Transplacental immune modulation with a bacterial-derived agent protects against allergic airway inflammation

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Chronic allergic inflammatory diseases are a major cause of morbidity, allergic asthma alone affecting over 300 million people worldwide. Epidemiological studies demonstrate that environmental stimuli are associated with either promotion or prevention of disease. Major reductions in asthma prevalence are documented in European and US farming communities. Protection is associated with exposure of mothers during pregnancy to microbial breakdown products present in farm dusts and unprocessed foods, and enhancement of innate immune competence in the children. We sought to develop a scientific rationale for progressing these findings towards clinical application for primary disease prevention. Treatment of pregnant mice with a defined clinically-approved immune-modulator was shown to markedly reduce susceptibility of their offspring to development of the hallmark clinical features of allergic airway inflammatory disease. Mechanistically, offspring displayed enhanced dendritic cell-dependent airway mucosal immune surveillance function, which resulted in more efficient generation of mucosal-homing T-regulatory cells in response to local inflammatory challenge. We provide evidence that the principal target for maternal treatment effects was the fetal dendritic cell progenitor compartment, equipping the offspring for accelerated functional maturation of the airway mucosal dendritic cell network following birth. These data provide proof-of-concept supporting the rationale for development of transplacental immune reprogramming approaches for primary disease prevention.
Transplacental immune modulation with a bacterial-derived agent protects against allergic airway inflammation.

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Abstract: Chronic allergic inflammatory diseases are a major cause of morbidity, allergic asthma alone affecting over 300 million people worldwide. Epidemiological studies demonstrate that environmental stimuli are associated with either promotion or prevention of disease. Major reductions in asthma prevalence are documented in European and US farming communities. Protection is associated with exposure of mothers during pregnancy to microbial breakdown products present in farm dusts and unprocessed foods, and enhancement of innate immune competence in the children. We sought to develop a scientific rationale for progressing these findings towards clinical application for primary disease prevention. Treatment of pregnant mice with a defined clinically-approved immune-modulator was shown to markedly reduce susceptibility of their offspring to development of the hallmark clinical features of allergic airway inflammatory disease. Mechanistically, offspring displayed enhanced dendritic cell-dependent airway mucosal immune surveillance function, which resulted in more efficient generation of mucosal-homing T-regulatory cells in response to local inflammatory challenge. We provide evidence that the principal target for maternal treatment effects was the fetal dendritic cell progenitor compartment, equipping the offspring for accelerated functional maturation of the airway mucosal dendritic cell network following birth. These data provide proof-of-concept supporting the rationale for development of transplacental immune reprogramming approaches for primary disease prevention.

Introduction

A series of prospective birth cohort studies on the children of European traditional farming families (1,2), now replicated in US studies contrasting Amish and Hutterite farming populations (3), have identified striking asthma-protective properties of oral and inhalation exposure to benign microbial stimuli present in dusts from farm barns. The target for these
exposures in the offspring appears to be the innate immune system, and involves modulation of both immunoregulatory and effector cell function(s) (3-6) resulting in markedly reduced susceptibility to the asthma-promoting effects of common respiratory allergies. The temporal window during which these environmental stimuli exert their immunomodulatory effects spans the period when the developing immune system is undergoing postnatal functional maturation, but susceptibility to these effects also appears particularly high during prenatal development as demonstrated by the strong impact of maternal microbial exposures during pregnancy on ensuing asthma resistance in their offspring (1,7).

A broad forerunner literature supports the general principle that maternal microbial exposures can result in transmission of transplacental signals that influence the functional phenotype of the developing fetal immune system (8-10), but these studies have focused almost exclusively on maternal infections, and usually on deleterious effects thereof. In contrast, in light of the findings from the farming family studies above (1,7), we posit that benign environmental microbial exposures during pregnancy can be read out by the maternal mucosal immune surveillance system and transcribed into positive “immune training” signals for transplacental transmission to their developing offspring, equipping them for more rapid adaptation after birth to the microbial-rich postnatal environment. Moreover, we posit that this natural mechanism can be harnessed therapeutically; notably, if these benign environmental exposure effects could be reproduced by an agent that could be safely administered during pregnancy, then this could open up novel possibilities for primary prevention of asthma. With this in mind we have recently completed a proof-of-concept study in pregnant mice with a microbial-derived therapeutic product OM-85, which has been in widespread use in Europe in human infants and adults for >30 years for boosting resistance to airways inflammation and attendant wheezing symptoms associated with lower respiratory infections (11-15).
initial investigations to establish the safety of OM-85 use during pregnancy we demonstrated that maternal treatment with this agent enhanced homeostatic control of innate immune and inflammatory functions in gestational tissues at baseline and in the face of challenge with microbial pathogens including live influenza infection and the bacterial mimic lipopolysaccharide (LPS). Specifically, OM-85 treatment attenuated inflammatory symptoms (which are typically exaggerated during pregnancy) and protected against fetal growth restriction and/or pregnancy termination which can follow maternal infection (16). In the study presented here we focus on the effects of maternal OM-85 treatment during healthy pregnancy on the immunocompetence of offspring during the postnatal weanling period when immune functions are typically developmentally compromised. In this regard, we focus on the effects of maternal OM-85 treatment on the capacity of offspring to regulate airways inflammatory responses associated with development of experimental atopic asthma during the weanling period, which in humans represents the age range at highest risk for initiation of what can be life-long asthma (17).

Results

Experimental model of allergic airways inflammation in sensitized weanling mice: study rationale

For this study we utilized an experimental system developed for induction of T helper (Th) 2-associated cell-mediated inflammation in the conducting airway mucosa in adult rodents, as a model for the main lesional site in human asthma. Additional (albeit less extensive) inflammation also develops in peripheral lung tissue, but the relative contribution of this to airflow limitation in the asthmatic state is uncertain. The principal features of this model, focusing mainly on the airway mucosa, are illustrated in Supplementary Figure 1A. Aeroallergen delivered to the airways of pre-sensitized animals via large-droplet aerosol is
captured by resident mucosal dendritic cells (DC) that are functionally quiescent in the steady-state (as marked by low-modest IAIE expression) and are specialised for antigen sampling only, which they subsequently transport to airway draining lymph nodes (ADLN) for presentation to allergen-specific T-memory cells (18-20). The resultant T-cell response generates a mixture of T-effector-memory (T\textsuperscript{m}eff) and T-regulatory (Treg) cells in proportions determined via DC programming. Representatives of these populations traffic back to the airway mucosa, where they encounter resident mucosal DC which have recently acquired aeroallergen, and bidirectional interactions between these three cell populations in situ determine the intensity and duration of the ensuing T-cell dependent inflammatory response within the airway mucosa (21,22). In particular, the capacity for local activation of incoming T\textsuperscript{m}effs is limited via the suppressive effects of Tregs on surface IAIE and CD86 expression by mucosal conventional DC (cDC) (18,22-24).

