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IKK β Activity Drives Fetal Lung Macrophage Maturation Along a Non-M1/M2 Paradigm

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Abstract

In preterm infants, exposure to inflammation increases the risk of bronchopulmonary dysplasia, a chronic, developmental lung disease. While macrophages are the key cells that initiate lung inflammation, less is known about lung macrophage phenotype and maturation. We hypothesized that fetal lung macrophages mature into distinct subpopulations during mouse development, and that activation could influence macrophage maturation. Expression of the fetal macrophage markers CD68, CD86, CD206, Ym1, fibrinogen-like protein 2 (FGL2), and indoleamine-2, 3-dioxygenase (Ido1) were developmentally regulated, with each marker having different temporal patterns. Flow cytometry analysis showed macrophages within the fetal lung were less diverse than the distinctly separate subpopulations in newborn and adult lungs. Similar to adult alveolar macrophages, fetal lung macrophages responded to the TLR4 agonist LPS and the alternative activation cytokines IL-4 and IL-13. Using a macrophage-specific constitutively active IKK β transgenic model (IKFM), we demonstrated that macrophage activation increased proinflammatory gene expression and reduced the response of fetal lung macrophages to IL-4 and IL-13. Activation also increased fetal lung macrophage proliferation. Fetal IKFM lungs contained increased percentages of more mature, CD11b^{lo}F4/80^{hi} cells that also expressed higher levels of the alternative activation markers CD204 and CD206. Development of fetal lung macrophages into mature alveolar macrophages may therefore include features of both proinflammatory and alternative activation paradigms.

INTRODUCTION

To maintain efficient gas exchange between the airspace and pulmonary circulation, the alveolar environment must remain dry, sterile, and free of particulates. As part of the lung innate immune system, macrophages protect the lung from inhaled pathogens, microbes, and harmful particulates. Within the alveolar environment, macrophages are the primary cells that kill pathogens and remove cellular and foreign debris. Expressing an array of pattern

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recognition receptors on their cell surface, lung macrophages detect and engulf inhaled microbes(1). Macrophages phagocytose and kill these pathogens by producing antimicrobial reactive oxygen and nitrogen species(2). When unable to completely kill and remove microbial pathogens, macrophages secrete cytokines and chemokines that recruit additional inflammatory cells to the alveolar space(3). Macrophages then remove both host and microbial cellular debris and promote tissue repair(4).

Like other tissues, the mature lung contains multiple macrophage subpopulations(5). These groups of macrophages appear to differ in their origin, phenotypic marker expression, and functional role in the immune response. During development, macrophages first originate in the yolk sac and later from hematopoietic precursors in the fetal liver(6, 7). Cells from both sources populate the lung, with additional bone marrow-derived monocytes migrating to the lung and differentiating into macrophages(7, 8). In addition, proliferation of differentiated cells can sustain macrophage populations within tissues(6). In addition to potentially deriving from different macrophage sources, the various macrophage subpopulations may have distinct functional roles.

Proinflammatory macrophages respond robustly to microbial organisms by phagocytosing infectious particles and releasing soluble inflammatory mediators(9). In addition to sensing extracellular microbes, infection of proinflammatory macrophages by intracellular pathogens elicits inflammatory cytokine and chemokine release(10). Also referred to as M1 or classically activated macrophages, these proinflammatory cells typically express the surface marker CD86 and cytokines IL-1 β and TNF α (10). TLR agonists, microbial products, and IFN- γ activate proinflammatory macrophages in slightly different ways, giving diversity to the inflammatory response(9, 11). In comparison, macrophages with an alternative phenotype can be classified as M2a, M2b, M2c, or M2d(12, 13). These alternatively activated or M2 macrophages express FGL2, Ym1, and the scavenger receptors CD204 and CD206(14-16). Alternatively activated cells are induced by IL-4 and IL-13 (M2a), TLR or IL1R ligands (M2b), IL-10 (M2c), or the tumor microenvironment (M2d) (12, 13, 17). M2 macrophages play roles in parasitic infections (M2a), atopic allergic disorders (M2a), Th2 differentiation (M2b), wound healing (M2c), and tumor progression (M2d)(12, 13). The relative differences and unique properties of M1/M2 macrophages have been investigated in cancer, diabetes, and chronic inflammatory disease(16, 18-21). How this M1/M2 paradigm applies to lung macrophages during both normal lung homeostasis and in disease processes is not completely clear.

Macrophages play important roles in both neonatal and adult lung immunity. However, neonatal lung macrophages, especially those found in preterm infants, may lack fully mature innate immune function. Neonates are particularly susceptible to pneumonia and inhaled pathogens, suggesting either immature killing or inability to control localized lung inflammation(22). Previous studies showed that neonatal monocytes responded normally to TLR agonists to produce inflammatory cytokines IL-6 and TNF α but macrophages from preterm infants have reduced IL-10 release(23-25). Recent studies reported an increase in M2 markers in the postnatal mouse lung(26). However, detailed characterization of fetal macrophages, how they respond functionally to proinflammatory and alternative activation stimuli, and the developmental signaling pathways regulating maturation are not understood.

Lung inflammation plays a key role in the pathogenesis of bronchopulmonary dysplasia (BPD) in preterm infants(27). Patients with elevated proinflammatory cytokines in their lungs are more likely to have arrested lung development and develop chronic lung disease(28-30). Macrophages are the primary cellular sources of these soluble inflammatory mediators in the lung. We demonstrated in mice that macrophage activation was required and sufficient for microbial products to cause arrested airway and alveolar morphogenesis(31). Targeted NF- κ B activation in fetal macrophages disrupted normal developmental gene expression in epithelial and mesenchymal cells and led to perinatal lethality(31). Interestingly, this disruption of lung morphogenesis occurred only during later stages of development, suggesting macrophage maturation might also be playing a role in the connections between lung inflammation and development.

We hypothesized that macrophages undergo significant maturational changes during fetal lung development, with mature macrophages having either a predominantly proinflammatory (M1) or alternative (M2) phenotype. We therefore measured expression of multiple macrophage markers across fetal mouse lung development. Transcriptional responses to LPS and IL-4/IL-13 in fetal and adult lung macrophages tested the effects of macrophage activation on maturation. The changes we observed both with normal development and following activation did not fit neatly within an M1/M2 paradigm, but instead demonstrated unique mixed phenotypes in lung macrophage populations. These data will be essential for future studies examining macrophage differentiation and function in neonatal and pediatric inflammatory lung diseases.

MATERIALS AND METHODS

Reagents

The following antibodies were used for immunofluorescence: rat anti-CD68 (Acris), rabbit anti-CD86 (Abcam), rat anti-Ym1 (R&D Systems), rabbit anti-CD206 (Abcam), goat anti-macrophage galactose N-acetyl-galactosamine specific lectin (Mgl1/2, R&D Systems), rat anti-Ecadherin (Zymed), mouse anti-alpha-smooth muscle actin-Cy3 (α -SMA, Sigma-Aldrich), rabbit anti-Ki67 (Abcam). ProLong Gold with DAPI mounting media and Alexa-conjugated secondary antibodies were purchased from Invitrogen. DRAQ5 (1,5-bis{[2-(dimethylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9,10-dione) was purchased from Biostatus Limited. Anti-CD11b microbeads and magnetic separation equipment were obtained from Miltenyi Biotec. Gel-purified *Escherichia coli* LPS (O55:B5), deoxyribonuclease I from bovine pancreas Type IV (DNase), and collagenase from clostridium histolyticum, Type XI were obtained from Sigma-Aldrich. Recombinant mouse IL-4 and IL-13 were purchased from R&D Systems. RPMI 1640 media was purchased from Invitrogen, and FBS was purchased from Thermo Fisher Scientific.

