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Metabolites and growth factors produced by airway epithelial cells induce tolerance in macrophages

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ABSTRACT

Macrophages play a role in preventing inflammation in the respiratory tract. To investigate the mechanisms that lead to tolerance in macrophages, we examined the crosstalk between airway epithelial cells (AECs) and macrophages using a 2D coculture model. Culture of macrophages with AECs led to a significant inhibition of LPS induced pro-inflammatory responses. More importantly, AECs induced the secretion of TGF-β and IL-10 from macrophages even in the absence of LPS stimulation. In addition, the expression of inhibitory molecule, CD200R was also upregulated on AEC exposed macrophages. Furthermore, the AECs exposed macrophages induced significantly increased level of T regulatory cells. Investigation into the possible mechanisms indicated that a combination of growth factor, G-CSF, and metabolites, Kynurenine and lactic acid produced by AECs is responsible for inducing tolerance in macrophages. Interestingly, all these molecules had differential effect on macrophages with G-CSF inducing TGF-β, Kynurenine elevating IL-10, and lactic acid upregulating CD200R. Furthermore, a cocktail of these factors/metabolites induced similar changes in macrophages as AEC exposure. Altogether, these data identify factors secreted by AECs that enhance tolerance in the respiratory tract. These mediators thus have the potential to be used for therapeutic purposes to modulate respiratory inflammation following local viral infections and lung diseases.

1. Introduction

Respiratory infections are a cause of major health concern as evident by the recent COVID-19 pandemic. Efficient immune responses at the respiratory mucosa are required for defense against the infections. Lung is also continuously exposed to innocuous inhaled antigens, many of which are harmless. Thus, innate immune cells need to be able to mount response against pathogens and be tolerant to harmless antigens. The airway epithelial cells (AECs), dendritic cells (DCs) and macrophages (Mac) are amongst the key components of respiratory innate immune responses. These cells together form a coordinated network to defend the respiratory mucosa. AECs form a natural barrier against infections [1,2]. They express pathogen recognition receptors (PRRs) to sense and respond to pathogens via production of mucus, antimicrobial peptides as well as production of various inflammatory mediators. In addition, AECs also modulate the function of innate immune cells in the airways. We and others have previously demonstrated that AECs enhance the immune surveillance and response functions of both myeloid (mDCs) and plasmacytoid DCs (pDCs) [3–6]. The expression of PRRs and downstream signaling molecules on DCs is increased at homeostasis that in turn enhances their response to pathogens. We also found that growth factors secreted by the AECs were able to enhance the type-I-IFN secretion from pDCs in response to influenza virus.

In contrast to DCs, studies indicate that alveolar macrophages (AM) may be playing a more important role in maintaining tolerance in the lung [7,8]. The AMs are the dominant immune cell in the lung at steady

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state. Granulocyte-macrophage colony-stimulating factor (GM-CSF) plays an important part in maintaining their presence in the lungs [9,10]. The AMs serve many functions at homeostasis including regulation of responses to epithelial damage and pathogens. Scavenger receptors on these cells help in cleaning the lung environment via removal of oxidized lipid molecules. For example, the expression of MARCO and class A scavenger receptors is upregulated on inhalation of oxidants that helps in reducing the inflammation [11]. AMs are also crucial in resolving injury and controlling inflammation after tissue damage [12]. Studies indicate that AMs are less efficient in antigen-presentation due to decreased expression of costimulatory molecules [13]. Active T cell suppression by AMs has also been reported in both mice and humans [11,13,14]. In addition, the immune-regulatory effect of AECs on the response of AMs to various pathogenic and other stimuli has also been reported from various groups. For instance, both rat RLE-6TN cells and human A549 cells were able to decrease the expression of inducible NO synthase (iNOS) and IL-6 in alveolar macrophages in response to LPS in a contact-independent mechanism [15].

The functions of immune cells in lung microenvironment are modulated both by direct contact and by factors secreted by AECs [8]. We have previously demonstrated that AECs can enhance the immune surveillance capacity of both mDCs and pDCs [3,4]. Recent studies indicate a role of exosomes in the interaction of cells [16,17]. Exosomes are 40–150 nm extracellular vesicles (EVs) released by all cell types [17–19]. Like cells, exosomes are composed of a lipid bilayer and, at any given point, can contain all known molecular constituents of a cell, including proteins, RNA, and DNA [17,20]. Upon release from the cell surface, exosomes possess the capacity to fuse with the plasma membranes of recipient cells to deliver their contents into the cytoplasm [21,22]. Alternatively, proteins present on the surface of the exosomes can engage cell surface receptors on recipient cells to induce intracellular signaling [22]. Exosomes have been reported to induce tolerance in cardiac allograft model [23]. Furthermore, placental derived exosomes have been shown to establish maternal immune tolerance [24]. We have shown that stem cell-derived exosomes are neuroprotective and may help reduce Alzheimer's disease associated neuropathology [25].

Despite these studies, information regarding the interaction between AECs and macrophages and its effect on macrophages is limited. The mechanisms by which AECs may be inducing tolerance in these cells are also not well understood. Recent COVID-19 pandemic has highlighted the problems associated with uncontrolled lung inflammation. Therefore, identification of factors/mechanisms that induce tolerance in the lung may be useful in development of novel therapeutic mechanisms to control inflammation in the lung. In the present study, using a 2D model of AECs and macrophage co-culture, we investigated the mechanisms by which airway epithelial cells induce tolerance in AMs.

2. Materials and method

2.1. Airway epithelial cells (AECs)

Primary bronchial epithelial cells (AECs) [Air–liquid interface (ALI)-tested] from three normal, healthy individuals (27–45 years) were obtained from Lonza Inc. (Basel, Switzerland) and were differentiated at the ALI on transwell plates as described [26]. Briefly, 5×10^4 AECs per insert were seeded into 24-well transwell plate with apical chamber coated with the rat tail collagen (BD Biosciences, San Jose, CA, USA). Cells were seeded in 100 μl B-ALI™ growth medium onto the collagen coated trans well inserts; 500 μl of B-ALI™ growth medium was added to the basal chamber of wells containing the inserts. On day 3 after seeding, once the monolayer of AECs was confluent, media were removed from the apical chamber and allowed for air–liquid differentiation. The media in the bottom chamber were changed every two days. Approximately 21 days post-differentiation, AECs were tested for the presence of cilia by staining for beta-tubulin and Mucus secretion as described [26].

