1-VLPs stimulation. We did not observe this effect in the HBoV2-VLPs-stimulated samples, although the highest dose of HBoV2-VLPs stimulation increased the percentage of $CD19^+$ B cells. While the B cell response mechanism specific to VLPs is yet to be fully understood, various studies have indicated that VLPs can stimulate a B cell response in both human subjects and mouse models. VLPs contain multimeric epitopes on their surface, which can facilitate crosslinking of B cell receptors (BCRs). Simple cross-linking of BCRs by VLPs can be strong enough for priming B cells.[34] HBoV-VLPs, which are similar in morphology and antigenicity to virions, have been successfully used as antigens for the detection of antibodies against HBoVs. Additionally, the homologies of the amino acid (aa) sequences of the HBoV1–4 VP2 are high – aa sequence identities of VP2 between HBoV1 and HBoV2–4 are about 77%–78%.[35] Amino acid sequence variation between HBoV1 and HBoV2-VLPs may be the reason for different B cell responses. The differences in aa sequences between HBoV1 and HBoV2-VLPs may affect the interaction with B cell receptors, which could potentially explain why the $CD19^+$ and $CD19^+CD25^+CD71^+$ B cell response, which increases with HBoV1-VLPs stimulation, is not observed with HBoV2-VLPs. In the literature, the effect of stimulation with parvovirus B19 on lymphocyte subpopulations was investigated in PBMCs cells on days 0, 14 and 210. No changes in the T cell numbers were recorded, while the total number of $CD19^+CD23^-$ and $CD19^+CD23^+$ B cells was increased on day 14. Further, increase in $CD19^+CD23^-$ B cell number was detected on day 210, and subsequent observations indicated that the number of cells remained the same.[36]

We used DyLight650-labelled HBoV1-VLPs to investigate the direct or indirect interaction of HBoV1 and $CD19^+$ B cells. It was demonstrated by imaging flow cytometry that HBoV1-VLPs were actively taken up by B cells. Red fluorescence-labelled VLPs overlapped with the CD19 signal, but no red signal was obtained from unlabelled VLPs. Xu et al. also showed that tonsillar, Raji, and GM12878 B cells have taken up HBoV1. They revealed that HBoV1 entry into the B-cell was enhanced with HBoV1-specific IgG through FcγRII mediated pathway, while entry was blocked by anti-FcγRII.[26] In that study, the B cells were ex vivo cultured from pediatric adenoids, whereas our B cells were from adult peripheral PBMCs. Since the cell entry pathways and receptors of HBoV1 have not been fully elucidated, it is possible that different cellular uptake mechanisms and receptors are involved in HBoV1-VLPs uptake in tonsils and PBMC-B cells. Within our study, we determined the time kinetics of the uptake of VLPs into $CD19^+$ B cells using flow cytometry. The uptake started in the first 3 min and reached the top at 2 h. To the best of our knowledge, up to this point, there is no published HBoV1 studies with uptake kinetic results.

The prevalence of RV and HBoV1 coinfection rates among respiratory diseases in children is high. The coinfection rate of HBoV1 with RSV, RV, HAdV and other respiratory viruses has been determined in approximately 60% to 75% of the cases.[22,37] RV internalization into $CD19^+$ B cells and the increased cell proliferation have been demonstrated before,[38] but the cellular effect of coinfection with HBoV1 on B subtypes has not been shown in the literature. One of the key findings of our study showed that single HBoV1-VLPs and RV infection enhanced the IL-10 synthesizing B-cell and Breg-cell percentage within the total B-cell population, and that this effect was stronger in HBoV1 and RV co-stimulated samples. IL-10 is an essential immunosuppressive cytokine that plays a key role in various immune tolerance models. Additionally, B-cell survival and class-switch recombination are positively affected by IL-10. IL-10 actively controls the B-cell responses to the virus. It has been shown that IL-10-producing B cells are crucial in inflammation and tolerance.[39] IL-10 expressing Breg cells have the ability to modulate responses to infection and the increased $IL\text{-}10^+$ Breg cells population has been demonstrated in various virus-caused diseases.[40,41]

As an interesting additional finding, $IL\text{-}10^+$ B cells showed significantly increased IL-1RA and PDL1 protein levels after single HBoV1 stimulation and in co-stimulated (HBoV1 and RV) samples, but not in $IL\text{-}10^-$ B cells. It has been shown that RV infection induces IL-1α, IL-1β, and IL-1RA into nasal secretions.[42] IL1-RA is known as an important inhibitor of inflammation. It binds to the IL-1 receptor and hinders the binding of IL-1α and IL-1β.[43] PDL1 synthesis plays an essential role in chronic inflammation via T and B-cell responses. PD-1 and its ligand PDL1 are expressed in many inflammatory cells such as activated B cells, T cells, dendritic cells and macrophages. The suppressive role of this ligand-receptor

interaction in acute virus infections has been studied in mice and humans.[44] The PD-1/PDL1 signalling pathway has a distinct immune regulatory role by Breg cells, such as suppression of the proliferation of $CD8^+$ T and $CD4^+$ T cells, and production of IL-10 and TGFβ.[45] The increased expression of IL-10 as well as IL-1RA and PDL1 suggests an active function in the control of inflammatory mechanisms over different pathways. Increased IL-10^+ B and Breg cell population with raised protein levels of IL-1RA and PDL1 in IL-10^+ B cells raise a question if HBoV1 may play a role in immunomodulatory function.

