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A1 β-CASEIN VARIANT OF COW MILK EXACERBATES HOUSE DUST MITE INDUCED ALLERGIC AIRWAY DISEASE IN MURINE MODEL

YADAV S[1, 2], GHEWARE A[3], YADAV NDS[4], SINGH VP[3*] AND SHARMA P[2*]

1: National Institute of Biologicals, Plot No. A-32, Sector-62, Institutional Area, Noida-201309, India

2: Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Sector 125, Noida-201313, India

3: CSIR-Institute of Genomics & Integrative Biology, Mathura Road, New Delhi- 110025, India, **4:** District Disease Diagnostic Laboratory, Nuh, Department of Animal Husbandry & Dairying, Haryana-122107, India

***Corresponding Author: Dr. Pankaj Sharma/ Dr. Vijay Pal Singh: E Mail: psharma8@amity.edu; psharma8.amity@gmail.com; vpsinghigib@gmail.com**

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ABSTRACT

Milk from cows is consumed by all age groups as an excellent source of high-quality protein and other nutrients. The β casein which constitutes up to 35% of the total protein in cow milk has been found to have 13 different genetic variants of which the A1 β casein variant of cow milk has been associated with higher incidence of diseases like type 1 diabetes, ischaemic heart disease and neurological disorders such as schizophrenia and autism. The objective of the present study was to evaluate the role of A1A1, A1A2 or A2A2 β casein variants of cow milk in modulating HDM induced allergic airway disease in murine model. We found that on inducing acute allergic airway disease with HDM in mice fed with A1A1, A1A2 and A2A2 milk for 30 weeks, the A1A1 and A1A2 milk fed BALB/c mice had significantly higher airway hyperresponsiveness as compared to those fed with A2A2 milk. The IL-4 levels in BAL as well as serum and IL-5 levels in serum were found to be significantly higher in the A1A1 milk fed mice as compared to those fed with A2A2 milk while the IFN-γ levels were not affected clearly indicating Th2 polarization of the immune response. The IgE and IgG levels were significantly

higher in BAL as well as serum of mice fed with A1A1 milk as compared to those fed with A2A2 milk which was also accompanied with significantly increased influx of inflammatory cells which was mainly due to increase in eosinophils, lymphocytes and neutrophils in BAL and eosinophils and lymphocytes in blood. Histology of lung tissue sections of mice fed with A1A1 milk also revealed increased airway inflammation, marked collagen tissue deposition and presence of mucus as compared to other milk groups. Based on these results it is concluded that the A1A1 β casein variant of cow milk is exacerbating HDM induced allergic airway disease in murine model.

Keywords: Airway Hyperresponsiveness; β Casein; Cow Milk; Oral Feeding; Th2 Response; Allergic Airway Disease

INTRODUCTION

The incidence of asthma and allergic diseases has increased dramatically in the recent decades and the burden due to allergic airway disease has been found to be on a rising trend in terms of both prevalence as well as severity of the disease. As per WHO estimates, nearly 300 million people of all ages worldwide have asthma and globally around 250,000 people die of asthma every year **[1]**.

Milk from cows is an excellent source of high-quality protein and other nutrients and is one of the first foods introduced early to infants. The β casein constitutes up to 35% of the total protein in cow milk and has 13 different genetic variants, of which A1 and A2 are the most common **[2]**. It has been well established that due to the change of just one amino acid from proline at 67^{th} position in A2 to Histidine in A1 variant, the gastrointestinal proteolytic digestion of A1 variant of β casein from both raw as well as processed milk results in production of beta- casomorphins, mainly BCM-7 which is a bioactive peptide established to have opioid and proinflammatory activity, which is not seen in A2 variant **[3-5]**. The yield of BCM-7 from the A1A1 variant has been found to be almost 3.2 times more than A1A2 variant of β-casein while it was not detected from A2A2 variant of β-casein **[6]**. In fact, consumption of the A1 β casein variant of cow milk and higher levels of BCM-7 has been associated with higher incidence of diseases like type 1 diabetes, ischaemic heart disease, sudden infant death syndrome, neurological disorders such as schizophrenia and autism while the A2 variant has been found to have a protective effect **[2, 7-14]**. In recent years, many studies

have focused on the gastrointestinal effects of A1/A2 forms of β-casein **[15]** and have reported A1A1 β-casein variant to be causing increased Th2-driven gut inflammation in mice **[16]** and worsened gastrointestinal symptoms in humans **[17-18]**.

Recently, we found that cow milk having A1 variant of β-casein was causing airway hyperresponsiveness and Th2 polarization of the immune response leading to establishing allergic airway disease in murine model **[19]**. In the present study, we investigated the role of A1 and A2 variants of cow milk in individuals who develop respiratory allergies by evaluating their effects in a mouse model of allergen-induced pulmonary inflammation. We used house dust mite (HDM), *Dermatophagoides pteronyssinus* to establish acute airway disease in the mice fed with the A1A1, A1A2 and A2A2 β casein variants of cow milk, as HDM is the most frequently implicated source of mite-related allergens in subjects with respiratory allergy **[20]**. In the present study, while HDM extracts were used which are more complex from an immunological perspective as compared to the single isolated allergen or their epitopes and represent the real-life aeroallergen exposure in humans.