2.2. Isolation and culture of human macrophages with AECs

Blood was collected from healthy volunteers under the protocol approved by the Institution Review Board of the University of California, Irvine. Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy subjects by density gradient centrifugation. Monocytes were enriched from the peripheral blood mononuclear cells (PBMCs) of young subjects by CD14 positive selection kit (Stemcell Sep, Vancouver). Purified monocytes were differentiated into macrophages by culturing them with GMCSF 50 ng/ml. After 6 days, macrophages were harvested and added to the bottom chamber of the transwell with ALI-differentiated AECs for 24 h. The macrophages were collected and used for stimulation and other studies.

2.3. LPS stimulation

Macrophages collected after overnight AEC exposure were washed and stimulated with *Klebsiella pneumonia* (Kp) LPS (Sigma- Aldrich, St. Louis, MO, USA), at 100 ng/ml for 24 h. Subsequently, the cells were collected and stained for surface markers CD86 (clone BU63), HLADR (clone L243) and CD200R (clone OX-108) (Biolegend). The supernatants collected were assayed for TGF-β, TNF-α and IL-10 by specific ELISA (R&D systems). Macrophages cultured in AEC media without AEC were used as controls. For TGF-β samples, total TGF was measured. To activate latent TGF-β, samples were acidified using 1 N HCL for 10 min and RT and subsequently neutralized by adding 1.2 N NaOH/0.5 M HEPES.

2.4. Induction of T regulatory cells

For co-culture experiments, purified macrophages were cultured without or with AECs differentiated at ALI. After overnight co-culture, purified CD4 T cells (purified by negative selection using magnetic beads-based kits from Stemcell sep, Vancouver, Canada) were added to Macrophages from both groups at a ratio of 1:5 (Mac:CD4 T) and cultured together for 6 days. Subsequently, the cells were collected and stained with specific antibodies for CD4, CD25 and FoxP3 (BD Biosciences, San Jose). Cells were acquired in BD FACSCalibur. Gated CD4 cells were analyzed for the expression of CD25 and FoxP3 using Flowjo. Supernatants collected were assayed for TGF-β, IFN-γ and IL-10 by ELISA.

2.5. Exosome isolation and culture with macrophages

Conditioned media was collected from the bottom wells of AECs grown at ALI. Exosomes were purified from AECs conditioned medium (CM) using exosome isolation kit (Takara Bio) following the manufacturer's protocol. Flow through after exosome removal was also collected (Flow). The size and concentration of isolated exosomes were evaluated by nanoparticle tracking analysis or (NTA). Exosomes (Exo) were around 76 nm. Macrophages were cultured with exosomes (1×10^6 exo/ml), flow through and the conditioned media overnight and subsequent stimulated with LPS. Supernatant collected were assayed for TGF-β, TNF-α and IL-10.

2.6. Multiplex assay of factors secreted by AECs

PBECs from four different subjects were differentiated at ALI. The conditioned basal medium in the bottom chamber was collected once the AEC differentiation was complete. PBEC differentiation media alone was used as control. Multiplex detection of 30 factors was done using the MILLIPLEX Human 30-Plex Cytokine Panel 1 (Cat# HCYTMAG-60K-PX30) which contains the following analytes- GM-CSF, G-CSF, IFNγ, IL-1α, IL-1rα, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-12p40, IL13, IL15, IL-17, MCP-1, TNFα, TNFβ, Eotaxin, type I IFN, IP-10, MIP-1α, MIP-1β, EGF, VEGF and RANTES (Millipore, USA).

2.7. Metabolites analysis

The AEC conditioned media (**CM**) and differentiation media (**DM**) were subjected to non-targeted GC-profiling that captures ~500 metabolites at the UC Davis West Coast Metabolomics core facility as described [27]. **CM** from AECs from three different subjects was used for the analysis and **DM** served as control. Each sample was run in triplicate.

2.8. Culture of macrophages with GCSF, VEGF, Kynurenine, and lactic acid

Purified Macrophages were cultured in the presence of Kynurenine (10 and 100 nM/ml) or lactic acid (0.1, 0.5 μM/ml) (R&D systems) or G-CSF (10 ng/ml) or VEGF (10 ng/ml) (Biolegend) or a cocktail containing all of them for 24 h. Supernatants collected were assayed for the cytokines, TGF-β, TNF-α and IL-10. Cells were stained for CD200R using flow cytometry.

2.9. Statistical analyses

Statistical analysis for cell culture experiments was performed using GraphPad Prism version 9.1.2 (GraphPad Inc., San Diego, CA, USA). *t*-Tests for two groups and one way ANOVA followed by Tukeys' or Dunnett's multiple comparison test was used for the analysis of three or more groups. Two way ANOVA was used when examining the effect of two factors. A p-value of <0.05 was considered statistically significant. The tests used for each figure are mentioned in figure legends. Unpaired *t*-tests were used for the tables (1 and 2).

