Transplacentally mediated immune training to accelerate fetal immune competence

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Abstract

Non-pathogenic environmental microbial exposures during pregnancy can be transplacentally transcribed into beneficial immune training signals for the developing fetus. These signals equip offspring for more rapid adaptation to the microbe-rich postnatal environment by optimising immunoregulatory innate cell function. We have previously identified that maternal treatment with a microbial-derived therapeutic (OM-85) can protect offspring against allergic airways inflammation. Here, we show that oral treatment of pregnant mice with OM-85 induces transplacental signals that manifest in fetal bone marrow as an enriched population of conventional dendric cells (cDC) displaying enhanced functional maturation. Moreover, the myeloid progenitor populations directly upstream of this cDC pool were significantly boosted in response to maternal treatment. Transcriptomic analysis of fetal bone marrow identified maternal OM-85-induced activation of X-box binding protein 1 (XBP1), with upregulation of active XBP1 restricted to cDC precursors. These data provide direct evidence that transplacental immune training with a microbial-derived therapeutic can accelerate functional immune competence of the fetal bone marrow myeloid compartment.

Introduction

The neonatal period represents a time of immunological plasticity during which the functionally immature immune system is highly susceptible to environmental manipulation (1,2). Moreover, the seeds for development of predisposition to a range of non-communicable diseases are frequently sewn during this early window period (3), suggesting that developmental-associated deficiencies in immune function(s) may be risk factors for these diseases. There is also compelling evidence that certain environmental exposures can potentially play protective roles in the pathogenesis of these diseases. In particular,

epidemiological data (4,5) suggests that benign environmental microbial exposures of mothers during pregnancy can transplacentally influence prenatal immune education processes within the fetus, leading to the optimisation of functional immune maturation within the neonate and subsequent protection against inception of chronic inflammatory diseases such as asthma and allergies. This capacity for microbial exposures to modulate immune development is consistent with the newly emerging paradigm of "immune training", whereby exposure to certain classes of microbial stimuli can alter the functional state of innate immune cells, likely occurring at the progenitor level in the bone marrow (6), leading to optimised immune responsiveness to other unrelated microorganisms (7). With this in mind, there is now a growing acceptance of the concept that immune training can be therapeutically harnessed, particularly during prenatal development, to enhance functional immunocompetence within the offspring.

In this regard, we recently reported in *The Journal of Clinical Investigation* that oral treatment of pregnant mice with the microbial-derived immunomodulatory agent OM-85 reduces susceptibility of their offspring to the development of Th2-driven allergic airways inflammation, and identified the offspring bone marrow as the primary target for maternal treatment effects (8). In the study presented here, we sought to identify the transplacental immune training mechanisms underpinning this protection, focusing on the fetal bone marrow as a potential mechanistic target.

Results and Discussion

To elucidate the mechanisms-of-action of maternal OM-85 treatment, we examined the *in utero* fetal response at gestation day (GD) 18.5 (Figure 1A), 2 days prior to expected natural term delivery.

Phenotypic analysis of the fetal bone marrow myeloid compartment using multicolour flow cytometry revealed significant expansion of the total dendritic cell (DC) pool in fetuses from OM-85 treated mothers as compared to equivalent fetuses from untreated mothers (Figure 1B). Further characterisation of the bone marrow DC response demonstrated that this increase was restricted to the conventional DC (cDC) subset as previously described (8), with no parallel changes observed in plasmacytoid DC (pDC) (Figure 1B). These fetal changes mirror our recent observations of increased cDC yields from bone marrow cultures and peripheral lung from the offspring of OM-85-treated mothers in the early postnatal period[1]. We next turned our attention to fetal DC maturation state as determined by surface IAIE expression. As shown in Figure 1C, maternal OM-85 treatment for the last half of gestation enhanced IAIE expression on cDC in fetal bone marrow when compared to fetal bone marrow cDC from untreated mothers. Collectively, these observations suggest that transplacental signals generated at the feto-maternal interface following OM-85 treatment during pregnancy can "train" the developing fetal immune system via accelerating the development of functional competence within the fetal bone marrow cDC compartment.

