

Fetal Phagocytes Take up Allergens to Initiate T-Helper Cell Type 2 Immunity and Facilitate Allergic Airway Responses

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Abstract

Rationale: Actively acquired tolerance occurs when foreign antigens come into contact with the immature fetal immune system.

Objectives: Armed with the knowledge of actively acquired tolerance, we attempted to prenatally abolish or diminish allergic responses.

Methods: *In utero* injection of adjuvant-free ovalbumin (OVA) was conducted in Gestational Day 14 FVB/N mouse fetuses. Postnatally, mice were evaluated for their resistance to intraperitoneal OVA sensitization and oral or aerosolized OVA challenge, and then they were examined for humoral and cellular immunological profiles, airway hyperresponsiveness to bronchospastic stimuli, and lung histology. Fluorescent conjugates of OVA were used for further studies of mechanisms.

Measurements and Main Results: This presumed tolerogenic action turned out to be a sensitization process with the development of anaphylaxis or heightened recall, T-helper cell

type 2-skewed responses to postnatal encounter with OVA. Further postnatal aerosolized OVA stress triggered allergic lungs with functional and structural alterations of airways. The unintended consequence resulted from macrophage-like fetal phagocytes that took up OVA and differentiated toward dendritic cells. These fetal dendritic cell progenitors attenuated proteolysis of endocytosed OVA for delayed presentation in postnatal life. This specialty of fetal phagocytes effectively retains the memory of antigens internalized early before full development of the immune system, leading to an event of *in utero* sensitization.

Conclusions: Our results have mechanical implications for prenatal imprinting of atopy and shed light on the importance of fetal phagocytes in shaping the developing immune system and initiating allergic airway diseases.

Keywords: allergic airway disease; antigen presentation; dendritic cell progenitor; *in utero* sensitization; macrophage

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Author Contributions: J.-C.C. formulated the idea of this work; performed *in utero* injections, pulse-chase studies, and adoptive transfer; analyzed the data; prepared the figures; wrote the manuscript; and coordinated the overall research efforts. C.-C.C. performed adulthood sensitization, cell culture, ELISA, and assessment of airway responsiveness. C.-J.W. generated recombinant Derp II and analyzed Derp II IgE. L.-S.O. measured lymphocyte proliferation. H.-Y.Y., H.-L.C., and L.-Y.T. assisted in experiments as well as animal surgery and care. M.-L.K. designed and supervised the experiments of allergic airway reactions and helped with data analyses and manuscript preparation.

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At a Glance Commentary

Scientific Knowledge on the

Subject: The timing of initiating atopy to allergens is imperative for allergy prevention strategies in public health. Whether allergen sensitization may develop *in utero* remains controversial because there is a shortage of direct evidence for the causal relationship between fetal allergen contact and T-helper cell type 2-skewed atopy initiation.

What This Study Adds to the

Field: This prospective animal study demonstrates that allergen contact in preimmune fetuses might initiate T-helper cell type 2-skewed atopy. Allergic airway diseases developed following repetitive aerosolized allergen stress in postnatal life. Delayed presentation of allergens endocytosed by fetal phagocytes was responsible for the event of *in utero* sensitization to allergens.

Immune tolerance was first conceptualized on the basis of acceptance of reciprocal skin grafts between dizygotic cattle twins with sharing of fetal blood (1). It stimulates the discovery of “actively acquired tolerance” (2), suggesting that self-nonself discrimination is not an inherited property but rather gradually learned before full immune development during fetal or early neonatal life. Thus, the preimmune period is ideal for the implementation of medical intervention that may later be hampered by immune responses. However, years of effort toward developing clinical prenatal cellular therapy led to only limited success in inherited immunodeficiency diseases (3), far less than the theoretically expected benefits for a variety of congenital hematopoietic and metabolic disorders. Despite disappointing clinical application of prenatal cellular therapies, the concept of immune tolerance contributes to evolving procedures for successful solid organ transplantation (4), which have now flourished to the untold benefit of human beings. Tolerance induction has also been an attractive allergen-specific immunotherapy to modulate immune responses to allergens in allergic diseases (5), either eliminating hypersensitivity or reducing medication

requirements or symptom severity. It prompted us to experimentally test the feasibility of allergy prevention through prenatal exposure to allergens. However, our attempt at allergen desensitization turned out to be an event of *in utero* sensitization characterized by allergen-specific T-cell responses. We found that fetal phagocytes as dendritic cell (DC) progenitors sequestered internalized antigens for delayed presentation, thereby bridging toward adaptive T-cell immunity later in postnatal life. These studies have previously been reported in part in abstract form (6).

Methods

Additional methods used for ovalbumin (OVA) sensitization and challenge in adult animals, assessment of airway responsiveness, lung histopathology, determination of antigen-specific immunoglobulins, proliferative response and cytokine production of lymphocytes, fetal organ harvest, fetal cell culture, cell phenotyping, intracellular staining, and acidification are described in the online supplement.

Mouse Husbandry and Reagents

Inbred FVB/N and BALB/c mice (6–8 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan), and DO11.10 transgenic mice were bred in the animal care facility at Chang Gung University. Females were caged with males in the afternoon and checked for vaginal plugs the following morning. The day the plug was observed was called day 0 of the pregnancy. The laboratory autoclavable rodent diet (LabDiet, St. Louis, MO) given to the mice was OVA free. All experiments were performed according to a Chang Gung University Institutional Animal Care and Use Committee-approved protocol. Antibodies and their isotypes were purchased from Abcam (Cambridge, UK), BioLegend (San Diego, CA), eBioscience (San Diego, CA), or BD Pharmingen (San Jose, CA).