The present study is the first to investigate the effect of HBoV1 on global transcriptome differences in PBMCs. β-diversity analysis of global PBMCs genes showed the impact of HBoV1 on the transcriptome. Variation is much higher in the 24-h stimulated conditions compared to the 6-h stimulation. According to the heat map analysis, we noticed that adaptive and innate immune-system genes, as well as virus-mediated response and expression of interferon signalling genes were affected by the VLPs stimulation. Heatmap results showed that Breg cell markers' gene expression was upregulated after HBoV1-VLPs stimulation for both 6 and 24 h. Especially the expressions of CD274, IL-1RN (IL-1RA), IL6 and CD38 genes that were highly increased after 24 h. Breg cell-marker genes including CD25 (IL-2RA), CD38, IL-1RA and CD274 (PDL1) were also increased supporting our flow cytometry results. Similarly, the upregulated synthesis of IL-1RA, CD25, PDL1 and CD38 was also shown in IL-10-expressing Breg cells.[46]

The Reactome pathway and String network analysis results indicated upregulated gene expression in the IL-10, interferon, and immune system-signalling pathways. These results mainly support B and IL-10^+ B cell analysis outcomes derived from flow cytometry.

Recent studies have shown conflicting results about the HBoV1 effect on IFN-β production. Zhang et al. demonstrated an inhibiting effect of HBoV1 on IFN-β, whereas Luo et al. pointed out to an upregulatory effect of HBoV1 on the IFN-β pathway.[47,48] In the literature, single RV, single HBoV1 and coinfection effect on cytokines were investigated only in children. Proinflammatory, Th1 and Th2 type responses were found in higher values in RV infections compared to HBoV1 infections, and cytokine synthesis was determined to be lower in the coinfection group compared to the RV only infected group. Anti-inflammatory cytokine IL-10 levels were highest in sole HBoV1 infection.[49] Our results indicated that HBoV1 stimulation significantly induced anti-inflammatory, proinflammatory and inflammatory cytokines. As supporting evidence, induced anti-inflammatory responses, increased IL-10 and IL-1RA synthesis in PBMCs cultures, stimulated with HBoV1-VLPs for 24 and 48 h, were observed in both NGS and flow cytometry results.

Within our study we used HBoV1 and HBoV2-VLPs instead of live viruses. In the antiviral immunity, nucleic acid sensors such as TLRs, RIG-1 and MDA-5 play essential roles in recognition and response to the viral genome.[50] The nucleic acid content of HBoV1 may activate different cellular and humoral immune responses. VLPs are self-assembled viral proteins without virus genomes and infectious features. VLPs have no potential for replication inside living cells and their size ranges from 20 to 200 nm. VLPs can be synthesized in various expression systems, such as prokaryotic and eukaryotic cells (from yeast, insects, plants and mammalian). VLPs are potent immune stimulators that trigger both humoral and cellular immune responses by interacting with innate and adaptive immune cells. VLPs stimulation creates an efficient immune response because the size and structure of VLPs resemble the actual size and structure of native viruses.[51,52] It has been shown in the literature that a strong HBoV-specific IgG response occurs after injection of HBoV1 and HBoV2 VLPs into the mice. Strong reaction is caused by VLPs efficiently binding to B-cell receptors, which subsequently induces high levels of neutralizing antibodies. Immunization with HBoV1 VLPs resulted in a higher IgG titre compared to immunization with HBoV2.[53]

Also, in this study, all B cell data, showing the effects of HBoV1, come from PBMC cultures. Further studies are needed to determine the effect of HBoV1 infection on pure isolated B and Breg cells.

In conclusion, the present study demonstrates HBoV1 interaction with B and Breg cells for the first time. The effects of HBoV1-VLPs on global gene expression profile was also investigated for the first time in this study. Potential immunomodulatory roles of HBoV1 infection was shown by flow cytometry, NGS and protein data from Luminex ELISA. In this study, we also demonstrated active uptake of HBoV1-VLPs by B cells. In order to understand the interaction between HBoV1 and B cells better, it is necessary to conduct further detailed cellular and molecular studies.

AUTHOR CONTRIBUTIONS

C.K. was involved in experiment design, data analysis, data interpretation, manuscript preparation and review; O.W., A.G. and T.D., were involved in the experiment, data analysis and manuscript review; G.T. was involved in data analysis and manuscript preparation; K.H., S.V., M.S.V., T.J. and M.A. were involved in data interpretation and manuscript review; C.A.A. was involved in experimental design, data interpretation, manuscript preparation and review.

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CONFLICT OF INTEREST STATEMENT

C. A. Akdis reported grants from the Swiss National Science Foundation, European Union (EU CURE, EU Syn-Air-G), Novartis Research Institutes, (Basel, Switzerland), Stanford University (Redwood City, Calif), Seed Health (Boston, USA) and SciBase (Stockholm, Sweden); is the Co-Chair for EAACI Guidelines on Environmental Science in Allergic diseases and Asthma; Chair of the EAACI Epithelial Cell Biology Working Group is on the Advisory Boards of Sanofi/Regeneron (Bern, Switzerland, New York, USA), Stanford University Sean Parker Asthma Allergy

Center (CA, USA), Novartis (Basel, Switzerland), Glaxo Smith Kline (Zurich, Switzerland), Bristol-Myers Squibb (New York, USA), Seed Health (Boston, USA) and SciBase (Stockholm, Sweden); and is the Editor-in-Chief of Allergy. The rest of the authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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