MATERIALS AND METHODS

Genotyping of cows

To obtain the A1A1, A1A2 and A2A2 combinations of milk for the study, Holstein Friesian and Hariana cows from organised dairy farm were genotyped to screen for A1 and A2 variants of β casein. Blood samples were collected in sterile tubes containing EDTA as anticoagulant. Genomic DNA was isolated by using Ultra clean BloodSpin DNA Isolation Kit (MOBIO) and purity and concentration of DNA was established using nanodrop method (Thermo Scientific Equipment). Allele Specific- PCR was carried out on Agilent Technologies Sure Cycler 8800 as described previously [21]. Forward primers with either A (IGBhF 5' CTT CCC TGG GCC CAT CCA 3') or C (IGBpF 5' CTT CCC TGG GCC CAT CCC 3') at the 3' end and a common reverse primer (IGBR 5' AGA CTG GAG CAG AGG CAG AG 3') were used to amplify a 244 bp fragment of B-casein gene. Primer pairs IGBhF (forward) -IGBR (reverse) and IGBpF (Forward)-IGBR (reverse) were intended to give rise to Histidine (A1) and Proline (A2) specific amplicon, respectively. PCR was carried out using 50 ng of genomic DNA in a final reaction volume of 10 µl. The PCR conditions were: initial denaturation at 95°C for 5 min, followed by 30 cycles of

95°C for 30 sec, 65°C for 45 sec and 72°C for 45 sec, and a final extension of 72°C for 10 min. The PCR products were visualized after electrophoretic separation in 2.0 % agarose gel.

Milk Collection and Processing

The A1A1, A1A2 and A2A2 β casein variants of cow milk were obtained from the genotyped cows every week, transported at 2-8°C and boiled at 100°C for 2 minutes. After the milk samples were cooled to room temperature, they were stored overnight at 4-6 °C. The fat layer was then removed and milk was aliquoted and stored at 4-6°C and used for feeding the BALB/c mice under this study.

Mice Housing and Maintenance

Specific Pathogen Free male BALB/c mice of 3-4 weeks age were obtained from National Institute of Biologicals (Noida, Uttar Pradesh, India) and experiments were performed at Animal Facility of CSIR-Institute of Genomics and Integrative Biology (IGIB), Delhi. The mice were housed in sterile individually ventilated cages (Citizen) with corncob as bedding and 12:12 hour day and night cycle. Autoclaved pellet diet and RO water were provided ad libitum. All animals were housed at a temperature of 22-25°C and at relative humidity of 50-60%.

Ethical Statement

All animals used in this study were maintained according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA) and the procedures performed on animals in this study were in strict compliance with the approvals granted by the Institutional Animal Ethics Committee (IAEC) of CSIR-IGIB, Delhi and guidelines provided by CPCSEA.

Experimental Design

BALB/c mice were randomized into 5 experimental groups (8 mice/group) which were named according to the treatment given to them i.e. Sham Control, HDM Control, A1A1 Milk + HDM, A1A2 Milk + HDM and A2A2 Milk +HDM. The two control groups received drinking water and other 3 groups received A1A1, A1A2 and A2A2 β casein variants of milk respectively at a dose rate of 10ml/kg body weight by oral gavage for 5 days per week.

HDM‑induced Mouse Asthma Model

After feeding A1A1, A1A2 and A2A2 β casein variants of milk for a period of 28 weeks, mice were sensitized with PBS (Sham control) or 1 µg HDM/100 µl PBS (Greer Laboratories, USA) intraperitoneally (i.p.) on day 0 followed by an intra nasal (i.n.) challenge with PBS or 10 µg HDM/40 µl PBS under short term isoflurane

anaesthesia on days 7–11 **[22]**. The different β casein variants of cow milk were continuously provided throughout this period also, making the total duration of milk feeding to 30 weeks. The airway resistance was measured on day 12 after which the animals were sacrificed to collect the body fluids and organs. The schematic details are provided in **Figure 1**.

Measurement of Airway Hyperresponsiveness (AHR)

The airway resistance was estimated using the FlexiVent system (SCIREQ, Montreal, Canada), which integrates the computer-controlled mouse ventilator with the measurements of respiratory mechanics under anesthesia (Xylazine 10mg/kg and Thiopentone 60mg/kg, intraperitoneal) in tracheostomized mice as described previously **[23]**. The results were expressed as airway resistance (fold change) with increasing concentrations of methacholine (Sigma).

Collection of Blood and Bronchoalveolar Lavage (BAL) Fluid

Immediately after measurement of airway resistance by FlexiVent system, mice were sacrificed using an overdose i.p of thiopentone sodium at a dose of 100mg/kg. Blood samples were collected by intra-cardiac route; serum was obtained by centrifugation at 3,000 rpm for 10 min and stored at –80°C until analyzed for cytokines and immunoglobulin levels. After cannulating the trachea, BAL fluid was collected by washing lungs with 1.0 ml of PBS from each mouse which was then centrifuged at 400*g* at 4°C for 10 min and the supernatant was stored at –80°C until analyzed for cytokines and Immunoglobulin levels while pellets were used to analyze BAL cells.

Cell Counts and Lung Histology

BAL cells were washed three times with PBS and the pellet was resuspended in 250 µl of cold PBS and a small volume was loaded on the Neubauer's chamber for total cell counts. Cytospin preparations were made and stained with Leishman's stain for differential counts. The cells were identified by standard morphology; 200 cells were counted and the absolute number of lymphocytes eosinophils and neutrophils were calculated.