Mouse strains

The Institutional Animal Care and Use Committees from Vanderbilt University and the University of California at San Diego approved all animal experiments. C57Bl/6 mice were obtained from Harlan Laboratories. For transgenic expression of enhanced green fluorescent protein (EGFP) in macrophages, we used C57BL/6J-Tg(Csf1r-EGFPNGFR/FKBP1A/

TNFRSF6)2Bck/J mice from the Jackson Laboratory. These mice express EGFP downstream of the *Csf1r* promoter and have no obvious phenotype in the absence of FKBP dimerization agents(32). We have referred to this strain as *Csf1r*-EGFP in the text. For studies using inducible expression of a constitutively active IKK β mutant (cIKK β) in macrophages, female *Csf1r-rtTA* mice were mated with male *Tet-O-caIKK β* mice to produce IKFM offspring(33). To induce cIKK β expression, timed pregnant dams were given doxycycline-containing water (2g/L) ad libitum beginning at E14 until E18. For timed pregnant matings, embryonic day 0 (E0) was defined as the morning of vaginal plug discovery.

Macrophage Isolation

For isolation of fetal macrophages in gene expression and flow cytometry experiments, fetal lungs were dissected free of surrounding tissues and placed in cold PBS. Lung tissue was dissected, minced, and enzymatically digested with DNase (30ug/ml) and collagenase (0.7mg/ml) at 37° C for 30 min. The cell suspension was passed through a 70 μ m cell strainer and collected by centrifugation. For gene expression, fetal cells were resuspended in the presence of anti-CD11b-conjugated microbeads and passed through a magnetic separation column (Miltenyi Biotec). CD68 staining confirmed that this isolation technique resulted in >95% macrophages. For IKFM samples, macrophages were isolated from each embryo and cultured separately in the presence of doxycycline.

Adult lung macrophages were isolated by bronchoalveolar lavage. Lung lavage was repeated three times with cold PBS. Cells were cultured and treated the same as fetal macrophages. To isolate peritoneal macrophages, adult mice received an intraperitoneal injection of 2 mL 3% thioglycollate medium. After 72h, the peritoneum was lavaged three times with cold PBS to collect macrophages. All macrophages were plated in RPMI 1640 with 10% FBS. After 1h in culture, macrophages were gently washed with PBS and treated with LPS (250 ng/ml), IL-4 (5 ng/ml), or IL-13 (10 ng/ml) for 4h.

RNA isolation and real-time PCR measurement

RNA isolation, cDNA synthesis, and real-time PCR were performed on whole lung or isolated macrophages using standard techniques. Tissue and cells were stored and homogenized (TissueRuptor, Qiagen) in TRIzol (Invitrogen). After total RNA isolation, first strand cDNA was synthesized using an oligo(dT) primer and Superscript III (Invitrogen). Real time PCR was performed using either unlabeled oligonucleotides with SYBR Green or TaqMan probes. PCR was performed using either an iQ5 or CFX96 thermocycler (Bio-Rad). Gene expression comparisons were represented using 2^{-C_t} method. Experiments were performed at least three independent times. Data between groups were analyzed by comparing C_T values using a multiple regression analysis model or the Mann-Whitney U test.

Tissue processing and immunolabeling

Mouse lung tissue was fixed, processed, and immunolabeled using standard techniques. Dissected lung tissue was fixed in 4% paraformaldehyde (Electron Microscopy Sciences), processed through a sucrose gradient, and frozen in OCT media (Tissue-Tek). Frozen tissue

sections (10 μm thick) were postfixed in 2% paraformaldehyde, permeabilized in 0.1% TritonX-100 (Thermo Scientific), and blocked with 5% normal donkey serum for 1h at room temperature. Tissue sections were incubated with antibody overnight at 4° C. Alexa-conjugated secondary antibodies (Invitrogen) were used for visualization. Following antibody labeling, nuclei were labeled with DAPI or DRAQ5.

Imaging and image analysis

Confocal images were acquired using a Leica TCS SPE (Leica Microsystems) laser scanning confocal microscope. Widefield fluorescence images were obtained using an Olympus IX81 microscope equipped with Hamamatsu Orca ER CCD monochrome camera and Slidebook software (Olympus). All microscopy images were saved in Tagged Image Format and imported into Photoshop (Adobe Systems) for processing. Images for comparison were identically processed. Immunofluorescence intensity measurements (relative fluorescent units) of individual macrophages were performed in Slidebook. A reference circle outlined a masked area over each macrophage. Relative fluorescent units for each channel were measured within the masked area and plotted against CD68 intensity values.

Flow cytometry and FACS

The following antibodies were used for flow cytometry and FACS experiments: CD11b-V450, CD86-PE-Cy7, Gr1 PerCP-Cy5.5, and CD45 APC-Cy7 (BD Bioscience); CD204-AF647, CD68-FITC, and CD206-PE (AbD Serotec); F4/80-PE (Invitrogen); CD11c-Pe-Cy5 (BioLegend). Viable cells were identified using the LIVE/DEAD Fixable Red Dead Cell Stain Kit or the LIVE/DEAD Fixable Blue Dead Cell Stain kit (Invitrogen). Fetal and adult macrophages were digested as previously described. Cells were resuspended in ACK Lysing buffer (Invitrogen) to lyse red blood cells. Cells were blocked with 3% FBS for 15min and incubated with primary antibodies against surface markers for 30-60min on ice. For intracellular antibody staining, we used the Intracellular Fixation and Permeabilization kit (eBioscience). Flow cytometry measurements were conducted using a BD Fortessa, and flow sorting was conducted on a BD FACSAria III. For flow cytometry gating, the initial gate was based on FSC-A against SSC-A. Doublets were excluded with two gates: FSC-A against FSC-H, followed by SSC-A against SSC-H. Viability gating selected cells that were negative for the LIVE/DEAD fixable Red or Blue Dead Cell stain. Out of viable cells, CD45⁺ cells identified the population of hematopoietic cells. For FACS gating, the initial gate was FSC-A against SSC-A, followed by doublet exclusion with FSC-A against FSH-A and SSC-A against SSH-A. CD45⁺ cells were separated into CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi}. These cell populations were sorted directly into Trizol.