The principal DC population involved comprises the network of cDC within the airway mucosa, that are responsible for the major aspects of local immune surveillance (18). Plasmacytoid DC (pDC) have also been implicated in this process (25), particularly in relation to pathogen surveillance (26). The airway mucosal cDC population has the property of uniquely rapid turnover in the steady-state with 85% of the resident population turning over every ~24 hours, being continuously depleted by migration of antigen-bearing cells to ADLN and simultaneously replenished via incoming precursors recruited from bone marrow (27). This orderly and highly dynamic process is rapidly accelerated during airway challenge events, during which cDC numbers can expand markedly within the airway epithelium and ADLN (18,28-30). This airway mucosal cDC network is developmentally compromised in immature humans (31,32) and experimental animals (33,34), and this partially explains the high risk of respiratory infections and aeroallergen sensitization associated with the infant.
period (35). Our hypothesis underlying this murine study is that maternal OM-85-treatment during pregnancy can enhance the functional maturation of this mucosal immune surveillance system in their offspring, and as a result reduce susceptibility to initiation of inflammatory airway disease during the high-risk early postnatal period.

To test this hypothesis, we have utilised a variant of the above mentioned allergic airways inflammation model, modified from earlier studies assessing farm-related exposures (36), involving sensitization of 21 day old weanling BALB/c mice to ovalbumin (OVA) employing a prime-boost schedule followed by subsequent airways challenge with aerosolised OVA (Supplementary Figure 1B, C). Details of the ensuing response are discussed below.

**Aeroallergen-induced cellular response in the airways: baseline characteristics**

Sensitized animals display high levels of OVA-specific serum IgE 24 hours following repeated OVA-aerosol challenge (Figure 1A). The challenged animals display gross hypertrophy of ADLN involving in particular T-cells (see below), which is accompanied by intense inflammatory cell infiltration into the airways encompassing eosinophils, neutrophils and lymphocytes detectable by bronchoalveolar lavage (BAL; Figure 1B), increased levels of Th2 cytokines in lung homogenates (not shown), and airways hyperresponsiveness (AHR) manifesting as increased airways resistance ($R_{aw}$) to methacholine (MCh; Figure 1C).

Further characterization of the phenotype of the cellular response within the airways compartment by multi-colour flow cytometry (see Methods for gating strategies) revealed significant increases in the total cellularity of parathymic and mediastinal ADLN and trachea, with no observable difference in peripheral lung (Supplementary Figure 1D). This cellular response was dominated by changes in the CD3$^+$ T-cells (Supplementary Figure 1E) and
especially the CD4+ T-cell compartment (Supplementary Figure 1F). These changes in particular involved increases in the numbers (Supplementary Figure 1G), proportions (Figure 1D) and activation status (Figure 1E, F) of CD3+CD4+CD25+FoxP3+ Tm eff cells within the ADLN and in tracheal tissue, with much smaller parallel changes in peripheral lung parenchyma (Figure 1D) which is consistent with deposition of the bulk of aerosol droplets in the large and central airways. ADLN Tm effs displayed a heightened state of activation (Figure 1E), whereas lung and especially tracheal Tm effs demonstrated high levels of Ki67 expression suggesting very recent (possibly local) proliferation (Figure 1F; see corresponding cDC data below). In conjunction with the CD25+FoxP3+ Tm eff response, a decrease in CD3+CD4+CD25+FoxP3+ Tregs within the T-cell compartment of ADLN was observed, accompanied by a large increase within trachea and a smaller (but significant) increase in peripheral lung tissues (Figure 1G), presumably derived by migration of these cells from ADLN. Characterisation of Treg function-associated markers revealed increased CTLA-4+ Tregs within trachea (74.47 ± 3.78; 46.41 ± 6.05) and peripheral lung (51.16 ± 2.50; 34.9 ± 2.54) compared to naïve controls (Supplementary Figure 1H). Furthermore, an increase in CD69+ Tregs was identified within peripheral lung samples (15.68 ± 0.53; 12.98 ± 1.54), while CD69+ Tregs were reduced within the ADLN (28.19 ± 0.69; 32.5 ± 1.19) compared to naïve controls (Supplementary Figure 1I). Airways Tregs additionally displayed enhanced CD25 expression (Figure 1H) and proliferative capacity (Figure 1I) following OVA challenge compared to naïve controls.

Forerunner studies from our lab (18,22,37) suggest that in the early stage of recall responses to inhaled antigen, the limiting factor determining the efficiency of generation of airway mucosal homing Tregs is the efficiency of DC-mediated transport of antigen-specific signals from the airway to ADLN. We therefore turned our attention to characterising the myeloid
cell populations localised within the airways of early life OVA sensitised and aerosol challenged animals. For these analyses, IAIE$^+$F4/80$^-$CD11c$^+$ conventional DCs (cDC) were subdivided into CD11b$^+$CD103$^-$ and CD11b$^+$CD103$^+$ populations, representing the two dominant cDC subsets localised within the airways, having specialised roles in immunogenic and tolerogenic responses respectively (38). We additionally quantified IAIE$^+$Ly6G/C$^{lo/+}$/F4/80$^-$CD11c$^+$B220$^-$CD11b$^+$ pDC which represented a much smaller proportion of the CD45$^+$ population compared to cDC (Figure 1J, K). Consecutive aerosol challenges of pre-sensitised mice induces a minor response in pDC in peripheral lung only (Figure 1K), in contrast to a significant influx of both cDC subsets across all airways tissues sampled, in particular the tracheal mucosa and its ADLN where numbers of these cells displayed a logfold increase (Figure 1L, M). Characterisation of cDC based on expression levels of surface IAIE demonstrated marked enhancement in expression intensity across the entire cDC population within the trachea following challenge (Figure 1N, O). This observation is consistent with allergen-driven functional maturation in situ of these cells from strict antigen-sampling to antigen-presentation phenotype as previously observed (18,19), and this provides a plausible mechanism for local activation of T$^{mefs}$ within the mucosa during repeated challenge (Figure 1D, F). In parallel, ADLN displayed modest IAIE upregulation in the CD103$^+$ subset, while both subsets remained at baseline in the peripheral lung (Figure 1N, O). Furthermore, upregulation of pDC IAIE expression occurred both in ADLN and peripheral lung (Figure 1P). Additionally, we observed increased numbers post challenge of rare IAIE$^+$F4/80$^{int}$/Ly6G/C$^{hi}$CD11b$^-$CD11c$^+$ inflammatory DCs (infDC) which have been implicated in driving Th2-mediated inflammation to antigen exposure (39,40), within ADLN and tracheal tissue (Figure 1Q).
Role of the bone marrow in the development of an experimental allergic airways inflammatory response

The granulocytic and DC subsets identified above as participants in the airways response to aerosol challenge in sensitized mice are derived from bone marrow, and we posited that (as inferred from earlier studies on eosinophils in aeroallergen challenged human asthmatics (41)) the dynamic changes detailed in these populations in our murine model of allergic airways inflammation above should be mirrored by changes in respective bone marrow precursor populations.