For lung histology, the lungs were fixed in 10% buffered formalin and then paraffin embedded tissues were cut into 5 µm sections and stained with Hematoxylin and Eosin (HE) to assess inflammation, Mason Trichrome (MT) for lung tissue remodelling and collagenisation and Periodic Acid-Schiff (PAS) for mucus secretion. Histopathological

analysis was performed for 4 mice from each group.

Measurement of Cytokines and Immunoglobulin Levels

The levels of cytokines IL-4, IL-5 and IFN-γ and immunoglobulins IgG and IgE were measured in triplicate in BAL fluid supernatant and serum by sandwich ELISA (RayBiotech Inc, USA), as per manufacturer's instructions.

Statistical Analysis

Results are presented as mean ± standard error of mean (SEM) and analyzed by one-way ANOVA. Significance has been denoted by $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.000$ using GraphPad prism.

RESULTS

Allergic airway disease is characterized by airway inflammation, reversible airway obstruction and airway hyperresponsiveness. Eosinophilic airway inflammation and the imbalance of T helper type I (Th1)/Th2 lymphocytes are generally recognized as pivotal factors in the pathogenesis of asthma **[24]**. In the present study, we were interested to investigate whether the A1A1, A1A2 and A2A2 β casein variants of cow milk modulated the immune function in the lungs of HDM-induced murine model of allergic airway disease. Therefore, we assessed airway hyperresponsiveness, levels of Th2 (IL-4 and IL-5) and Th1 (IFN-γ) cytokines, inflammatory cells, IgE and IgG levels in bronchoalveolar lavage (BAL) fluid and blood and lung tissue pathology.

Determination of A1 & A2 β casein variants by Allele Specific PCR

A total of 15 cattle, including 9 Holstein Friesian (HF) cross breed cattle and 6 Hariana cattle from organized farms were genotyped using Allele Specific PCR to obtain all the three β casein variants of cow milk i.e A1A1, A1A2 and A2A2 **(Figure 2)**. These cattle were individually identified by their ear tags and used for providing milk for feeding the mice throughout the study.

A1A1 milk exacerbated HDM-induced AHR

To evaluate the effect of A1A1, A1A2 & A2A2 β casein variants of cow milk on airway hyperresponsiveness, airway resistance was measured by FlexiVent after feeding all the three β casein variants of milk for 30 weeks and inducing acute allergic airway disease in mice in last 2 weeks using HDM. The A1A1/HDM and A1A2/HDM group showed significantly higher airway resistance as compared to the A2A2/HDM group in which the resistance was found to be lesser than the HDM control group **(Figure 3)**.

A1A1 milk increased HDM-induced inflammatory cells in lungs and blood

To evaluate any influx of inflammatory cells into the airways of HDM-allergic mice, BAL of mice fed for 30 weeks with the A1A1, A1A2 and A2A2 variants of cow milk was examined. Challenge with HDM induced a significant increase in the total number of inflammatory cells, eosinophils, lymphocytes and neutrophils in the mice as compared to PBS/Sham group. The A1A1/HDM group had a significantly higher number of total cells **(Figure 4A)**, eosinophils **(Figure 4B)**, lymphocytes **(Figure 4C)** and neutrophils **(Figure 4D)** as compared to the A2A2/HDM group. A similar trend was observed in systemic circulation for total white blood cells **(Figure 4E)** as also increased number of eospinophils **(Figure 4F)** and lymphocytes (Figure 4G); though no significant difference was observed in the neutrophil count in any of the groups fed with different β casein variants of cow milk **(Figure 4H)**.

A1A1 milk increases Th2 cytokines levels in mice on induction of allergic airway disease

To evaluate any shift in the immune response towards Th1 or Th2 after HDM challenge in mice fed with A1A1, A1A2 & A2A2 β casein variants of cow milk, we measured the levels of Th1 cytokine IFN γ and Th2 inflammatory cytokines IL-4 and IL-5 in both BAL as well as serum collected from the five groups of mice using ELISA. The A1A1/HDM group showed significantly higher levels of IL-4 in BAL as compared to the HDM and A2A2/HDM groups, **(Figure 5A)**. The IL-5 levels in BAL of A1A1/HDM were also significantly higher than HDM alone, A1A2/HDM as well as A2A2/HDM **(Figure 5B)**. There was no significant difference in the levels of IFN γ in BAL of any of the five experimental groups **(Figure 5C)**.

In serum, the IL-5 levels in A1A1 /HDM group were significantly higher than the levels in A1A2/HDM and A2A2/HDM groups, which in turn were found to be significantly lower as compared to the HDM control **(Figure 5D)**. There was no significant difference in the levels of IFN γ in serum of any of the milk fed groups. Interestingly, the IFN γ levels in the A2A2/HDM group were found to be significantly lower as compared to the HDM control group **(Figure 5E)**.

A1A1 milk increases IgE and IgG levels in mice

In the current study, we found that the IgE as well as IgG levels in the BAL of A1A1/HDM group were significantly more than the A1A2/HDM as well as A2A2/HDM

group (Figure 6A & B). The serum IgE levels in the A1A1/HDM group were also found to be significantly higher than the A1A2/HDM and A2A2/HDM group **(Figure 6C)**. The IgG levels in serum of the A1A1/HDM group were found to be significantly more than A2A2/HDM group **(Figure 6D)**. Interestingly, in BAL the levels of IgG in the A2A2/HDM group **(Figure 6B)** and in serum, the levels of IgG in both A1A2/HDM and A2A2/HDM groups **(Figure 6D)** were found to be significantly lesser than the HDM control which may be suggesting some protective role of the A2 β casein variant.