RESULTS

Fetal lung macrophages differentially express phenotype markers during development

Lung macrophages are clearly important in neonatal lung immunity and disease. However, the differentiation and maturation of lung macrophages during fetal development is just now being characterized. We measured the expression of various macrophage population markers in developing mouse lungs. As shown in Figure 1, cells expressing the pan-macrophage

marker CD68 were present in the fetal lungs throughout development. The pro-inflammatory macrophage marker CD86, however, was rarely detected at E13 and E15. Cells expressing CD86 were more prevalent later in gestation and in the adult lung. Ym1 and CD206 are markers of alternatively activated macrophages, found commonly associated with tumors or in chronic inflammation models. While we did not detect Ym1 expression in E13 and E15 mouse lungs, staining appeared at E18 and was abundant in adult lungs. In contrast, CD206 was widely present at E13, and appeared to label similar numbers of cells as CD68 throughout mouse lung development. Macrophage galactose N-acetyl-galactosamine-specific lectin 1 (Mgl1/2) was expressed both by macrophages and airway dendritic cells in the adult lung. Interestingly, Mgl1/2 expression in the fetal lung appeared at E15 and was restricted to cells with a more round macrophage morphology. Macrophage markers were therefore differentially expressed throughout fetal lung development and with distinct temporal patterns.

Gene expression experiments showed similar patterns of macrophage marker expression throughout lung development (Fig. 1F-H). CD68 expression increased in postnatal and adult lung, suggesting either a higher CD68 expression or an increase in number of CD68⁺ macrophages. The pro-inflammatory markers CD86 and Ido1 also increased during development, being most highly expressed in adult lung. Similar to the immunofluorescence data in Fig. 1D, CD206 gene expression was relatively constant throughout lung development (Fig. 1G). In contrast, expression of other alternatively activated macrophage markers was higher in more mature lungs. While Ym1 expression first increased at P7, fibrinogen-like protein 2 (FGL2), another marker of alternatively activated macrophages, increased earlier during development, plateauing around E17. Markers of alternatively activated macrophages and airway dendritic cells (Mgl1/2, CD11c, CD103) were more highly expressed in postnatal and adult lungs (Fig. 1H). Both immunofluorescence and gene expression data therefore support developmental regulation of macrophage differentiation and maturation. The temporal changes in expression were distinct for each marker tested and did not clearly follow pro-inflammatory or alternative activation paradigms.

Because multiple macrophage markers were expressed in the developing lung, we next tested if these various markers represented distinctly separate macrophage populations. We immunostained multiple sections of developing mouse lungs obtained at E15 with both CD68 and various population-specific markers. Individual cell fluorescence intensity was then measured for all cells expressing either macrophage marker. As shown in Figure 2, CD86 expression in CD68⁺ macrophages was variable in E15 lungs. The separation of CD68 and CD86 expression was more apparent in adult lung, with CD86-expressing cells having lower CD68 staining intensity. Consistent with data in Figure 1, only a minority of CD68⁺ macrophages in E15 lungs expressed Mgl1/2, while a larger population of adult macrophages and airway dendritic cells expressed both CD68 and Mgl1/2 (Fig. 2C-D). CD206 was expressed throughout lung development. However, the intensity of CD206 staining in E15 lungs varied (Fig. 2E). In contrast, CD206 expression in adult CD68⁺ cells was relatively low. Using this approach to identify macrophage populations in situ revealed differences in relative marker expression between E15 and adult lung macrophages. However, this immunostaining approach was not able to clearly identify distinct populations of macrophages in the fetal lung with relative differences in expression levels or patterns.

We next tested for the presence of subpopulations of fetal mouse lung macrophages by flow cytometry (Figure 3). Lung macrophages were isolated from single cell suspensions obtained from fetal (E15 and E18), postnatal day 7 (P7), and adult *Csf1r*-EGFP lungs. In these mice, GFP-positive cells uniformly expressed CD68 (Fig. 3A). Viable, CD45⁺, *Csf1r*-EGFP⁺ cells (Fig. 3B) were analyzed based on expression of Gr1, CD86, CD11c, CD11b, and F4/80. Cells from E15 lungs expressed Gr1, CD11b, and F4/80, but the patterns of expression suggest less diversity when compared to macrophages from more mature lungs. Expression of CD11c was low in E15 lungs, increased at E18, and clearly present by P7 (Fig. 3D). Distinct populations of CD11c⁺ cells were observed in P7 and adult lungs, consistent with previous data showing CD11c expression in mature alveolar macrophages(34, 35). This flow cytometry expression pattern followed the real time PCR data, where CD11c expression was low in fetal lung but increased at birth (Fig. 1H).

The most consistent separation of E15 macrophages into distinct subpopulations was observed with gating CD11b⁺F4/80⁺ cells (Fig. 3E). The distribution of CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} cells in E15 lungs was consistent with recently reported data suggesting these two subpopulations may arise from distinct cellular precursors(8). Interestingly, macrophage subpopulations based on CD11b and F4/80 expression appeared to be more diverse in P7 and adult lungs. These data confirmed that the fetal lung does contain subpopulations of macrophages, but with less diversity than more mature lungs. To test if the CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} subpopulations had uniquely different proinflammatory markers, we compared CD86 expression in both populations. As seen in Figure 3F, both CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} macrophages expressed CD86 in E15 lungs.

We next tested if fetal lung macrophages responded to the proinflammatory stimulus LPS and the alternative activation cytokines IL-4 and IL-13 (Figure 4). LPS treatment of E15 fetal lung macrophages increased expression of CXCL-10, CCL-3, IL-1 β , and TNF α , consistent with a robust proinflammatory response. IL-4 and IL-13 each inhibited expression of CXCL-10 and CCL-3, but did not dampen the response to LPS. Treating E15 macrophages with IL-4 or IL-13 caused only a modest and statistically insignificant increase in the alternative activation markers CCL-17 and CD206. However, adding both LPS and IL-4/IL-13 did increase CCL-17 expression above levels seen with any agonist alone. In adult lung macrophages, IL-4 and IL-13 both increased CCL-17 and CD206 expression (Fig. 4B). Interestingly, IL-4 and IL-13 also upregulated expression of CCL-3 and IL-1 β , although much less than LPS (Fig. 4B). As an additional comparison, IL-4 and IL-13 decreased CXCL-10 and increased CCL-17 and CD206 in adult peritoneal macrophages (Fig. 4C). Therefore E15 fetal lung macrophages responded similarly to LPS as adult macrophages, but appeared to have reduced sensitivity to the alternative activation cytokines IL-4 and IL-13. This reduced sensitivity to IL-4 and IL-13 could be due to low receptor expression in E15 cells compared to adult cells, as suggested by gene expression data for IL4Ra, IL13R1, etc. (Fig. 4D). LPS did not significantly change receptor gene expression in E15, E18, or adult lung macrophages (Fig. 4D).

We used a transgenic approach to further test if inflammatory signals and specifically NF- κ B activation could regulate fetal lung macrophage development and maturation. IKFM

double transgenic mice express a constitutively active IKK β mutant in macrophages upon doxycycline exposure(31, 33). Pregnant dams were given doxycycline from E14-E18; lung macrophages were isolated from E18 IKFM and littermate controls. We then measured expression of the pro-inflammatory mediators CXCL-10, CCL-3, IL-1 β , and TNF α in addition to the alternative activation markers CCL-17 and CD206 (Figure 5). IKFM macrophages expressed higher levels of CXCL-10, CCL-3, IL-1 β , and TNF α , consistent with NF- κ B activation (Fig. 5A-B). While E18 control macrophages expressed increased CCL-17 and CD206 following IL-4 and IL-13 treatment, IL-4 and IL-13 had less effect on IKFM macrophages (Fig. 5C). These data suggested that fetal lung macrophage NF- κ B activation could promote functional resistance to IL-4 and IL-13.

