Introduction

Allergic diseases among children and youth are today one of the most common chronic diseases in westernized countries, and the prevalence has increased dramatically during the last decades. The great increase in allergy prevalence is commonly explained by the hygiene hypothesis, postulating that a clean lifestyle increases the risk of developing allergy, and that exposure to microbes early in life may protect against allergic disease. The hygiene hypothesis was initially based on the observation that children that are more consistently exposed to infections, such as children from large families and those attending day care, are statistically less likely to develop atopic disease than e.g. first-born or single children [1]. However, it is not yet clear whether it is bacterial or viral exposure that plays the main inhibitory role during the development of allergic manifestations.

Some common herpes virus infections have been linked to a reduced incidence of IgE sensitization and/or development of allergic disease, including HHV-6 [2] and Epstein-Barr virus (EBV) [3,4]. These viruses are transmitted via saliva and close contact between children or between the child and the parents. The virus transmission usually occurs during childhood, where HHV-6 is one of the first viruses to infect the young child and to establish a lifelong infection in the host [5,6]. HHV-6 is the causative agent of exanthema subitum, also known as roseola infantum or sixth disease, which is a benign disease characterized by 3-5 days of high fever, occasionally followed by a self-limiting rash. In Sweden, more that 90% of the adult population is infected, of which 60% is infected before 1 year of age [7]. HHV-6 replicates most efficiently in activated T-cells but the virus has a broad host cell range and infection in vivo occurs in a variety of cells including macrophages, dendritic cells, B-cells and NK cells [8].

The hallmark of atopic allergy is the presence of allergen specific IgE antibodies in serum and high infiltration of cells into the allergic lung. These infiltrating cells include a high proportion of eosinophils, as well as a lower weight of T cells, macrophages and monocytes. Eosinophils are recruited and activated during allergen challenge in both human subjects [9] and experimental animal models of disease [10]. During the allergic inflammation in the lung, eosinophils contribute to bronchi constriction, increased vascular permeability and, hence, an increased recruitment of inflammatory cells to the site of inflammation [11]. This process is accompanied by the interaction between the inhaled allergen and IgE, which activates mast cells to secrete inflammatory cytokines including the Th2 cytokines IL-4 and IL-13, leading to increased cellular infiltration and airway inflammation [12].

The allergic inflammation is dependent on the antigen specific activation of CD4+ Th2 cells and their production of IL-4, IL-5 and IL-13 [13]. Recruitment of eosinophils from the bone marrow to an inflammatory site is selectively regulated by Th2 cell derived IL-5, and eotaxin produced by epithelial cells [14]. In the lung, the eosinophilic inflammation is driven and regulated by IL-13 [15]. IL-13 regulates the mucus secretion and airway hyper responsiveness [15] and allows the eosinophils to participate in the maintenance and development of the Th2 response [16]. However, the most prominent role of IL-13 is to induce the class-switch and the secretion of allergen-specific IgE, a process that carried out together with IL-4 [17].

The allergic response can be blocked by type 1 interferons. This inhibition is mediated by IFN-α, which direct the CD4+ T cell response from an already established Th2 response into a more Th1-like response [18]. Additionally, human pDC from allergic individuals have impaired ability to produce IFN-α, a process that is inversely related to expression of the IgE receptor FcεRI [19]. Being the main producer of IFN-α in the body, pDC has been suggested a role in allergic disease by preventing allergic sensitization in vivo [20].
We have previously shown that infection with HHV-6 before 18 months of age is associated with a reduced incidence of IgE sensitization in young children [2]. This study was undertaken to test the hypothesis that HHV-6 exposure can block the development of allergic disease. For this purpose we have set up an animal model where mice were exposed to HHV-6 intra-peritoneal at the time of allergen sensitization. The effect of HHV-6 exposure on the development of allergic lung disease was then evaluated following repeated inhalation of allergen. We found that HHV-6 blocked the characteristic allergic response, characterized by allergen specific IgE, high infiltration of eosinophils to the allergic lung, and high production of Th2 specific cytokines.

**Material and Methods**

**Animals**

Male BALB/c mice, 6-8 weeks of age, were purchased from Scanbur AB (Sollentuna, Sweden). Male A129 (Interferon α/β receptor knockout (IFNα/βR)) mice, 8-10 weeks of age, were bred at the animal facilities at the department of Rheumatology and Inflammation Research. All mice were kept under standard conditions of temperature and light, in the animal facilities at the department of Rheumatology and Inflammation Research, University of Gothenburg. This study was approved by the Animals Ethics Committee in Gothenburg, Sweden.