The scheme in Supplementary Figure 2 summarizes current understanding of the interrelationships between relevant bone marrow progenitor compartments in the development pathway leading to production of granulocytes, pDC and cDC. We assessed the impact of repeated challenge with aeroallergen on the size of relevant compartments at or beyond the myeloid precursor (MP) stage, employing multicolour flow cytometry, targeting the markers shown. These analyses (Figure 2A-D) demonstrate firstly that early myeloid precursor compartments up to and including the granulocyte-macrophage progenitor (GMP) population, which are a major source of both DC and granulocyte populations (42,43), and the macrophage-dendritic cell progenitor (MDP) compartment which is committed to pDC and cDC production (44-46), expand significantly in response to repeated aeroallergen challenge of sensitized animals. This finding is consistent with the data shown in Figure 1, demonstrating the buildup of these populations in the challenged airways. Beyond this stage the cDC compartment appeared reduced relative to baseline (Figure 2F), and this may be expected in light of the accumulation of these cells in tracheal mucosa and at their ultimate destination in ALDN, which displays a logfold increase in cDC numbers post-challenge (Figure 1L, M).
Furthermore, cDCs remaining within the bone marrow post challenge displayed reduced IAIE surface expression relative to baseline controls (Figure 2G), which may indicate preferential recruitment of cDC from the more functionally mature end of the developmental spectrum.

**Maternal OM-85 treatment during pregnancy: effects on experimental allergic airways inflammation susceptibility in sensitized offspring**

We posited that treatment of pregnant mice with the microbial-derived immunomodulatory agent OM-85 would enhance the resistance of their offspring to development of allergic airways inflammation during the early post-weaning period. To test this hypothesis, we utilised an OM-85 treatment protocol we have recently demonstrated to protect pregnant mice and their fetuses against the toxic effects of bacterial and viral infections (16), comprising oral administration of OM-85 from gestation day 9.5-17.5, followed by natural delivery of offspring 2-3 days later. Age-matched offspring from OM-85 treated and untreated control mothers were sensitized at weaning (21 days of age) and aerosol challenged as per Supplementary Figure 1B and C, and their airways responses compared (Figure 3). As previously demonstrated, early life OVA sensitisation and ensuing aerosol challenge initiates granulocytic and lymphocytic infiltration of the airways, and these cellular responses (Figure 3A), together with accompanying development of AHR to MCh (Figure 3B), were markedly attenuated in the offspring of mice treated during pregnancy. Of note, treatment did not affect OVA-specific IgE levels (log2 titres 5.44 ± 0.21 and 5.54 ± 0.34 in treated versus untreated groups respectively), implying that OM-85 treatment influences mechanism(s) downstream of sensitization *per se*. 
**Treg function in offspring as a potential target for maternal OM-85 treatment effects**

Previous studies from our lab and others have identified mucosal homing Tregs as a potential target for OM-85-mediated treatment effects in adult non-pregnant (47,48) and pregnant (16) animals, prompting an initial focus on this population. We accordingly phenotyped the T-cell response within the airways compartment using multi-colour flow cytometry. The magnitude of the overall CD4+ T-cell response to OVA aerosol was reduced in the treatment group, particularly in the tracheal mucosa (Figure 4A, B). Following challenge, the proportion of Tregs within the ADLN CD4+ T-cell population declined in both groups (Figure 4C) and correspondingly increased in respective tracheal tissues (Figure 4F), consistent with their migration to the airway mucosal challenge site. However, both the proportional decline in Tregs in ADLN and the corresponding increase in trachea were significantly higher in the OM-85-treatment group (Figure 4F). Treg: TmEff ratios post challenge remained higher in ADLN in offspring from treated mothers (Figure 4E) but did not differ significantly between the groups in trachea (data not shown). Furthermore, the relative expression levels of Treg function-associated molecules CD25 (Figure 4G), CTLA-4 (Figure 4I) and FoxP3 (Figure 4K), along with activation/proliferation-associated markers CD69 (Figure 4J) and Ki67 (Figure 4H), were significantly elevated in tracheal Tregs from the treated group, consistent with activation and enhanced functionality. Similar patterns were also observed for Treg populations from peripheral lung tissues (Supplementary Figure 3).

**Maternal OM-85 pre-treatment modulates the functional phenotype of airway-associated DC populations in offspring**

The accumulation of cDC in airway-associated tissue compartments in response to airways challenge was generally reduced in the treated group, and these differences were statistically significant for CD103+ cDC in the trachea, and for CD11b+ cDC in ADLN and lung (Figure
5A, B). However, the most notable finding related to cDC maturational status as measured by surface IAIE expression, which was reduced at baseline in both CD103+ and CD11b+ subsets in ADLN (Figure 5C). Moreover, the antigen-induced surge in IAIE expression levels on both subsets in the rapidly turning over cDC population in the tracheal mucosa, which is a hallmark of functional activation of these cells, was likewise attenuated (Figure 5D). This contrasted with the picture in the peripheral lung (Figure 5E), which is dominated by cDC populations with much longer half-lives, and which displayed minimal upregulation of IAIE in response to challenge. However as noted above, aerosol challenge does elicit a small but significant increase in pDC in peripheral lung tissue, and this response was attenuated in the treated group (Figure 5F, G). We also screened the groups for treatment effects on the rare inflammatory DC subset in airway tissues, however none were detected (data not shown).

**Offspring bone marrow as the primary target for maternal OM-85 treatment effects**

The final series of experiments tested the hypothesis that maternal OM-85 treatment mediated effects on cellular immune function(s) in offspring respiratory tract tissues in this model may be associated with upstream effects on relevant precursor populations in bone marrow. Figure 6 directly compares the aeroallergen-induced bone marrow responses of offspring from treated versus untreated mothers. Firstly, while baseline output of pre-cDC and cDC was comparable between groups, there were small but significant increases in the resting GMP and MDP populations in the control offspring from treated mothers (Figure 6C, D). However, the major treatment-associated differences were revealed by aeroallergen challenge, notably a consistent attenuation of the expansion in all precursor compartments spanning the MP–MDP stages which was observed in the challenged offspring from untreated mothers (Figure 6A-D). Secondly, at the end of this developmental spectrum, the post challenge depletion of bone marrow cDC reserves that occurs in the offspring of
untreated mothers was significantly attenuated (Figure 6E, F), consistent with the reduced draw on this pool resulting from reduced recruitment to airway mucosa and ADLN in the OM-85-treated group (Figure 5A, B).