A1A1 milk increases inflammation, collagen and mucus deposition in lungs

The H&E staining of the lung tissue sections showed increased airway inflammation in the A1A1/HDM group as compared to control as well as A2A2/HDM groups **(Figure 7A)** which further confirms the presence of increased inflammatory cell counts seen in this group. The MT staining of lung tissue sections showed marked collagen tissue deposition in the mice of A1A1/HDM group as compared to the mice of A1A2/HDM and A2A2/HDM groups **(Figure 7B)**. Further, PAS staining revealed significant hypersecretion of mucus in lung tissue sections of mice in A1A1/HDM group indicating goblet cell hyperplasia and mucin overexpression in the mice fed with the A1 β casein variant of cow milk whereas no mucous was seen in the mice of the A1A2/HDM and A2A2/HDM groups **(Figure 7C)**.

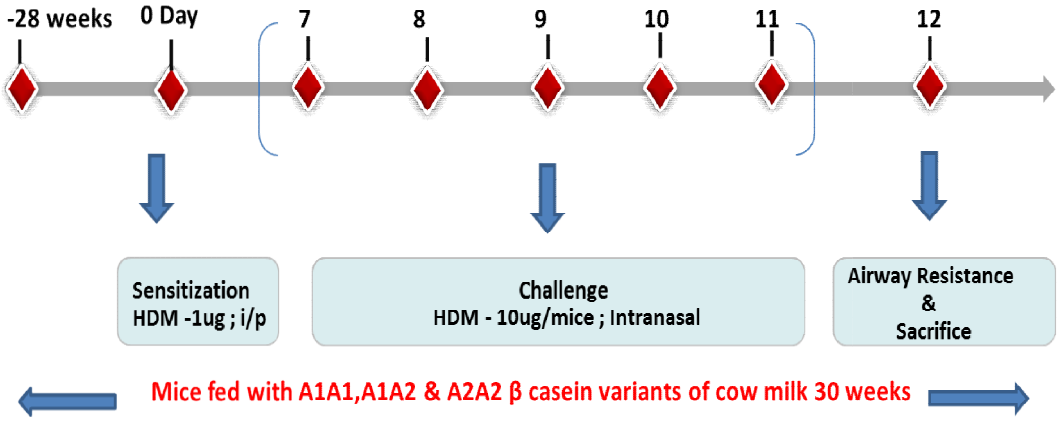

Figure 1: Induction of Acute Model of Allergic Airway Inflammation after 28 weeks of feeding milk

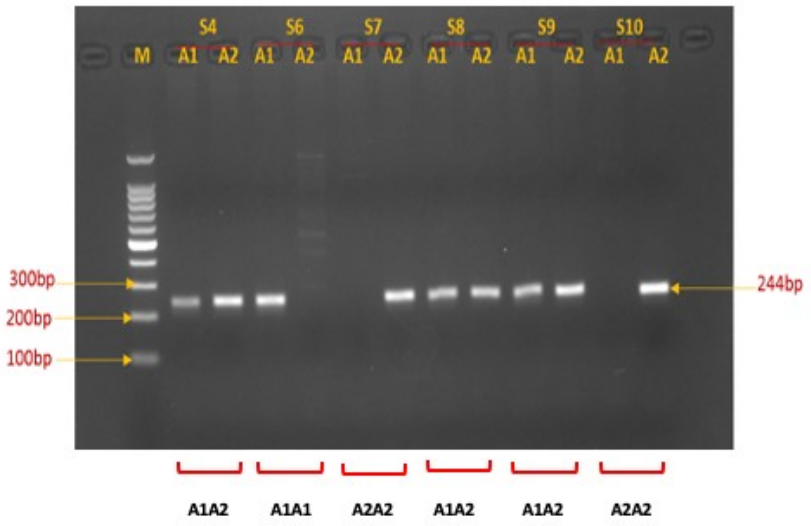

Figure 2: Determination of A1A1, A1A2 and A2A2 genotypes of cows by allele-specific PCR.
A1 and A2 specific PCR product for each cow have been indicated above each lane and the genotype shown below the image

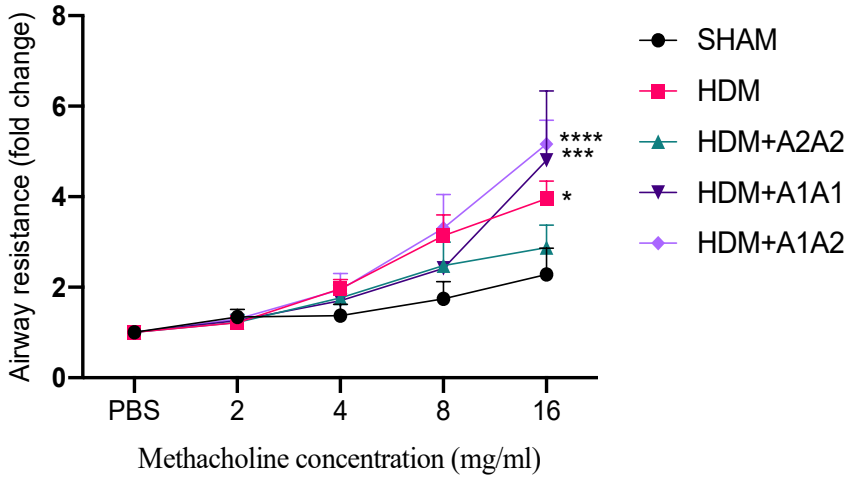

Figure 3: A1A1 milk exacerbates HDM-induced airway hyperresponsiveness. Airway Resistance (n=8-10 mice/group) were measured at different doses of methacholine after 30 weeks of feeding A1A1, A1A2 and A2A2 milk. Results are presented as mean ± standard error of mean (SEM) and analyzed by one-way ANOVA. Significance denoted by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.000$