In Utero Injection

On Gestational Day 14 (GD14), inocula were intraperitoneally injected into each fetus in a litter using a transuterine approach (7, 8). *In utero* inocula (5–20 μ l) included OVA-pulsed fetal cells, fluorescent conjugates of OVA, aluminum-free OVA, endotoxin-free OVA (EndoGrade; Hyglos GmbH, Bernried am Starnberger See, Germany),

or recombinant *Dermatophagoides pteronyssinus* group II (Derp II).

Adoptive Transfer of OVA-pulsed Fetal Cells into the Murine Fetus and Adult

Pooled cells from GD14 fetal liver and peritoneum were pulsed with OVA and washed vigorously with phosphate-buffered saline (PBS), then they were injected intraperitoneally into GD14 murine fetuses at a dose of $5\text{--}10 \times 10^6$ cells per fetus in 5–10 μ l of saline or intravenously into male adults (to avoid OVA release from H-Y-related cytotoxicity) at a dose of $5\text{--}10 \times 10^7$ cells per mouse in 200 μ l of saline. After cell transfer, recipients were examined for serum OVA-specific IgG1/IgE (OVA-IgG1/IgE) levels by ELISA. Sera of adult recipients were additionally collected before cell transfer for paired comparison. Controls were fetal cells maintained in OVA-free medium.

In Vivo Measurement of Antigen Presentation by Carboxyfluorescein Succinimidyl Ester Labeling

Pooled BALB/c OVA-pulsed fetal cells were prepared, and DO11.10 splenocytes were labeled with carboxyfluorescein succinimidyl ester (9). They were either simultaneously or sequentially (the former on Day 1 [D1] of cell transfer and the latter on D5 or D9) injected into male BALB/c adults via tail veins at a ratio of 1 ($5\text{--}6 \times 10^7$):2–3 ($1\text{--}1.6 \times 10^8$). After 3 days, recipients' splenocytes were enriched by density gradient and stained with anti-CD4 phycoerythrin. Cells were then analyzed by flow cytometry to the exclusion of dead cells by high propidium iodide staining.

Pulse-Chase Studies

Cells from GD14 fetal liver or peritoneum (5×10^6 cells per milliliter of RPMI 1640 medium) were pulsed with 50 μ g/ml fluorescein isothiocyanate-, Alexa Fluor 647-, or Alexa Fluor 488-labeled OVA (OVA-fluorescein isothiocyanate, OVA-647, or OVA-488, respectively) or with DQ-OVA (Molecular Probes, Eugene, OR) at 37°C over an indicated period. After being washed vigorously with PBS, cells were collected for enumeration of OVA-laden cells and intracellular staining, or chased in granulocyte-macrophage colony-stimulating factor-containing RPMI 1640 medium. Then, all were subjected to cytometric analyses.

Statistical Analyses

The χ^2 test was used to determine the significance of differences in offspring survival. Numerical data were compared by using Student's *t* test or the Mann–Whitney *U* test (see SUPPLEMENTARY METHODS section in the online supplement) between two independent groups or, for three or more groups, one-

way analysis of variance with a *post hoc* multiple comparison test using the least significant difference. We performed paired samples *t* tests to compare paired measurements taken on the same subject before and after certain treatments. Lethality curves were constructed by calculating 1 minus Kaplan–Meier cumulative survival and compared by

log-rank test. Differences were regarded as significant in all tests at $P < 0.05$.

Results

Susceptibility of FVB/N Adults to OVA

We first examined the susceptibility of FVB mice to OVA-induced allergic reactions.