Immunostaining of IKFM and control lung sections did not reveal dramatic differences in the relative populations of cells expressing CD86, Mgl1/2, or CD206 in situ (Figure 6). However, flow cytometry showed shifts in relative CD11b and F4/80 expression. The increased relative percentage of CD11b^{lo}F4/80^{hi} macrophages in IKFM lungs was consistent between litters (Fig. 7A-C). These CD11b^{lo}F4/80^{hi} cells also expressed higher levels of IL-1 β (Fig. 7D). Differences in gene expression were not due to selective transgene expression, as the cIKK β transgene was expressed in both CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} cell populations (Fig. 7E). We previously measured increased macrophage cell number in IKFM lungs(31). This increase could be due to proliferation of resident lung macrophages. Immunostaining showed that IKFM lungs contained a higher percentage of macrophages expressing phospho-HistoneH3 (Fig. 7F) and Ki67 (Fig. 7G). The increase in proliferation was associated with the relative increase in cyclinD1 expression in CD11b^{lo}F4/80^{hi} macrophages (Fig. 7H). Therefore, in addition to stimulating expression of inflammatory mediators, NF- κ B activation increased macrophage proliferation within fetal lung tissue.

The increase in CD11b^{lo}F4/80^{hi} macrophages in IKFM lungs is consistent with increased differentiation, as mature alveolar macrophages are also CD11b^{lo}F4/80^{hi} (34). Similarly, we measured increased CD204 and CD206 expression in IKFM lungs, both of which are expressed on the surface of mature alveolar macrophages (Fig. 7I). IKFM lungs also contained higher numbers of Gr1⁺ macrophages (Fig. 7J), suggesting possible recruitment of fetal monocytes. Importantly, Gr1 expression was only detected in CD11b^{hi}F4/80^{lo} populations. Increased macrophage differentiation in IKFM lungs did not lead to large increases in CD11c, which remained very low in both CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} cells (Fig. 7K). Therefore, NF- κ B stimulated changes in macrophage marker expression were most consistent with lung macrophage maturation and did not follow a clear proinflammatory/alternative activation or M1/M2 paradigm.

DISCUSSION

Fetal lung macrophages undergo significant differentiation and maturational changes during development. While fetal lung macrophages expressed relatively consistent levels of CD68 and CD206 at each stage tested, other macrophage lineage markers increased significantly. Both proinflammatory and alternative activation markers increased during development without following a consistent M1/M2, proinflammatory/alternative activation paradigm.

Developing lung macrophages were functional, as immature macrophages from E15 mouse lungs responded to the proinflammatory stimulus LPS. The partial response to IL-4 and IL-13 suggest that E15 lung macrophages may indicate a relative functional polarization toward a proinflammatory phenotype.

The notion of M1/M2 macrophage polarization derives largely from studies using tumor-associated macrophages and chronic inflammation models(36-38). So while activation of M1 macrophages and M2 polarization may contribute to disease, this classification paradigm may not directly apply to the developmental phenotypes of lung macrophages. Adult alveolar macrophages express both M1 and M2 markers(34). We speculate that the constant exposure to inhaled pathogens and particulates requires that lung macrophages possess both robust killing activity (typically thought of as M1) and the ability to resolve or suppress inflammation and injury (an M2 process). Recent work demonstrated that macrophages in the postnatal lung express the alternative activation or M2 genes, Arg1, CCL-17, and Mrc1(26). These studies also showed a downward trend of expression for some proinflammatory genes in later postnatal stages(26). However, mature alveolar macrophages also possess the ability to mount a strong proinflammatory immune response. Recent publications have in fact expanded M1/M2 classification into a spectrum model for classifying macrophage phenotype(11). Additionally, tissue macrophages in various developing organs appear to acquire unique phenotypic features(5, 39). How these unique expression patterns might regulate differential or tissue-specific macrophage function remains unclear.

During lung development, the relative abundance of CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} macrophages undergo dynamic shifts. Distinct subpopulations are seen early in lung development and again around the time of birth as fetal monocytes/macrophages develop into postnatal and adult lung macrophages. Consistent with Williams et al. (35), we also observed increasing CD11c expression when fetal macrophage subpopulations differentiated into alveolar macrophages. Maturation changes in macrophage marker expression correlated with postnatal migration of macrophages into the alveolar air space. Our data showing differences in CD11b and F4/80 expression with maturation and activation suggest that NF- κ B signaling accelerates macrophage differentiation. IKK β activity in fetal macrophages increased the percentage of CD11b^{lo}F4/80^{hi} cells within the developing lung. The CD11b^{lo}F4/80^{hi} subpopulation expressed higher levels of IL-1 β mRNA, suggesting macrophage activation leads to the developmental maturation or recruitment of a proinflammatory subpopulation. However, the overall increase in the M2-like scavenger receptors CD204 and CD206 again emphasizes that lung macrophage differentiation is more complex than a simple M1/M2 classification. Previous studies show NF- κ B activation also influences dendritic cell maturation. Deleting both RelA and p50 subunits of NF- κ B prevented normal CD11c⁺ dendritic cell development in the livers of mice in adoptive transfer studies(40). In studies using GM-CSF and IL-4 to induce dendritic cell differentiation and maturation in human monocytes, NF- κ B inhibition reduced expression of CD1a and DC-SIGN and reduced dendritic progenitor cell number(41). How NF- κ B signaling might interact with other factors regulating macrophage development (i.e. PU.1, CEBP, Myb, Maf) also remains unclear(42).

Recent findings demonstrating proliferation and self-renewal of resident tissue macrophages have challenged the notion that macrophage populations are solely comprised of recruited monocytic cells(6). Local production of the macrophage mitogens CSF-1 and CCL-2 stimulate macrophage proliferation within tissues via JAK/STAT signaling pathways(43-46). Here we showed that transgenic expression of a constitutively active IKK β transgene in macrophages also stimulated proliferation within fetal mouse lungs. NF- κ B activation could increase production of intermediate factors that drive cell proliferation via autocrine loops or via neighboring cells residing within a multicellular niche. Alternatively, increased IKK β activity could facilitate signaling through additional intracellular pathways connected with cell proliferation. In cancer cell models, NF- κ B promotes cyclinD1 expression and is required for cell growth and proliferation (47, 48). While both CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} macrophage populations in IFKM fetal mouse lungs expressed the cIKK β transgene, macrophages with the highest F4/80 and IL-1 β expression also contained the highest expression of cyclinD1. These data suggested that more mature CD11b^{lo}F4/80^{hi} cells in the fetal lung may have increased capacity for both cytokine expression and proliferation.

Our data demonstrate that immature fetal lung macrophages respond robustly to LPS exposure. The relative pro-inflammatory and anti-inflammatory functions of developing lung macrophages remain unclear. How IL-4 and IL-13 regulate these functions may be important in disease, as IL-13 induces Th2 inflammation in asthmatic lungs (49, 50). Previous studies comparing adult and neonatal monocytes from different sources measured variable cytokine responses following exposure to TLR agonists (51-53). Consistent differences were reported primarily in interferon production in response to co-cultures and macrophage activators (54-56). However, relative differences in production of cytokines such as IL-1 β or TNF α and changes to the kinetics of transcriptional activation and subsequent silencing could still lead to important physiological differences in the innate immune response within immature lungs.

