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Serine protease allergen favours Th2 responses via PAR-2 and STAT-3 activation in murine model

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Short title: Per a 10 favours Th2 response via PAR2/STAT3 pathway

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Abstract

Background: Protease activity of Per a 10 favours Th2 responses by differential regulation of IL-12p70 and IL-23 cytokine subunits. The present study aims to elucidate the underlying mechanism of differential regulation of IL-12p70 and IL-23.

Methods: PAR-2 activation was blocked in murine model by administering SAM11 before each sensitization. CD11c⁺ p-STAT3⁺ cells were measured in lungs by flow cytometry. BMDCs were pre-treated with SAM-11 or isotype control or static and stimulated with Per a 10. p-STAT3 levels were measured using Western blot. Transcript levels of IL-12p35, IL12/23p40 and IL-23p19 were measured using RT PCR. Cytokine levels were analysed using ELISA.

Results: Protease activity of Per a 10 increases p-STAT3 levels in mice lungs, which gets reduced on PAR-2 blockage. Percentage of p-STAT3⁺ CD11c⁺ cells was higher in Per a 10 administered mice and gets reduced upon PAR-2 blockage. IL-12p35 and IL-12p70 levels were higher, IL-23p19 and IL-23 levels were lower in both SAM-11 treated mice and BMDCs indicating a role of PAR-2 mediated signalling. IL-4, TSLP, IL-17A, EPO activity, total cell count and specific IgE and IgG1 levels were lower in SAM-11 administered mice. Inhibiting STAT3 activation via static also leads to lower levels of IL-23p19, IL-23 and higher level of IL-12p35.

Conclusions: Per a 10 leads to PAR-2 activation on BMDCs resulting in downstream activation of STAT3 to regulate the balance between IL-12/IL-23 subunits causing a cytokine milieu rich in IL-23 to favour Th2 polarization.

Introduction:

Allergic inflammation is characterized by aberrant Th2 immune response to an otherwise innocuous protein resulting in higher levels of IL-4, IL-5, IL-13, infiltration of immune cells into the airways and increased airway resistance [1]. The prevalence of allergic diseases is

increasing at an alarming rate during past few decades raising public health concerns globally [2].

The immune response observed following exposure to allergens is a result of complex interplay between various signalling pathways resulting from activation of a wide array of cells, antigen presenting cells, particularly, dendritic cells takes the centre stage in deciding the type of T cell polarization [3, 4]. The cytokine milieu generated by activation of antigen presenting cells is a crucial factor governing the type of T cell polarization and adaptive immune response, Th2 being the hallmark of allergic diseases [5].

Protease activity of allergens from a variety of clinically relevant sources like HDM, fungi, cockroaches, pollen etc. have been reported to play a critical role in the allergenicity of these proteins [6-8]. Proteases act via cleavage of tight junction proteins like ZO-1 and occludin increasing epithelial permeability, a number of cell surface receptors like CD25, CD23 and DC-SIGN influencing the functions of a wide array of cells [9-12]. Protease allergens activate dendritic cells and leads to upregulation of CD86, cleavage of CD40 to favour Th2 responses [13-15]. Furthermore, proteases can also act as adjuvants for other proteins present in the same microenvironment [16]. Several studies have shown the role of proteolytic activity of allergens in potentiating Th2 responses but the underlying mechanisms remain to be elucidated.

Sensitization to cockroach allergens has been a major risk factor for asthma development and rate of hospitalization has been reported to be 3.4 times higher than that of HDM or cat allergen [17]. Per a 10, a major serine protease allergen purified from *Periplaneta americana* induces Th2 responses in mice [18]. We have recently shown that protease activity of Per a 10 increases IL-23 secretion from dendritic cells and its neutralization lead to reduced levels of Th2 cytokines. Proteolytically active Per a 10 leads to a downregulation of IL-12p35 and an upregulation of IL-23p19 at transcript level. Inactivation of protease activity attenuates allergic immune responses [13]. In the present study, we have elucidated the mechanism by which protease activity of Per a 10 activates dendritic cells to create a cytokine milieu rich in IL-23 and deficient in IL-12p70 and leads to Th2 polarization.

Materials and Methods:

A detailed description of the methods is provided in the online supplement.

Mice immunization protocol and sample collection: Female BALB/c mice of 6–8 weeks weighing 18–22 grams were purchased from National Institute of Nutrition, Hyderabad (India). The study protocol was approved by animal ethics committee of Institute of Genomics and Integrative Biology, Delhi.

Per a 10 induced mice model of allergic airway inflammation was generated as described elsewhere [13]. PAR-2 activation was blocked by intranasal administration of 10 ng of SAM-11, or isotype control (mouse IgG2a), 30 mins prior to Per a 10 administration on days 0, 2 and 4. Mice were sacrificed on 15th day by *i.p.* injection of sodium thiopentone (100 mg/kg). BALF, blood and lungs were collected from each mouse. To study the role of PAR-2 signalling in STAT3 activation, mice were administered with Per a 10 or PBS on days 0, 2, and 4 in which SAM11 or isotype control (10ng) was administered 30 mins before each exposure and mice were sacrificed after 16 hrs. Lungs were isolated and cells were stained for CD11c-APC and p-STAT3-Alexa 488 and acquired using BD FACS Jazz. The percentage of double positive cells was analysed.