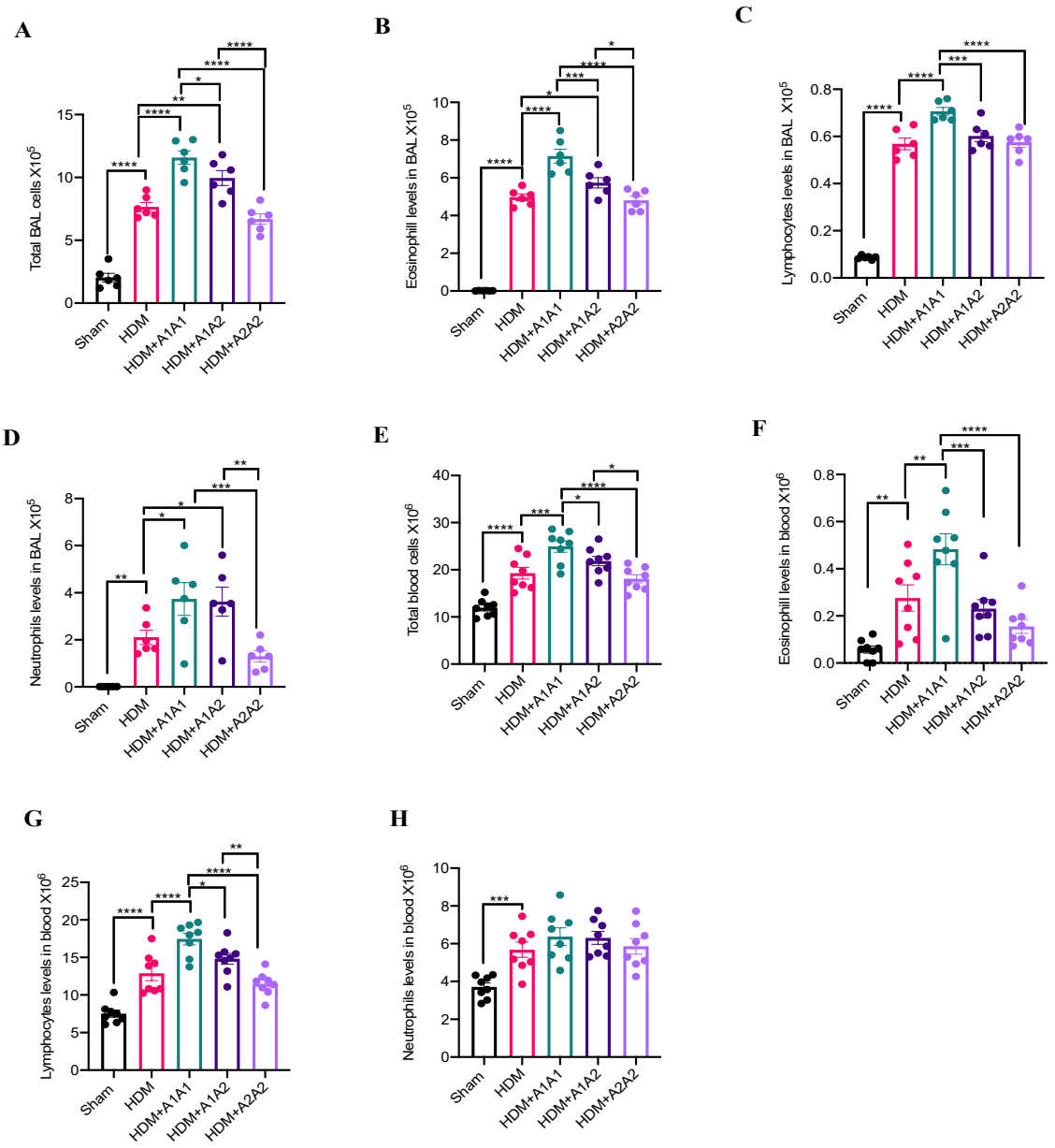

Figure 4: Total and differential cell counts in bronchoalveolar lavage (BAL) fluid and blood of HDM-induced allergic airway disease murine model after feeding A1A1, A1A2 and A2A2 β casein variants of milk for 30 Weeks. A) Total BAL cells, B) Eosinophils in BAL, C) Lymphocytes in BAL, D) Neutrophils n BAL, E) Total Blood cells, F) Eosinophils in Blood, G) Lymphocytes in blood, H) Neutrophils in blood. Results are presented as mean ± standard error of mean (SEM) and analyzed by one-way ANOVA. n=6-8 mice/group. Significance denoted by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.000$

