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Acetaminophen modulates the ratio of Group 2 innate lymphoid cells and regulatory Innate lymphoid cells in Ovalbumin sensitization mice model

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Acetaminophen (APAP) is a widely used drug known for its analgesic and antipyretic properties. However, its exact mechanism of action is still unclear and has been primarily associated with its effects on COX enzymes and serotonergic pathways. Till date, the immunological mechanisms affected by APAP has gained only inadequate attention. A distinct group of immune cells called Group 2 Innate Lymphoid Cells (ILC2s) are known to play an important role in type 2 cytokine-mediated immunity and their regulatory dysfunction is associated with numerous type 2 pathologies, such as allergy. Conversely, another class of recently characterized Innate Lymphoid Cells called Innate Regulatory Cells (ILCregs) suppress the activation of ILC2s. Maintaining a balance between ILC2s and ILCregs is vital for achieving a well-controlled immune response and tissue homeostasis. Recent studies have highlighted the critical role of prostaglandin D2 (PGD2) in the maturation and development of ILC2 functions. Since APAP also suppresses PGD2 synthesis, it was speculated that APAP treatment could reduce the number of ILC2s while potentially increasing the number of ILCregs. To investigate this hypothesis, here, we administered therapeutic doses of APAP to OVA-sensitized mice, a well-established model of type 2 pathological inflammation. The mice received oral doses of 200 mg/kg body wt. of APAP twice weekly, along with weekly OVA sensitizations for six weeks. The mice were sacrificed at different time points (days 14, 28 and 42) to assess the kinetics of ILC2s, ILCregs and immunoglobulins (IgE and IgG1). The results demonstrated that APAP treatment effectively suppressed OVA-induced ILC2s while significantly increasing the number of IL-10+ ILCregs. APAP exposure also led to decreased levels of serum PGD2, OVA-specific IgE and IgG1, and enhanced the level of IL-10 in OVA sensitized mice. Moreover, OVA sensitized mice treated with APAP did not develop pathological changes in spleen, when compared to OVA sensitized mice. Additionally, APAP treatment did not cause any adverse effects on mice liver in treatment groups. These preliminary findings suggest that APAP exhibits the capacity to modulate type 2 immune responses by suppressing ILC2s and inducing the expansion of IL-10+ ILCregs.

Keywords: Analgesic, Antipyretic, Immunoglobulins, Inflammation, Ovalbumin sensitization, Paracetamol, Prostaglandin D2

Acetaminophen (APAP) or paracetamol is one of the most popular analgesic and anti-inflammatory drug, as it exhibits a remarkable safety profile at therapeutic doses[1]. During the recent COVID-19 crisis, APAP and similar drugs regained prominence. Despite decades of usage for treatment of fever and acute pain, the mechanism of action of APAP is still unclear. The antipyretic and analgesic effects of APAP are largely attributed to inhibition of COX enzymes and prostaglandin synthesis[2-4], however, several other reports did not attribute the therapeutic effects of APAP to inhibition of prostaglandin synthesis alone[5,6]. Few studies have documented that the analgesic action of APAP is mediated through activation of descending inhibitory serotonergic pathway, which plays an important role in reducing the pain signal through neuronal inhibition[7,8]. On the other hand, another investigation reported that effects of APAP cannot be understood by inhibition of serotonergic pathway alone, as APAP did not exhibit affinity for serotonergic receptors or enzymes involved in the pathway[9]. Till date, there is no definitive consensus on the mechanism of action of paracetamol[10]. Notably, during the course of a pathological disorder, fever, pain and inflammation are a consequence of a complex interplay of immune cells and their interaction with other organ systems. Incidentally, the research so far has largely focused on the effect of APAP on COX enzymes and serotonergic pathway, and little emphasis has been

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laid upon the immunological mechanisms impacted by APAP.

As an integral part of adaptive or specific immune system, the important role of T-cells (Th1, Th2, Th17, Tregs, etc.) has been well appreciated over the decades. Of late, a group of immune cell populations called "Innate Lymphoid Cells" (ILCs) have been discovered as a part of innate or non-specific immune system, which shares characteristics and functions with T-cells[11]. ILCs are found in all the tissues of body and play a crucial role: (i) as a first line of host defense; (ii) generate initial signals for fine orchestration and strengthening of appropriate adaptive immune response against foreign threats; (iii) tissue remodeling; and (iv) maintenance and repair of hematopoietic as well as non-hematopoietic cells[12]. Broadly, three distinct populations of ILCs have been characterized: Group 1 ILCs or "ILC1" analogous to Th1 cells; Group 2 ILCs or "ILC2s" analogous to CD4+ Th2 cells; and Group 3 ILCs or "ILC3s" analogous to Th17 cells[13]. Among the ILC populations, ILC2s promote type 2 cytokine mediated immunity; play an important role in protection against helminth parasites; induce lung epithelium repair following respiratory infections; promote antitumor immunity; regulate obesity, feeding and circadian rhythms; and also regulate functional homeostasis in liver and adipose tissue[14-20]. However, ILC2s have also been implicated in the development of several diseases and disorders involving pathologic type 2 inflammation, such as allergic asthma and airway inflammation, colitis, gastrointestinal allergy, influenza, pulmonary fibrosis, chronic rhinosinusitis, atopic dermatitis, liver inflammation and liver cirrhosis[16,21-30]. Type 2 immune disorders are characterized by enhanced levels of Th2 cytokines, and a deregulated Th2 and ILC2 response[31]. It has been reported that PGD2 plays a very important role in maturation and development of ILC2 responses[32]. APAP is well known to inhibit the synthesis of PGD2[33], however, the effect of APAP on the distribution of ILC2s has not been investigated until now.