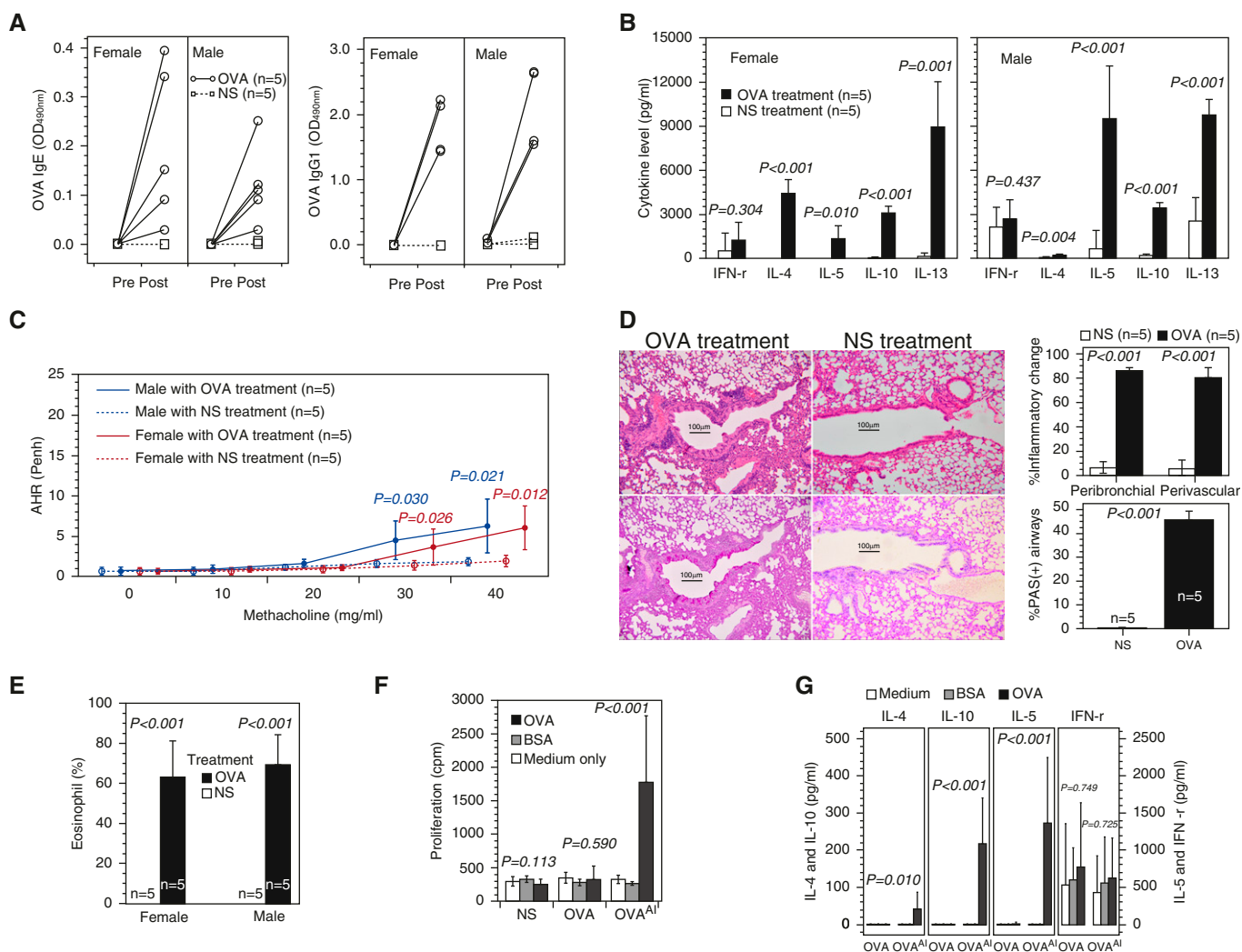


Figure 1. Allergic responses of FVB/N mice to ovalbumin (OVA). (A) FVB/N adult mice were subjected to intraperitoneal Al(OH)₃-emulsified OVA sensitization and aerosolized OVA challenge. This treatment elicited serum OVA-IgE ($P = 0.047$ for females and $P = 0.029$ for males)/OVA-IgG1 ($P = 0.001$ for females and males), in contrast to the response in controls that received normal saline (NS) injections and inhalations. Coupled circles or squares represent pre- and post-treatment paired data from an individual mouse. (B) In OVA-stimulated lymphocyte culture, there was significant expression of IL-4, IL-5, IL-10, and IL-13 cytokines. (C) Airway hyperresponsiveness (AHR), expressed as enhanced pause (Penh) values, significantly increased upon bronchospastic aerosolized methacholine stimuli at 30–40 mg/ml. (D) Histological examination of lungs revealed mainly peribronchial and perivascular inflammation (upper panels; hematoxylin and eosin staining) and extensive goblet cell hyperplasia of airways (lower panels; periodic acid–Schiff [PAS] staining). The severity of peribronchial and perivascular inflammation was quantified as the ratio of airways and vessels with surrounding leukocyte infiltration to all the airways and vessels counted in hematoxylin and eosin-stained, paraffin-embedded lung sections, and goblet cell hyperplasia was quantified as the ratio of airways with the presence of PAS-stained goblet cells to all the airways counted in PAS-stained lung sections. (E) The severity of eosinophilia was measured as the ratio of eosinophils to total cell counts in bronchoalveolar lavage fluid. (F) In cell cultures, lymphocytes proliferated specifically to OVA stimulation in mice subjected to intraperitoneal sensitization with Al(OH)₃-emulsified OVA (OVA^{Al}; $n = 5$), as opposed to those that received injection of aluminum-free OVA (OVA; $n = 5$) or saline (NS; $n = 5$). (G) Proliferative lymphocytes significantly secreted IL-4, IL-5, and IL-10, indicating a T-helper cell type 2-skewed phenotype. All bar data are shown as 95% confidence intervals for the means. BSA = bovine serum albumin; OD = optical density.