Western blot analysis: The protein levels of p-STAT3 and STAT3 were analysed in the lung protein of mice. The intensity of bands was quantified using Image J software and the relative expression was expressed as a ratio to STAT-3.

Quantitative Real time (RT) PCR: The mRNA levels of IL-12p35, IL-23p19 and IL-12/23p40 were quantified in mice lungs. The Ct value for the gene was normalized with an internal control β -actin. The relative gene expression was calculated as $2^{-\Delta Ct}$.

Generation of bone marrow derived dendritic cells: BMDCs were generated from femur and tibia of Balb/c mice using protocol previously described [13]. PAR-2 activation was blocked by treating cells with SAM-11 or isotype control for 2.5 hr and stimulated with Per a 10 whereas, media stimulated cells served as control. Cells were treated with 5 μ M stattic acid for 1 hr and then stimulated with Per a 10 for STAT-3 activation blockade experiments.

Statistical analysis: Statistical significance was determined by student's t-test to compare between two groups. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for data analysis. Data are expressed as mean $\pm$ SEM.

Results:

Protease activity of Per a 10 leads to STAT3 phosphorylation and activation: To assess the status of STAT-3 activation during Per a 10 induced allergic immune responses, levels of p-STAT3 (Y705) were measured in the lung sections of mice immunized with proteolytically active Per a 10 and heat inactivated Per a 10 (Δ Per a 10). The levels of p-STAT3 were significantly higher in lung sections of active Per a 10 immunized mice as compared to inactive Per a 10 administered mice or PBS control group (Fig. 1A,B), suggesting a role of protease activity in phosphorylation and activation of STAT3 during allergic immune responses. To assess the role of PAR-2 signalling in downstream activation of STAT-3 in dendritic cells, levels of dual positive cells (CD11c⁺ and p-STAT3⁺) were measured in mice lungs in which PAR-2 activation was blocked by administering SAM11. The number of dual positive cells was significantly reduced in SAM11 administered group as compared to isotype control (Fig. 1C, D), indicating involvement of PAR-2 mediated signalling in downstream STAT3 activation in dendritic cells.

PAR-2 signalling regulates balance between IL-12p70 and IL-23: To study the role of PAR-2 activation in regulating the balance between IL-12p70 and IL-23, we analysed the mRNA levels of cytokine subunits in lungs and bioactive cytokine levels in BALF of mice. Transcript levels of IL-12p35 (Fig. 2A) were higher and IL-23p19 (Fig. 2B) was lower in the lungs of SAM11 administered mice as compared to isotype control. There was no difference in the levels of IL-12/23p40 (Fig. 2C). Furthermore, an increase in levels of IL-12p70 (Fig.

2D) and a decrease in levels of IL-23 (Fig. 2E) were seen in the BALF of SAM11 administered mice as compared to isotype control, pointing towards role of PAR-2 signalling in regulating the levels of both cytokines. Also, p-STAT3 levels were significantly lower in the lungs of SAM11 administered group as compared to isotype control (Fig. 2F,G), pointing towards a critical role of PAR-2 activation in the downstream activation of STAT3. Blocking of PAR-2 activation also attenuates allergic immune responses as observed by a decrease in levels of Th2 cytokines, cellular infiltration and serum IgE and IgG1 levels (Fig. S1).

PAR-2 signalling regulates STAT3 activation and cytokines secretion from BMDCs: An increase in intracellular Ca^{2+} levels was observed upon Per a 10 stimulation which was attenuated upon blocking PAR-2 activation (Fig. S3A,B). The levels of p-STAT3 were reduced in Per a 10 stimulated BMDCs pre-incubated with SAM11 as compared to that treated with isotype control (Fig. 3A, S3C,D) indicating a role of PAR-2 signalling in downstream activation of STAT3 in dendritic cells, corroborating the results in mice model. PAR-2 activation blocked BMDCs were further analysed for secretion of cytokines and ability to prime naïve T cells. Levels of IL-12p35 (Fig. 3B) were higher and IL-23p19 (Fig. 3C) levels were lower in SAM-11 treated BMDCs upon Per a 10 stimulation as compared to isotype control. There was no difference in the levels of IL12/23p40 (Fig. 3D). This was also further corroborated as an increase in the levels of IL-12p70 (Fig. 3E) and a decrease in the levels of IL-23 (Fig. 3F) were observed in the supernatant of PAR-2 activation blocked Per a 10 stimulated BMDCs as compared to control. Levels of Th2 cytokines IL-4 (Fig. 3G), IL-13 (Fig. 3H) were lower in the supernatant of T cells co-cultured with PAR-2 signalling blocked Per a 10 stimulated BMDCs as compared to isotype control. However, there was no change in levels of IFN γ (Fig. 3I), indicating reduced Th2 priming potential of PAR-2 signalling blocked dendritic cells. Administration of PAR-2 activation blocked Per a 10 pulsed BMDCs to naïve mice also led to reduction in allergic parameters as compared to Per a 10 pulsed BMDCs upon Per a 10 challenge (Fig. S2).