Among the innate lymphoid cells, another class of cells called "regulatory innate lymphoid cells" (ILCregs) play an important regulatory role in maintaining immune homeostasis and containment of tissue inflammation. ILCregs are basically functional analogs of Treg cells which were first described for their role in maintenance of mucosal and tissue immunity[34,35]. Interestingly, several studies have reported that ILC2s may transform into IL10+ ILCregs in the presence of inhibitory or unfavorable signals not suitable for ILC2 activation[36,37]. Therefore, in the current investigation, we sought to examine the effect of APAP treatment on the distribution of ILC2s and ILCregs in type 2 inflammation, and further analyzed its impact on OVA specific immunoglobulins and regulatory cytokine IL-10. Precisely, to determine the impact of APAP treatment on ILC2s and ILCregs, here, we investigated the effect of therapeutic doses of APAP in OVA sensitized Balb/c mice which is a well-established model of "type 2 pathological inflammation".

Materials and Methods

Animals

Female BALB/c mice of about 6-7 weeks of age, obtained from the animal breeding colony (IITR, Lucknow, India) were kept in well-controlled, pathogen-free animal housing facilities and fed ad libitum. The Animal Ethics Committee of the IITR approved the study protocol and the animal ethical approval number for this manuscript is #IITR/IAEC/50/19. A total of 12 mice per group were randomized into four different groups like; naive mice (control), mice that had only been administered acetaminophen, mice that had only been administered OVA (OVA), and mice that were administered both APAP and OVA (APAP + OVA).

Acetaminophen treatment and OVA immunization protocol

Schedules of treatment are shown in Fig. 1. Mice from APAP and APAP + OVA groups received Acetaminophen [200 mg/kg body wt., (human therapeutic dose) Cayman #10024] orally twice a week from day 0 to day 42. APAP was given 1 h before OVA sensitization and challenge. The mice from the APAP+OVA and OVA groups, were sensitized with OVA. These mice received intraperitoneal injections of 100 µL of 10 µg OVA (Sigma, #A5503) absorbed in Imject™ alum adjuvant (Thermo #77161) on days 0, 7, 14, 21 and 27. The mice from the control group received only PBS by means of oral administration during the sensitization phase. Subsequently, after the sensitization phase, mice from all the groups were challenged with 100 µg of OVA intraperitoneally in 0.1 mL saline on days 28 and 35. The higher dose of OVA (500 µg per injection in 0.1 mL saline) was administered to all

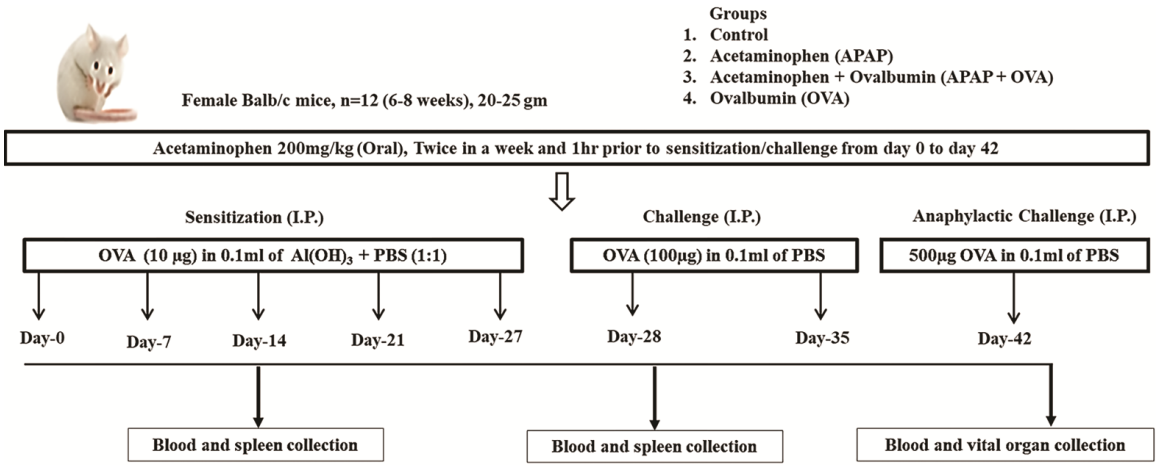


Fig. 1 — Experimental design to investigate the effect of acetaminophen (APAP) treatment on ILC2s and ILCregs in OVA-sensitized mice, and the consequent impact on Type 2 inflammation. [Schedule of APAP treatment, OVA sensitization and challenge. Blood samples and spleens were collected at the indicated time points. i.p., intraperitoneal]

animals on day 42. Different sets of mice were also sacrificed intermittently on days 14, 28 and 42 to examine the kinetics of ILC2s, ILCregs and immunoglobulins IgE and IgG1.