3. Results

3.1. AECs induce tolerance in macrophages

Recent studies from human lung samples suggest that macrophages may be playing a more important role in maintaining tolerance in the lung while DCs are more important for generating an immune response against pathogens [7]. Therefore, we investigated the response of macrophages to AECs using a similar model system as we have employed

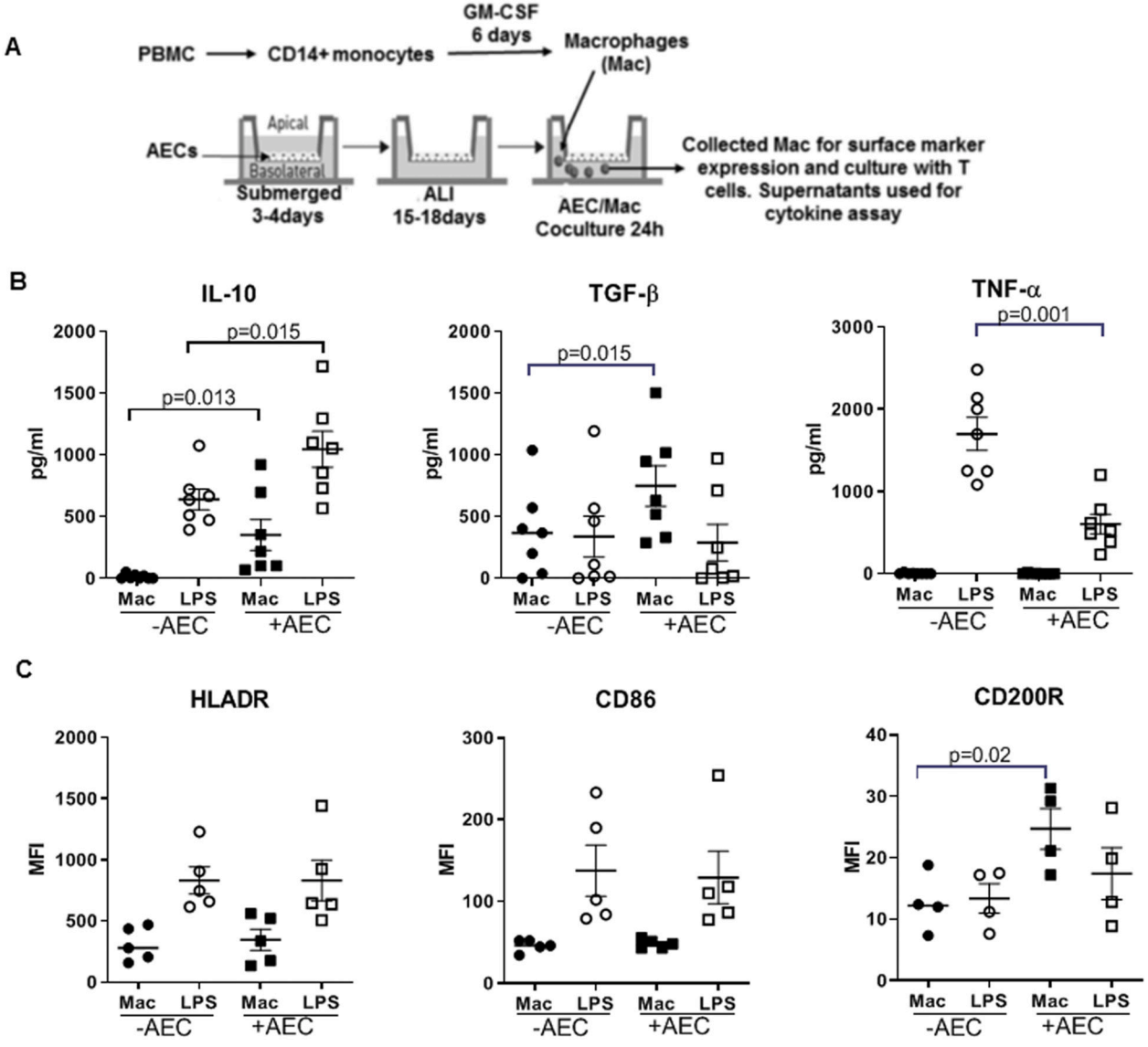

Fig. 1. AECs induce tolerance in macrophages. Monocyte derived macrophages were exposed to AECs for 24 h and subsequently stimulated with LPS. A. Experimental design; B. dot plot depicts the quantitation of cytokines in the supernatant; C. surface markers on cells. Two way ANOVA was used to determine significance. Mean ± S.E. $N = 7$ (A); $N = 4$–5 (B).

for studying DC-AEC interactions (Fig. 1A). Briefly, AECs were cultured on transwell plates and differentiated at air-liquid interphase (ALI) for about 21 days. Subsequently, monocyte–derived macrophages were added in the bottom chamber of the transwell. Since macrophages differentiated from monocytes in the presence of GM-CSF have been shown to be similar to alveolar macrophages [28] we used this method to generate macrophages. After overnight exposure to AECs, macrophages were collected and stimulated with *Klebsiella pneumonia* LPS as described for mDCs [3]. Supernatants collected after overnight LPS stimulation were assayed for cytokines. It is evident (Fig. 1A) that macrophages co-cultured with AECs secreted significantly increased levels of anti-inflammatory cytokines, IL-10 and TGF-β at homeostasis compared to controls. LPS stimulation led to significantly ($p < 0.05$) increased secretion of IL-10 and suppression of TNF-α in macrophages cultured with AECs as compared to those without AECs. IL-6 and IL-1β levels were below the detection limits (data not shown). We also examined the effect on surface markers of antigen presentation, HLADR and CD86 on the macrophages after exposure to AECs and LPS. Stimulation with LPS resulted in upregulation of both these molecules, however, there was no significant difference in the expression between AECs exposed and non-exposed groups (Fig. 1B). We also investigated changes in the expression of inhibitory molecules CD206, B7H3 and CD200R that are known to be present on tolerized macrophages. Amongst these, there were no differences in CD206 and B7H3 expression (data not shown). However, the expression of CD200R was found to be significantly upregulated on macrophages exposed to AECs as compared to those that were not exposed even in the absence of any stimulation (Fig. 1C). The difference was not significant between the groups after stimulation with LPS. CD200R is known to be present on alveolar macrophages and has been reported to be involved in inhibition of innate immunity in the lung via interaction with AECs [8,29]. These data demonstrate that AECs inhibit macrophage activation and induce the secretion of tolerance inducing cytokines, IL-10 and TGF-β and enhance the expression of the inhibitory receptor, CD200R.