The ability of macrophage activation to increase maturation as well as release inflammatory mediators could have significant implications in disease. Preterm infants are often exposed to multiple agents capable of activating immature but functional lung macrophages (57-59). Early macrophage activation in IKFM mice disrupts structural lung morphogenesis, possibly modeling BPD pathogenesis in human patients. In addition, inflammation-induced macrophage maturation might sensitize the lung innate immune response to repeated infectious challenges. This two-hit hypothesis has been frequently suggested as important in the pathogenesis of neonatal chronic lung disease and BPD(60, 61). Increased macrophage maturation in the immature lung and reduced responsiveness to IL-4 and IL-13 could limit the ability to resolve lung inflammation and promote wound healing. Investigating these new paradigms in macrophage biology and development will lead to new therapeutic strategies for pediatric lung disease.

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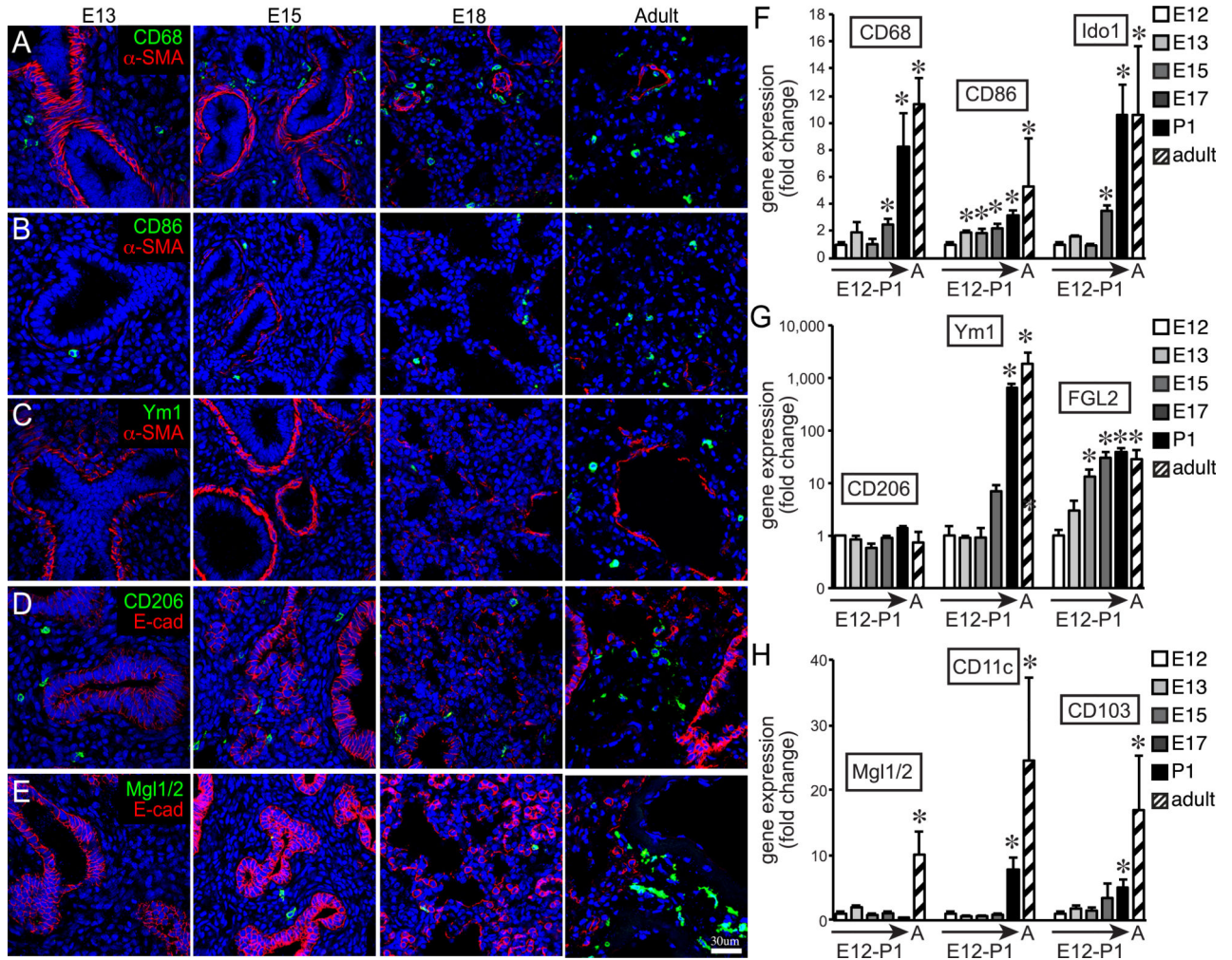


Figure 1. Macrophage marker expression during mouse lung development. (A-E) Frozen sections of E13, E15, E18 or adult mouse lungs were immunostained with Abs against the pan-macrophage marker CD68 (A) the pro-inflammatory macrophage marker CD86 (B) the alternative activation markers Ym1 (C), CD206 (D) and Mgl1/2 (E). Smooth muscle and myofibroblasts immunolabeled with anti- α -SMA in (A-C; red); epithelial cells immunolabeled with anti-E-cadherin in (D,E; red). Nuclei were labeled with DRAQ5. Images obtained by laser scanning confocal microscopy. (F-H) Gene expression of macrophage markers in E12, E13, E15, E17, P1 and adult mouse lungs. (F) Expression of the pan-macrophage marker CD68 and proinflammatory markers CD86 and INDO increased during later stages of development and following delivery. (G) Expression of the alternative activation markers CD206, Ym1, and FGL2 each had distinct temporal expression patterns. (H) The alveolar macrophage/dendritic cell markers Mgl1/2, CD11c, and CD103 were expressed highest in postnatal and adult lung. (n = 4 litters for each embryonic time point, 4 lungs from individuals at P1 and adult; * $p < 0.05$).