Specific IgE and IgG1 estimation in serum

Indirect ELISA was used to measure OVA-specific IgE and IgG1 in mouse serum samples at different time points (days 14, 28 and 42) of sensitization and challenge. We coated the individual wells of microtiter ELISA Nunc MaxiSorp, flat-bottom plates (Thermo Scientific, Waltham, MA) with 2 µg OVA in 100 µL carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Thereafter, the wells were washed with PBS containing 0.05% Tween-20 (PBST), and then blocked with 3% bovine serum albumin (Genetix Puregene # PG-2330) in PBS. The excess blocking reagent was removed by washing with PBST before adding mouse serum to the blocked wells. The sample plates were kept at 37°C for 2 h. Afterwards, the OVA-specific immunoglobulins were detected using biotin-conjugated anti-mouse IgE (Abcam ab99574) and IgG1 (Abcam ab97240), followed by incubation of plates for 2 h at room temperature (25ºC). The plates were then incubated with streptavidin-peroxidase for 30 min at RT and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermofisher scientific 3402) for 30 min in the dark. The reaction was terminated using 2N H_2SO_4 and optical density was measured using the Fluorescence multi-mode microplate reader VarioskanTM LUX.

PGD2 and IL-10 estimation

The serum concentrations of PGD2 and IL-10 were assessed by ELISA. We used the PGD2 Abbkine # KTE70766 ELISA kit to determine the concentrations of PGD2 in blood serum. The concentration of IL-10 (R&D # DY417-05) was measured in the blood serum using ELISA Duo-Set kit, R&D systems. The experimental procedure for ELISA was performed as per manufacturer's instructions.

Characterization of ILC2s and ILCregs population in splenocytes

On days 14, 28 and 42, single-cell suspensions of splenocytes were prepared for flow cytometry analysis. For staining and fixation, the splenocytes were centrifuged at 300×g for 5 min in PBS. In order to identify the target population, we suspended the splenocytes in the staining buffer (3% FBS and 1% sodium azide in 1x PBS) and incubated them with surface marker antibody cocktail at room temperature (25ºC, dark condition) for 45 min. After staining, all the procedures were conducted in the dark. Subsequently, 1x PBS was used to wash and pellet the samples. Afterwards, the pellet was resuspended in 1 mL cold PBS with 3% freshly prepared paraformaldehyde and incubated at room temperature for 20 min. After that, we added 1 mL of cold PBS with 0.2% BSA and centrifuged the sample at 300 ×g for 5 minutes. After fixation, we resuspended the pellet in 300 µL cold PBS and added 700 µL chilled absolute ethanol, and vortexed briefly to prevent cell clumping. Next, the sample was kept at –20°C for 1 h. The cells were then centrifuged at 400 ×g for 5 min and washed twice in PBS with 0.2% BSA. For immunostaining of intracellular transcription factors, the cells were resuspended in the staining buffer (3% FBS and 1% sodium azide in PBS) and

incubated for 1 h at room temperature with the desired intracellular antibody cocktail. Again, the cells were centrifuged at 400 ×g for 5 min and washed twice in PBS with 0.2% BSA. A PBS wash was used to remove the unbound excess antibodies. The percentage of each population type was measured with an Attune NxT flow cytometer. In order to identify the ILC2s/ILCregs populations, FITC-conjugated anti-mouse Lineage Cocktail (FITC anti-mCD3ε/FITC anti-mGr-1/FITC anti-mCD11b/FITC anti-mCD45R(B220)/ FITC anti-mTer-119) with Isotype control (FITC Armenian hamster IgG/ FITC Rat IgG2b/FITC Rat IgG2a) (BioLegend #133301) antibody, Alexa Fluor™ 647-conjugated mouse CRTH2 (Invitrogen 51-2941-82) antibody, PE-conjugated mouse IL-10 (BD Pharmingen™ 554467) antibody, PE-Cyanine7 conjugated anti-mouse Gata-3 Monoclonal Antibody (TWAJ), and Brilliant Violet 421™ conjugated anti-mouse TSLPR (TSLP-R) antibody were used.

Histopathological analysis

The effects of acetaminophen and ovalbumin on spleen and liver tissue histology were observed. We collected random sections of each spleen and liver and fixed them in 10% buffered formalin. Afterward, fixed specimens were dehydrated in ascending grades of ethyl alcohol (30, 50, 70, 90 and 100%), subsequently cleared twice with propylene oxide, and finally embedded in paraffin. The 5 μm thick sections of tissues were stained with H & E dyes and then examined under a light microscope (10×40 magnification) (Olympus, Tokyo, Japan) by a pathologist who was blinded to the mouse treatments. Hepatic sections stained with H&E were examined for lobular fibrin ring granulomas.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The normally distributed data was analyzed using 1-way ANOVA for data with single variable and 2-way ANOVA for data with two variables; followed by Bonferroni post hoc test. The non-normally distributed data was compared using Kruskal Wallis test and Dunn's multiple comparison test. The co-relation analysis was carried out using (n = 36) data points using 9 animals from each group. Subsequently, Pearson R and R^2 were calculated to measure the degree of positive or negative association among variables. The results were expressed as the mean ± SEM and the P-value of less than 0.05 was considered significant.