3.2. Macrophages exposed to AECs induce T regulatory cells (Tregs)

Since macrophages exposed to AECs secreted increased levels of IL-10 and TGF-β at homeostasis, their Treg induction capacity was determined as a measure of tolerance. When the macrophages were cultured with purified T cells, the macrophages exposed to AECs induced significantly increased percentages ($p < 0.05$) of Tregs compared to control macrophages (Fig. 2A & B). The secretion of IL-10 was also significantly upregulated ($p < 0.05$) and IFN-γ downregulated in the culture (Fig. 2C). TGF-β levels displayed no significant difference. In summary, the AEC-exposed macrophages generated Tregs that may help induce airway tolerance.

3.3. Exosomes from AECs can induce only partial tolerance in macrophages

We explored the possible mechanisms of the immunosuppression of macrophages by AECs. Several studies have demonstrated that miRNA and/or proteins in exosomes can exert an inhibitory or stimulatory effect

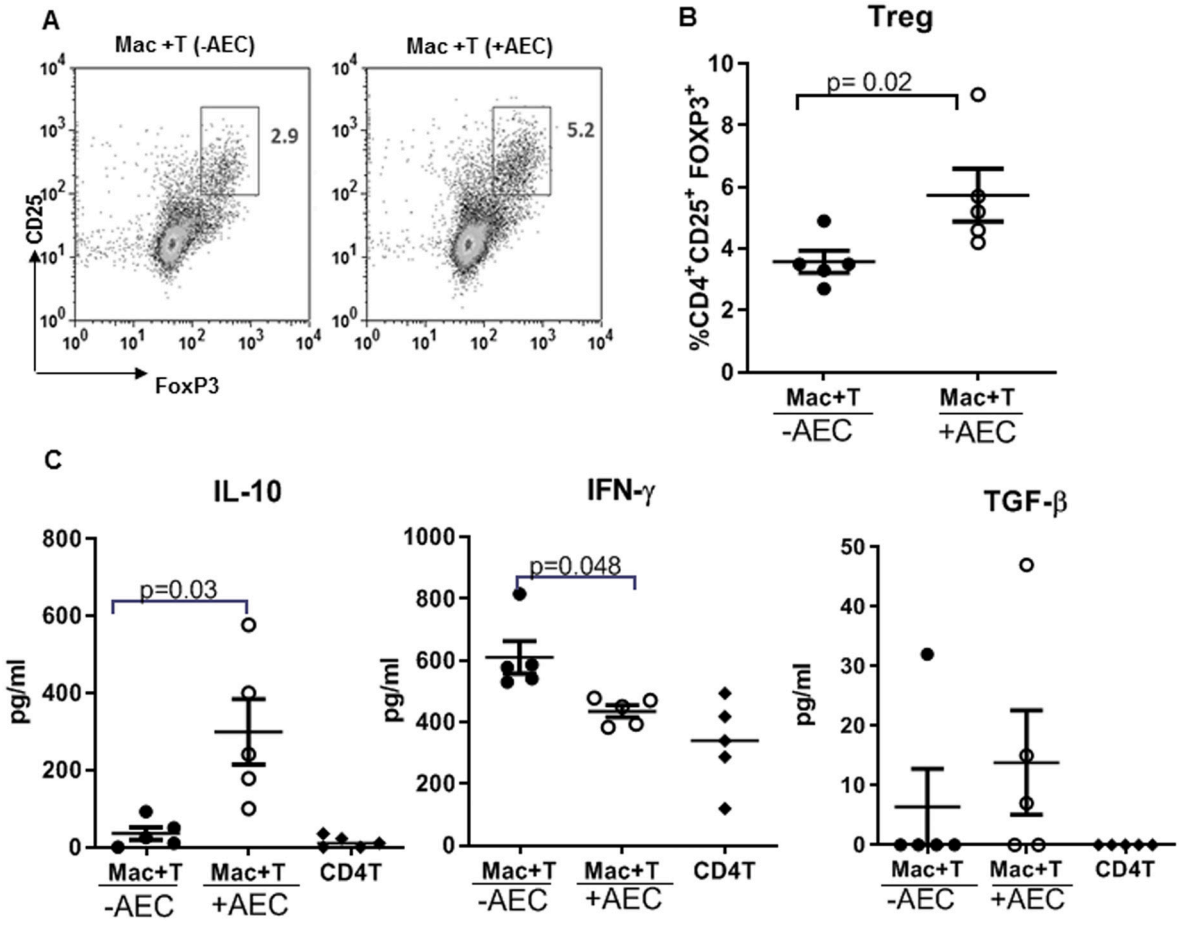

Fig. 2. Macrophages exposed to AECs induce Tregs. Macrophages exposed to AECs were cultured with purified CD4 T cells to determine the induction of Tregs by flow cytometry. Supernatants collected were assayed for T cell cytokines. A. Dot plot indicates the percentage of $CD4^+CD25^+FoxP3^+$ Tregs in culture; B. Mean ± S.E. of the same; C. cytokines quantification in the supernatant. Paired *t*-test was used to determined significance. CD4 T alone is just provided as a reference and was not used to calculate significance. Mean ± S.E.

on cells [30,31]. Therefore, we examined whether exosomes from AECs can inhibit pro-inflammatory cytokine secretion in macrophages. Exosomes were purified from AECs conditioned medium (CM). Flow-through was also collected (Flow). The size and concentration of isolated exosomes was evaluated by nanoparticle tracking analysis. Exosomes (Exo) were around 81 nm (Fig. 3A). Macrophages were cultured with exosomes (1×10^6 exo/ml), flow-through and the conditioned media overnight and then stimulated with LPS. IL-10 secretion by macrophages was significantly increased ($p < 0.005$) in the flow-through and CM group while there was no significant change in the exosomes exposed group (Fig. 3B) at homeostasis. Lower concentration of exosomes (1×10^5/ml) was not effective (data not shown). Stimulation with LPS also led to significant increase ($p < 0.05$) in IL-10 only in flow-through and CM groups compared to controls. TNF-α was also significantly inhibited only in these two groups and not the exosome treated group (Fig. 3B). These data indicate that the major inhibitory activity is present in the exosome flow-through and CM as the exosomes induced low levels of IL-10 and were unable to inhibit LPS induced TNF-α.