In addition to effects on fetal myeloid cDC, maternal OM-85 treatment during pregnancy enhanced the total $CD3^+$ T cell pool within fetal bone marrow, when compared to equivalent fetuses from untreated mothers (Figure 1D). Furthermore, these $CD3^+$ T cells displayed a

lymphoblastic morphology based on their size and granularity (Figure 1E), as determined using multicolour flow cytometry, consistent with current or recent activation.

Previous studies from our laboratory have additionally identified postnatal expansion of bone marrow myeloid progenitor (MP) cell populations as an effect of maternal treatment with OM-85 during pregnancy (8). Based on these findings, we hypothesised that the enhanced cDC population within fetal bone marrow following maternal OM-85 treatment would also be accompanied by concomitant upregulation of upstream MP subsets. Analysis of fetal bone marrow Lin^{-}IL-7Rα^{-}c-$Kit^{+}Sca\text{-}1^{-}$ MP (Figure 2A), Lin^{-}IL-7Rα^{-}c-$Kit^{+}Sca\text{-}1^{-}CD16/32^{hi}CD34^{+}$ granulocyte-macrophage progenitor (GMP; Figure 2B) and Lin^{-}IL-7Rα^{-}c-$Kit^{+}Sca\text{-}1^{-}CD16/32^{hi}CD34^{+}CX3CR1^{+}Flt\text{-}3^{+}$ macrophage-dendritic cell progenitor (MDP; Figure 2C) populations demonstrated a total increase in all 3 progenitor subsets within the bone marrow compartment following maternal OM-85 treatment, when compared to fetal bone marrow from untreated mothers. Consistent with the findings in Figure 1B and C, these data provide further evidence that maternal OM-85 treatment selectively modulates the offspring bone marrow myeloid lineage *in utero*, beginning at the early-stage myeloid progenitor level through to the terminal cDC populations which are responsible for seeding peripheral tissues during early postnatal life to provide local DC-mediated immune surveillance.

To gain further insight into the molecular mechanisms underpinning the maternal OM-85-treatment effects, we employed transcriptomic profiling of fetal bone marrow cells and also thymic tissue, given the findings in Figure 1D and E that maternal treatment may enhance trafficking of activated $CD3^{+}$ T cells into fetal bone marrow. Comparison of the transcriptomic profiles in the two groups indicated that maternal OM-85 treatment resulted in 152

differentially expressed genes (DEG) in fetal bone marrow (119 upregulated, 33 downregulated; Figure 3A left panel, Supplemental Table 1), versus a total of 67 DEG in fetal thymus (42 upregulated, 25 downregulated; Figure 3B left panel, Supplemental Table 2). We then interrogated the DEGs for enrichment of biological pathways employing *InnateDB* (9). In fetal bone marrow, upregulated DEGs were enriched for genes involved in multiple aspects of protein metabolism, the endoplasmic reticulum (ER) stress response, and the unfolded protein response (UPR; Supplemental Table 3). Similar signatures were identified with the thymic response, however, this did not include the UPR (Supplemental Table 4).

Upstream regulator analysis was then performed to identify putative molecular drivers of the observed DEG. The data for bone marrow revealed X-box binding protein 1 (XBP1), a transcription factor central to the UPR (10) and crucial in the development, survival and function of multiple cell types including plasma cells (11), eosinophils (12), natural killer (NK) cells (13), T cell subsets (14) and DCs (15,16), as the most strongly activated molecular driver within fetal bone marrow associated with maternal OM-85 treatment effects (*P*-value = 3.81×10^{-17}, Z-score = 4.427, Figure 3A right panel; Supplemental Table 5). In addition to XBP1, Endoplasmic Reticulum To Nucleus Signalling 1 (ERN1) was also identified as a candidate driver of the maternal OM-85-induced response in fetal bone marrow (*P*-value = 3.32×10^{-6}, Z-score = 2.156; Figure 3A right panel; Supplemental Table 5). Identification of ERN1 is noteworthy given that during the ER stress response, this gene encodes the ER stress sensor inositol-requiring enzyme 1 (IRE1α), responsible for the unconventional cleavage of a 26 nucleotide fragment from *Xbp1* mRNA, resulting in the generation of the active spliced variant of XBP1 (XBP1s) and enabling it to function as a potent transcription factor (17). Additional candidate drivers in fetal bone marrow following maternal OM-85 treatment included CD38,