Results and Discussion

Acetaminophen treatment inhibits prostaglandin production

Earlier studies have reported that APAP weakly reduces the levels of PGD2[33,38,39]. To test these findings in our model system, we measured the levels of PGD2 in sera of APAP treated OVA sensitized mice. The results showed that compared with the OVA sensitized mice, the APAP + OVA group exhibited a reduced level of serum PGD2 at almost all the time points of sensitization and challenge phases (day-14, 28 and 42) (Fig. 2).

Acetaminophen diminished expansion of ILC2s in OVA-sensitized mice

We sought to investigate the effect of APAP on the number of ILC2s in OVA-immunized mice. The OVA sensitized animals were treated with APAP twice weekly up to 42 days and their spleens were collected on day 14, 28 and 42 to study the time kinetics of ILC2s over this period. In accordance with the recently published reports on ILC2s, the Lineage$^-$/CRTH2$^+$/TSLPR$^+$/GATA3$^+$ cells were considered as ILC2s in our study[40].We observed a gradual increase in the number of ILC2s from day 14 to day 42 in mice sensitized with OVA in the presence or absence of APAP, however the number of ILC2s was significantly diminished in (APAP + OVA) treated mice in comparison to OVA sensitized mice (Fig. 3). A similar ILC2 kinetics was also reported in a previous study[41]. Together, our data suggests that APAP treated OVA sensitized mice showed a decrease in the number of ILC2s, when compared to mice sensitized with OVA alone.

Acetaminophen induced expansion of IL-10$^+$ILCregs

Recent studies suggest that alternative activation of ILC2s can transform them into IL-10 secreting

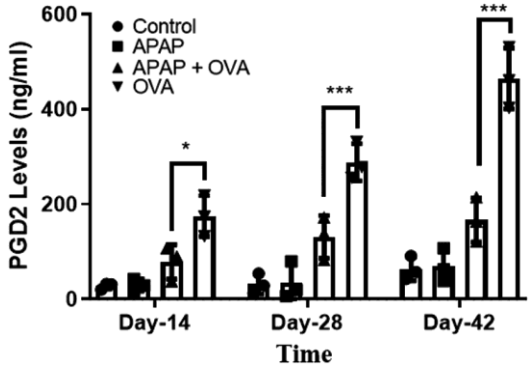
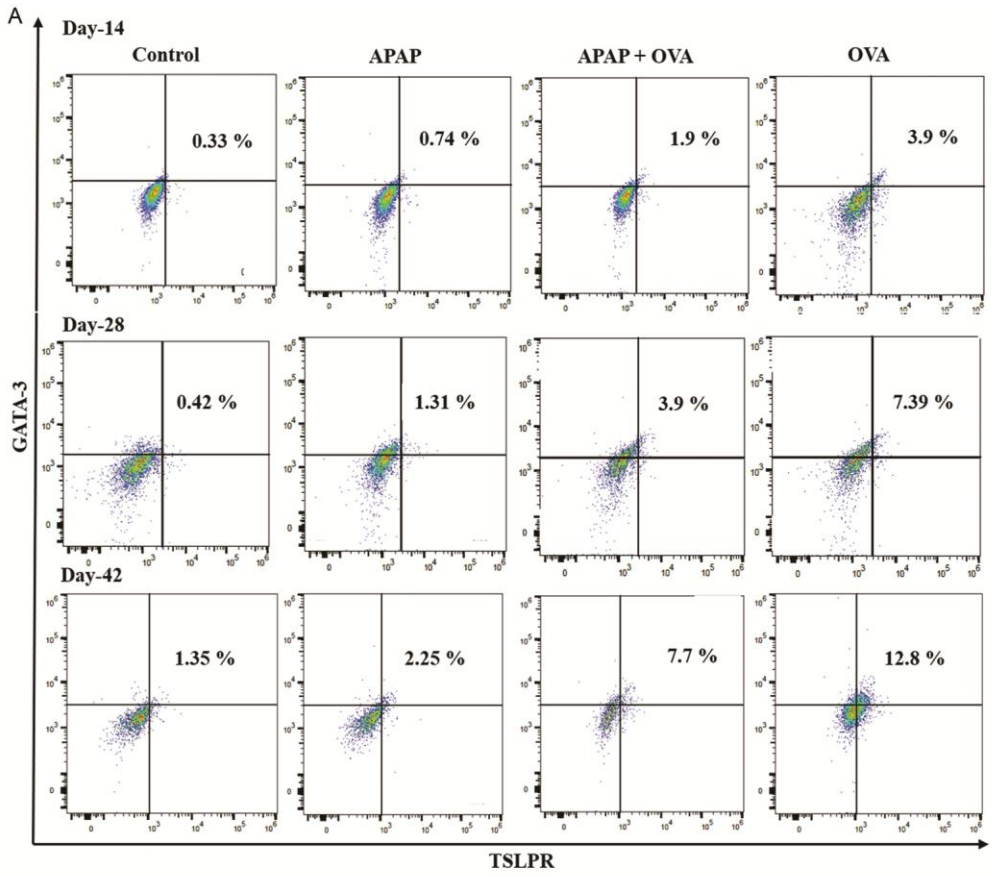