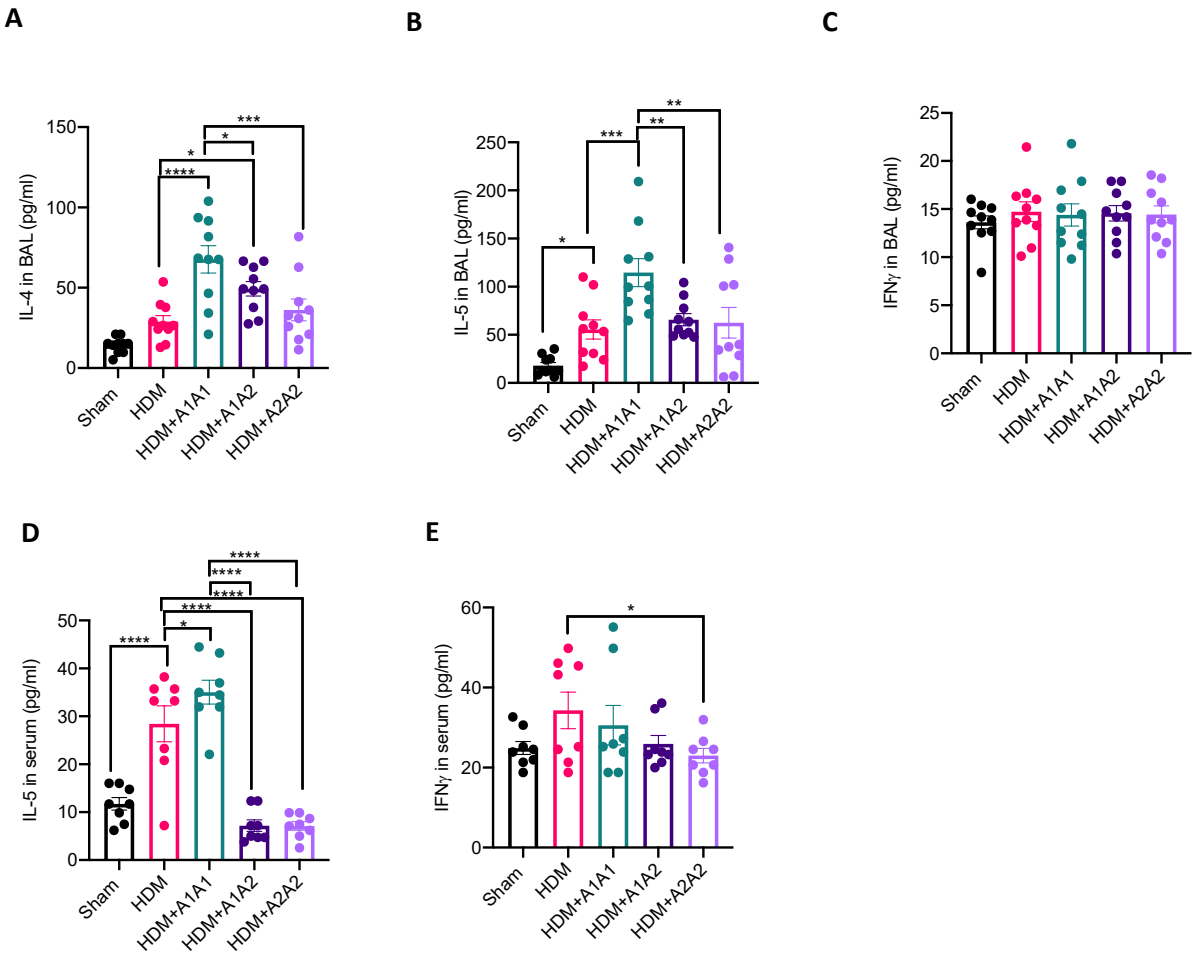

Figure 5: Cytokine profiling in HDM-induced allergic airway disease murine model after feeding A1A1, A1A2 and A2A2 β casein variants of milk for 30 Weeks. A) IL-4 in BAL, B) IL-5 in BAL, C) IFNγ in BAL, D) IL-5 in serum and E) IFNγ in serum. All values are expressed in pg/ml and results are presented as mean ± standard error of mean (SEM) and analyzed by one-way ANOVA. n = 8-10 mice/group, Significance denoted by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.000$