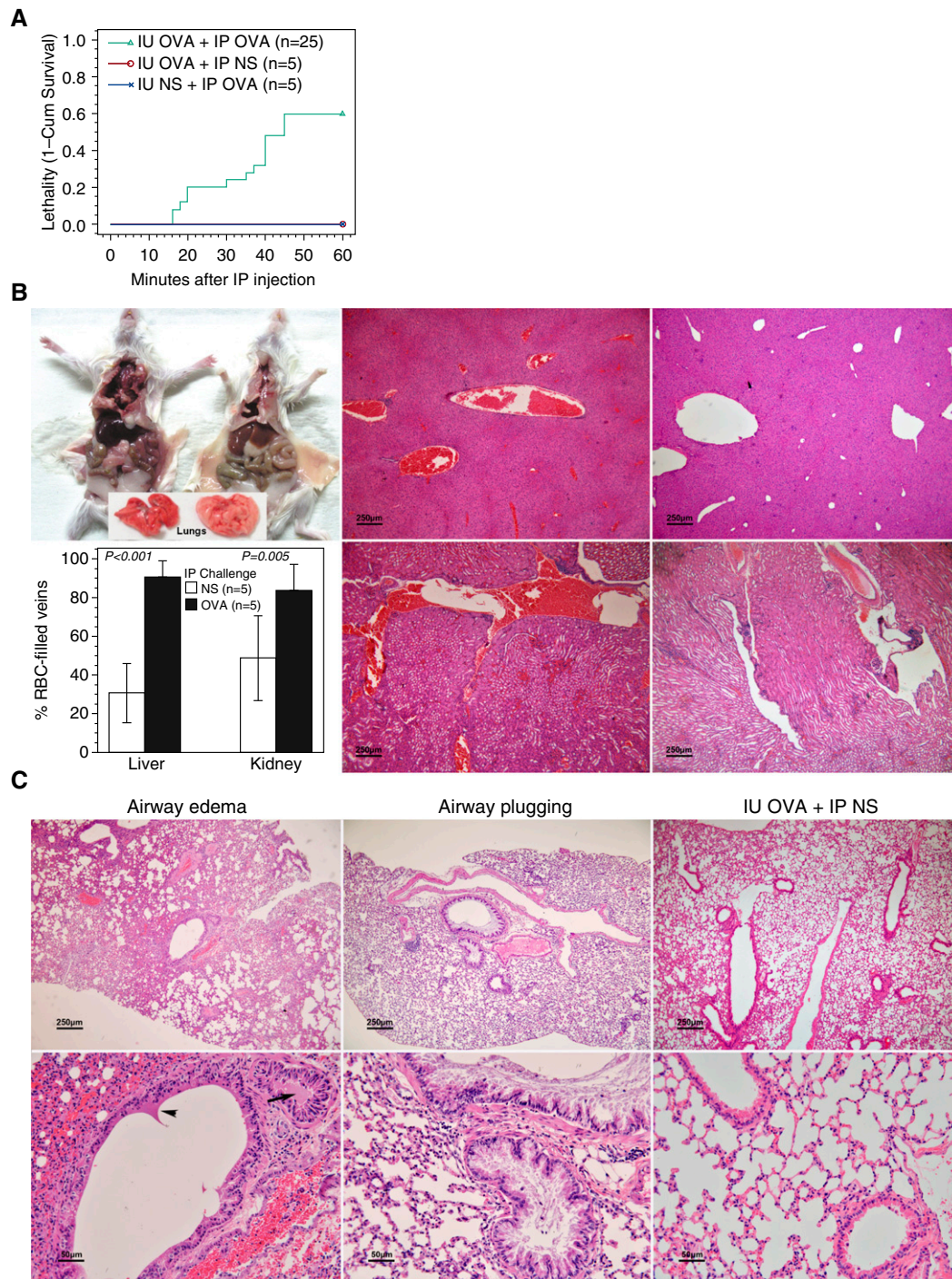


Figure 2. Anaphylactic reactions in FVB/N mice prenatally exposed to ovalbumin (OVA). (A) Following intraperitoneal (IP) aluminum-emulsified OVA (50 µg) injection, FVB/N mice exposed to OVA prenatally (intrauterine [IU] OVA + IP OVA) developed anaphylaxis and had a mortality rate of 60% within 1 hour, as compared with IU OVA mice subjected to normal saline (NS) injection (IU OVA + IP NS; $P = 0.033$) and IU NS mice subjected to OVA injection (IU NS + IP OVA; $P = 0.033$). (B) Autopsy revealed visceral congestion (*upper left panel*, left mouse). Microscopically, there is venous blood pooling in the liver (*upper middle panel*) and kidney (*lower middle panel*), as compared with the controls (*right panels*; IU OVA + IP NS). The severity of venous congestion was quantified as the ratio of veins filled by red blood cells (RBCs) to all veins counted in hematoxylin and eosin-stained, paraffin-embedded sections of livers and kidneys (*lower left panel*). (C) For lung sections, one representative mouse (*left panels*) exhibited alveolar wall thickening due to mural inflammation and vascular congestion, as well as a fluid-filled (*arrow*) or fluid-coated airway with a crescent air–fluid interface (*arrowhead*) blurring the apical cilia of epithelia. The other case (*middle panels*) had no airway or alveolar edema, but rather partial or almost complete airway occlusion by excessive mucus in association with neighboring atelectasis. The lung histology of a mouse from the IU OVA + IP NS group (*right panels*) is shown for comparison. Life-threatening anaphylaxis remained following intraperitoneal challenge of aluminum-free OVA but not aluminum alone.