3.4. Effect of G-CSF and VEGF produced by AECs on macrophages

Since we observed inhibitory activity in the flow-through we performed multiplex analysis of the AEC conditioned medium to identify possible inhibitory molecules. We have previously reported that amongst the thirty factors tested, only GM-CSF, G-CSF and VEGF levels were significantly different ($p < 0.05$) between AEC conditioned medium and the control differentiation medium [4] (Fig. 4A). We examined whether G-CSF or VEGF were inducing tolerance in macrophages. GM-CSF was not used because the macrophages were already differentiated in the presence of GM-CSF. As is evident from Fig. 4B, G-CSF induced high levels of TGF-β secretion from macrophages but had no effect on IL-10 secretion and CD200R expression. VEGF had no significant effect on either the cytokines or the CD200R. Thus, G-CSF is responsible for partial induction of tolerance in macrophages.

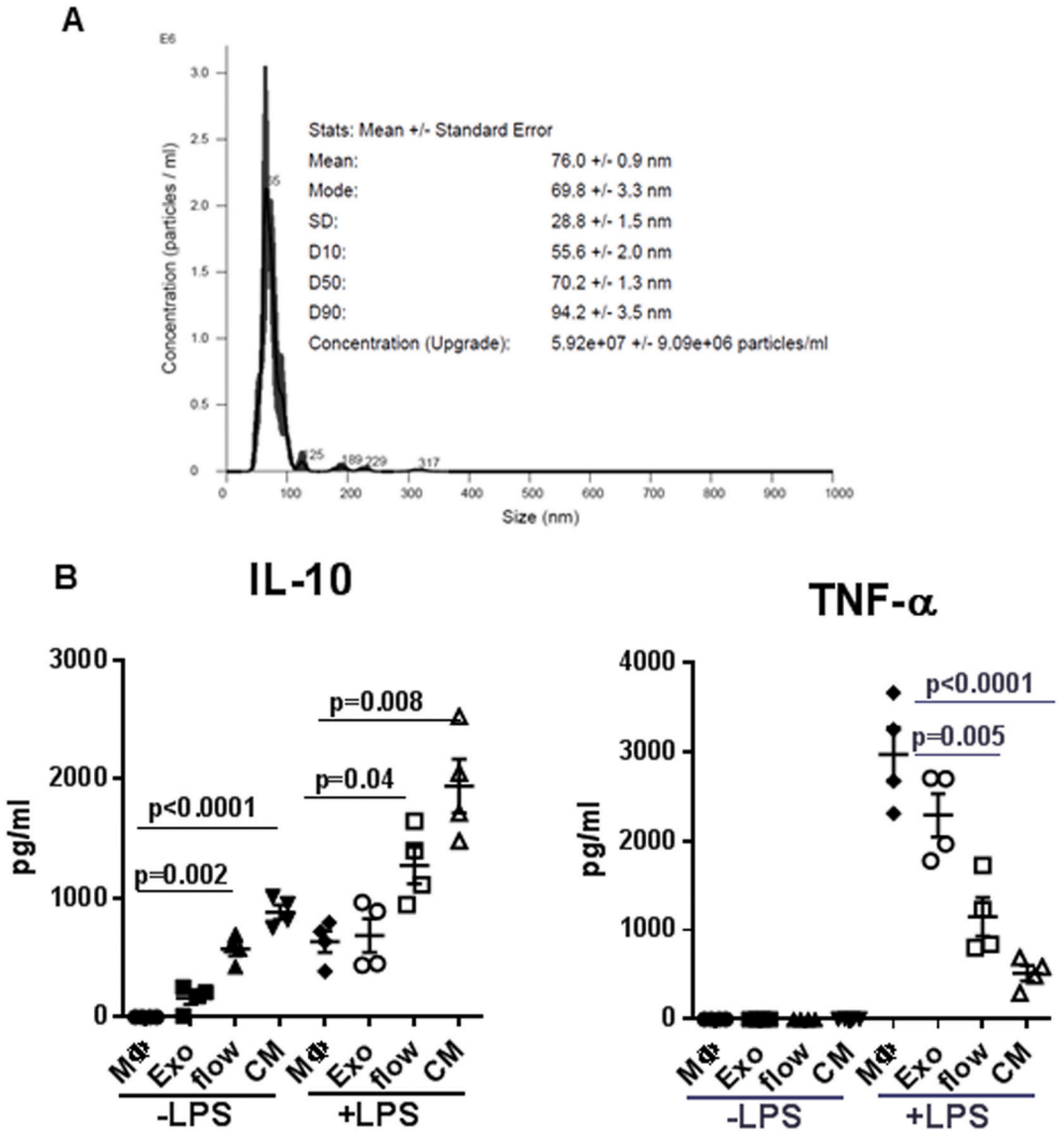

Fig. 3. Exosomes from AECs do not induce tolerance in macrophages. Exosomes were purified from AEC conditioned media. A. Histogram depicts the size distribution of exosomes as determined by nanoparticle tracking analysis; macrophages were exposed to exosomes (Exo) or the flow-through (Flow) or conditioned media (CM) for 24 h and subsequently activated with LPS. B. Dot plots depict the level of cytokines in the supernatants. Two way ANOVA was used to determine significance. Mean ± S.E.