It is additionally pertinent to note that expression levels of IAIE on cDC were reduced in the treated group both at baseline and in particular post-challenge (Figure 6G), suggesting maintenance of a more tightly regulated/quiescent functional state, mirroring the picture seen for trachea and ADLN cDC in Figure 5. A similar pattern was also observed in relation to the bone marrow pDC reservoir (data not shown).

**Maternal OM-85 treatment effects at earlier ages**

The data above pertains to animals sensitized at 3 weeks and challenged/sacrificed at 6 weeks. In the studies presented in Figure 7, we assessed the extent to which treatment effects on DC populations were demonstrable at younger ages. Looking firstly at age 3 weeks, we observed that the offspring of OM-85-treated mothers displayed higher numbers CD11b+ and CD103+ cDC in lung tissue at baseline (Figure 7A, B) and higher levels of attendant IAIE expression (Figure 7C), consistent with treatment-mediated acceleration of postnatal maturation of DC networks in the respiratory tract. In a preliminary experiment we also compared total cDC yields in granulocyte-macrophage colony-stimulating factor (GM-CSF)-driven bone marrow cultures derived from the same animals, and the increased yields from the treated group (Figure 7D) again point to the marrow as the likely primary site of action of maternal OM-85 treatment.

To further extend this finding, we characterised the progenitor pool within freshly harvested fetal bone marrow at 18.5 days gestation, 24 hours following the last maternal OM-85 oral
The marked increase in total bone marrow cDC (Figure 7E) accompanied by parallel expansion in the upstream MDP compartment in the treated group (Figure 7F) is consistent with the conclusion that the bone marrow is the ultimate target for OM-85 treatment effects.

**OM-85-mediated attenuation of the responsiveness of bone marrow DC precursors to environmental inflammatory stimuli: validation of OM-85 treatment effects in an independent inflammatory model**

In the experiments illustrated in Figure 8 we cultured bone marrow from 6-week old offspring of OM-85-treated/untreated mothers in GM-CSF-enriched medium for 7 days, adding the archetypal pro-inflammatory agent bacterial LPS to half the cultures for the last 24 hours. Comparison of resultant activation levels of cDC by surface expression of IAIE (Figure 8A) and the costimulator CD86 (Figure 8B) indicated marked attenuation of upregulation of these function-associated markers, consistent with enhanced capacity for homeostatic regulation of inflammatory responses in general in cDC from the treated group.

**Discussion**

In this experimental model, allergen challenge of early-life sensitized animals via aerosol triggers accumulation of a Th2-associated inflammatory cell infiltrate in respiratory tract tissues, and ensuing AHR, mimicking some of the hallmark features of atopic asthma. Consistent with earlier reports (18,19), prominent within these infiltrates were activated CD4+ Tmefs and Treg cell populations, with the accompanying buildup of an expanded population of cDC and their transition (especially in the mucosa) from the passive/antigen surveillance phenotype (IAIElow) to a functionally mature (IAIEhigh) state. We also demonstrate for the first time that these rapid changes in the population dynamics of cDC in the challenged airways of sensitized animals are accompanied by concomitant depletion of
committed cDC from bone marrow, and parallel (presumably compensatory) expansion of upstream multipotent precursor compartments.

We further demonstrate that susceptibility to this aeroallergen-induced asthma-like response in the airways is markedly attenuated in young animals born to mothers given repeated doses of OM-85 during pregnancy. These findings mirror those previously reported by Conrad et al. (36) in a model of controlled maternal exposure to Acinetobacter lwofii F78, which the authors asserted to changes in Th1/Th2 balance (49). In contrast, in the present study, accompanying this acquired resistant state following OM-85 treatment is increased capacity for expansion and functional activation of Tregs in the airway mucosa in response to aeroallergen challenge, with parallel attenuation of local cDC recruitment, activation and trafficking to ADLN. It is pertinent to note that this OM-85 treatment is not accompanied by overt attenuation of allergen-specific IgE production, similar to the situation described in humans undergoing successful allergen immunotherapy in which treated patients frequently display reduced inflammatory symptoms without major reduction in IgE production, and this has been ascribed inter alia to boosting of Treg functions (50,51).

Moreover, underpinning these OM-85 treatment effects in respiratory tract associated tissues are a series of parallel changes in bone marrow DC progenitor populations at various stages of DC commitment, which are collectively consistent with the reduced draw on bone marrow cDC reserves in the treated group as a result of more effective control of the inflammatory milieu within the challenged mucosa. The key question is whether these changes in bone marrow of offspring from treated mothers are primary or secondary in this process.
In this regard, we (48) and Navarro et al. (47) have previously demonstrated that oral OM-85 treatment of rodents can directly promote generation in gut-associated tissues of mucosal-homing DC that can bolster systemic natural Treg populations (including those in the airway mucosa) at baseline. Moreover, Navarro et al. (47) has demonstrated that these effects of OM-85 are Toll-like receptor (TLR)-dependent, and similar findings have been reported in in vitro studies in a murine model (52). In the current model, airway mucosal Treg density and functional phenotype do not differ between OM-85- and non-treated groups at baseline, and the stimulatory effects of treatment are only evident following aeroallergen challenge (Figure 4C-K). We posit therefore that OM-85 treatment has affected the functionality of the airway mucosal cDC that are responsible for programming Treg:Teff balance in the aerosol induced T-cell response, prior to their migration into the airway mucosa i.e. at bone marrow precursor stage. Several lines of indirect evidence support the possibility of OM-85 treatment effects in bone marrow: (i) baseline IAIE expression on 6-week old bone marrow cDC is significantly reduced in offspring from treated mothers (Figure 6G), and corresponding GMP and MDP precursor compartments are expanded in the same animals (Figure 6C, D); (ii) at age 3 weeks when airway mucosal cDC networks are normally developmentally compromised with respect to baseline density, offspring from treated mothers display higher frequency of CD103+ and CD11b+ cDC in lung tissue digests (Figure 7A, B) and higher cDC yields from bone marrow cultures (Figure 7D); (iii) the frequency of cDC and their MDP precursors in fetal bone marrow are already increased in those from treated mothers by late gestation (Figure 7E, F). Moreover, preliminary studies (Supplementary Figure 4.) have demonstrated a direct stimulation effect of OM-85 feeding on the myeloid precursor compartment of adult non-pregnant mice, and our forerunner studies (16) have demonstrated a range of effects on myeloid and regulatory cell populations in maternal gestational tissues. However, the strongest evidence for treatment related effects at the DC precursor stage comes from the
studies on cDC from 6-week old GM-CSF-driven 7-day bone marrow cultures (Figure 8). The introduction of the archetypal pro-inflammatory stimulus bacterial LPS for the final 24 hours of these cultures triggers ultra-high expression on cDC from untreated controls of both IAIE and CD86, at levels likely to result in potentially pathologic T-cell hyperstimulation. However, this upregulation was more tightly controlled in cDC from offspring of treated mothers, suggesting enhanced capacity to maintain homeostasis and avoid bystander damage to host tissues during immunoinflammatory responses to environmental stimuli.