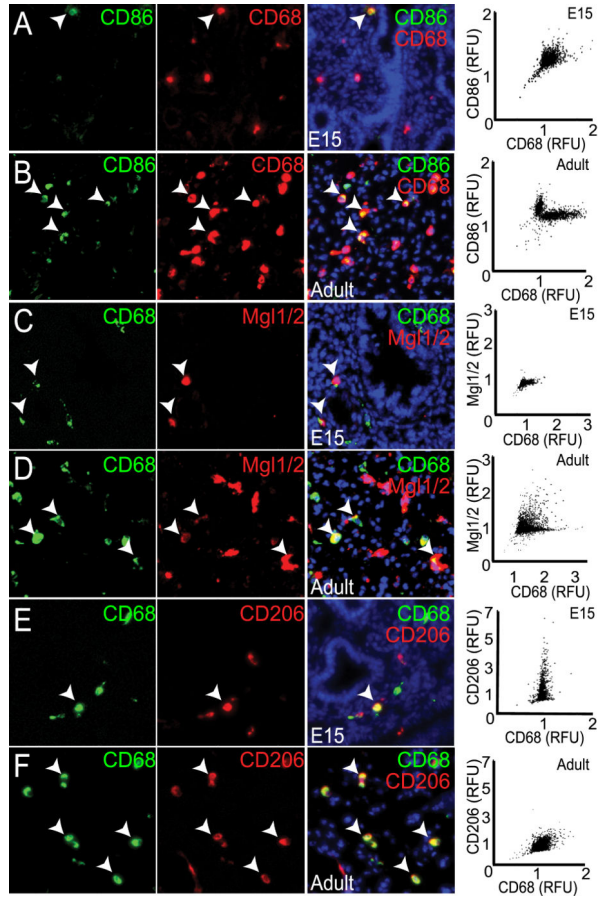


Figure 2. Comparison of macrophage populations in fetal and adult mouse lungs. (A-F) Frozen sections of E15 and adult mouse lungs were immunostained with Abs against CD68 and CD86 (A-B), Mgl1/2 (C-D), or CD206 (E-F). Nuclei were labeled with DAPI. Macrophages labeled in were identified by widefield fluorescence microscopy. Multi-channel fluorescence of each cell was measured and plotted to demonstrate potential populations of cells with differing expression levels. Macrophages in E15 mouse lungs expressed relatively similar marker levels, with the exception of CD206 (E), which had a larger expression range. Adult lungs contained more clearly identified populations of macrophages based on CD86 (B) and Mgl1/2 (D) expression. (n = 4 different lung samples from each stage, 20 images were measured per lung)

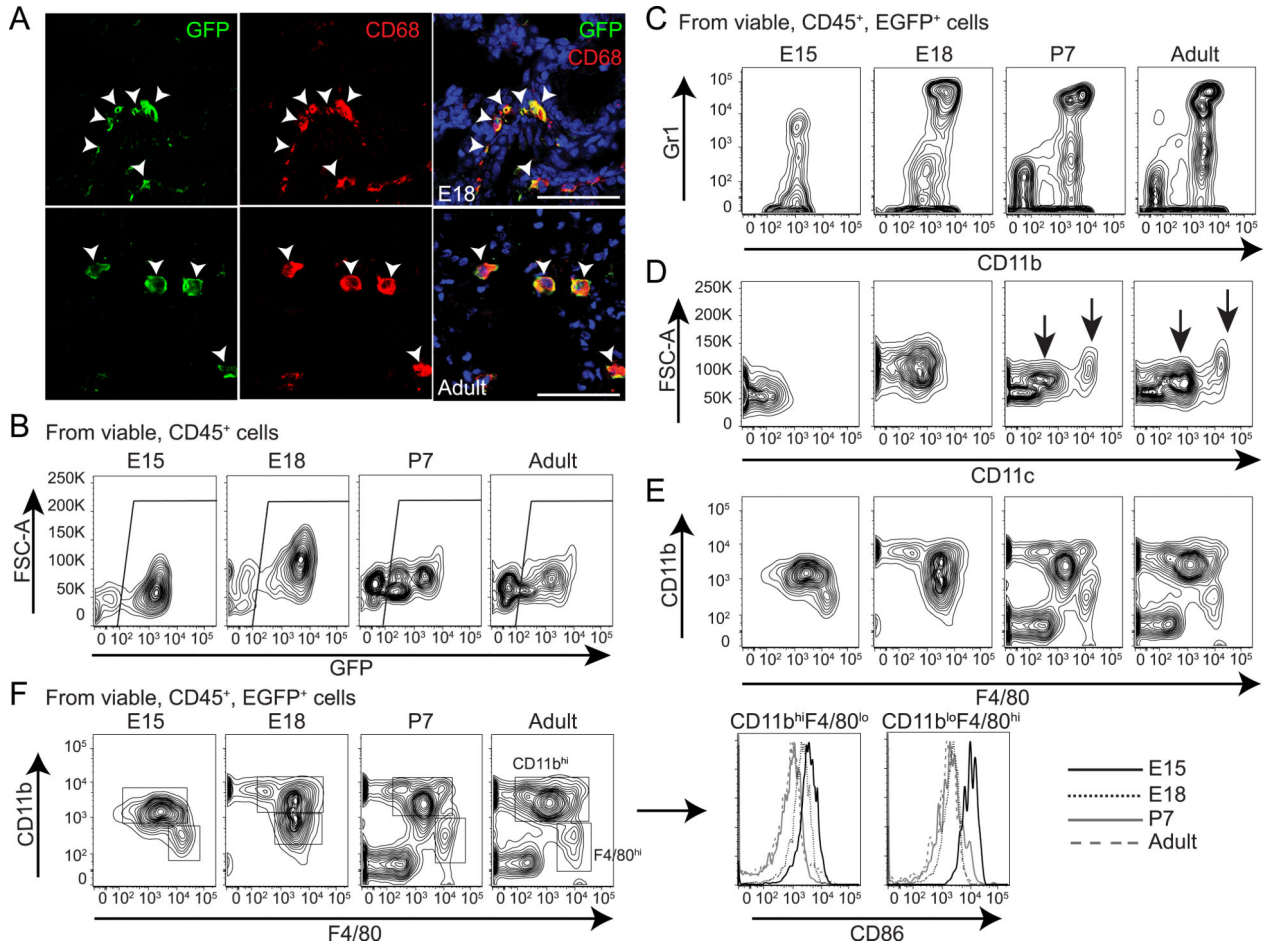


Figure 3. Developmental maturation of lung macrophages occurs after birth. *Csf1r*-EGFP⁺ cells were positive for CD68 (A). CD45⁺ cells were gated for GFP expression (B). (C-F) Flow cytometric analysis of *Csf1r*-EGFP⁺, CD45⁺ macrophages from E15, E18, P7, and adult mouse lungs. CD11b and Gr1 expression show potential neutrophil populations in E18, P7, and adult lungs (C). Broad expression of CD11c⁺ (D), and CD11b⁺/F4/80⁺ (E) cells were detected in P7 and adult lungs, consistent with mature alveolar macrophage differentiation. CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} populations were gated and analyzed for CD86 expression (F). (n = 3 adult mice, 3 E15 litters, 3 E18 litters, 3 P7 pups)