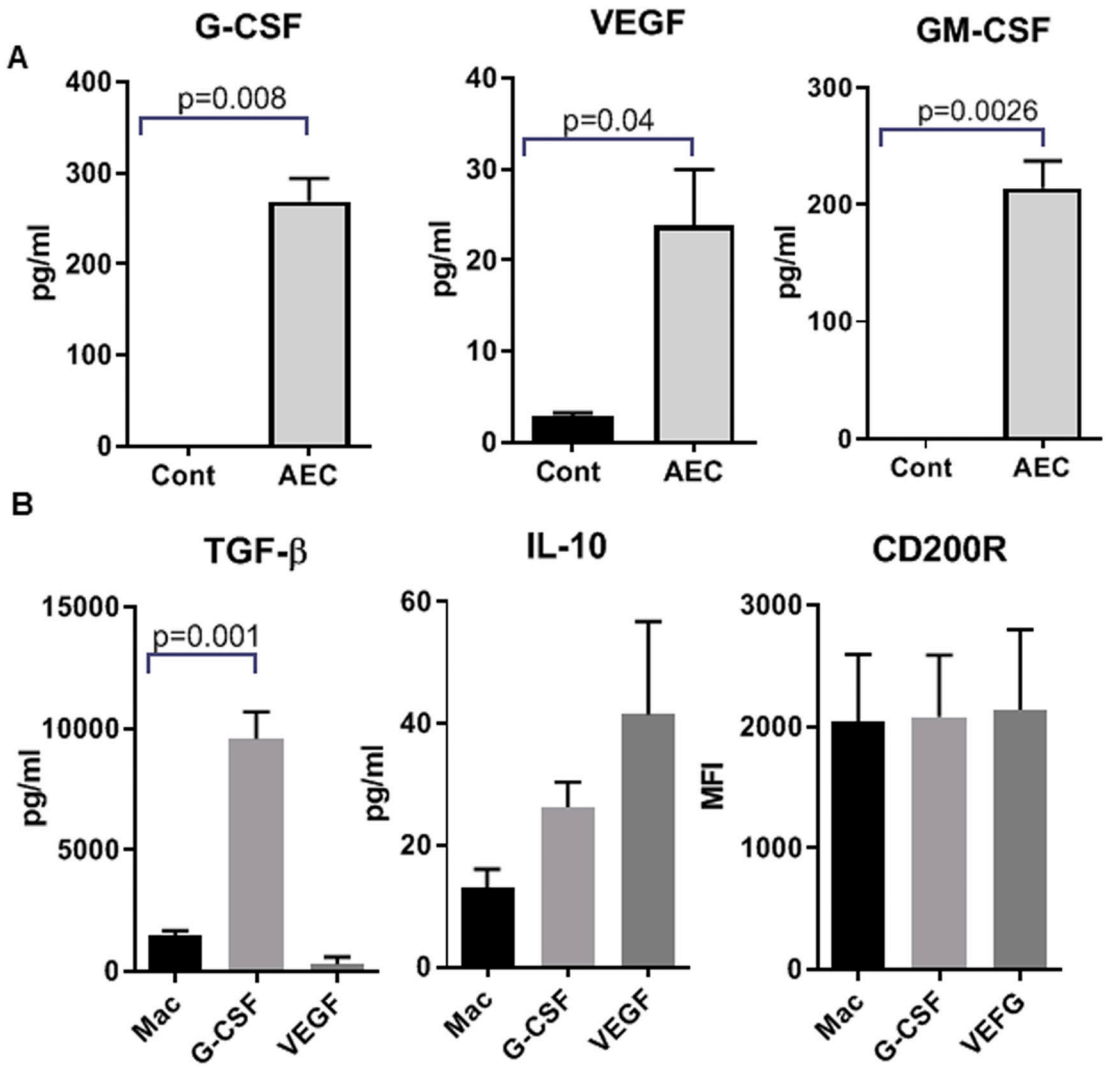

Fig. 4. Effect of G-CSF and VEGF produced by AECs on macrophages. The level of cytokines, chemokines growth factors in the AEC conditioned media were determined by multiplexing. A. Bar graph depicts the factors that were different between AEC CM and control media; unpaired *t*-test was used to determine significance. B. Macrophages were exposed to G-CSF or VEGF for 24 h. Bar graphs depict the level of IL-10 and TGF-β in the supernatant and expression of CD200R on cells. One way ANOVA followed by Tukey's test was used to determine significance. Mean ± S.E. of 4 experiments.

3.5. Metabolite profiling of AEC conditioned medium (CM) identifies possible factors that may be tolerogenic

Next, we performed metabolomics analysis of the AEC conditioned medium to identify other possible inhibitory molecules. The conditioned media (CM) and differentiation media (DM) was subjected to GC-profiling Table 1 depicts the fold changes between CM/DM metabolites that were significantly upregulated ($p < 0.05$) between CM and DM. Most of the metabolites that were increased such as Kynurenine, lactic acid, were metabolic products of amino acids and glucose utilization by cells. Interestingly, both Kynurenine (tryptophan metabolite) [32], lactic acid [33] (Glucose oxidation) have been reported to inhibit macrophage functions. Isothreonic acid also displayed a substantial increase in CM. It is a metabolite of vitamin C and its function on immune system has not been studied. Keto-isocaproate, a key leucine metabolite has been shown to inhibit macrophage foam cell formation [34]. Sugar alcohols and amino acids were the major metabolites that were

Table 1
Metabolite profiling of AEC CM. Metabolites displaying increase between CM and DM are shown in the table.

Metabolite	Fold change (increase)	*p* value
Lactic acid	184.8	0.0002
Isothreonic acid	32.3	0.0062
Kynurenine	16.3	0.0027
Lactamide	14.2	0.0223
2-Ketoisocaproic acid	2.4	0.0001
Urea	1.7	0.0124
Hexadecane	1.5	0.0276

Table 2
Metabolite profiling of AEC CM. Metabolites displaying decrease between CM and DM are shown in the table.

Metabolite	Fold change (decrease)	*p* value
Tryptophan	0.7	0.0302
Serine	0.8	0.0167
Pyruvic acid	0.4	0.0080
Malic acid	0.2	0.0000
Leucine	0.8	0.0129
Isoleucine	0.8	0.0293
Erythritol	0.4	0.0119
Aspartic acid	0.6	0.0323
Asparagine	0.7	0.0147
Arabitol	0.2	0.0000

downregulated (Table 2). These were probably used up by the cells for growth and differentiation.

3.6. Effect of metabolite kynurenine and lactic acid on macrophages

Next, we investigated whether kynurenine (Kyn) and lactic acid (LA) produced by AECs were inducing tolerance in macrophages. Macrophages were cultured with varying concentrations of Kyn (0–1000 nM) and LA (0–1 μM) for 24 h (Supplementary Fig. 1). Concentrations of Kyn1000nM and LA 1 μM were found to be toxic (data not shown). Therefore, we used Kynurenine at (10 and 100 nM) and lactic acid at (0.1 and 0.5 μM) in the experiments. IL-10 secretion was significantly increased ($p < 0.05$) in Kyn 100 nM treated macrophages compared to controls (Fig. 5A). However, Kyn had no significant effect on TGF-β and CD200R at the same concentration. Lactic acid had no significant effect on IL-10 or TGF-β production but led to significant increase in the expression of CD200R at 0.5 μM (Fig. 5A).