STAT-3 activation regulates cytokine secretion from BMDCs: To investigate the role of activated STAT-3 downstream of PAR-2 signalling, we next stimulated BMDCs with Per a 10 pre-incubated with 5 μM of stattic for 1 hr. p-STAT3 levels were reduced at 5 μM concentration (Fig. S4). There was a decrease in IL-23p19 (Fig. 4A) and increase in IL-12p35

(Fig. 4B) transcript levels when BMDCs were stimulated with Per a 10 pre-incubated with static, indicating a role of STAT3 activation in regulating the transcript levels of these subunits. IL-23 levels (Fig. 4C) in the supernatant were below detection level of kit in static treated BMDCs, however, there was no difference in the levels of IL-12p70 (Fig. 4D).

Discussion

The allergic immune response involves a complex interplay between various cells, dendritic cells playing central role in governing the type of T cell polarization, Th2 responses being the hallmark of allergic diseases. The cytokine milieu created upon activation of dendritic cells acts as major factor driving the type of T cell polarization [19]. There is mounting evidence showing that protease activity of allergens play a critical role in initiation and exacerbation of allergic immune responses by activation of protease activated receptors, cleavage of cell surface molecules like CD23, CD25, thereby, modulating the function of a wide array of cells, yet the underlying mechanisms remain elusive [10,11,20,21]. Proteolytically active allergens activate dendritic cells to bias T cell polarization towards Th2 via higher expression of CD86, OX40L, cleavage of CD40 and an increased secretion of pro-inflammatory cytokines. Previously, we have shown that Per a 10, a major serine protease allergen from *Periplaneta americana*, activates DCs to secrete higher levels of IL-23 and a lower level of IL-12p70. A downregulation of IL-12p35 and an upregulation of IL-23p19 levels were observed in mice lungs upon intranasal administration of Per a 10 and blocking of IL-23 led to reduction in levels of Th2 cytokines in DC-T cell co-culture studies [13]. However, the mechanisms which differentially regulate the secretion of cytokines by dendritic cells upon activation by protease allergens are not understood. In the present study, we studied the mechanism by which proteases activate DCs to secrete higher IL-23 and lower IL-12p70.

Protease activated receptors are member of G protein coupled receptor (GPCR) family which are activated upon cleavage of its extracellular N-terminal domain. PAR-2 is activated by trypsin like serine proteases and expressed on a wide array of cells including epithelial cells, endothelial cells, smooth muscle cells, monocytes, leukocytes etc. Numerous studies have demonstrated a critical role of PAR-2 signalling to favour allergic immune responses [22]. Increased expression of PAR-2 on PBMCs has been observed in the severe asthmatics [23].

Previous study has shown activation of PAR-2 on epithelial cells by Per a 10 leads to secretion of pro-inflammatory cytokines [24]. In the present study, PAR-2 on dendritic cells is activated via Per a 10 as seen by increase in intracellular calcium mobilization, a characteristic feature of GPCR activation, this was reduced on blocking PAR-2 activation. Studies have reported an increase in levels of p-STAT3 as a result of PAR-2 activation and downstream signalling [25,26]. Consistent with these findings, we show an increase in levels of p-STAT3 in multiple cell types in lungs of proteolytically active Per a 10 administered mice and get reduced in both PAR2 blockage and inactive Per a 10 administered group, pointing towards a critical role of protease activity and PAR-2 mediated signalling in downstream activation of STAT-3. This was further corroborated by *in vitro* studies in BMDCs where blocking of PAR-2 activation leads to lower p-STAT3 levels.

STAT3 is a member of signal transducer and activation of transcription family activated by a number of mechanisms including IL-6, IL-23 signalling [27]. A recent study has shown STAT-3 can differentially regulate the levels of IL-23 and IL-12p70 by causing transcriptional activation of IL-23p19 and downregulation of IL-12p35 [28]. Previous reports also suggest APCs expressing activated STAT3 leads to lower IL-12 secretion [29]. The mechanism could be explained by a study which shows STAT-3 binds upstream of the start site of p35 promoter, inhibiting recruitment of CDK9/T-TEF b and prevents elongation by RNA pol II, whereas, it acts as co-activator for p19 promoter along with p65/NFkB [28,30]. STAT-3 has been shown to be critical for STAT6 to bind at target site and mice with STAT3 deficiency in T cells had attenuated allergic immune responses [31]. Our results show an increase in levels of IL-12p35, IL-12p70 and a decrease in levels of IL-23p19 and IL-23 upon PAR-2 blockade in both *in vitro* studies in BMDCs and *in vivo* mice model. This could possibly be a due to reduced levels of activated STAT3 as a result of blockage of PAR-2 mediated signalling in SAM-11 administered group. Previous studies have suggested PAR-2 activation on myeloid dendritic cells plays an important role in favouring Th2/Th17 immune responses upon activation via allergens [32]. In the present study, it was further validated in *in vitro* studies on BMDCs where blocking STAT-3 activation via stattic leads to lower levels of IL-23p19, IL-23 and higher levels of IL-12p35, pointing towards a direct role of STAT3 in the transcriptional regulation of both subunits. No change in the levels of IL-12p70 suggests involvement of additional contributing pathways. A recent study has shown blocking STAT-3 leads to abrogation of HDM induced allergic airway inflammation by reducing both Th2 and

Th17 responses [33]. Our results show attenuation of allergic immune responses upon PAR-2 activation blockage during sensitization, similar to previous reports demonstrating reduced allergic immune responses in PAR-2 receptor blockade in ovalbumin and cockroach model of allergic airway inflammation [34].