**Virus preparation**

HHV-6 strain Z 29 (variant B) was grown for four weeks on Molt-3 cells in isocove medium, supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Gibco), 1% gentamycin (Essex Läkemedel AB) and 0.01% cortisone (Upjohn). After primary infection, fresh Molt-3 cells were added to the culture once a week in a concentration of 2 × 10^6 cells/ml. After four weeks of culture, the suspension was centrifuged at 1500 rpm for 5 min to remove cell debris. The virus-containing supernatants were then ultra centrifuged in a Ti-70 rotor for one hour at 36000 rpm (Optima™ L-100 XP Ultracentrifuge). The virus was gently resuspended in isocove medium complete (supplemented with 10% FBS, 1% L-glutamine, 1% gentamycin and 1% 2-ME) at 4°C over night. The number of HHV-6 DNA copies was determined with PCR at the virology routine facility at Sahlgrenska University hospital. Virus was inactivated at 2500 rad and stored at -70°C. The stock of HHV-6 was a kind gift from Helena Dahl, Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden.

**Allergy protocol and HHV-6 exposure**

Mice were sensitized intra-peritoneal (i.p) at two occasions, with 10 days in between, with 10^6 cells in iscove medium, supplemented with 10% FBS (Sigma-Aldrich), 10% BSA diluted in PBS) and stored at -20°C until further use. After 10 days in between, with 10^6 cells/ml in a total volume of 100 μl. This procedure was followed by staining with May-Grünwald-Giemsa (Merck). Approximately 250-300 cells were then differentially counted in a light microscope (Leica).

Lungs: 20% saponin (Sigma) diluted in PBS were added to the lung extracts and incubated at 4°C overnight, as previously described [21]. The samples were then centrifuged at 13000 rpm for ten minutes at 4°C and the obtained supernatants were stored at -20°C until analyzed for cytokine content.

**Antibody detection**

Passive Cutaneous Anaphylaxis (PCA): The levels of OVA-specific IgE in serum were determined by PCA in Sprague-Dawley rats [22,23]. The rats were briefly anaesthetized with Isoflurane followed by i.p. injection of 8 mg/kg xylazine and 40 mg/kg ketamine. Mouse sera were diluted serially in PBS and injected intra-dermal into the shaved dorsal skin of the rats. After 72 hours, the rats were challenged intra-venous (i.v) with 5 mg of OVA diluted in 1 ml of PBS containing 1% Evans blue (Sigma). 30 minutes later the rats were sacrificed and the skin areas were examined for the appearance of blue dots. The PCA titers were defined as the reciprocal of the highest dilution of serum giving a readable (>2 mm in diameter) blue spot.

OVA-specific IgG1 ELISA: The levels of OVA-specific IgG1 in serum were analyzed by ELISA. A 96-well microplate (Nunc) were coated with 20 μg OVA in a volume of 100 μl/well and incubated at 4°C over night. The plate was washed in 0.01% PBS-Tween. Serum samples were diluted in 0.5% BSA-PBS and added in 3-fold serial dilutions in a total volume of 100 μl/well. The plate was then incubated for 1 h at room temperature. After washing, a HRP-labeled goat anti-mouse IgG1 antibody (Southern biotech), diluted 1:2000 in PBS, were added, and the plate was incubated as previously. The plate was washed and developed for 20 min using 100μl of 0.1 mg/ml tetramethylbenzidine in 0.05% phosphate-citrate buffer pH 5.0 and 0.04% H₂O₂. Development was stopped with 25 μl of 1M H₂SO₄, and the absorbance was measured at 450 nm. The OVA-specific IgG1 titer was defined as the reciprocal sample dilution giving an absorbance of 0.4 above the background level.