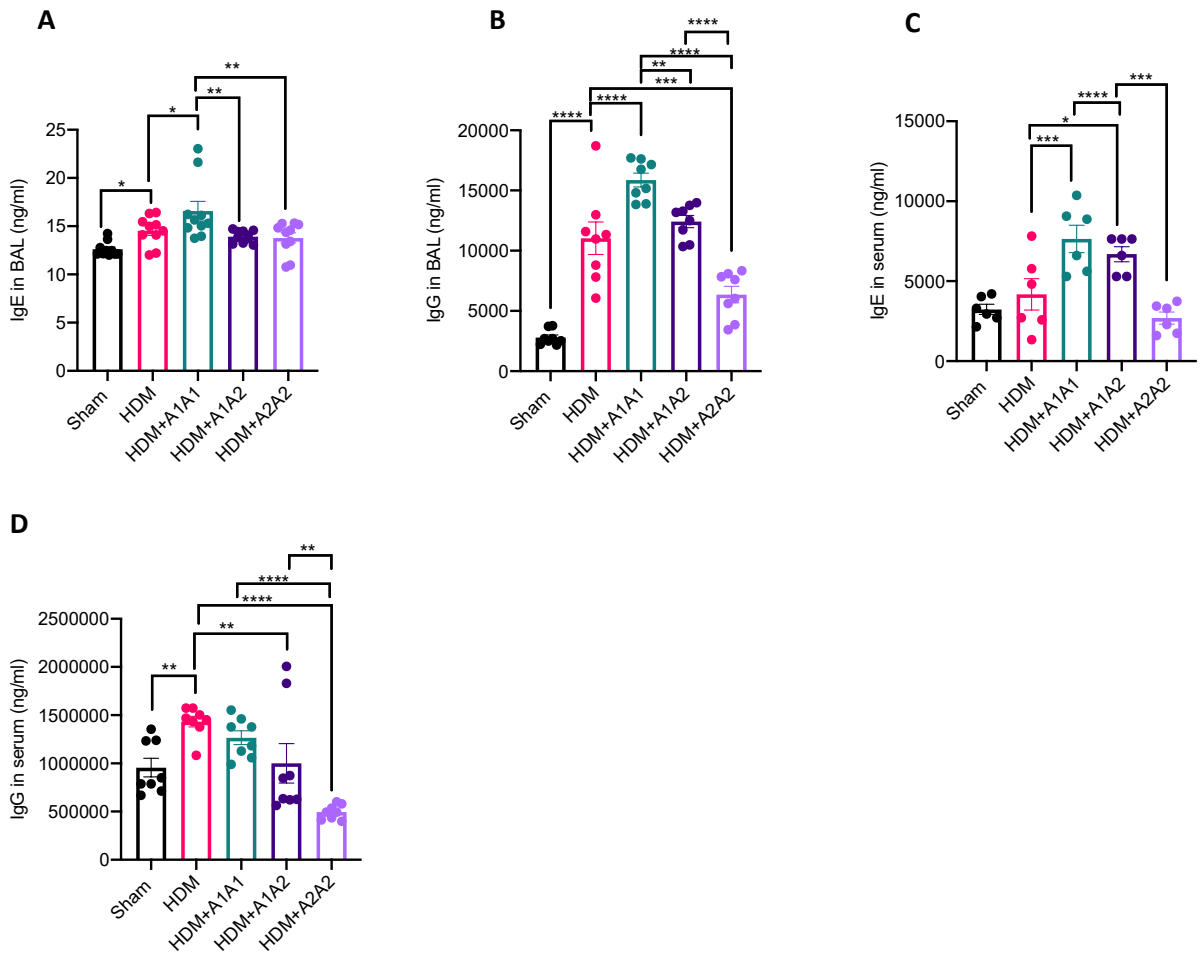

Figure 6: Humoral immune response in HDM-induced allergic airway disease murine model after feeding A1A1, A1A2 and A2A2 β casein variants of milk for 30 Weeks. A) IgE in BAL, B) IgG in BAL, C) IgE in serum and D) IgG in serum. All values are expressed in ng/ml and results are presented as mean ± standard error of mean (SEM) and analyzed by one-way ANOVA. n = 6-10 mice/group, Significance denoted by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.000$.