Collectively, these results show that PAR-2/STAT-3 axis in DCs play a critical role in creating the cytokine milieu rich in IL-23 and deficient in IL12p70 leading to priming of T cells towards Th2 by Per a 10 activated DCs. Further studies on the role of STAT-3 activation would pave the development of novel therapeutic approaches for combating allergic diseases.

Authors Contributions: KA and NA designed the experiments, KA performed the experiments and analysed the results. KA and NA wrote the manuscript.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Figure legends:

Figure 1: Per 10 increases p-STAT3 levels in mice lungs. BALB/c mice were administered with either, Per a 10, inactive Per a 10 (Δ Per a 10) or PBS via intranasal route and were sacrificed on 15th day. (A) Western blot of p-STAT3 (Y705); (B) Densitometry analysis showing relative expression (ratio of p-STAT3 levels to STAT3 levels) in mice lungs. Mice were administered via intranasal route with 10ng of SAM11 or isotype control 30 min prior to Per a 10 administration on days 0, 2 and 4 and sacrificed 16 hr after last dosage. (A) Dot

plot (B) bar graph showing percentage of double positive (CD11c⁺p-STAT3⁺) cells in mice lungs. Data are presented as mean $\pm$ SEM of 5-6 mice per group from one of the two independent mice experiments performed. * P < 0.05.

Figure 2: PAR-2 signalling regulates levels of IL-12 and IL-23: Cytokine subunits at transcript level were analysed by RT PCR. (A) IL-12p35, (B) IL-23p19, (C) IL-12/23p40 levels in lung section of PAR-2 blocked Per a 10 induced mice model. (D) IL-12p70 (E) IL-23 levels in BALF of mice. (F) Western blot of p-STAT3 (Y705) in lungs; (G) Densitometry analysis showing relative expression (ratio of p-STAT3 levels to STAT3 levels). Data are presented as mean $\pm$ SEM of 5-6 mice per group from one of the two independent mice experiments. * P < 0.05, ns: not significant.

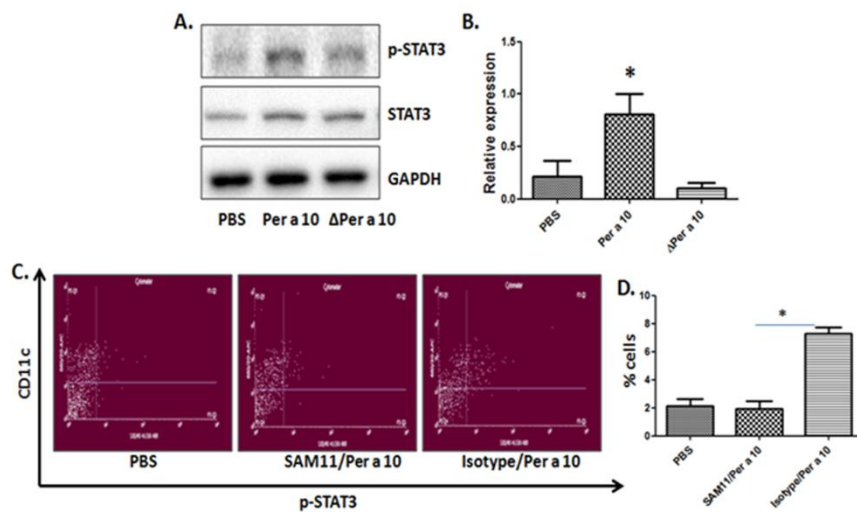
Figure 3: PAR-2 signalling regulates STAT3 activation and cytokines secretion from BMDCs: Cells were treated with 0.1 μ g/ml SAM-11 or isotype control for 2.5 hrs and stimulated with Per a 10. (A) p-STAT3 levels; Transcript Levels of (B) IL-12p35, (C) IL-23p19, (D) IL-12/23p40 in BMDCs stimulated for 24 hrs. (E) IL-12p70, (F) IL-23 levels in the supernatant of BMDCs stimulated for 48 hrs. (G) IL-4, (H) IL-13 (I) IFN γ levels in the supernatant of DC-T cell co-culture after 72 hrs. Data are presented as mean $\pm$ SEM for three independent experiments. * P < 0.05.

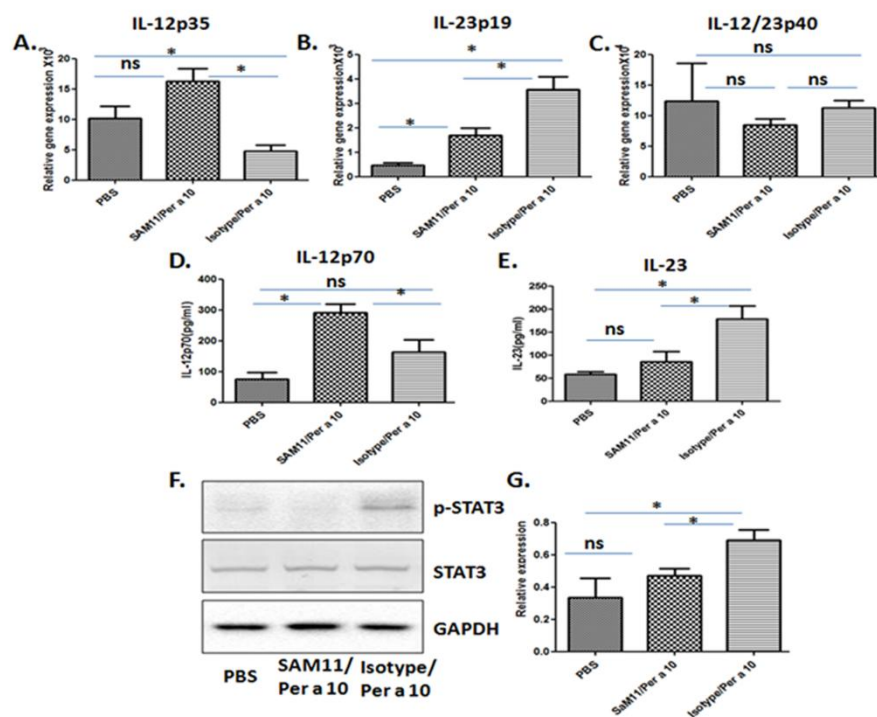
Figure 4: STAT-3 regulates levels of cytokine subunits: BMDCs were treated with stattic for 1 hr and stimulated with Per a 10. Transcript levels of (A) IL-23p19, (B) IL-12p35 in the BMDCs stimulated for 24 hrs; (C) IL-23, (D) IL-12p70 levels in the supernatant 48 hrs post stimulation. Data are presented as mean $\pm$ SEM for three independent experiments. * P < 0.05, ns: not significant; nd: not detectable.