Fig. 2 — APAP suppresses the production of PGD2. [Kinetics of PGD2 serum concentration was determined by ELISA. The data format is expressed as the mean ±SEM (n = 3), where statistically significant differences: *P* values <0.05*, and <0.001***]



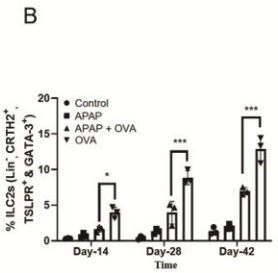
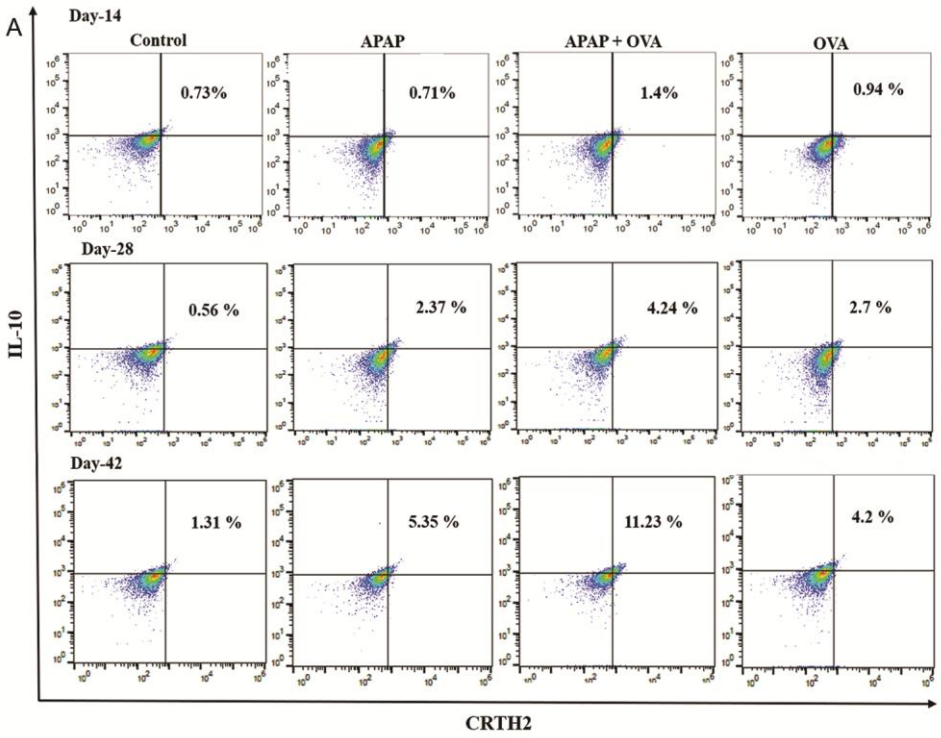


Fig. 3 — Time kinetics of ILC2s. (A) Depicts flowcytometry analysis of % ILC2s (Lin^-, $CRTH2^+$, $GATA3^+$ and $TSLPR^+$) with respect to healthy control for each time point. The percentage of the selected population is shown in the quadrants; and (B) shows a comparative bar graph trend of % ILC2s with respect to healthy control for each time point and the data format is expressed as the mean ±SEM (n = 3). [*P* values <0.05*, <0.01** and <0.001***]



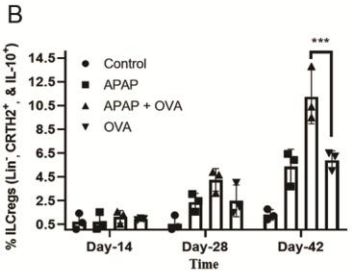


Fig. 4 — Acetaminophen-induced expansion of IL-10 secreting ILCregs. Time kinetics of ILCregs. (A) depicts flowcytometry analysis of % ILCregs (Lin^-, $CRTH2^+$, $IL\text{-}10^+$) with respect to healthy control for each time point. The percentage of the selected population is shown in the quadrants; and (B) shows a comparative bar graph showing trend of % ILCregs with respect to healthy control for each time point and the data format is expressed as the mean ±SEM (n = 3). [***P values <0.001]

$ILC2_{10}$s, referred to as "ILCregs"[36,37]. Therefore, we asked whether APAP treatment could induce the expansion of IL-10 secreting ILCregs in OVA sensitized mice. To study ILCregs kinetics, immunophenotyping was carried out in mice spleen on day 14, 28 and 42, and $Lineage^{-}$ /$CRTH2^{+}$/ $IL\text{-}10^{+}$ cells were considered as ILCregs. Of interest, the increase in the percentage of ILCregs with time in OVA-sensitized positive control groups was far less pronounced in comparison to (APAP + OVA) treated mice. Comparatively, the number of ILCregs between OVA-sensitized PC groups and (APAP + OVA) treated groups did not show significant difference on day 14 and day 28. However, on day 42, the (APAP + OVA) treated mice exhibited a notably higher number of ILCregs with respect to OVA-sensitized mice (Fig. 4).