IL5 and an array of microRNAs (miR) (Figure 3A right panel; Supplemental Table 5) recognised principally in the context of cancer-associated functions (18-20). It is important to note however, that miR-149-3p has been shown to negatively regulate Toll-like receptor (TLR) 4 expression in murine monocytic cells *in vitro* (21) and it is possible that other miRs may have similar (but as yet undefined) innate immune regulatory functions (22). In fetal thymus, the top-ranking drivers associated with maternal OM-85 treatment were primarily involved in cellular metabolic processes and ER function (CYP51A1, INSIG2, CYP7A1, INSR, PPARGC1B, ACACB, NR1H3 and MTORC1), including overlap with several drivers (SCAP, SREBF1, SREBF2, INSIG1, POR, SIRT2; Figure 3B right panel, Supplemental Table 6) identified within the bone marrow. A crucial observation however was the complete absence of XBP1 as a driver within the thymic response.

Finally, to obtain experimental evidence confirming that the activated form of XBP1 was upregulated, we measured expression of the active spliced variant of XBP1 (XBP1s) at the protein level within fetal bone marrow. Using multicolour flow cytometry, we identified significant upregulation of intracellular XBP1s expressing $CD11b^{+}CD11c^{+}$ pre-cDCs within fetal bone marrow following maternal OM-85 treatment (Figure 4). While intracellular XBP1s expression was additionally localised within $CD19^{+}B220^{+}$ B cells, $NKp46^{+}CD11b^{+}B220^{+}CD11c^{lo}$ NK cells and $CD3^{+}$ T cells (Supplemental Figure 1), as previously described in the literature (11,13,14,23), maternal OM-85 treatment had no impact on expression levels in these cell types.

Our objective in this study was to characterise the fetal response to maternal OM-85 treatment over the last half of pregnancy. We provide evidence that signals generated via

oral treatment of pregnant mice with OM-85 can act transplacentally to modulate the *in utero* development of the fetal bone marrow myeloid compartment. Consistent with our previous reports in 6-week-old offspring (8), maternal OM-85 treatment was associated with an enhanced baseline pool of GMPs and MDPs in fetal bone marrow. Furthermore, maternal OM-85 treatment selectively amplified the overall abundance of fetal bone marrow cDCs (but not pDCs at this stage of development) directly downstream of these progenitor subsets, with concomitant upregulation of their functional maturation (IAIE) state. This ability for OM-85-induced education of the fetal myeloid compartment *in utero* parallels previous reports of enhanced levels of human leukocyte antigen (HLA)-DR on DCs of neonates whose mothers endured high microbial burden during gestation (24). Moreover, these HLA-DR^{++} DCs had a heightened capacity to regulate the intensity of the inflammatory response to *in vitro* TLR4 stimulation, implying that maternal microbial exposure prepared neonatal DCs for the strength of microbial challenges experienced in the postnatal environment by optimising their response to TLR stimuli, which is a key component of the anti-microbial defence response.

We provide further evidence of the transplacental stimulation effect of OM-85 on fetal bone marrow cDCs following identification of the transcription factor XBP1 as a putative molecular driver of the maternal OM-85-induced response, with enhanced protein-level expression of XBP1s restricted to the pre-cDC pool. The recent inclusion of XBP1 to the expanding list of transcription factors involved in DC development and function (15,16,25) provides valuable insight into the mode-of-action of OM-85 treatment. In this regard, seminal studies demonstrate a significant reduction in CD11c^{+} cells (mirroring that of our pre-cDC phenotype) within XBP1$^{-/-}$ bone marrow cultures (15). Furthermore, forced overexpression of XBP1s in XBP1$^{-/-}$ DC precursors has been shown to rescue and subsequently drive significant expansion of the total DC pool *in vitro*. The findings by Iwakoshi et al. (15) therefore support

our hypothesis that OM-85-induced upregulation of XBP1s in fetal bone marrow pre-cDCs is responsible for maintaining the enriched population of bone marrow cDCs displaying enhanced functional immunocompetence by late gestation. Moreover, maternal OM-85-induced activation of XBP1 may in part explain the increased population of $CD103^+$ cDCs previously observed in the peripheral lungs of 3-week-old offspring from treated mothers (8), given the homeostatic regulation XBP1 exerts on lung mucosal cDC1s (26).