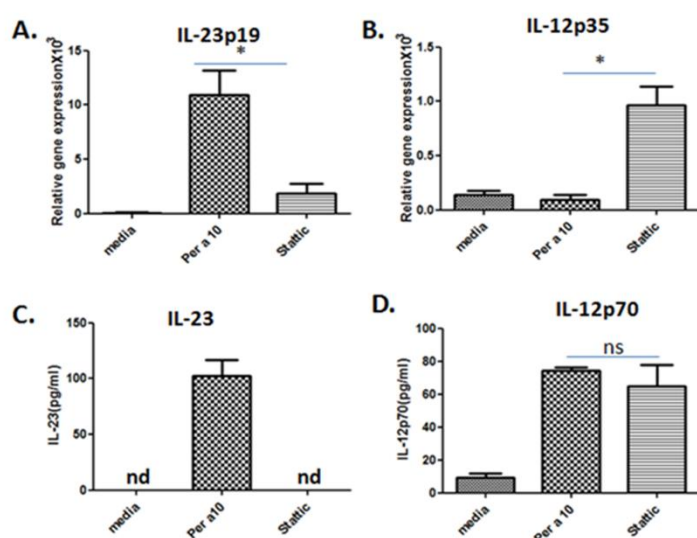
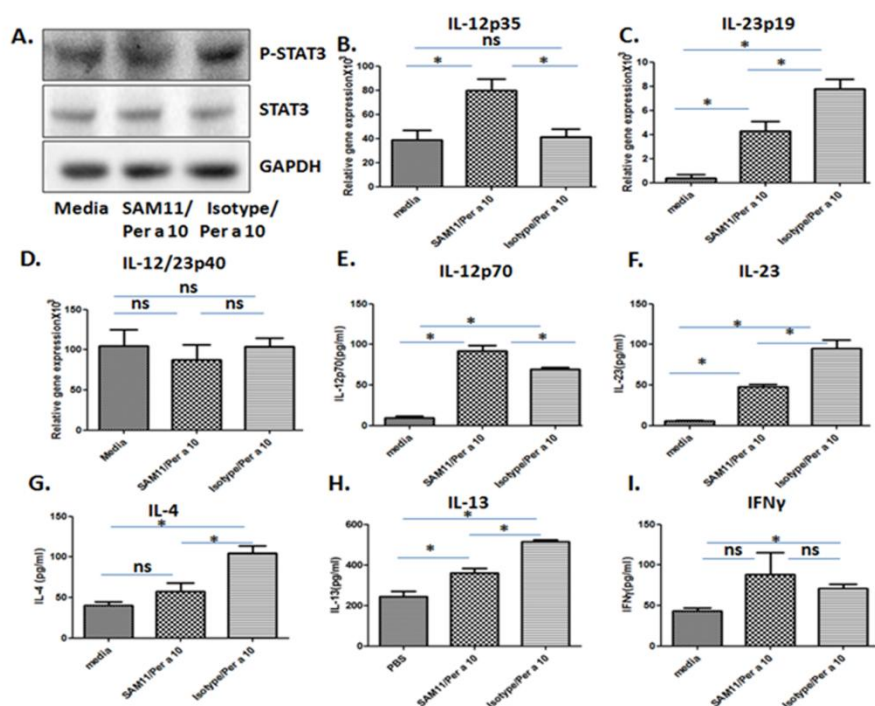
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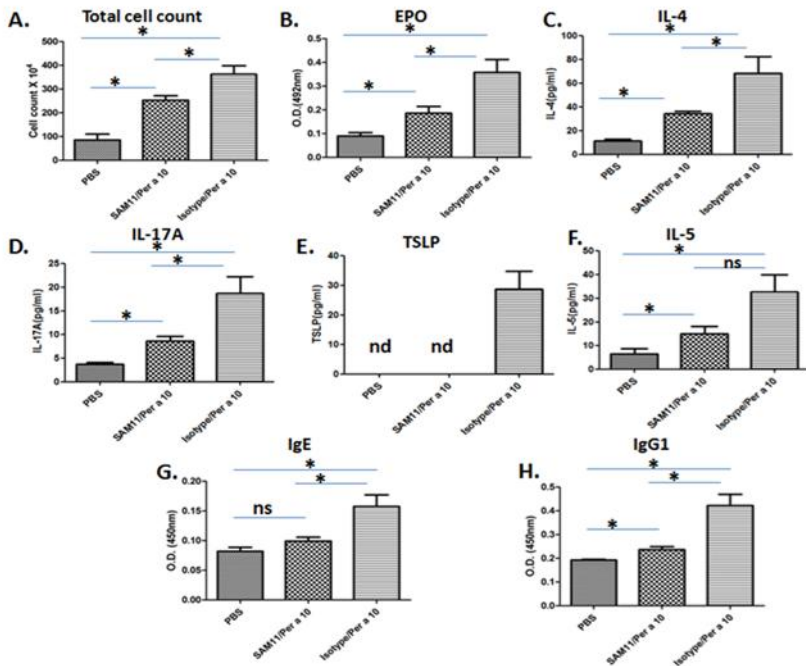
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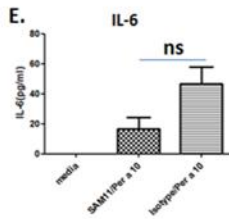
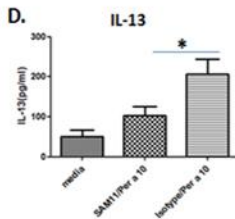
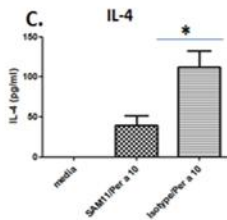
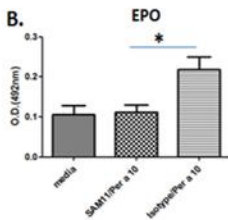
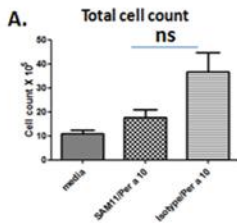
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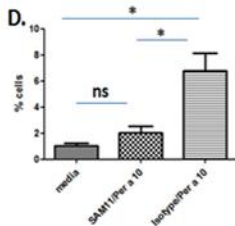
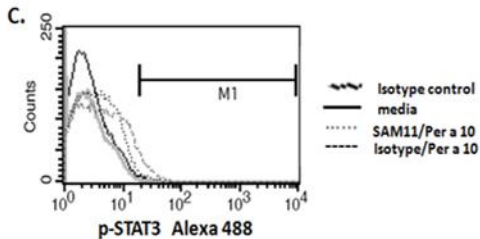
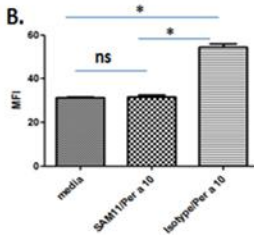
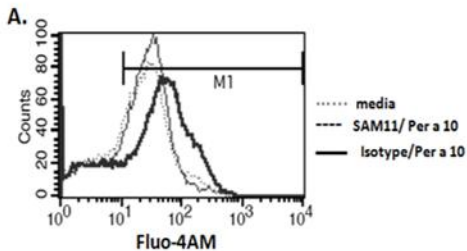