Since in the AEC conditioned media, the metabolites Kyn and lactic acid and G-CSF, VEGF are all present together, we tested the effect of this cocktail on macrophages (Fig. 5B). Exposure to the cocktail led to significant increased secretion of IL-10 and TGF-β from macrophages. The expression of CD200R also displayed significant upregulation.

We also examined whether presence of Kyn, lactic acid or cocktail were able to inhibit LPS induced inflammatory responses. Kyn and cocktail both induced significantly higher level of IL-10 compared to LPS-treated controls (Fig. 5C). In keeping with this, we also observed a significant decrease in TNF-α levels in the cocktail group (Fig. 5C). As before (Fig. 1B), treatment with LPS led to comparable levels of TGF-β in all groups (Fig. 5C). Altogether, these results indicate that G-CSF, Kyn and lactic acid induce tolerance in macrophages via different mechanisms. The results also suggest that the effect is not synergistic since the levels of the cytokines and CD200R are either lower or similar to the factor/metabolite alone.

4. Discussion

Airways are exposed to a wide variety of substances during inhalation. Majority are harmless and thus no immune response is required against them. To distinguish between harmless and pathogenic antigens, the immune system in the airways exists in an immunosuppressed state known as airway/mucosal tolerance. AMs have emerged as major players in maintenance of airway tolerance. Recent studies from human lung samples suggest that Mφs may be playing a more important role in maintaining tolerance in the lung while DCs are more important for generating an immune response against pathogens [7]. Baharom et al. [7] examined the phenotype of monocytes in the airways and blood on samples from the airways via bronchial washing and broncho-alveolar lavage as well as mucosal tissue (endobronchial biopsies) from 20 healthy subjects with ages ranging from 18 to 40 years. They observed that lung monocytes were less inflammatory than blood monocytes. Here we examined whether AECs induce tolerance in macrophages and the mechanisms responsible for the tolerance. Identification of soluble factors that can induce airway tolerance may lead to development of novel therapeutics to control airway inflammation. Increased basal level airway inflammation is a major risk factor for asthma, COPD as well as respiratory viral infections [35,36].

Our results indicate that AECs are able to induce tolerance in macrophages (Figs. 1 & 2). The macrophages exposed to AECs in our 2D cell culture model secreted enhanced levels of IL-10 and TGF-β and induced Tregs. Soroosh et al. have shown that lung resident macrophages express TGF-β and induce T regulatory cells to promote respiratory tolerance [37]. The expression of CD200R was also upregulated on the macrophages. Previous studies have demonstrated that CD200 on airway epithelium interacts with CD200R on the macrophages to limit inflammatory cytokine production [38,39]. Our results indicate that AECs also induce the expression of CD200R on macrophages via soluble factors. In this regard, both IL-10 and TGF-β have been reported to upregulate the expression of CD200R on macrophages.

Recent studies have highlighted the role of exosomes in modulating the functions of immune cells. Our results suggest that exosomes produced by AECs do not play a role in inducing tolerance in macrophages at homeostasis. Previously [4] and here also, we have examined a range of cytokines and chemokines produced by AECs and observed that only growth factors, GM-CSF, G-CSF and VEGF were increased (Fig. 4A). Amongst these, GM-CSF is known to enhance inflammation in macrophages [40] while certain reports indicate that G-CSF and maybe VEGF may induce polarization of macrophages towards an M2 phenotype [41,42]. Our data indicates that G-CSF induces TGF-β secretion from macrophages (Fig. 4B). We have previously observed that G-CSF enhances the production of type I interferons from pDCs [4]. Thus, G-CSF can potentially be used to prevent inflammation and enhance immune response during a respiratory viral infection such as the SARS-CoV-2 where excessive lung inflammation is a major cause of concern [43–45].

In addition to cytokines, chemokines and growth factors, cells also produce several metabolites. The metabolites can also influence immune cell functions. We found increased production of lactic acid, tryptophan metabolite, Kynurenine, 2-ketoisocaproic acid and urea (Table 1). Amongst these, α-ketoisocaproic acid (KIC), an obligatory metabolite of leucine, is known to foam cell formation and urea has been reported to enhance macrophage proliferation [46]. Both Kynurenine, lactic acid have been reported to inhibit macrophage functions. Kynurenines are major metabolites produced by the catabolism of amino acid tryptophan by indoleamine 2,3-dioxygenase (IDO). Kynurenine activates the aryl hydrocarbon receptor (Ahr) in macrophages that induces Treg cells [47,48]. Lactic acid is a byproduct of glycolysis that has immunomodulatory functions. In tumors, lactic acid has been shown to induce polarization of macrophages towards a M2 (immunosuppressive) phenotype. Several mechanisms have been suggested for M2 polarization ranging from ERK-STAT3 signaling, HIF-1α stabilization, induction of G-protein-coupled receptor 132 (GPR132) and post-translation modification of histone proteins that enhances expression of M2 genes. Our study confirms this, but we also find that the two metabolites act differentially. Kynurenine induces IL-10 secretion from macrophages while lactic acid enhances the expression of CD200R [49] (Fig. 5A).

Interestingly, a combination of the metabolites and growth factors were able to induce TGF-β, IL-10 and CD200R albeit at a lower level than when each molecule was used alone (Fig. 5B). The AECs induced tolerance in macrophages is reversible and does not seem to affect their capacity to respond to infections, particularly antigen presentation, since LPS exposure resulted in the upregulation of HLADR and CD86 to a similar level in both AEC exposed and unexposed macrophages. Previous reports also indicate that alveolar macrophages suppress inflammation at steady state but are still capable of responding to infections [50–52].