We acknowledge several limitations to this study. Firstly, we have not addressed the question of whether OM-85 treatment influences susceptibility to primary allergic sensitization to inhalant allergens, and this merits future investigation, given that this process has also been shown to be controlled by ADLN-derived Tregs (53). Secondly, we have no information on the mechanism of transmission of orally delivered OM-85-associated signals to the bone marrow. Earlier studies from our group (48) and others (47) have demonstrated local activation of both T-cells and myeloid cells in the gut wall and associated lymphoid tissues following OM-85 feeding, and it is possible that trafficking of representatives of either or both of these populations, or transmission of soluble signals generated at these sites, may be involved. Likewise, information on the gene target(s) in fetal bone marrow DC progenitors is not available, and will be the subject of future studies, along with possible effects on fetal thymus. We also have not formally demonstrated TLR-dependency of OM-85, although (as discussed above) this has been established in related models. Additionally, OM-85 dosing of mothers in this study spanned only the second half of gestation, and future studies need to investigate the impact of extending feeding to earlier stages. Moreover, additional dose-response studies are required to determine the minimal dosage of OM-85 required to mediate these effects. In this regard, recent studies (54) suggest that effective attenuation of allergic
airways inflammation via direct OM-85 treatment may be attainable at a log-fold lower dose than that used in the current study.

Our goal in this study was to provide a scientific rationale for subsequent use of OM-85 during pregnancy in human mothers whose progeny are at risk of postnatal development of persistent atopy and/or asthma. The prime initial candidates for therapy in this regard are atopic asthmatic mothers (55,56). The severity of asthma symptomatology in this group is exaggerated during pregnancy (57-59), likely as a consequence of the generalized Th2-skewing of immune functions associated with the pregnant state, and asthma exacerbations during pregnancy further increase asthma risk for their offspring (60). Moreover, susceptibility to respiratory infections exemplified by influenza virus and its associated symptom severity is likewise increased during pregnancy (61,62), and the latter (along with bacterial infections) is a risk factor for fetal growth restriction (63) which in turn is associated with increased risk for postnatal development of a range of non-communicable diseases including asthma (64,65). In this regard, our forerunner studies on OM-85 use in pregnant mice have demonstrated strong protection against the effects of pathogen-associated challenge during pregnancy employing high dose LPS or live influenza, both with respect to preservation of pregnancy per se, and maintenance of normal maternal weight gain and associated fetal growth trajectories (16). In this system the principal treatment effect of OM-85 involved selective attenuation within gestational tissues of the intensity of the pro-inflammatory (particularly TNFα/IL-1/IL-6) components of the myeloid innate immune response to pathogen, with parallel preservation of vigorous Type 1 interferon (IFN)-associated host defense networks (16). Moreover, we (48) and others (47) have also previously demonstrated marked attenuation of airways inflammation in OM-85-treated adult animals in models of experimental allergic airways inflammatory disease. On this basis, it
can be argued that OM-85 use during pregnancy has potential direct short-term benefits to mothers, as well as long-term benefits to their offspring in regards to reduced susceptibility to asthma development. The latter may also include enhanced resistance to early postnatal respiratory infections, given the OM-85-associated effects demonstrated above on increased airway mucosal cDC numbers and IAIE expression at baseline in 3-week old weanlings, and studies are in progress to test this possibility.

A series of additional steps are required before progression to human trials with OM-85 in pregnancy. The first involves an independent assessment by regulators of relevant safety issues. In this regard there is a wide body of data available on safe use during pregnancy in experimental animals, including our own (16). Direct human safety data for pregnancy is not presently available. However, there is a >30-year history of safe use in non-pregnant adult humans and children down to age 6 months, and this has proven sufficient for relevant US (66) (National Institute of Health) and Australian (67) (National Health & Medical Research Council) authorities to endorse and publicly fund multicenter clinical trials in infants, targeting protection against wheezing symptoms (including those associated with early infections) and subsequent asthma development. In this regard, it is evident that airways inflammation associated with infections and inhalant allergy act in synergy to drive asthma pathogenesis during childhood (35), and moreover that the earlier these episodic inflammatory events commence after birth, the greater is the risk for subsequent asthma (68-70). This provides a compelling argument for development of protective therapeutic strategies that can reduce susceptibility to either or both of these environmental stressors, from birth onwards, and the possibility that this may be achievable prenatally with a readily available therapeutic such as OM-85 merits further detailed investigation.
Methods

Animals

Specified pathogen-free BALB/c mice and Sprague Dawley (SD) rats were obtained from the Animal Resource Centre (Murdoch, WA, Australia). All animals were housed under specified pathogen-free conditions at the Telethon Kids Institute Bioresources Facility, with a 12h:12h light-dark cycle and access to an ovalbumin-free diet and water ad libitum. In-house bred BALB/c offspring of both sexes were used in these studies.

Time-mated pregnancies

Female BALB/c mice 8 – 12 weeks of age were time-mated with male studs between 8 – 26 weeks of age. Male studs were housed separately in individual cages. One-to-two females were housed in an individual male cage overnight, with the presence of a vaginal plug the following morning used as an indicator of mating. The day of vaginal plug detection was designated gestation day (GD) 0.5.

OM-85

OM-85 (OM Pharma, Geneva, Switzerland) is an endotoxin-low lyophilised extract containing multiple TLR ligands derived from 8 major bacterial pathogens (Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, and Neisseria catarrhalis) frequently associated with respiratory tract infections (12,71).

Maternal OM-85 treatment protocol

Based on previously optimised dosing concentrations (16,48), time-mated pregnant female BALB/c mice selected at random received daily feeding of lyophilised OM-85 reconstituted
in phosphate-buffered saline (PBS) to a concentration of 400mg.kg$^{-1}$ body weight for the second half of gestation (GD9.5-17.5). Controls were left untreated. All treatments were performed from a single batch of OM-85 (Batch no. 1812162).