HHV-6 specific IgG ELISA: HHV-6 specific IgG antibodies were analyzed by an in-house IgG ELISA. A 96-well microplate (Nunc) were coated with 8.5x10⁶ qeq of irradiated HHV-6 diluted in PBS in a total volume of 100 μl/well and incubated at 4°C overnight. The plate was washed three times in PBS and blocked with 0.5% BSA diluted in PBS for one hour at room temperature. Serum samples were diluted in 0.5% BSA-PBS and added in 3-fold serial dilutions in a total volume of 100 μl/well. After washing with 0.01% PBS-Tween, the plate was then incubated as previously with (ⅰ) biotin sheep antimouse IgG (Jackson) and (ⅱ) HRP-Streptavidin (Sigma-Aldrich), diluted 1:200 and 1:500, respectively, in 0.5% BSA. After each incubation the plate was washed...
as described. The plate was developed for 20 min using 100 μl of 0.1 mg/ml tetramethylbenzidine in 0.05% phosphate-citrate buffer pH 5.0 and 0.04% H₂O₂. Development was stopped with 25 μl of 1M H₂SO₄, and the absorbance was measured at 450 nm. The HHV-6-specific IgG titer was defined as the reciprocal sample dilution giving an absorbance of 0.4 above the background level.

**Cytokine measurements**

Lung extracts and BAL were assayed for cytokine content using the Th1/Th2 Cytokine Bead Array (CBA) kit for FACS (BD Biosciences) and the eotaxin ELISA DuoSet® kit (R&D Systems), both according to manufacturer’s instructions.

**Statistical analysis**

Non-parametric statistical analyses were performed with Mann-Whitney U-test using GraphPad Prism version 5.

**Results**

**HHV-6 exposure induce virus-specific IgG titers in mice**

We have previously attempted to establish a mouse model for clinical HHV-6 infection in mice that are transgenic for the HHV-6 receptor, human CD46. However, we could not create a clinical infection with HHV-6 in these mice (data not shown). Even though we were not able to establish a clinical infectious model for the virus, exposure to HHV-6 do induces a distinct virus-specific immune response. We found that exposure of mice to irradiated HHV-6 give rise to high HHV-6-specific IgG titers. Mice that were subjected to HHV-6 had significantly increased titers of HHV-6 specific IgG in serum, compared to control mice (Figure 1). The group of virus exposed mice had a median IgG titer of 105, ranging from 48 to 272, while the unexposed mice had a median IgG titer of 0 (range 0-12) (Figure 1).

**Exposure to HHV-6 decrease the allergic inflammation in the lung**

Allergic asthma is characterized by the infiltration of inflammatory cells to the lung, with eosinophils as the hallmark cell in the allergic inflammation. To evaluate the effect of HHV-6 in the allergic immune response, lung-fluids from HHV-6 exposed and OVA challenged mice was obtained and analyzed for cell profile. Exposure to HHV-6 significantly reduced the infiltration of inflammatory cells to the lung, with significantly lower numbers of both inflammatory cells (Figure 2A) and eosinophils (Figure 2B) in the BAL fluid, compared to BAL fluid of control mice. The proportion of eosinophils corresponded to 72% and 40% of the total cell numbers in lung fluid from HHV-6 treated mice and untreated control mice, respectively. The reduced infiltration of eosinophils to the lung of HHV-6 exposed mice was associated with significantly reduced levels of the eosinophil-attracting chemokine eotaxin in both BAL fluid (Figure 3A) and lung extracts (Figure 3B).

**Exposure to HHV-6 decreases the serum levels of allergen-specific IgE**

We have previously shown that childhood infection with HHV-6 protects against IgE sensitization [2]. To experimentally prove that HHV-6 can block allergen specific IgE formation, we analyzed the allergen-specific IgE prevalence in serum of OVA challenged mice that had been exposed to HHV-6 during OVA sensitization. We found that exposure to HHV-6 significantly decrease the levels of OVA-specific IgE; the OVA-specific IgE titer was reduced by half in virus exposed mice compared to control mice (Figure 4A). Furthermore, the serum levels of OVA-specific IgG1 were also significantly reduced in mice that were subjected to HHV-6 (Figure 4B).