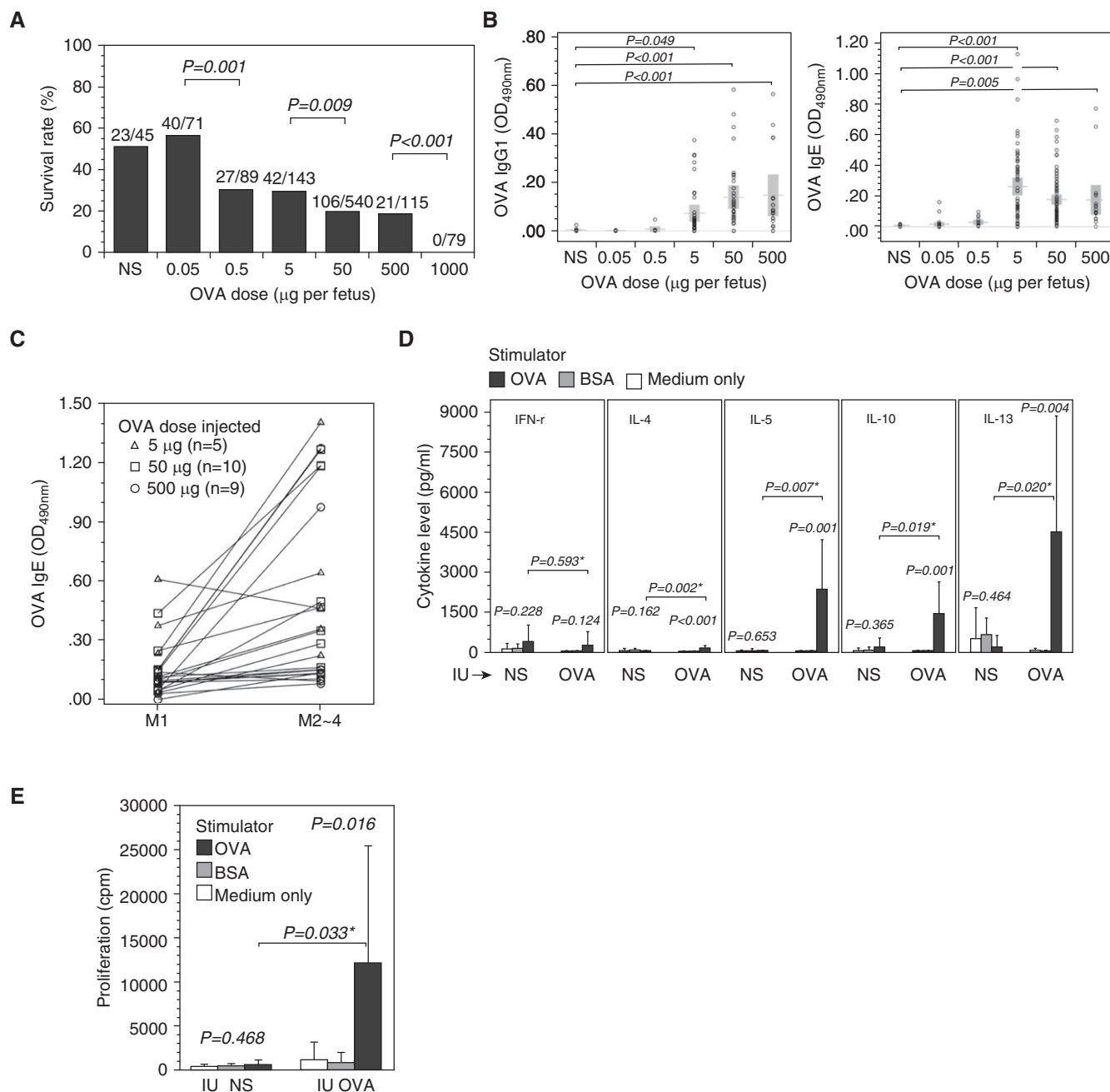


Figure 3. Immunological consequences of *in utero* ovalbumin (OVA) exposure. (A) Following *in utero* injection of aluminum-free OVA or normal saline (NS), offspring survival at 2 weeks old significantly differed among the doses used. Injection itself evoked about 50% fetal loss (NS group), mainly due to placental injury, fetal liver puncture, amniotic leakage, or postnatal cannibalization. (B) Mean serum OVA-IgG1/IgE differed among all groups ($P < 0.001$), with significantly higher levels for 5, 50, and 500 μg than for NS control. Each circle represents the optical density (OD) of OVA-IgG1/IgE at 490 nm for an individual mouse at 4–6 weeks old. The means and their 95% confidence intervals are shown as horizontal lines and shaded boxes, respectively. (C) At the age of 2–4 months (M2–4), serum OVA-IgE was significantly increased ($P = 0.001$, paired comparison). (D and E) Prenatally OVA-sensitized mice (intrauterine [IU] OVA; $n = 5$) had OVA-specific proliferation of T-helper cell type 2 cytokine-secreting (IL-4, IL-5, IL-10, and IL-13) lymphocytes (one-way analysis of variance), as opposed to prenatally NS-exposed mice (IU NS; $n = 5$). * P values derived from t tests between IU NS and IU OVA for cytokine secretion and lymphocyte proliferation under OVA stimulation. BSA = bovine serum albumin.

FVB/N adults were subjected to intraperitoneal OVA sensitization followed by aerosolized OVA challenge. Irrespective of sex, they accurately demonstrated

the immunological events of allergy, including OVA-IgG1/IgE production (Figure 1A) and T-helper cell type 2 (Th2)-skewed lymphocyte differentiation

(Figure 1B). Allergic mice had an enhanced pause increment following high-dose methacholine inhalation (Figure 1C), as well as pulmonary inflammation and