**Offspring antigen sensitisation and aerosol challenge**

Offspring from OM-85 treated or naïve mothers were experimentally sensitised to OVA at the age of 21 and 35 days via intraperitoneal (i.p.) inoculation of 20µg OVA (grade V; Sigma-Aldrich, MO, USA) emulsified in 1.3mg aluminium hydroxide (Alu-Gel-S, SERVA, Heidelberg, Germany) in a total volume of 200µl. On days 42, 43 and 44, sensitised offspring were exposed OVA aerosol challenge (1% weight/volume in PBS) for 30 minutes, delivered via ultrasonic nebuliser. Mice for airways hyperresponsiveness assessment received a single OVA aerosol challenge on day 42 for 30 minutes. All experimental mice were sacrificed 24 hours post final aerosol.

**Measurement of airway hyperresponsiveness**

Airway hyperresponsiveness (AHR) to inhaled methacholine (MCh) was assessed 24 hours after a single OVA (1% weight/volume in PBS) aerosol on day 42 following pre-sensitisation. The low-frequency forced oscillation technique (LFOT) was used to measure respiratory system input impedance ($Z_{rs}$), as determined by previously optimised protocols (72). Briefly, BALB/c mice were anesthetised (40% ketamine 100mg.ml$^{-1}$, 10% xylazine 20mg.ml$^{-1}$, 50% saline; 1% body weight), tracheotomised and ventilated (Legacy flexiVent, SCIREQ, Montreal, Canada) at 450 breaths/min with a tidal volume of 8ml.kg$^{-1}$ and 2cmH$_2$O positive end-expiratory pressure (PEEP). Lung volume history was standardised for each individual mouse prior to measurement of experimental lung mechanics. $Z_{rs}$ was measured during 16-second periods of apnea using a signal containing 19 mutually prime sinusoidal
frequencies ranging from 0.25 to 19.625 Hz. The constant-phase model was fit to $Z_{rs}$ in order to calculate changes in airways resistance ($R_{aw}$). Ventilated BALB/c mice had 5x baseline measurements recorded, with 10-second aerosol challenge of saline followed by semi-log-fold increasing dose concentrations of MCh ranging from $0.1 – 30\text{mg.ml}^{-1}$ to assess for AHR. Five LFOT measurements were recorded after each MCh dose at 1 minute intervals. Dose-response curves were generated using the maximum response recorded for $R_{aw}$.

**Tissue collection**

*Fetal.* Pregnant BALB/c mice were sacrificed on GD18.5. Both horns of the uterus were removed and fetuses were sacrificed via decapitation. Hind legs were removed, cleaned of remaining tissue and stored in cold PBS + 0.1% BSA. Dead fetuses were excluded. *3-week old offspring.* Offspring were sacrificed as 21 days of age. Lungs were perfused via cardiac flush with 2ml cold PBS + 0.1% BSA. Peripheral lung and the femur and tibia from both hind legs were collected. *6-week old offspring.* Offspring were sacrificed at 45 days of age. Lungs were perfused via cardia flush with 2ml cold PBS + 0.1% BSA. Parathymic and mediastinal (airways draining) lymph nodes (ADLN), trachea, peripheral lung and the femur and tibia from both hind legs were collected. Blood was collected via cardiac puncture at time of autopsy.

**Passive cutaneous anaphylaxis IgE assay**

In vivo passive cutaneous anaphylaxis assays were performed using male Sprague Dawley rats >10 weeks of age. Individual BALB/c serum samples were prepared as serial 1:2 dilutions with a final sample volume of 55µl. SD rats were anaesthetised via i.p. injection of 4ml 5.71% chloral hydrate (Sigma-Aldrich, MO, USA) in PBS. Once anesthetised, rats had their back closely shaved to remove all hair and 50µl of each sample was subcutaneously
injected down the back. 24 hours later, rats were anaesthetised with chloral hydrate and intravenously (i.v.) injected with 2ml of a 1:1 antigen-dye solution containing 4mg.ml\(^{-1}\) OVA in PBS and 1% Evans blue dye (Sigma-Aldrich, MO, USA). Blue subcutaneous injection sites after 15-30 minutes indicate serum samples positive for OVA-specific IgE. The highest positive serum dilution for each sample was recorded and animals were euthanised with 600µl Lethabarb i.v.

**Single cell suspension preparation**

**Airways tissue and fetal bone marrow.** ADLN, trachea, peripheral lung and fetal bone marrow single cell suspensions were prepared by mincing excised tissue/bone into smaller pieces and resuspending in 10ml GKN (11mM D-glucose, 5.5mM KCl, 137 mM NaCl, 25mM Na\(_2\)HPO\(_4\)) + 10% fetal calf serum (FCS; Serana, Bunbury, WA, Australia) with collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase (Sigma-Aldrich, MO, USA) for enzymatic digestion at 37°C under gentle agitation for 30 minutes (ADLN and trachea), 60 minutes (fetal bone marrow) or 90 minutes (peripheral lung). Following digestion, tissues were disaggregated via manual pipetting and filtered through sterile cotton wool columns. Cell suspensions were centrifuged and pellet resuspended in red blood cell (RBC; 17mM Tris-HCl, 0.14M NH\(_4\)Cl at pH 7.2) lysis buffer for 3 minutes. Cell were washed with cold PBS and pelleted. Supernatant was removed and pellet resuspended in PBS + 0.1% BSA for total cell counts. **3- and 6-week old bone marrow.** Long bones were flushed with 10ml GKN + 5% FCS using a 25g needle. Cells were disaggregated by manual pipetting and filtered through a sterile cotton wool column. Filtered cells were washed with GKN + 5% FCS and centrifuged at 1800rpm for 8 minutes at 4°C. Supernatant was removed and pellet resuspended in RBC lysis buffer for 5 minutes. Cells
were washed in cold PBS, centrifuged and pellet resuspended in PBS + 0.1% BSA for total cell counts.

**Bronchoalveolar lavage and differential cell counts**

Bronchoalveolar lavage (BAL) fluid was collected via tracheal cannula flushing the lungs three times with 800µl cold PBS + 0.1% BSA. BAL cells were resuspended in 300µl RBC lysis buffer for 4 minutes. Cells were washed with cold PBS, spun and pellets resuspended in 100µl cold PBS + 0.1% BSA for counting. BAL samples were counted with Trypan Blue (LabChem; Thermo Fisher Scientific, MA, USA) using a haemocytometer and counting to a minimum of 100 leukocytes. 1x10^5 cells for each individual sample were spun onto Superfrost® Plus microscope slides (LabServ; Thermo Fisher Scientific, MA, USA). Cytospin cell preparations were stained using Diff-Quik (Rapid Stain Kit; Perth Scientific, WA, Australia) and differential cell counts performed by counting ≥300 cells per cytospin.