Acetaminophen suppresses OVA specific IgG and IgE in OVA-sensitized mice

ILC2s play an essential role in allergic disorders and other pathologies with type 2 inflammation[21,42]. As APAP suppressed the number of ILC2s, we hypothesized that APAP treatment might reduce the serum levels of OVA specific IgG and IgE in OVA sensitized mice. To evaluate the impact of APAP exposure on humoral responses, we examined the serum OVA specific IgE and IgG1 on day 14, 28 and 42. We found a prominent reduction of IgE and a partial reduction of IgG1 levels in the sera of (APAP + OVA) treated mice when compared to OVA sensitized positive controls (Fig. 5).

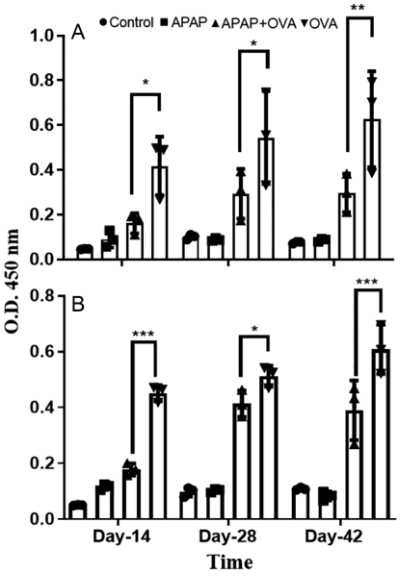


Fig. 5 — Acetaminophen attenuates Th2 immune responses in OVA-sensitized mice. Time kinetics of OVA-specific serum (A) IgE; and (B) IgG1 were determined by ELISA. The data format is expressed as the mean ±SEM (n = 3). [*P* values <0.05*, <0.01** and <0.001***]

Acetaminophen increases production of serum level of IL-10

Further, we assessed the serum levels of IL-10 in our experimental groups, because IL-10 is central to the induction of allergen tolerance[43,44]. IL-10 can suppress the production of immunoglobulin E (IgE), which is a key mediator of allergic reactions in Type 2 immune disorders. Interestingly, we found that up to day 14, the IL-10 concentration was similar in all groups, but subsequently on days 28 and 42, it showed a time dependent increase in the (APAP + OVA) group which was significantly higher than OVA allergic positive control group (Fig. 6).

Acetaminophen did not affect liver but protects spleen from allergic effects

The histopathological analysis of spleen sections stained with H&E showed a marked increase in white pulp proliferation in OVA-sensitized mice compared to the control group. However, in APAP-treated OVA-sensitized mice, the increment of white pulp was significantly suppressed compared to the OVA-sensitized group (Fig. 7A). These findings suggest that APAP treatment may have a potential inhibitory effect on the development of OVA-induced Th2 immune responses. The adverse effect of acetaminophen on the liver in clinical practice is not rare. As the animals in the APAP and (APAP + OVA) groups were given 200 mg/kg body wt. of acetaminophen twice a week, so to determine whether the drug may cause hepatotoxicity, histopathological analysis of the liver was performed. According to the histological analysis of liver tissue, the dose of acetaminophen was non-toxic, and no substantial change was observed in the

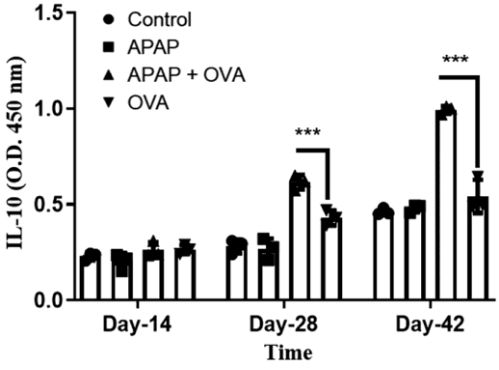


Fig. 6 — Acetaminophen induces IL-10 production. Time kinetics of tolerogenic cytokine IL-10 serum concentration was determined by ELISA. The data format is expressed as the mean ±SEM (n =3). [*P* values <0.05*, <0.01** and <0.001***]