A secondary finding within the transcriptomic analysis was the maternal OM-85-induced inhibition of miR-149-3p within fetal bone marrow, the downregulation of which has previously been shown to enhance both gene- and protein-level expression of TLR4 (21). The upregulation of TLR4 is currently recognised as an important immune training mechanism involved in protecting offspring from allergic disease onset following exposure of human mothers during pregnancy to the myriad of microbes frequently encountered in traditional farming environments (4). As such, characterisation of TLR expression profiles on fetal bone marrow myeloid cells following maternal OM-85 treatment warrants further investigation.

An unexpected observation from this study was the OM-85-induced increase in fetal bone marrow of $CD3^+$ T cells displaying a lymphoblastic morphology, in conjunction with upstream activation of IL5. It is interesting to note in this regard that the intracellular IRE1α-XBP1 pathway is engaged upon naïve T cell activation and controls both gene- and protein-level expression of IL5 in $CD3^+CD4^+$ T-helper type 2 (Th2) cells (14). However, despite their lymphoblastic morphology indicating recent activation, we did not observe increased protein-level XBP1s expression in $CD3^+$ T cells following maternal OM-85 treatment. Nevertheless, the enhanced $CD3^+$ T cell population still remains the likely source of the IL5 signature within the bone marrow, although our data suggests this response is not under the direct control of XBP1. In conjunction with $CD3^+$ T cells, $CD34^+$ bone marrow progenitors, in which both GMPs

and MDPs were upregulated following maternal OM-85 treatment, are also recognised to produce IL5 *in vitro* (27,28). Maternal OM-85 treatment also resulted in the upstream activation of CD38 in fetal bone marrow. CD38 is a known marker of T cell activation and proliferation (29,30) and we therefore speculate that maternal OM-85-induced activation of CD38 may be involved in the lymphoblastic $CD3^+$ T cell response. Moreover, recent thymic emigrants (RTE) express CD38 (31), of which the lymphoblastic $CD3^+$ T cell population within the bone marrow at this stage of development would represent, given murine RTE survive in the peripheral T cell pool for 2-3 weeks following release from the thymus (32,33).

In summary, the data presented here builds on forerunner human (4,24) and experimental animal (8,34,35) studies providing evidence that non-pathogenic maternal microbial-associated exposures during pregnancy can be transcribed transplacentally into beneficial immune training signals for the developing fetus. We provide direct evidence that maternal OM-85 treatment during pregnancy transplacentally accelerates functional immunocompetence of fetal bone marrow cDCs and for the first time identify the transcription factor XBP1 as a putative driver of this immune training mechanism.

Methods

See Supplemental Materials for a detailed description of all experimental procedures.

Study approval

All animal experiments were formally approved by the Telethon Kids Institute Animal Ethics Committee, operating under the guidelines developed by the National Health and Medical Research Council of Australia for the care and use of animals in scientific research.

Author contributions

KTM, PGH and DHS designed the study. KTM, MB , NMS and JFLJ performed the experiments. KTM, ACJ, MB and DHS analysed the data. PAS and AB contributed to the project design and discussions on data interpretation. KTM, PAS, PGH and DHS wrote the manuscript. All authors reviewed the final version of the manuscript.