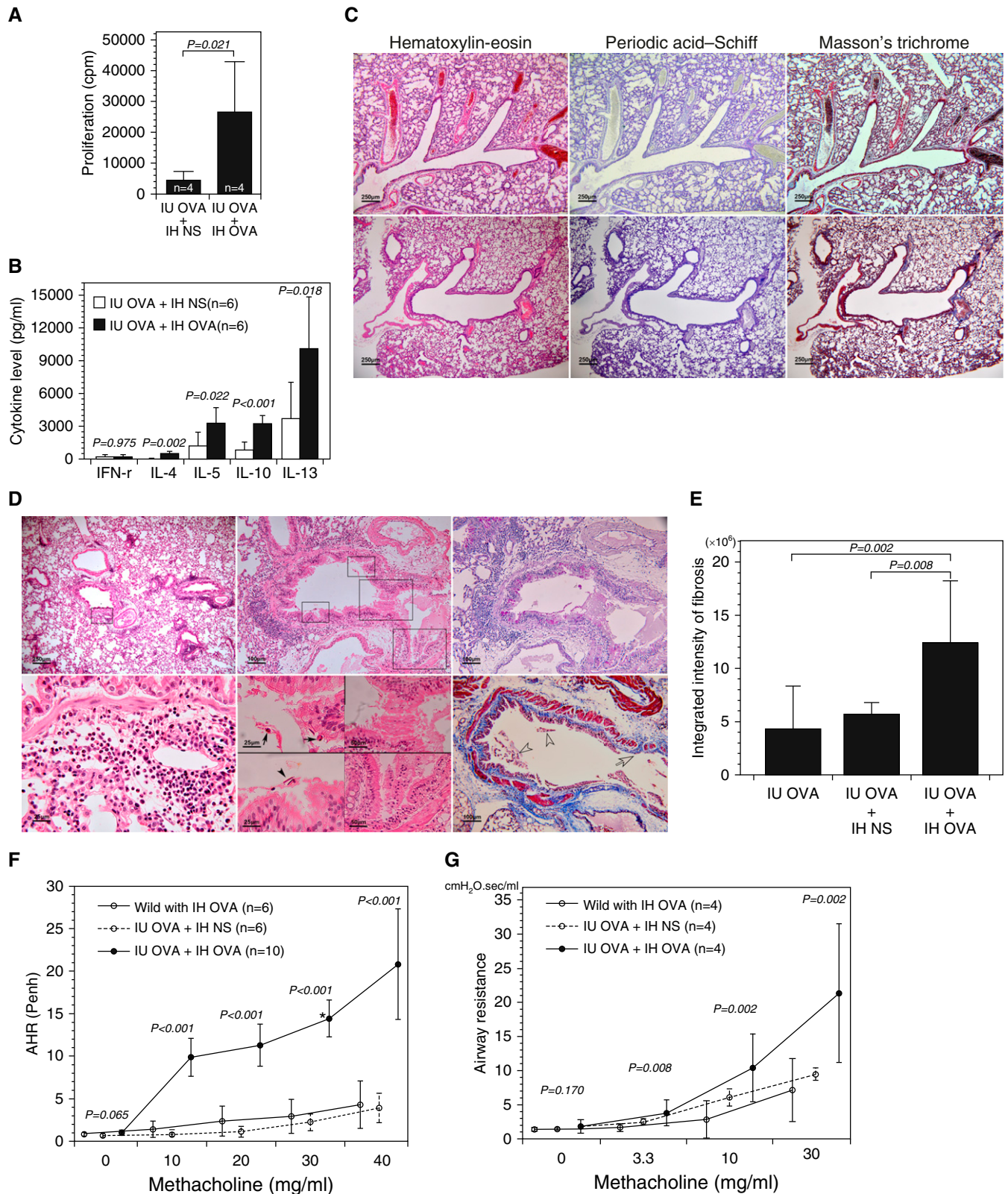


Figure 4. Postnatal aerosolized ovalbumin (OVA) stress in prenatally OVA-primed FVB/N mice. We subjected prenatally OVA-primed (intrauterine [IU] OVA) FVB/N mice to aerosolized OVA (inhaled [IH] OVA) challenge twice per week for a total of five times. Postnatal OVA inhalation (IU OVA + IH OVA) boosted (A) lymphocyte proliferation and (B) T-helper cell type 2 cytokine production in OVA-stimulated culture, as opposed to postnatal normal saline (NS) inhalation (IU OVA + IH NS). (C) Following *in utero* OVA exposure, neither FVB/N neonates (5–7 days after *in utero* injection; upper panels) nor adults

goblet cell metaplasia (Figure 1D) with eosinophilia in bronchoalveolar lavage fluid (Figure 1E). Aluminum adjuvant was indispensable to lymphocyte sensitization in FVB/N adults (Figures 1F and 1G).

Perverse Effect of *In Utero* OVA Exposure

To examine the feasibility of prenatal allergen desensitization, we subjected GD14 FVB/N fetuses to aluminum-free OVA (50 μ g) exposure. Postnatally, 25 mice were tested for their resistance to intraperitoneal OVA sensitization. It caused sluggish movement, shallow breathing, and cyanosis of mice. Eventually, 15 mice died (Figure 2A) as a result of either circulatory collapse due to profound shock (Figure 2B) or asphyxia due to airway edema or mucus plugging (Figure 2C). Apparently, postnatal OVA injection triggered a catastrophic type I hypersensitivity.