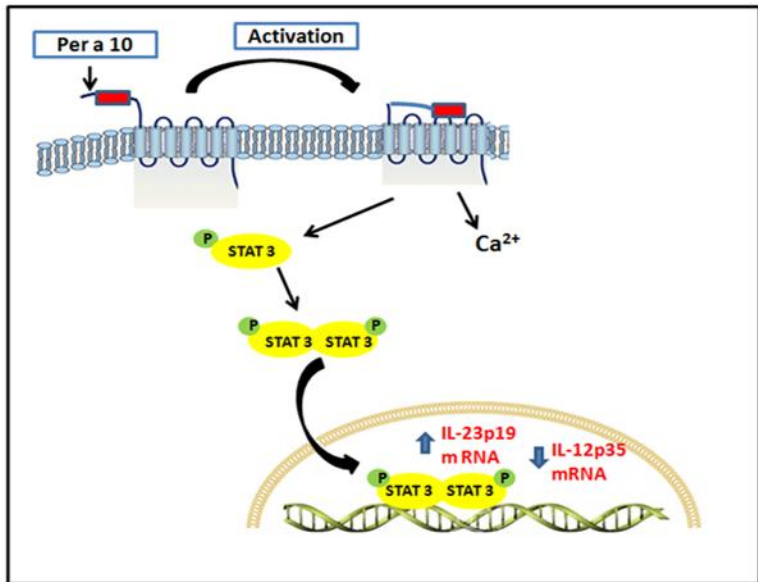




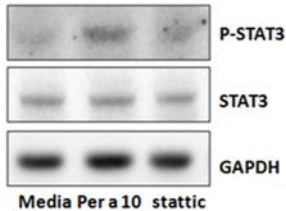




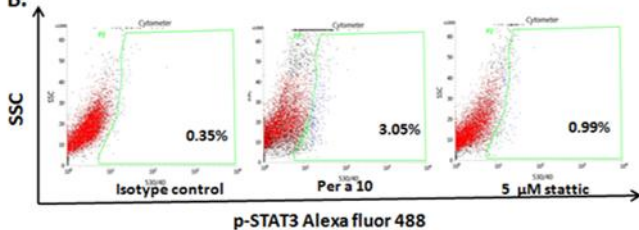




A.