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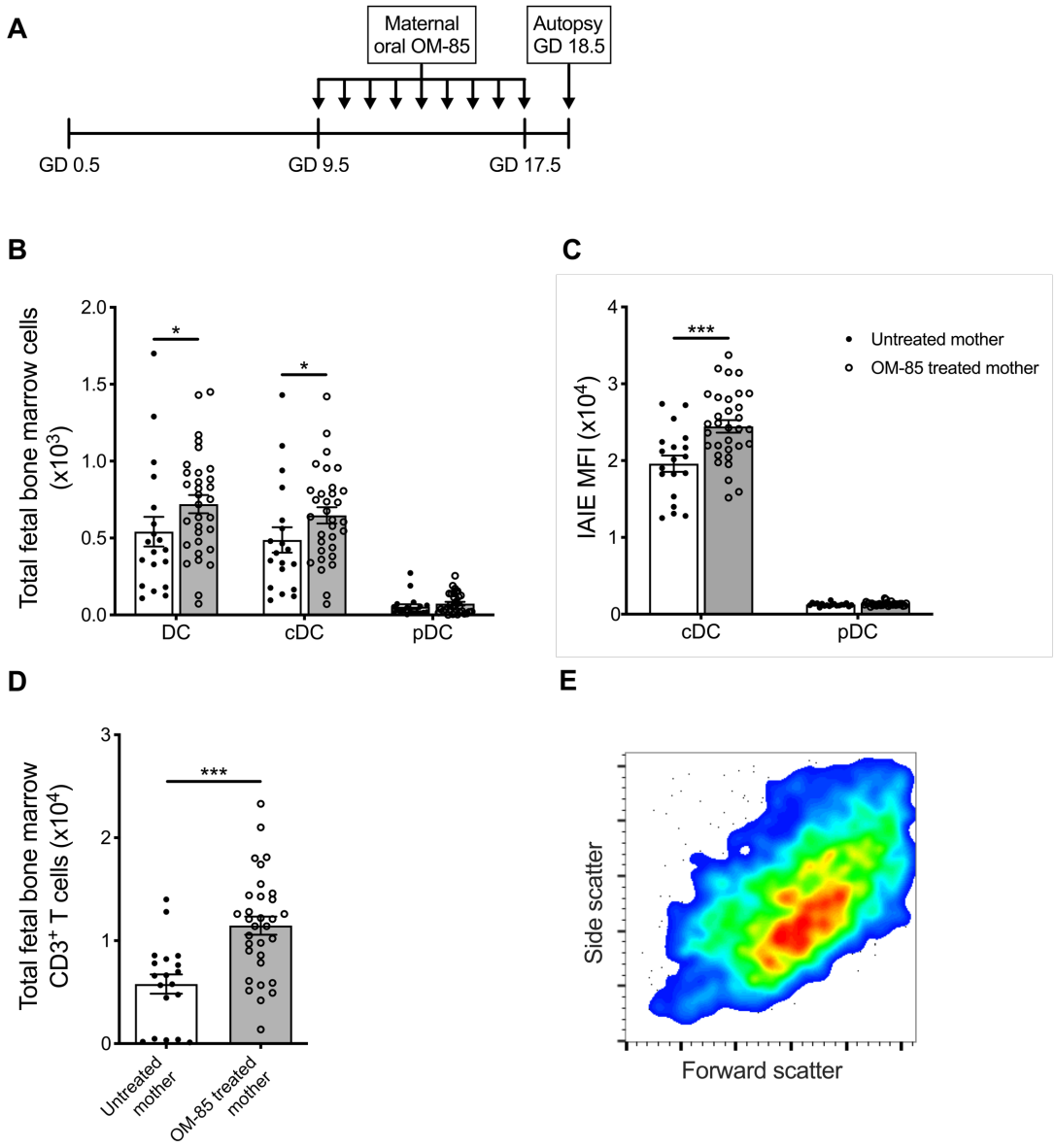

Figure 1. Maternal OM-85 treatment during pregnancy selectively modulates the fetal bone marrow conventional DC subset. (A) Kinetics of maternal OM-85 treatment beginning at gestation day (GD) 9.5 with daily oral treatment until GD 17.5 and autopsy 24 hours post final treatment at GD 18.5. **(B)** Absolute numbers of dendritic cells (DC), $CD11b^{+}B220^{-}CD11c^{+}Gr\text{-}1^{-}SIRP\text{-}\alpha^{+}IAIE^{+}$ conventional dendritic cells (cDC) and $CD11b^{-}B220^{+}CD11c^{+}Gr\text{-}1^{+}IAIE^{+}$ plasmacytoid dendritic cells (pDC) in fetal bone marrow. **(C)** Mean fluorescence intensity (MFI) of IAIE expression on cDCs and pDCs in fetal bone marrow. **(D)** Absolute numbers of $CD3^{+}$ cells in fetal bone marrow. **(E)** $CD3^{+}$ T cell lymphoblastic morphology as based on side and forward scatter parameters. Data are presented from individual animals comparing fetuses from OM-85-treated and untreated mothers and displayed as bar graphs showing mean ± SEM of $n = 8$ independent experiments. Statistical significance was determined using Student's t test and presented as $^{*}P < 0.05$, $^{***}P < 0.001$.