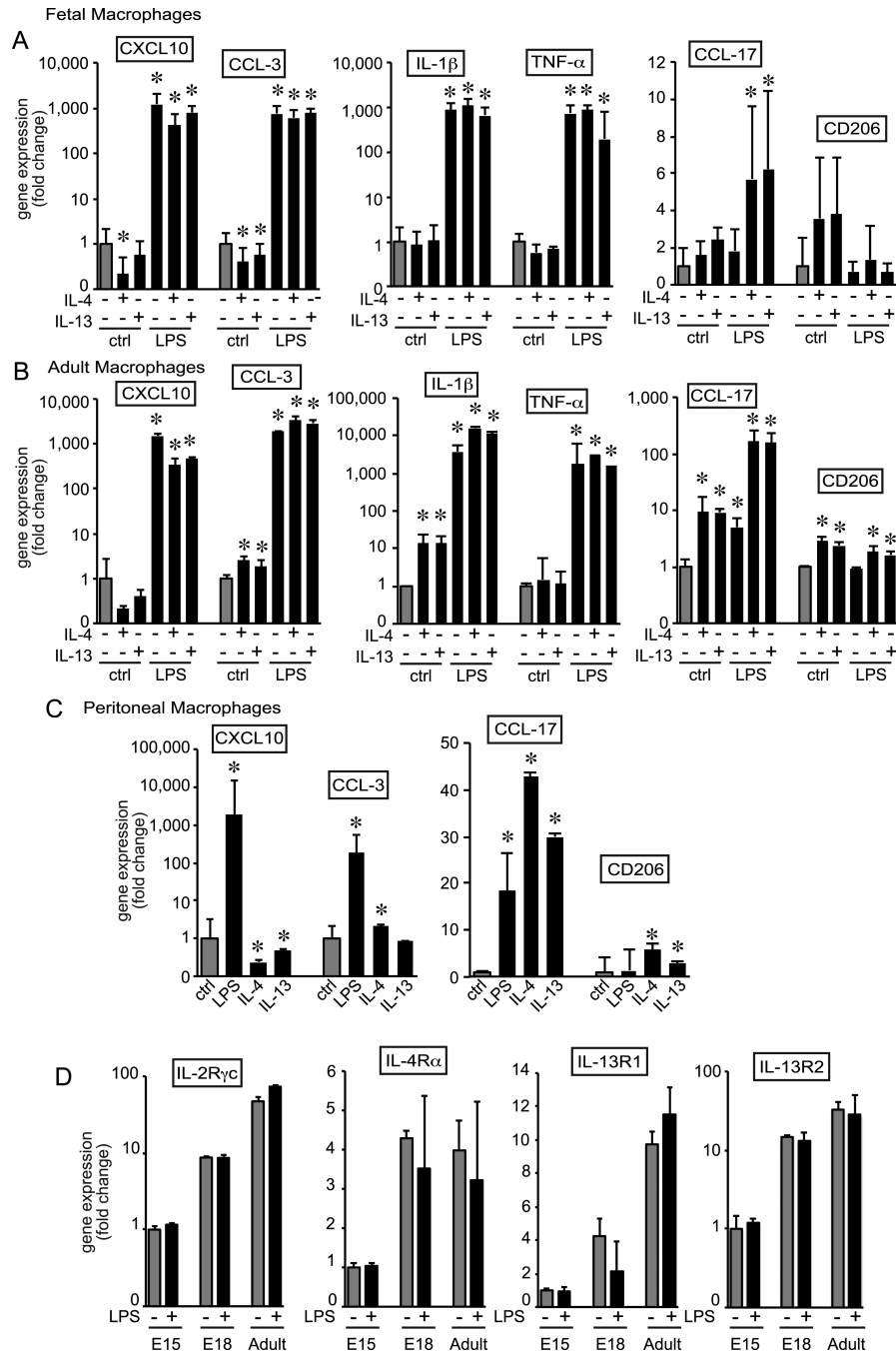


Figure 4. Transcriptional response of fetal mouse lung macrophages in response to LPS, IL-4, and IL-13. **(A)** Macrophages were isolated from E15 fetal C57/BL6 mouse lungs and treated with LPS (250 ng/ml), IL-4 (5 ng/ml), and IL-13 (10 ng/ml) for 4 h as indicated. Following RNA isolation, real time PCR measured expression of proinflammatory markers (CXCL10, CCL-3, IL-1 β , TNF α) and alternative activation markers (CCL-17, CD206). For each marker, expression was compared to control, untreated samples (* $p < 0.05$; $n = 6$ litters). **(B)** Adult mouse alveolar macrophages were isolated by bronchoalveolar lavage and

cultured with LPS, IL-4, and IL-13 as in (A) (* $p < 0.05$; $n = 3$). (C) To demonstrate control macrophage response to both LPS and IL-4 and IL-13, peritoneal macrophages were isolated from thioglycollate-injected C57/BL6 adult mice. Isolated cells were cultured in the presence of LPS, IL-4, or IL-13 for 4 h and gene expression measured by real time PCR (* $p < 0.05$; $n = 3$ mice for adult and peritoneal samples, 6 litters used for E15 samples). (D) IL-4R components were measured in macrophages from different stage lungs by real-time PCR. (* $p < 0.05$; $n = 3$)

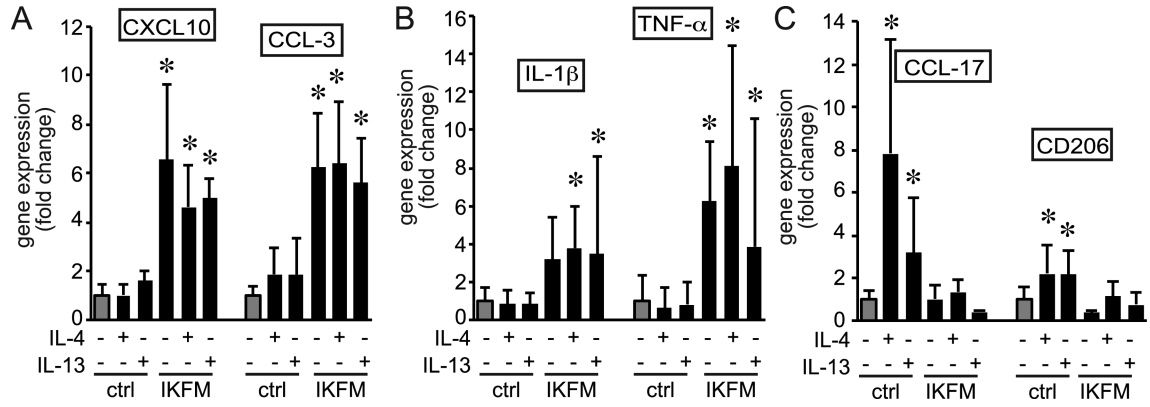


Figure 5. NF-κB activation in fetal mouse lung macrophages stimulated a proinflammatory response and reduced the response to IL-4 and IL-13. IKFM mice expressing a tetracycline-inducible constitutively active IKKβ mutant were given doxycycline from E14-E18. Fetal lung macrophages from E18 IKFM fetal mouse lungs and littermate controls were cultured with IL-4 or IL-13. (A-C) RNA was isolated and expression of proinflammatory markers (CXCL10, CCL-3, IL-1β, TNFα) and alternative activation markers (CCL-17, CD206) were measured by real time PCR (□ *p* < 0.05; n = 5 litters).

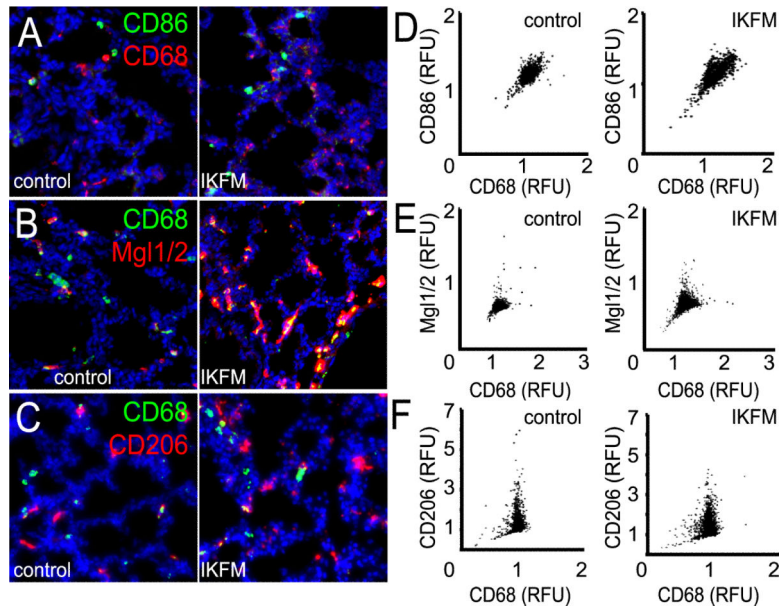


Figure 6.