B.



Serine protease allergen favours Th2 responses via PAR-2 and STAT-3 activation in murine model

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Short title: Per a 10 favours Th2 response via PAR2/STAT3 pathway

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Materials and Methods:

Animal and ethics: Female BALB/c mice of 6–8 weeks weighing 18–22 grams were purchased from National Institute of Nutrition, Hyderabad (India). Standard chow diet (*ad libitum*) and purified water were provided to mice, kept at 40–70% humidity with 12 hour controlled light: dark cycle. The study protocol was approved by animal ethics committee of Institute of Genomics and Integrative Biology, Delhi.

Immunization protocol and sample collection: Per a 10 was purified from whole body *Periplaneta americana* extract by affinity chromatography using benzamidine Sepharose as described elsewhere [E1]. Per a 10 induced mice model of allergic airway inflammation was generated as described elsewhere [E2]. Briefly, mice were administered PBS, Per a 10, ΔPer a 10 (25μg/mice) in 15ul PBS through intranasal route by anesthetizing mice with 3% isofluorane on days 0, 2, 4, 10, 12, 14. To study the role of PAR-2, mice model of allergic airway inflammation was developed using Per a 10, activation of PAR-2 was blocked by intranasal administration of 10 ng of SAM-11, a PAR-2 cleavage blocking antibody or isotype control (mouse IgG2a), 30 mins prior to each sensitization with Per a 10 on days 0, 2 and 4. Mice were sacrificed on 15th day by *i.p.* injection of sodium thiopentone (100 mg/kg). BALF was collected from each mouse by instillation of 0.5 ml of chilled PBS, thrice into the lung. BALF was centrifuged at 400 g for 10 min at 4°C and supernatant was used for analysis of cytokines and cells for total cell count. Blood was collected, allowed to clot and sera separated by centrifugation at 400 g for 10 min. Lungs were incised from thoracic cavity of mice and lobes were preserved at -80°C for RNA and protein isolation. To study the role of PAR-2 mediated signalling in STAT3 activation, mice were administered with Per a 10 or PBS on days 0, 2, and 4 in which PAR-2 activation was blocked using SAM11 or isotype control (10ng) 30 mins before each exposure and mice were sacrificed after 16 hrs. Lungs were isolated and single cell suspension was prepared. Cells were stained for CD11c-APC and p-STAT3-Alexa 488 and acquired using BD FACS Jazz. The percentage of double positive cells was analysed. BMDCs were stimulated with Per a 10 previously treated with SAM11 or isotype control for 24 hrs. Media stimulated DCs served as control. These were washed with PBS twice and 1X10⁴ cells per 20μl was intranasally administered to mice on day 0. Mice were challenged with 1μg of Per a 10 on days 12, 14 and sacrificed on day 15 [E3]. Blood and BALF were collected.

Western blot analysis: The lung lobe was homogenised and protein estimation in samples was done by Bicinchoninic acid method. Protein (50 µg) per sample were fractionated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked in TBST (Tris buffered saline with 0.05% of Tween-20) containing 5% BSA for 2hrs washed and incubated with rabbit anti mice p-STAT3 (Y705) at 1:3000 dilution, rabbit anti mice STAT3 at 1:1000 dilution (abcam) or goat anti mice GAPDH at 1:500 dilution (Santa cruz biotechnology) overnight at 4°C. It was washed with TBST and incubated with secondary antibody (anti-rabbit IgG, anti-goat IgG respectively) conjugated to HRP for 1hr at RT. The membrane was washed 4 times with TBST at 15 min interval each and was developed using enhanced chemiluminescence method. The intensity of bands was quantified using Image J software and the relative expression was expressed as a ratio to STAT-3.

Quantitative Real time (RT) PCR: Total RNA was isolated from lung tissue by homogenizing in 1ml of Trizol (Sigma) and quantified using nanodrop. cDNA was prepared from 1µg/sample of RNA using superscriptII (RNase H-) and random hexamer primers using cDNA synthesis kit (Fermentas) as per manufacturer's instructions. Real time PCR was conducted using KAPA SYBR green master mix (KAPA biosystems) on a Roche lightcycler 480II system. The reaction mixture had, 1µl cDNA corresponding to 50ng of total RNA from reverse transcription reaction; 0.12µM of sense primer; 0.12µM of anti sense primer, 3µl of SYBR green mix and volume was made up to 6µl using nuclease free water. Melt curve analysis was done. Negative controls were included in each assay to ensure the Ct values obtained were not due to reaction contamination. Primers used were IL-12p35 (FP: 5' ATGACCCTGTGCCTTGGTAG3'; RP: 5'CAGATAGCCCATCACCTGT3'), IL-23p19 (FP: 5' GCCCCGTATCCAGTGTGA3'; RP: 5'GCTGCCACTGCTGACTAG3'), IL-12/23p40 (FP: 5'AGGTGCGTTCCTCGTAGAGA3'; RP: 5' AAAGCCAACCAAGCAGAAGA3') and β -actin (FP: 5' CGGTTCCGATGCCCGAGGCTCTT3'; RP: 5'CGTCACACTTCATGATGGAATTGA3'). The Ct value for the gene was normalized with an internal control β -actin. The relative gene expression was calculated as $2^{-\Delta Ct}$.