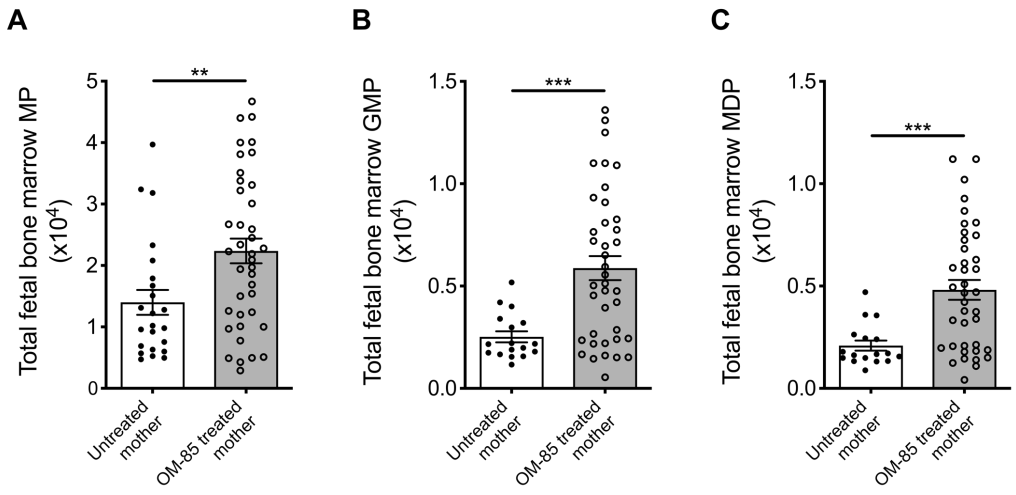

Figure 2. Treatment of mothers with OM-85 during pregnancy boosts myeloid progenitor subsets within fetal bone marrow. Absolute numbers of **(A)** $Lin^{-}IL\text{-}7R\alpha^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}$ myeloid progenitors (MP), **(B)** $Lin^{-}IL\text{-}7R\alpha^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD16/32^{hi}CD34^{+}$ granulocyte-macrophage progenitors (GMP) and **(C)** $Lin^{-}IL\text{-}7R\alpha^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD16/32^{hi}CD34^{+}CX_3CR1^{+}Flt\text{-}3^{+}$ macrophage-dendritic cell progenitors (MDP) in fetal bone marrow. Data are presented from individual animals comparing fetuses from OM-85-treated and untreated mothers and displayed as bar graphs showing mean ± SEM of $n = 8$ independent experiments. Statistical significance was determined using Student's *t* test (B) or Mann-Whitney *U* test (A, C) based on distribution of the data as determined by D'Agostino-Pearson omnibus normality test and presented as $^{**}P < 0.01$, $^{***}P < 0.001$.