NF- κ B activation in fetal mouse lung macrophages did not cause major shifts in macrophage populations. IKFM female mice were given doxycycline from E14-E18. Lungs from IKFM and control littermates were isolated at E18. (A-C) Frozen sections of littermate control and IKFM fetal lungs were co-immunostained with Abs against the pan-macrophage marker CD68 (green) and proinflammatory marker CD86 (A) or the alternative activation markers Mgl1/2 (B) and CD206 (C). Nuclei were labeled with DAPI. (D-F) Macrophages labeled in (A-C) were identified by widefield fluorescence microscopy. Multi-channel fluorescence of each cell was measured and plotted to demonstrate potential populations of cells with differing expression levels. Sum fluorescent intensity of individual macrophages was measured using Slidebook software. The patterns of macrophage marker intensity were similar between IKFM and control littermates. (n = 3 control littermate and 3 IKFM lungs, 20 images were counted from each lung)

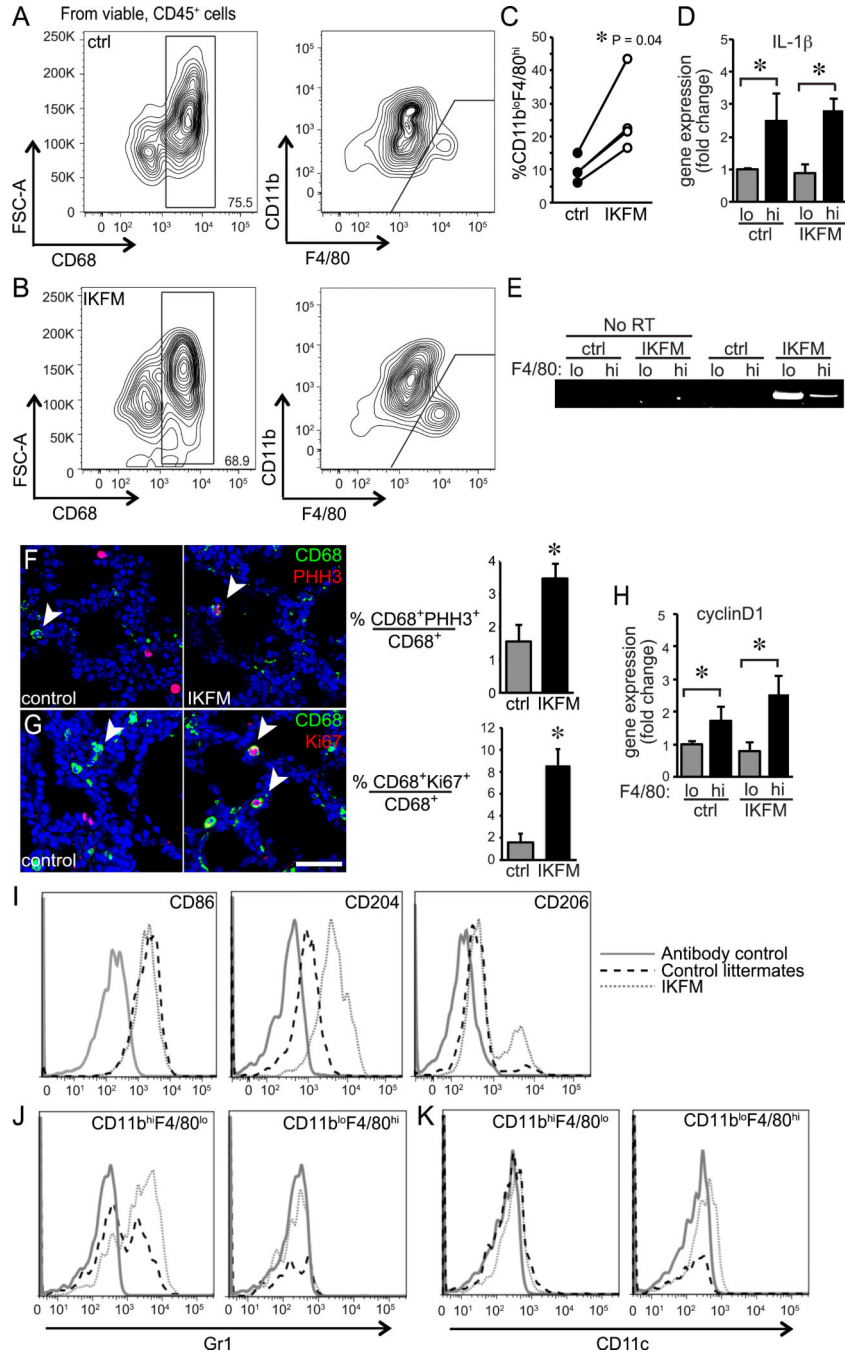


Figure 7. NF- κ B activation increased alveolar macrophage phenotype markers in fetal mouse lung. Single cell suspensions were isolated from E18 IKFM and control-littermate fetal lungs. (A,B) Viable, CD45⁺CD68⁺ cells were gated on F4/80 and CD11b expression. IKFM lungs contained a more identifiable subpopulation of CD11b^{lo}F4/80^{hi} macrophages (B). (C) Percentage of CD11b^{lo}F4/80^{hi} macrophages within the CD68⁺ population was increased in IKFM lungs compared to control littermates. Four independent litters are shown (* p = 0.04). (D) CD11b^{lo}F4/80^{hi} macrophages produced higher mRNA levels in both IKFM and

littermate-controls. **(E)** Both CD11b^{hi}F4/80^{lo} and CD11b^{lo}F4/80^{hi} macrophages from IKFM fetal lungs expressed the cIKK β transgene when exposed in utero to doxycycline. **(F-G)** Frozen sections of E18 control littermate and IKFM lungs were immunostained stained with the macrophage marker CD68 and phosphohistone H3 (PHH3) or Ki67. IKFM lungs had increased number of PHH3⁺ and Ki67⁺ macrophages that were not due to overall increased cell proliferation throughout the lung. **(H)** CD11b^{lo}F4/80^{hi} macrophages expressed increased cyclinD1 mRNA in both IKFM and control littermate fetal lungs (* $p < 0.05$; $n = 3$ control and 3 IKFM). **(I)** IKFM macrophages expressed increased CD204 and CD206. **(J-K)** Increased Gr1 expression in IKFM macrophages was only detected in the CD11b^{hi}F4/80^{lo} subpopulation (J), while CD11b^{lo}F4/80^{hi} macrophages from IKFM lungs expressed slightly elevated levels of CD11c (K). ($n = 4$ litters for A-C, I-J; 3 control and IKFM samples for D, E, H; 4 individual control and IKFM lungs were used in F-G, with approximately 10 images per lung)