Generation of bone marrow derived dendritic cells: BMDCs were generated from femur and tibia of Balb/c mice using protocol previously described [E2]. Cells (1×10^6 cells/ml) were stimulated with 1µg/ml of Per a 10 in a 12 well plate containing 2ml media/well. For blocking of PAR-2 activation, cells were treated with 0.1µg/ml SAM-11 or isotype control for 2.5 hr and stimulated with 1 µg/mL of Per a 10 whereas media stimulated cells (in serum-

free RPMI-1640 medium) served as negative control. Cells were treated with 5 μ M stattic at for 1 hr and then stimulated with 1 μ g/mL of Per a 10 for STAT-3 activation blockade experiments.

DC: T cell co-culture: Naive T cells were purified from spleen of Balb/c mice by CD4⁺ CD62L⁺ T cell isolation kit using MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) following manufacturer's instruction. The stimulated DCs and naive T cells were co-cultured in 96 well flat bottom tissue culture plates at a ratio of 1:10 with 0.2 ml of serum free RPMI-1640 media per well in presence of 0.5 μ g/ml of anti-CD3 antibody. Cells were allowed to proliferate at 37°C in 5% CO₂. Culture supernatant was collected after 72 hr and used for cytokines analysis.

Flow cytometry for p-STAT3 expression: BMDCs (1X10⁶ cells) were treated with 0.1 μ g/ml of SAM-11 or isotype control for 2.5 hr. Cells were washed and resuspended in serum free RPMI-1640 and stimulated with Per a 10 for 30 min, whereas, unstimulated cells served as control. Cells were lysed and fixed using Phosflow Lyse/Fix buffer (BD Biosciences) by keeping on water bath at 37°C for 10 min. Cells were then washed with PBS and permeabilized using Perm buffer III (BD Biosciences) for 30 min on ice. Cells were washed and resuspended in stain buffer (BD Biosciences) and incubated with p-STAT3-Alexa fluor488 (BD Biosciences) for 1hr at room temperature in dark. Cells were washed and resuspended in stain buffer and acquired using FACS calibur (BD Biosciences) and analysed using cell quest pro software.

Mobilization of intracellular Calcium: Levels of intracellular Ca²⁺ as a consequence of PAR-2 activation via Per a 10 was assessed using a Ca²⁺ binding fluorescent dye, Fluo-4AM. Briefly, BMDCs were harvested and treated with 0.1 μ g/ml of SAM-11 or isotype control for 2.5 hr. Cells were washed and resuspended in serum free RPMI-1640 and loaded with Fluo-4AM for 30 min at 37°C. Cells were exposed to Per a 10 or media only. The change in fluorescence intensity was measured using BD FACS calibur flow cytometer and analysed using cell quest pro software.

Enzyme linked immunosorbent assay: The cytokines GMCSF, IL-4, IL-6, IL-12p70, IL-13, IL-23(R&D), IL-10, IL-25, IL-17A (Biolegend), IFN γ , TNF α , TSLP, (eBioscience, San Diego, CA, USA), were detected in supernatant by using paired antibodies following manufacturer's protocol.

Figure legends

Figure S1: PAR-2 blockade attenuates Th2 responses: (A) Total cell count (B) EPO activity (C) IL-4 (D) IL17A (E) TSLP (F) IL-5 in BALF; Per a 10 specific (G) IgE (H) IgG1 in sera of PAR2 activation blocked mice model of allergic airway inflammation. Data are presented as mean $\pm$ SEM of 5-6 mice per group from one of the two independent mice experiments. * $P < 0.05$, ns: not significant

Figure S2: Blocking PAR-2 activation in BMDCs reduces its allergenic potential: BMDCs were stimulated with Per a 10 prior exposed to SAM11 or isotype control. Pulsed BMDCs (1×10^4 cells/20 μ l) were intranasally administered to mice on day 0 and challenged with 1 μ g Per a 10 on days 12,14 and sacrificed on 15th day. (A) Total cell count (B) EPO activity (C) IL-4 (D) IL-13 (E) IL-17A and (F) IL-6 levels in BALF. Data are presented as mean $\pm$ SEM of 5-6 mice per group. * $P < 0.05$, ns: not significant

Figure S3: PAR-2 signalling in BMDCs leads to STAT3 activation: Cells were treated with 0.1 μ g/ml SAM-11 or isotype control for 2.5 hr, loaded with Fluo-4AM at 37°C for 30 mins and stimulated with Per a 10. (A) Levels of intracellular Ca^{2+} as analysed by flow cytometry, (B) MFI showing Ca^{2+} levels, (C) Levels of p-STAT3 (D) percent cells expressing p-STAT3 as analysed by flow cytometry. Data are presented as mean $\pm$ SEM for three independent experiments. * $P < 0.05$

Figure S4: Stattic inhibits STAT-3 activation: BMDCs were treated with 5 μ M stattic for 1 hr and stimulated with Per a 10. p-STAT3 levels as analysed by (A) Western blot and (B) flow-cytometry.

Figure S5: Schematic diagram representing a central role of PAR-2/STAT3 signalling in regulation of IL-23 and IL-12p70 subunits by serine protease allergen in dendritic cells. Per a 10 activates PAR-2 on dendritic cells leading to downstream phosphorylation and activation of STAT-3 (Y705). STAT-3 acts to downregulate IL-12p35 levels and upregulate IL-23p19 mRNA levels.

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