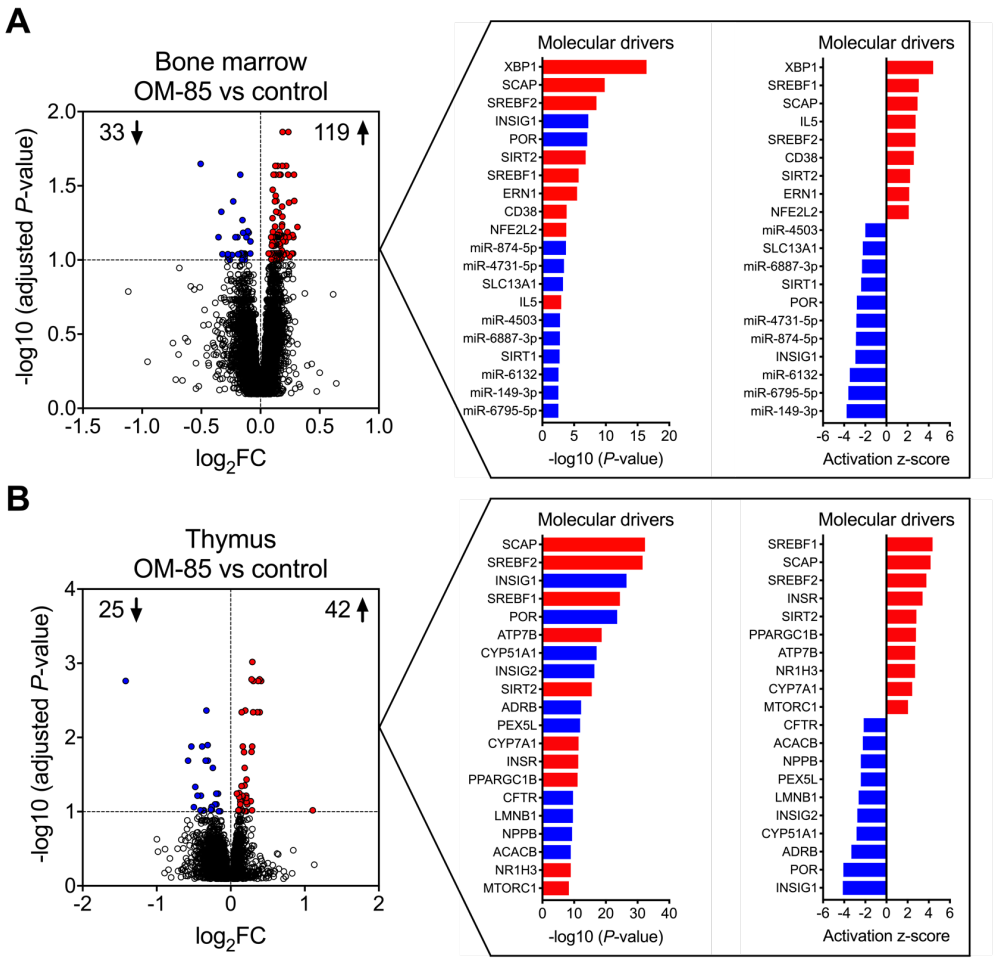

Figure 3. Maternal OM-85-induced changes in fetal bone marrow and thymus gene expression profiles. Left panels: differentially expressed genes (DEG) within fetal **(A)** bone marrow and **(B)** thymus comparing offspring from OM-85-treated and untreated mothers. DEGs are summarised as volcano plots showing data along axes of statistical significance (-log10 adjusted *P*-value) and differential expression magnitude (log_2 fold change) for $n = 16$ individual animals per group collected from $n = 7$ independent experiments. Dashed horizontal lines indicate a False Discovery Rate (FDR) adjusted *P*-value < 0.1. Genes shown in red were upregulated and those shown in blue were downregulated. Right panels: activated (red) and inhibited (blue) molecular drivers of the differential expression patterns were identified using Upstream Regulator Analysis.

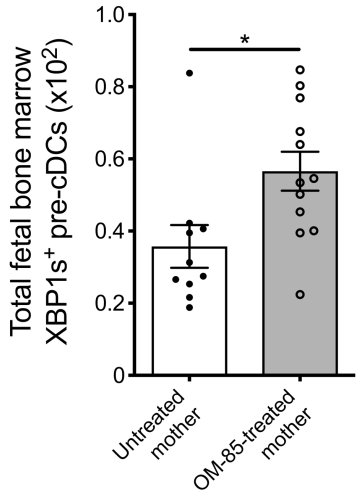

Figure 4. XBP1s expression on pre-cDCs within fetal bone marrow. Absolute numbers of $CD11b^{+}B220^{-}CD11c^{+}Gr\text{-}1^{-}IAIE^{-}XBP1s^{+}$ pre-conventional dendritic cells (pre-cDCs) in fetal bone marrow. Data are presented from individual animals comparing fetuses from OM-85-treated and untreated mothers and displayed as bar graphs showing mean ± SEM of $n = 4$ independent experiments. Statistical significance was determined using Mann-Whitney *U* test and presented as $*P < 0.05$.