**Exposure to HHV-6 decreases the Th2-driven immune response**

The allergic response is mediated by Th2 cells, which are characterized by the production of the inflammatory cytokines IL-4, IL-5 and IL-13. To strengthen our findings that HHV-6 reduces the allergic response in the lung of mice, the levels of Th2 associated cytokines was measured in BAL fluid and lung extracts. Indeed, mice that were subjected to HHV-6 prior to OVA challenge had significantly lower levels of all the Th2 related cytokines, compared to HHV-6 unexposed mice (Figure 5). This was evident in the lung fluid, as well as in lung extracts. The levels of IL-4 (Figure 5A) and IL-5 (Figure 5B) in BAL fluid were greatly diminished in HHV-6 exposed mice. Moreover, the inhibition of Th2 cytokine production by HHV-6 was most pronounced in the IL-13 levels, both in BAL fluid (Figure 5C) and in lung extracts (Figure 5F). The levels of IL-5 were also significantly diminished in the lungs of virus treated mice compared to mice that were not subjected to the virus (Figure 5E). No statistically significant differences in the levels of IL-2, IL-10 and IFN-γ was found between HHV-6 treated and untreated animals, and no measurable levels of TNF-α was found in any of the BAL samples or lung extracts (data not shown).

**The HHV-6 induced protection against allergic asthma does not require signaling trough the type 1 interferon receptor**

Our recent data show that plasmacytoid dendritic cells (pDC) can induce high levels of type 1 IFN in response to HHV-6, leading to a diminished production of the Th2-related cytokines IL-5 and IL-13 [2]. Thus, we hypothesized that IFN signaling could regulate the HHV-6 induced protection against OVA-induced allergic asthma in mice. For this purpose we used knockout mice lacking the IFN-α/β receptor (IFN-α/βR⁻). Mice were subjected to the OVA-induced allergy protocol and exposed to HHV-6 as previously described. We noticed a 2.7-fold decrease in serum titers of OVA-specific IgG in HHV-6 exposed IFN-α/βR⁻ mice compared to IFN-α/βR⁺ mice that were not exposed to the virus (Figure 6A). Furthermore, the infiltration of eosinophils to the lung was lower in HHV-6 exposed mice, since the numbers of eosinophils in the BAL fluid of these mice were reduced compared to HHV-6 unexposed mice (Figure 6B). However, the observed decrease in eosinophil accumulation in virus exposed mice was not statistically
**Discussion**

It is generally held that the large increase in allergic diseases seen in western countries is in part a consequence of our clean environment.

significant (p=0.06). Even though the reduction in eosinophil number in HHV-6 exposed mice did not reach statistical significance, we conclude that IFN-α/β signalling is not involved in the regulation of the HHV-6 mediated protection against allergic asthma in mice.
**Figure 5:** HHV-6 exposure decreases the Th2 cytokine production. BAL fluid and lung extracts from HHV-6 exposed mice (filled circles) contain lower levels of the Th2 associated cytokines IL-4, IL-5 and IL-13 compared to control mice (empty circles). Data represent the individual (circles) and the median (line) of IL-4 (A and D), IL-5 (B and E) and IL-13 (C and F) in BAL fluid (A, B and C) and in lung extracts (D, E and F) from 4 independent experiments with at least 5 animals/group.

**Figure 6:** Type I IFN-signaling is not required for HHV-6 induced protection against allergic inflammation. Levels of OVA-specific IgE in serum (A) and eosinophil number in BAL fluid (B) from HHV-6 exposed (filled circles) and control exposed (empty circles) IFN-α/βR−/− mice. Data represent the OVA-specific IgE PCA titer (A) and the number of eosinophils /ml BAL fluid (B) from each individual (circles) and the median value for each group (line) from 1 experiment with 6 animals/group.
lifestyle leading to a reduced childhood exposure to microbes. In this paper, we show in an experimental model of allergic asthma that exposure to HHV-6 protects against allergic inflammation in the lung. This data suggest that infection with, or exposure to, HHV-6 may protect against the development of allergic disease. We thus confirm experimentally our previous observation that children exposed to HHV-6 before 18 months of age are less likely to be IgE sensitized [2].

Several epidemiological studies illustrate that viral infection, including the herpes virus EBV, correlates with a reduced incidence of allergic sensitization [3,4]. Both HHV-6 and EBV induce a Th1 driven immune response [24,25], which may direct the adaptive immune response away from the pre-programmed Th2 response into more Th1-like immunity. In addition, infection with orofecal and foodborne microbes including Hepatitis A, endotoxin, Toxoplasma gondii, or Helicobacter pylori also indicate an inverse relationship with the incidence of allergy [26-29]. HHV-6 and EBV colonize the child in early age, with an 84% seroprevalence for HHV-6 at the age of 2 years [3]. HSV-1, Hepatitis A, Toxoplasma gondii and Helicobacter pylori on the other hand, infect the host later in life, during late childhood or adulthood.