5. Conclusions

In summary, our study identifies G-CSF, Kynurenine and lactic acid as factors produced by AECs at homeostasis that induce tolerance in macrophages and prevent sterile inflammation. Identification of these factors is not only important from a therapeutic standpoint to control lung inflammation but also suggests novel modalities that may be altered in elderly and those with underlying conditions including diabetes, obesity and hypertension that render them more prone to inflammatory damage caused by viral infections.

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Credit authorship contribution statement

SA performed the Mac-AEC interaction, Mac-G-CSF and metabolite experiments and analysis, CM performed the exosome isolation and

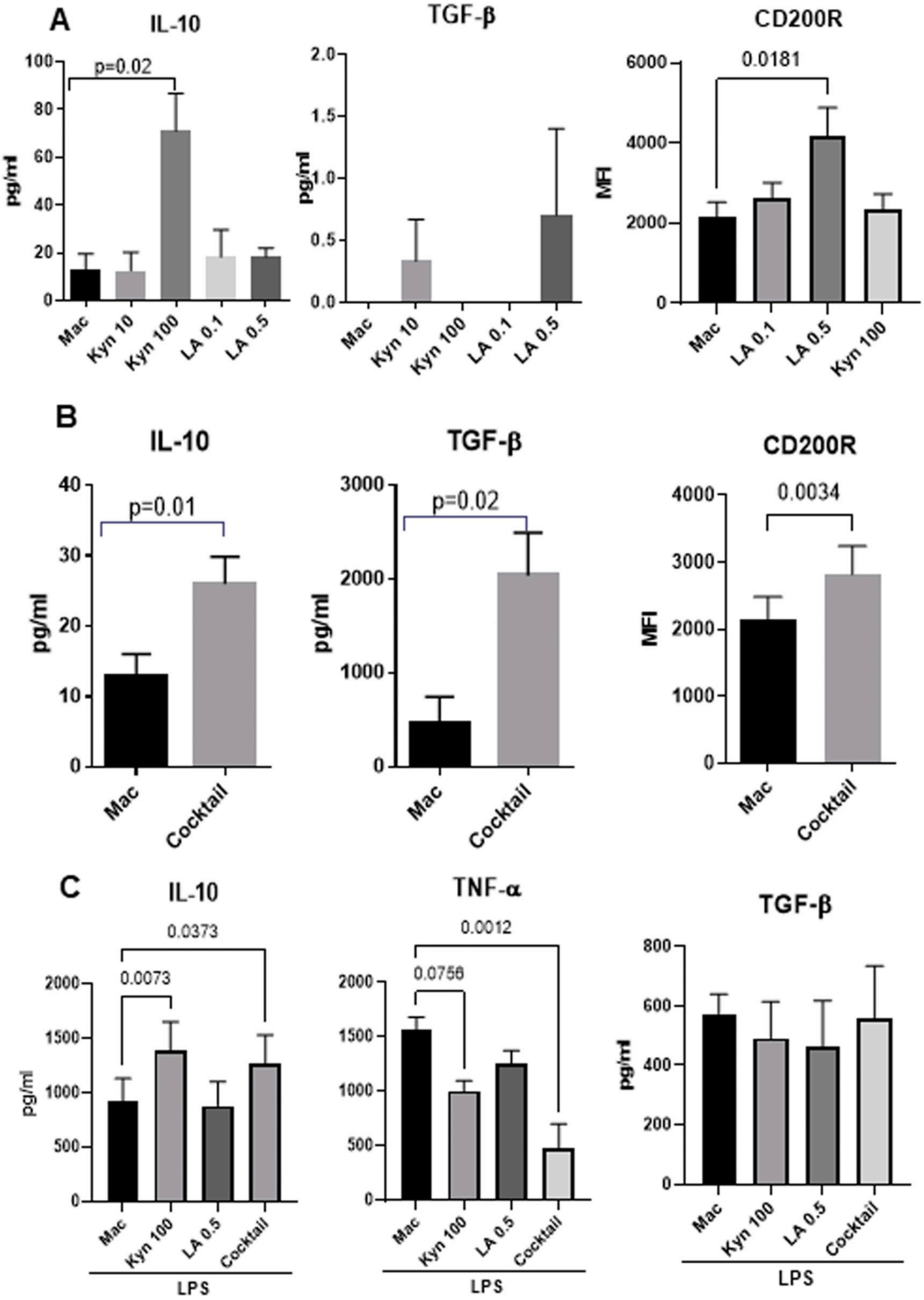

Fig. 5. Effect of metabolites, kynurenine and lactic acid on macrophages. Monocyte derived macrophages were exposed to Kynurenine or lactic acid for 24 h. A. Bar graphs depict the level of IL-10 and TGF-β in the supernatant and expression of CD200R on cells. One way ANOVA followed by Tukey's test was used to determine significance. B. Bar graphs depict the level of IL-10 and TGF-β in the supernatant and expression of CD200R on cells when macrophages were exposed to a cocktail of Kynurenine, lactic acid, G-CSF and VEGF for 24 h. Paired *t*-test was used to determined significance. C. Bar graphs depict the level of IL-10, TNF-α and TGF-β in the supernatants when macrophages were exposed to Kynurenine, lactic acid and cocktail and were subsequently stimulated with LPS. One way ANOVA followed by Dunnett's test was used to determine significance. Mean ± S.E. of 4 experiments.

Mac-exosome experiments, RM performed exosome isolation, FB helped with exosome characterization, CB and VS helped in analysis and discussion, AA wrote the manuscript and supervised the experiments. All authors helped in the discussing and editing of manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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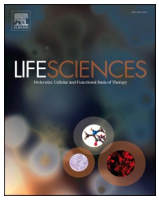

Corrigendum

Corrigendum to "Metabolites and growth factors produced by Airway epithelial cells induce tolerance in macrophages" [Life Sci. 302 (2022) 120659]

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The authors regret the incorrect publication of the name of the co-author 'Veedamali S Subramanian' in the original article. The correct name 'Veedamali S Subramanian' is updated in the authors list.

The authors would like to apologise for any inconvenience caused.

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