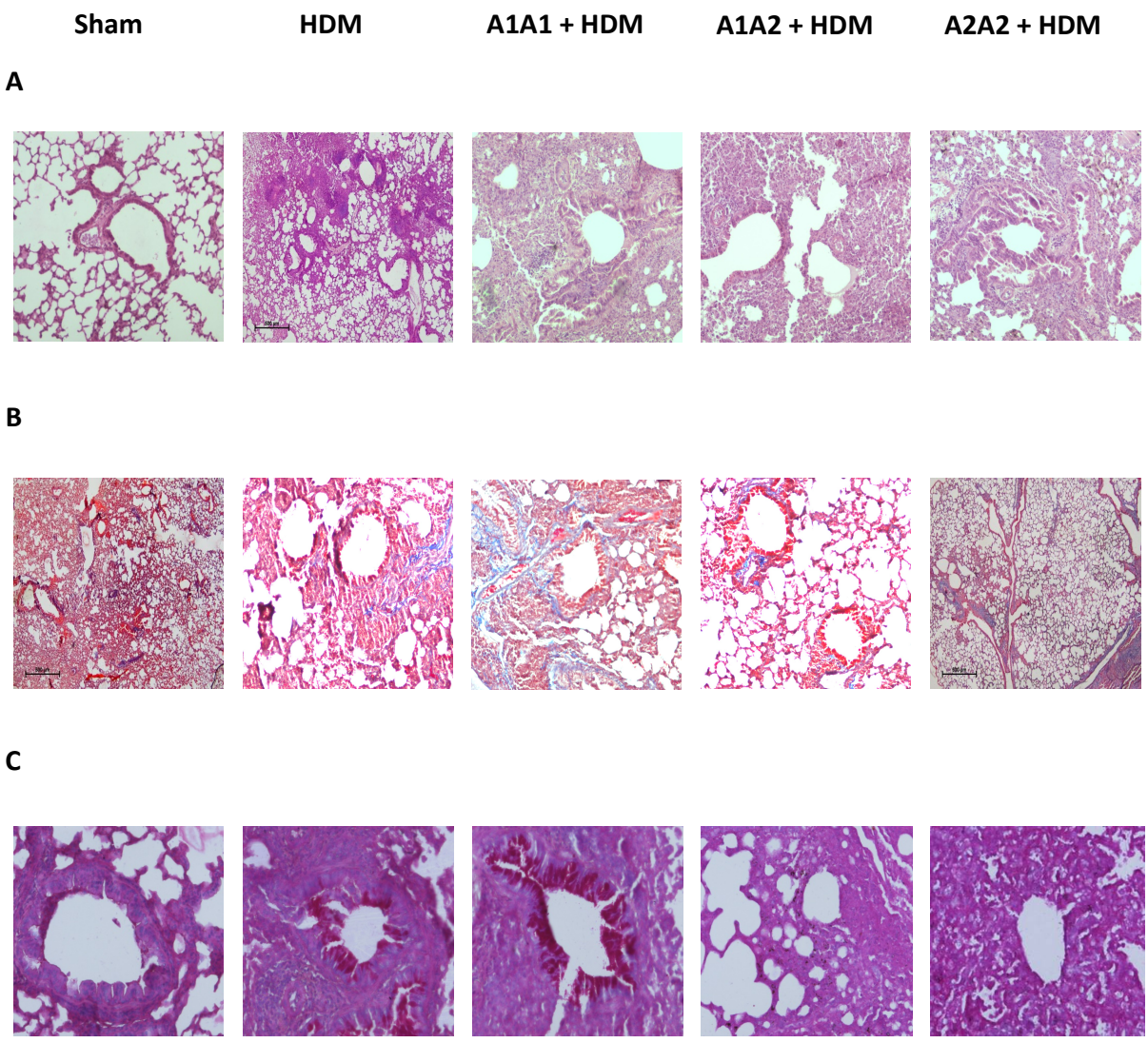

Figure 7: Lung histology in HDM-induced allergic airway disease murine model after feeding A1A1, A1A2 and A2A2 β casein variants of milk for 30 Weeks. Mouse lungs stained with A) Hematoxylin & Eosin (H&E) 10x, B) Mason Trichrome (MT) 10x and C) Periodic acid-Schiff (PAS); 40x magnification. The images are representative of 4 animals /group

DISCUSSION

The A1 variant β-casein of cow milk has been reported to be a potential etiological factor in a number of diseases like diabetes mellitus-1, ischemic heart disease, autism, schizophrenia **[7-14]**. Several studies have also focused on the gastrointestinal effects of A1and A2 forms of β-casein **[15]** and on the protective effect of raw milk on allergies and asthma **[25-30]** but there are limited studies which have evaluated the effect of A1 and A2 β-casein variants of cow milk on

respiratory health. Based on recent observation that long-term feeding of A1A1 milk induced significant Th2-driven allergic airway inflammation in mice **[19]**, the focus of the current study was to evaluate the role of these β casein variants of cow milk in allergen-induced airway inflammation.

We found that mice fed with the A1A1and A1A2 milk exhibited significantly higher airway hyperresponsiveness along with increased recruitment of inflammatory cells (including eosinophils) in both lungs as well as systemic circulation. This was accompanied with enhanced lung inflammation, collagen deposition and mucous hypersecretion. All these changes are typical manifestations of increased pulmonary hypersensitivity characteristic of allergic individuals **[31-33]**. Further, increased IL-4 and IgE levels in mice fed with A1 milk observed in our study are hallmarks of allergen-induced type I hypersensitivity **[34-35]**. Interestingly, mice fed with A2A2 milk had significantly lower airway resistance along with reduced IgG levels in both blood and BAL compared to allergen-exposed controls, suggesting an immune-protective effect of A2 milk.

Our observations are in line with earlier studies that noted that the A1 variant of β casein induced inflammatory response in gut of mice by activating the Th2 pathway **[16]** and worsening of the gastrointestinal symptoms, increased gastrointestinal transit time and increased serum inflammation markers in humans **[18]**. These effects have been attributed to the opioid and proinflammatory activities of BCM-7 which is produced only on digestion of A1 variant of cow milk **[42-44]**, its yield from the A1A1 variant is almost 3.2 times more than that from the A1A2 variant of β-casein and is undetectable from A2A2 variant of β-casein **[6]**. Betacasomprhins such as BCM-7 are known to have opioid and proinflammatory activities and stimulate the mucin production **[38]**.

There is extensive cross-talk between the mucosal sites of intestine and lungs; changes in the gut play an important role in directing the immune responses in lungs **[45]**. In patients suffering from moderate to severe persistent asthma, food-induced allergic reactions may increase airway hyperresponsiveness even though they may not show any symptoms immediately after ingestion **[46]**. Also primary sensitization to one allergen is known to prime the immune system to mount a more intense response to a completely different inhaled allergen; compared to the mono-allergic mice, the bi-allergic ones develop significantly higher

airway hyperresponsiveness with increased levels of IL-4, IL-5 and IgE **[41]**. Oral feeding of A1 milk leads to gut inflammation resulting in increased intestinal permeability **[17-18]**. The generation of pro-inflammatory β-casomorphins like BCM-7 in the jejunum **[5]** may enter systemic circulation and transport to different parts of the body including the lung. In individuals predisposed to allergic pulmonary inflammation, immediate hypersensitivity on re-exposure to the allergen may further be exuberated by prolonged feeding of A1 milk that is known to generate peptides with pro-inflammatory effects. Although detailed investigations using appropriate animal models are needed, it may also explain the increased incidence like type 1 diabetes, cardiovascular and neurological disorders in humans consuming cow milk with A1 variant of β-casein.

CONCLUSION

Based on the results obtained, it can be concluded that the A1A1 β casein variant of cow milk is exacerbating HDM induced allergic airway disease in the murine model and provides the basis to understand the mechanistic details, pathways and mediators involved in this process. The A2A2 milk has shown some immune-protective role, which needs detailed investigation. The findings of this study are significantly important for public health, as milk is being provided as basic food to infants and children and also consumed by adults all over the world.

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