**Bone marrow cultures**

**3-week offspring.** Single-cell bone marrow suspensions (previously described) were washed with 20ml cold PBS + 0.1% BSA and centrifuged at 1800rpm for 8 minutes at 4°C. Cells were resuspended in RPMI-10 Complete media (RPMI 1640, 10% FCS, 2mM L-glutamine, 50µM 2-β-mercaptoethanol (Sigma-Aldrich, MO, USA), 5µg.ml^{-1} gentamycin (Pfizer, NY, USA) and 10ng.ml^{-1} GM-CSF) at a concentration of 8x10^5 cells.ml^{-1}. 1ml aliquots were seeded onto 24-well treated cell culture plates and incubated at 37°C and 5% CO₂ in a water jacketed incubator. At 48 hours, culture media was aspirated and wells were washed with 1ml RPMI supplemented with 2mM L-glutamine, 50µM 2-β-mercaptoethanol and 5µg.ml^{-1} gentamycin. Wash was aspirated and 1ml of fresh RPMI-10 Complete media was added to wells. After 6 days, cells were harvested and wells washed twice with 500µl RPMI-10. Cells
were centrifuged and pellets resuspended in 500\(\mu\)l RPMI-10 to perform total cell counts. Following counts, cells were resuspended at a density of \(1\times10^6\) cells.ml\(^{-1}\) in RPMI-10 Complete media and 1ml aliquots were re-seeded on a 24-well treated cell culture plate. After 24 hours, cells were harvested for flow cytometric phenotypic analysis. **6-week offspring.**

Culture days 1-5 were as described for 3-week offspring. On day 6, cells were harvested into 15ml conical tubes and wells washed twice with 500\(\mu\)l RPMI-10. Cells were centrifuged and pellets resuspended in 500\(\mu\)l RPMI-10 to perform total cell counts. Following counts, cells were resuspended at a density of \(1\times10^6\) cells.ml\(^{-1}\) in RPMI-10 Complete media. 1ml aliquots were re-seeded on a 24-well treated cell culture plate and 1ng.ml\(^{-1}\) LPS was added to each well. Cells were cultured in the presence of LPS for 24 hours. After 24 hours, cells were harvested for flow cytometric phenotypic analysis.

**Flow cytometry**

Single-cell suspensions (as per above) were used for all immunostaining. Panels of monoclonal antibodies were developed to enable phenotypic characterisation of airways T cell, myeloid cell, bone marrow hematopoietic stem and progenitor cell and bone marrow committed myeloid cell populations, as summarized in Supplementary Tables 1-4. Intracellular staining for FoxP3, CTLA-4 and Ki67 was performed using a FoxP3 intracellular staining buffer set (eBiosciences, San Diego, CA, USA). Acquisition was performed on a four-laser LSR Fortessa\textsuperscript{TM} (BD Bioscience, San Jose, CA, USA). All samples were kept as individuals and not pooled. Immune cell phenotyping was analysed using FlowJo\textsuperscript{®} software (Version 10.1, Tree Star, Sanford, CA, USA) and associated gating strategies are outlined in Supplementary Figures 5-7. Fluorescent minus one (FMO) staining controls were used for all panels.
**visNE analysis**

ADLN, trachea and peripheral lung FCS files, with software compensation applied, were uploaded to the Cytobank platform (Cytobank Mountain View, CA, USA) and analysed using established methods (73,74). The software transformed the data to arcsinh scales. Antibodies listed as per above were used for T cell subset identification to create visNE maps using a total of 10,000 (ADLN and peripheral lung) or 3,000 (trachea) cells per sample.

**Statistics**

Statistical analysis and graphing was performed using GraphPad Prism (GraphPad software; version 7.0a). Statistical significance of p<0.05 was considered significant. Unpaired, two-tailed Student’s t-test or Mann Whitney test were used based on distribution of the data as determined by D’Agostino-Pearson omnibus normality test, unless otherwise stated. The data was not corrected for multiple testing because the analyses were not *ad hoc* but at each stage address a series of specific hypotheses, each of which is based on *a priori* knowledge of underlying mechanisms.

**Study approval**

All animal experiments were approved and performed in accordance with the Telethon Kids Institute Animal Ethics Committee and the National Health & Medical Research Council of Australia guidelines for use of animals for scientific research.

**Author contributions:** K.T.M., P.G.H. and D.H.S. designed the study. P.G.H. and D.H.S. supervised the study. K.T.M., N.M.S., J.-F.L.-J., J.L. and A.N.L. performed the experiments. K.T.M., N.M.S., J.-F.L.-J., J.L., A.N.L. and D.H.S. analyzed the data. P.A.S. contributed to the project design, methodology and discussions on data interpretation. S.A.R. contributed to
the project design and methodology. C.P. contributed to initial project design. K.T.M., P.G.H. and D.H.S. wrote the manuscript. All authors reviewed the final manuscript.