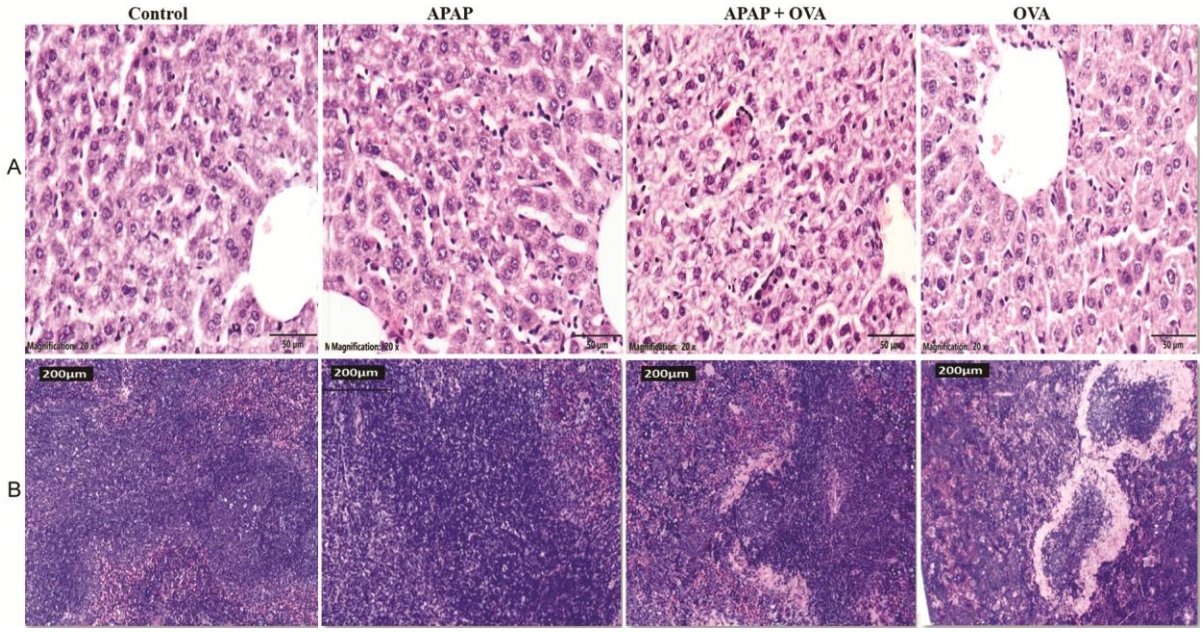


Fig. 7 — Histopathological changes in (A) spleen; and (B) liver. [H&E staining, original magnification 20X]

liver tissue histology after acetaminophen treatment (Fig. 7B).

Correlation between ILC2s, ILCregs and PGD2 in type 2 inflammation and modulation of ILC2 to ILCregs ratio by acetaminophen in OVA sensitized mice

Further, we sought to determine the co-relation between the ILC2s, ILCregs and PGD2 in different treatment groups, which play a pivotal role in Th2 immune responses. Among innate lymphoid cells, we found the strongest positive correlation of ILC2s population with PGD2 (Pearson R = 0.90; R2 = 0.82; P value <0.01). However, ILC2s showed a weak co-relation with ILCregs (Pearson R = 0.53; R2 = 0.28; P value = 0.001). In contrast, we found a weak association of ILCregs with PGD2 (Pearson R = 0.261; R2 = 0.069) (Table 1). These findings are indicative of important role of PGD2 in maturation and development of ILC2s.

Additionally, we also studied the ratio of ILC2s to ILCregs in OVA sensitized mice and APAP treatment groups. It was clearly observed that OVA sensitized mice exhibited a significantly high ratio of ILC2s to ILCregs throughout the study period (Table 2). However, APAP treatment downregulated the ILC2s to ILCregs ratio in OVA sensitized mice and almost restored it to the level of healthy controls by the end of day 42 (Table 2).

Prostaglandin D2 (PGD2) which is primarily released by activated mast cells, is crucial for the activation of ILC2s. It activates ILC2s via CRTH2 receptor, and blocking of CRTH2 receptor diminishes the ILC2 responses[32]. Interestingly, APAP and its metabolites have also been reported to suppress the production of PGD2 in different model systems[33,39,45]. Since APAP also acts as a COX-1/2 inhibitor, therefore, we explored the possibility of allergy attenuation by APAP treatment. Consistent with the

Table 1 — Co-relation between ILC2s, ILCregs and PGD2 in OVA sensitized mice

Parameters		Pearson	Coefficient of	P
1	2	R	determination (R^2)	value
% ILC2s	PGD2	0.90	0.82	<0.001
% ILC2s	% ILCregs	0.53	0.28	<0.001
% ILCregs	PGD2	0.26	0.069	0.12

[The co-relation was measured using (n = 36) data points including 9 mice in each group. Pearson R and R^2 were computed to measure the degree of positive or negative association among variables. The results are expressed as the mean ± SEM and the p values of less than.05 were considered significant]