Immunological Profiles Following Fetal or Neonatal OVA Exposure

To further study immunological consequences of *in utero* OVA exposure, we injected 0.05–1,000 μ g of aluminum-free OVA prenatally. Increased doses worsened offspring survival, with 1,000 μ g of OVA being entirely incompatible with life (Figure 3A). When mice were 4–6 weeks old, serum OVA-IgG1/IgE was not significantly detected until a dose of 5 μ g of OVA was reached (Figure 3B). At follow-up when mice were 2–4 months old, OVA-IgE persisted and was even significantly increased (Figure 3C). Endotoxin-free OVA (50 μ g) still hypersensitized the fetus to generate OVA-IgE (see Figure E1A in the online supplement), indicating the irrelevance of endotoxin contamination, if any, in OVA inocula to fetal immune reaction. GD16–GD17 and neonatal

(1–2 days old) exposure to 50 μ g of OVA elicited OVA-IgE in FVB/N or BALB/c mice. Neither adulthood nor maternal intraperitoneal injection of high-dose OVA (1 and 0.5 mg) induced OVA-IgE in adult recipients, dams, and offspring (Figure E1A). Derp II also generated the threshold-dependent patterns of antigen-specific IgE production in FVB/N fetuses (Figures E1B and E1C), indicating that *in utero* sensitization was not limited to OVA. Prenatal coinjection of OVA with sera from OVA-sensitized mice might abolish OVA-IgE secretion. However, this protective effect depended upon the OVA load imposed on the fetus (Figure E1D). Following fetal injection of 50 μ g of OVA, OVA clearance was so efficient that it left a peritoneal OVA residue of less than 5 ng at birth (Figure E2), far less than a dose of 5 μ g of OVA insufficient for neonatal sensitization (Figure E1A). Thus, leftover OVA, if any in neonatal peritoneum after fetal injection, could not cause sensitization. FVB/N mice with significant OVA-IgG1/IgE production had Th2-skewed lymphocytes (Figure 3D) that proliferated specifically to OVA rather than bovine serum albumin stimulation (Figure 3E), indicating the development of highly OVA-specific adaptive immunity. FVB/N mice without significant OVA-IgG1/IgE production were free from anaphylaxis following intraperitoneal OVA injection and remained responsive to adulthood OVA sensitization (Figure E3), representing the lack of a tolerance state to OVA.

Leukocyte Development of Mouse Fetuses

The unintended consequence of *in utero* sensitization to OVA impelled us to reappraise leukocyte development of GD14 murine fetuses by flow cytometry (Figure

E4A). We observed minimal expression, if any, of CD45R, but no expression of CD3 or CD11c in the fetus. The expression levels of F4/80, Gr-1, and major histocompatibility complex (MHC) classes I and II were organ specific at that time, mainly in the liver and thymus. CD4⁺CD8⁺ thymocytes did not emerge until GD17 (Figure E4B).

Postnatal Oral or Aerosolized OVA Stress

To mimic the human experience of allergen contact in airway allergy, we subjected prenatally OVA-primed mice to oral or aerosolized OVA challenge. Postnatal OVA gavage caused fatal anaphylaxis (Figure E5). Postnatal aerosolized OVA stress did not induce anaphylactic shock but boosted Th2 immune reactions (Figures 4A and 4B and Figure E6). It triggered allergic lungs with eosinophilic inflammation and airway structural alterations (Figures 4C–4E) in association with marked increment of airway enhanced pause (Figure 4F) and resistance (Figure 4G) to bronchospastic stimuli.

Antigen Uptake by Fetal Phagocytes

Using fluorescent conjugates of OVA to track injected OVA, we found that GD14 murine fetuses had been equipped with phagocytes capable of taking up OVA and migrating systemically (Figure E7A). OVA-laden cells showed a macrophage-like morphology (Figure 5A) and rapidly inhabited in the spleen, which developed on GD17 (Figure E7B). OVA endocytosis of fetal phagocytes necessitated a threshold level of OVA and displayed a dose-responsive pattern (Figure 5B). With an effective OVA dose, the proportion of OVA-laden cells gradually reached a plateau within 3–5 hours and then continued increasing with prolonged OVA

Figure 4. (Continued). (8 weeks old; *lower panels*) had inflammatory cell infiltration, goblet cell hyperplasia, or significant collagen deposition in the lung. (D) Further OVA inhalation in postnatal life caused peribronchial and perivascular inflammation (*upper left panel*) with abundant eosinophils featuring bilobed nuclei and eosinophilic cytoplasmic granules (*lower left panel*). Allergic lungs exhibited airway structural alterations (*upper middle panel*). *Lower middle panel* images represent magnifications of the boxed regions, showing intraluminal eosinophils (*solid arrows*), Charcot-Leyden crystals (*solid arrowhead*), airway plugging, and epithelial shedding. Periodic acid–Schiff staining at a neighboring section illustrates massive goblet cell metaplasia/hyperplasia (*upper right panel*). Masson's trichrome staining revealed dense subepithelial and peribronchial collagen deposition (*lower right panel*). The airway also showed focal epithelial denudation (*open arrow*) and intraluminal exfoliated epithelia (*open arrowheads*). (E) Fibrosis area of airways on captured images was further quantified in prenatally OVA-primed mice (IU OVA; $n = 5$) and IU OVA mice treated with postnatal NS (IU OVA + IH NS; $n = 5$) or OVA inhalation (IU OVA + IH OVA; $n = 5$). IU OVA + IH OVA compared favorably in integrated intensity of fibrosis with IU OVA and IU OVA + IH NS. (F and G) IU OVA + IH OVA had significantly increased enhanced pause (Penh) and airway resistance under methacholine aerosolization, in contrast to wild-type mice subjected to OVA inhalation (Wild with IH OVA) and IU OVA + IH NS. Five unrestrained mice died at a methacholine level of 30 mg/ml (*) following slowness to absence of spontaneous movement, labored breathing, and cyanosis. All data are shown as 95% confidence intervals for the means. AHR = airway hyperresponsiveness.