The beneficial effect of microbe exposure on allergic inflammation is also supported in several experimental animal models [30]. Mouse models of OVA-induced allergic asthma are widely used to study allergic inflammation in vivo [31]. Using the OVA-model we could show that mice that were administered systemically (i.p) with HHV-6 had significantly lower infiltration of inflammatory cells and eosinophils to the lung. This was accompanied by decreased serum levels of OVA-specific IgE, as well as decreased levels of the Th2 cytokines IL-4, 5 and 13. In a similar model, exposure to CpG ODN concurrent to OVA sensitization and prior to OVA challenge was shown to inhibit the Th2 driven allergic immune response [32]. i.p. administration of CpG reduced the levels of serum IgE, the production of IL-5 and IL-13, as well as the eosinophil accumulation in nasal cavity fluids. Additionally, exposure to endotoxin [33] or Influenza A virus [34] have also been shown to diminish the allergic response in mice, by shifting the Th2 response into a Th1 response and by blocking the Th2 cell infiltration to the lung, respectively. The latter finding is controversial thought, as other studies show that pre-exposure to Influenza A virus increase the sensitivity to allergic exposure and thus exacerbate the allergic disease [35,36].

Even though it is commonly accepted that certain infections can reduce the incidence of allergic sensitization and disease, the underlying mechanisms are poorly understood. Our results show that treatment of mice with HHV-6 inhibits the Th2 biased immune response, including Th2 cytokine production, eosinophilic inflammation and allergen-specific antibody production. One mechanism that has been suggested for the microbe-induced protection against allergic inflammation in mice is a shift from Th2 to Th1 immunity, which is characterized by high levels of IFN-γ [33,34]. However, we could not detect any increased levels of IFN-γ after exposure to HHV-6. A Th1 biased CD4+ T cell response is however not necessarily beneficial for the host and may instead worsen the allergic inflammation as a consequence of high levels of microbe-induced IFN-γ [35-37]. Furthermore, transfer of Th1 cells to the lung prior to OVA sensitization can cause inflammation in the lung and transfer of both Th1 and Th2 cells together further enhance the inflammation by evoking a robust eosinophilic inflammation [38]. Conversely, IFN-γ together with IL-10 has also been suggested to have a regulatory role in the inflamed lung [37], though this most likely is due to the regulatory and anti-inflammatory effect of IL-10. In our hands neither of these two cytokines was affected by the exposure to HHV-6. Still, we do not rule out a role for these two cytokines, as HHV-6 can induce a Th1 profile, as well as the secretion of IL-10, at least in human cells in vitro [2].

Type 1 IFNs represent the first line of defense against most viral infections, where large amounts of IFN-α/β are secreted in response to virus [18,39-42]. This is true also for HHV-6, which induces huge amounts of IFN-α in human cells in vitro [2]. Furthermore, HHV-6 activated pDC can inhibit production of the Th2 cytokines IL-5 and IL-13 in responding T cells [2], perhaps through the induction of IFN-α [18]. To assess the requirement of IFN-α/β signalling on the HHV-6 mediated anti-allergic effects in vivo, we used the OVA-induced allergic asthma model in mice that lack the IFN-α/β receptor. HHV-6 exposure decreased the levels of OVA-specific serum IgE and lowered the numbers of infiltrating eosinophils into the allergic lung of HHV-6 exposed IFN-α/β KO mice. Thus, IFN-α/β signalling is not an absolute requirement for HHV-6 induced protection against allergic disease in mice. This does not rule out the possibility that IFN-α/β and/or pDC may influence the HHV-6 induced protection against allergy. Administration of IFN-α can block the induction of IgE and allergic symptoms in vivo [43] and pDC have been ascribed a central role in the prevention of allergic sensitization in rodents [20].

In summary, we confirm experimentally our previous data showing that childhood infection with HHV-6 may protect against allergic sensitization. Exposure of mice to HHV-6 protects both against allergen-specific IgE and Th2 responses and the development of allergic inflammation in the lung. Given that HHV-6 is one of the first viruses to infect the young child, we suggest that HHV-6 might influence the maturation of the immune system, leading to the prevention of allergic sensitization and the development of allergic manifestations.

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