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References


Figure 1. Response in early life sensitised mice to aeroallergen challenge. (A) Serum titres of OVA-specific IgE as measured by in vivo passive cutaneous anaphylaxis assay. (B) Absolute numbers of macrophages, eosinophils, neutrophils and lymphocytes as determined by bronchoalveolar lavage (BAL) 24 hours post challenge. (C) Airways hyperresponsiveness to methacholine (MCh) challenge at a dose of 30mg.ml⁻¹ MCh. (D-F) Analysis of CD3⁺CD4⁺CD25⁺FoxP3⁺ T-effector cells (Teff) within airway draining lymph nodes (ADLN), trachea and peripheral lung showing (D) Teff as a proportion of total CD4⁺ T-cells, (E) mean fluorescence intensity (MFI) of CD25 on Teff and (F) proportion of Teff cells Ki67⁺. (G-I) Analysis of CD3⁺CD4⁺CD25⁺FoxP3⁺ T-regulatory cells (Treg) within ADLN, trachea and peripheral lung showing (G) Treg as a proportion of total CD4⁺ T-cells, (H) MFI of CD25 on Treg and (I) proportion of Treg Ki67⁺. (J) IAIE⁺F4/80⁺CD11c⁺ conventional DCs (cDCs) and (K) IAIE⁺Ly6G/Clo⁻F4/80⁺CD11c⁺CD11b⁻B220⁺ plasmacytoid DCs (pDCs) as a proportion of total CD45⁺ leukocytes in ADLN, trachea and peripheral lung samples. (L-M) Absolute numbers of (L) CD11b⁺ and (M) CD103⁺ cDCs within ADLN, trachea and peripheral lung samples. (N-P) MFI of IAIE expression on (N) CD11b⁺ cDCs, (O) CD103⁺ cDCs and (P) pDCs within ADLN, trachea and peripheral lung samples. (Q) Proportion of inflammatory DCs (infDC) within ADLN, trachea and peripheral lung samples. Data are presented from individual animals comparing naïve controls (white) versus OVA sensitised and aerosol challenged offspring (with sample collection 24 hours post challenge; red) and displayed as box and whisker plot showing median, Q₁ and Q₃ and min to max values of n≥6 independent experiments. Total peripheral lung cells displayed as cells per milligram of tissue (#; L, M) Mϕ = macrophage, Eos = eosinophil, Neut = neutrophil, Lymph = lymphocyte. Statistical significance was determined using Student’s t-test or Mann Whitney test (A-B, E-Q) or two-way ANOVA followed by Sidak’s multiple comparison test (C) and presented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 2. Bone marrow cellular response following aeroallergen challenge in mice sensitised at weaning. (A) Lin-IL7-Rαc-Kit+Sca-1+ myeloid progenitors (MP), (B) Lin-IL7-Rαc-Kit+Sca-1-CD16/32lo/CD34lo common myeloid progenitors (CMP), (C) Lin-IL7-Rαc-Kit+Sca-1-CD16/32hiCD34+ granulocyte-macrophage progenitors (GMP), (D) Lin-IL7-Rαc-Kit+Sca-1-CD16/32hiCD34+ CX3CR1+Flt-3+ macrophage-dendritic cell progenitors (MDP), (E) CD11c+CD11b+IAIE+ pre-cDCs and (F) CD11c+CD11b+IAIE+ cDCs in bone marrow as a proportion of total cells. (G) MFI of IAIE on bone marrow cDCs. Data are presented from individual animals comparing naïve controls (white) versus OVA sensitised and aerosol challenged offspring (with sample collection 24 hours post challenge; red). Data displayed as box and whisker plot showing median, Q1 and Q3 and min to max values of n≥8 independent experiments. Statistical significance was determined using Student’s t-test or Mann Whitney test and presented as **p<0.01, ***p<0.001, ****p<0.0001.
Figure 3. Maternal OM-85 treatment during pregnancy confers resistance to airways inflammation in sensitized and challenged offspring. (A) Absolute numbers of macrophages, eosinophils, neutrophils and lymphocytes within BAL 24 hours post challenge. (B) AHR to 30mg.ml\(^{-1}\) MCh challenge. Data are presented from individual animals comparing naïve controls versus OVA sensitised and aerosol challenged offspring from OM-85 treated/untreated mothers and displayed as box and whisker plot showing median, Q\(_1\) and Q\(_3\) and min to max values of n\(\geq\)6 independent experiments. Statistical significance was determined using Student’s t-test (A) or two-way ANOVA followed by Sidak’s multiple comparison test (B) and presented as *p<0.05, ***p<0.001, ****p<0.0001.
Figure 4. Maternal OM-85 treatment during pregnancy promotes Treg suppressive phenotypes in sensitized and challenged offspring. (A-B) Absolute numbers of CD3+CD4+CD8− T-cells in (A) ADLN and (B) trachea. (C-E) Analysis of Tregs within ADLN showing (C) Tregs as a proportion of total CD4+ T-cells, (D) MFI of CD25 on Tregs and (E) Treg:Teff ratio within total CD4+ T-cells. (F-J) Analysis of Tregs in the trachea showing (F) Tregs as a proportion of total CD4+ T-cells, (G) MFI of CD25 on Tregs, (H) proportion of Treg Ki67+, (I) proportion of Treg CTLA-4+ and (J) proportion of Treg CD69+.
(K) MFI of FoxP3 on tracheal Tregs. Data are presented from individual animals comparing naïve controls versus OVA sensitised and aerosol challenged offspring from OM-85 treated/untreated mothers and displayed as box and whisker plot showing median, Q₁ and Q₃ and min to max values of n≥9 independent experiments. Statistical significance was determined using Student’s t-test or Mann Whitney test and presented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 5. Treatment of mothers with OM-85 during pregnancy limits inflammatory airways mucosal DC responses in sensitized and aeroallergen challenged offspring. Shown are absolute numbers of (A) CD11b⁺ and (B) CD103⁺ cDCs within ADLN, trachea and peripheral lung. (C-E) MFI of IAIE expression on CD11b⁺ and CD103⁺ cDCs within (C) ADLN, (D) trachea and (E) peripheral lung. (F) peripheral lung pDCs as a proportion of total CD45⁺ cells. (G) MFI of IAIE on peripheral lung pDCs. Data are presented from individual
animals comparing naïve controls versus OVA sensitised and aerosol challenged offspring from OM-85 treated/untreated mothers and displayed as box and whisker plot showing median, Q₁ and Q₃ and min to max values of n≥5 independent experiments. Total peripheral lung cells displayed as cells per milligram of tissue (A, B). Statistical significance was determined using Student’s t-test or Mann Whitney test and presented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 6. Treatment of mothers with OM-85 during pregnancy alters ensuing responses in bone marrow cellular subpopulations. Shown are (A) MPs, (B) CMPs, (C) GMPs, (D) MDPs, (E) pre-cDCs and (F) cDCs as a proportion of total cells. (G) MFI of IAIE expression on bone marrow cDCs. Data are presented from individual animals comparing naïve controls versus OVA sensitised and aerosol challenged offspring from OM-85 treated/untreated mothers and displayed as box and whisker plot showing median, Q₁ and Q₃ and min to max values of n=14 independent experiments. Statistical significance was determined using Student’s t-test or Mann Whitney test and presented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 7. Maternal OM-85 treatment during pregnancy supports early life development of the myeloid compartment. (A-D) Data represent individual 3-week old naïve offspring from mothers treated or not with OM85 during pregnancy. Shown are (A) CD103⁺ and (B) CD11b⁺ cDCs as a proportion of total peripheral lung cells (C) MFI of IAIE expression on peripheral lung CD11b⁺ cDCs (D) Frequency of cDCs generated following in vitro 7-day GM-CSF-driven bone marrow cultures. (E-F) Data represent individual fetal bone marrow collected at gestation day 18.5 from mothers treated or not with OM85 during pregnancy. Shown are absolute numbers of fetal bone marrow (E) cDCs and (F) MDPs. Data are displayed as box and whisker plot showing median, Q₁ and Q₃ and min to max values of n≥5 independent experiments. Statistical significance was determined using Student’s t-test or Mann Whitney test and presented as *p<0.05, **p<0.01, ***p<0.001.
Figure 8. OM-85 treatment effects on bone marrow DCs in an independent inflammatory model. Shown is the MFI of (A) IAIE and (B) CD86 on bone marrow cDCs cultured for 7 days in the presence of GM-CSF ± lipopolysaccharide for the last 24 hours. Data are presented comparing offspring from OM-85 treated/untreated mothers and displayed as box and whisker plot showing median, Q1 and Q3 and min to max values of n=14 independent experiments. Statistical significance was determined using Student’s t-test or Mann Whitney test for intergroup comparisons and Paired Student’s t-test or Wilcoxon signed-rank test for intragroup comparisons and presented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.