Table 2 — Ratio of ILC2s to ILCregs

Group	Ratio of ILC2s to ILCregs
Control-14	0.45
Acetaminophen Only-14	1.04
Acetaminophen + OVA-14	1.72
Positive Control-14	4.20
Control-28	0.75
Acetaminophen Only-28	0.55
Acetaminophen + OVA-28	0.93
Positive Control-28	2.72
Control-42	1.03
Acetaminophen Only-42	0.42
Acetaminophen + OVA-42	0.69
Positive Control-42	3.03

earlier reports, we found significantly reduced levels of PGD2 in the sera of APAP treated mice. Next, we assessed the effect of APAP exposure on ILC2 population. Notably, we demonstrated that APAP downregulated the expansion of ILC2s in OVA sensitized mice and throughout the study period, the number of ILC2s in (APAP + OVA) treated mice was significantly higher than OVA group mice. Since ILC2s are the key players for development of "Type I hypersensitivity", we speculated that APAP dosing might attenuate the Th2 responses in OVA sensitized mice. Moreover, a recent study found that retinoic acid, a metabolite of vitamin A, could induce IL-10 producing ILCregs from ILC2s. In addition, it was shown that *in vitro* ILCregs derived from ILC2s acquire a regulatory T-cell (Tregs) like characteristic, and suppress the proliferation of T cells and other ILC2s via IL-10[36]. According to another study, alternative activation of ILC2s causes them to become IL-10 secreting anti-inflammatory $ILC2_{10}$ in the lungs (denoted as ILCreg in the intestine)[37]. This indicates that in the presence of inhibitory signals, ILC2s change their phenotype to regulatory ILCregs and perform anti-inflammatory function. APAP has been documented to reduce PGD2 synthesis which is a key underlying factor triggering ILC2s activation. Therefore, we hypothesized that APAP may suppress the number of ILC2s and concomitantly enhance the number of ILCregs. We assessed the effect of APAP exposure on ILC2 population. We demonstrated that acetaminophen downregulated the expansion of ILC2s in OVA sensitized mice, and throughout the study period, the number of ILC2s in (APAP + OVA) treated mice was significantly higher than OVA sensitized positive control mice. Furthermore, we found that APAP treatment induced an expansion of IL-10 secreting ILCregs in OVA sensitized mice. It was conspicuous that (APAP + OVA) treated mice had significantly higher number of ILCregs on day 42 in our study, in reference to mice sensitized with OVA alone. Upon comparison, it was clearly apparent that the kinetics of ILCregs and ILC2s showed an inverse pattern in (APAP + OVA) group and OVA sensitized mice group; ILC2s being the dominant population in OVA sensitized mice, whereas ILCregs numbers far exceeded ILC2s in (APAP + OVA) treated mice.

We further examined the implications of these APAP induced effects on the OVA specific immunoglobulins IgG1 and IgE. It was clearly observed that (APAP + OVA) treated mice had significantly lower levels of OVA specific serum IgE and IgG1 compared to OVA sensitized positive control group. Both IgE and IgG1 are type of antibody that play a crucial role in Th2 immune responses. When an antigen (allergen or helminths/ worms) enters the body, it binds to specific receptors on the surface of B cells, leading to the production of IgG1 and subsequently IgE antibodies that are specific to that antigen. These antibodies particularly IgE then bind to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils, which are well known to play important roles in the manifestation of clinical symptoms of the Th2 immune reactions[46]. From our study it was clearly evident that (APAP + OVA) treated mice had significantly lower levels of OVA specific serum IgE and IgG1, in comparison to OVA sensitized positive control group.

The role of Th2 cells in deregulated Type 2 immune responses such as IgE mediated allergy is well established, and recent evidence show the prominent role of ILC2s in development and maturation of Type 2 immunity[47,48]. Our study also suggests that APAP treatment may reduce the level of IgG1 and IgE through suppressing ILC2s in OVA sensitized mice. Furthermore, we noticed an increment of white pulp of spleen in OVA sensitized mice which was not evident in the (APAP + OVA) treated group. It clearly corroborated our outcomes, that APAP treatment attenuated the Th2 immune responses in the OVA sensitized mice. Finally, we analyzed the co-relation between the IL-10^{+}ILCregs, ILC2s and PGD2. Our data shows a remarkable association between PGD2 and ILC2s. It was also clearly evident that OVA sensitized animals had a significantly high ratio of ILC2s to ILCregs throughout the study period, however, APAP treatment restored this ratio to normal levels by day 42.

Since APAP is also reported to cause hepatotoxicity in certain dosing regimens, therefore we also investigated the effect of APAP treatment in our study on lever histopathology. According to the histological analysis of liver tissue, the dose of acetaminophen was nontoxic, and no substantial change was observed in the liver tissue histology after acetaminophen treatment. Conclusively, our data clearly shows that APAP exposure may suppress the number of ILC2s and induce an expansion of ILCregs

in type 2 pathological inflammation. The current findings could have implications for the immunological mechanism of action for acetaminophen and its prospective therapeutic use in type 2 immune disorders.

Conclusion

Overall, this study uncovers a novel aspect of acetaminophen (APAP), revealing its ability to influence immune responses beyond its well-known analgesic and antipyretic properties. Through careful experimentation in a mouse model of type 2 inflammation, we found that APAP effectively dampens ILC2s while promoting the expansion of ILCregs expressing IL-10. This suggests that APAP may hold promise in modulating allergic and type 2 immune-mediated conditions. It was also corroborated by reduced levels of IgG1 and IgE, and enhanced serum IL-10 in OVA sensitized mice treated with APAP. Additionally, the study highlights the safety of APAP, as it did not induce adverse effects on vital organs. These findings open avenues for further research, potentially leading to new therapeutic strategies for type 2 immune disorders.

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Conflict of Interest

Authors declare no competing interests.

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