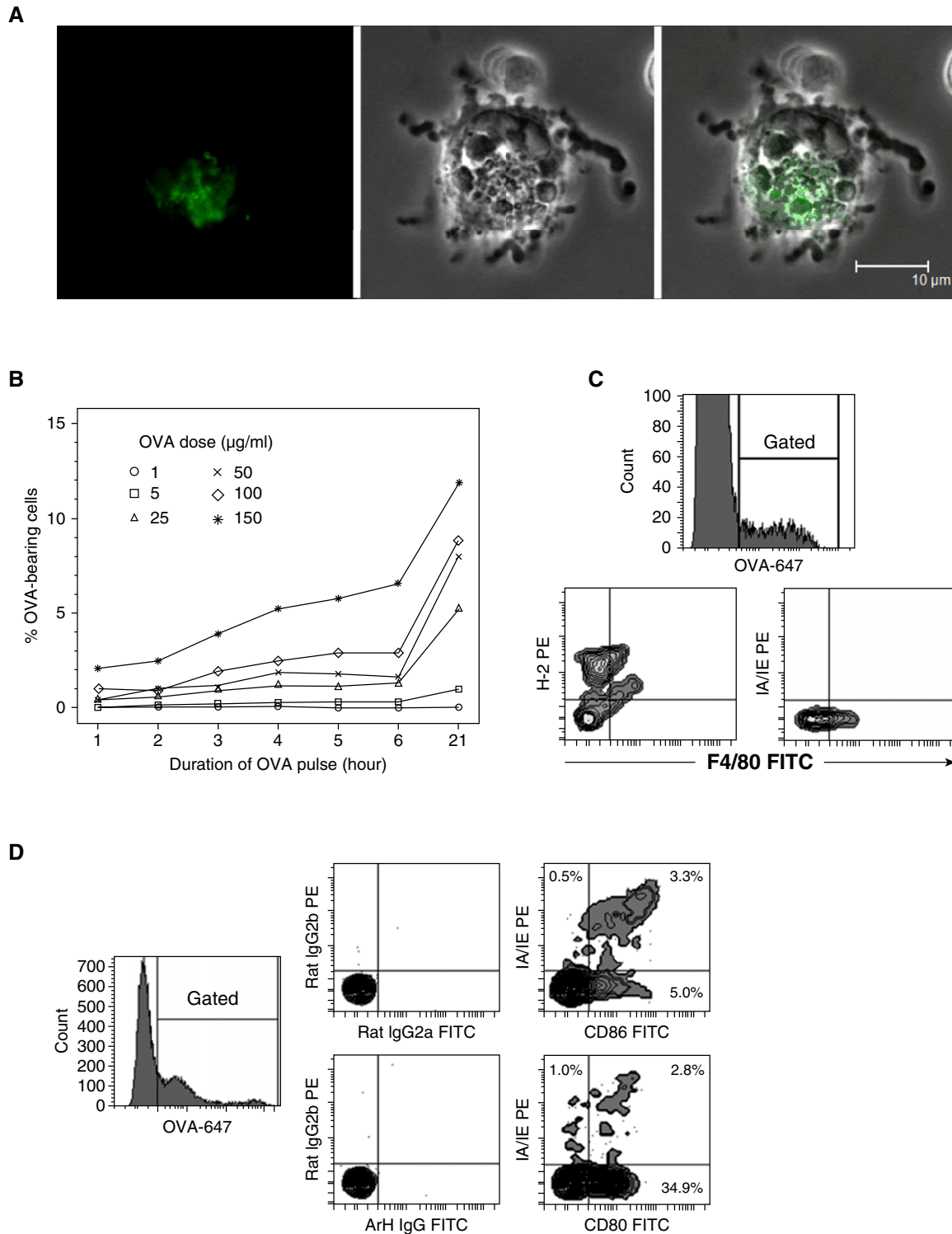


Figure 5. Characterization of fetal phagocytes. Fetal peritoneal cells (5×10^6 cells per milliliter of RPMI 1640 medium) were pulsed with ovalbumin–fluorescein isothiocyanate (OVA-FITC) or Alexa Fluor 647–labeled OVA (OVA-647) *in vitro* and subjected to various examinations. (A) Under a fluorescence microscope, OVA-endocytosing cells exhibited large intracytoplasmic vacuoles as well as ruffles and pseudopod-like processes on the surface, resembling macrophages. (B) After being pulsed with graded doses of OVA-FITC, fetal peritoneal cells were harvested to enumerate OVA-laden cells by flow cytometry at the indicated time points. The fraction of OVA-laden cells was dose responsive when the concentration of pulsed OVA was greater than 5 $\mu\text{g/ml}$. (C) Regardless of F4/80 expression, OVA-laden phagocytes might express major histocompatibility complex class I (H-2) but lacked major histocompatibility complex class II (I-A/I-E) on their surface. (D) Intracellular staining for OVA-laden phagocytes revealed cytoplasmic I-A/I-E and CD80/CD86. ArH = Armenian Hamster; PE = phycoerythrin.

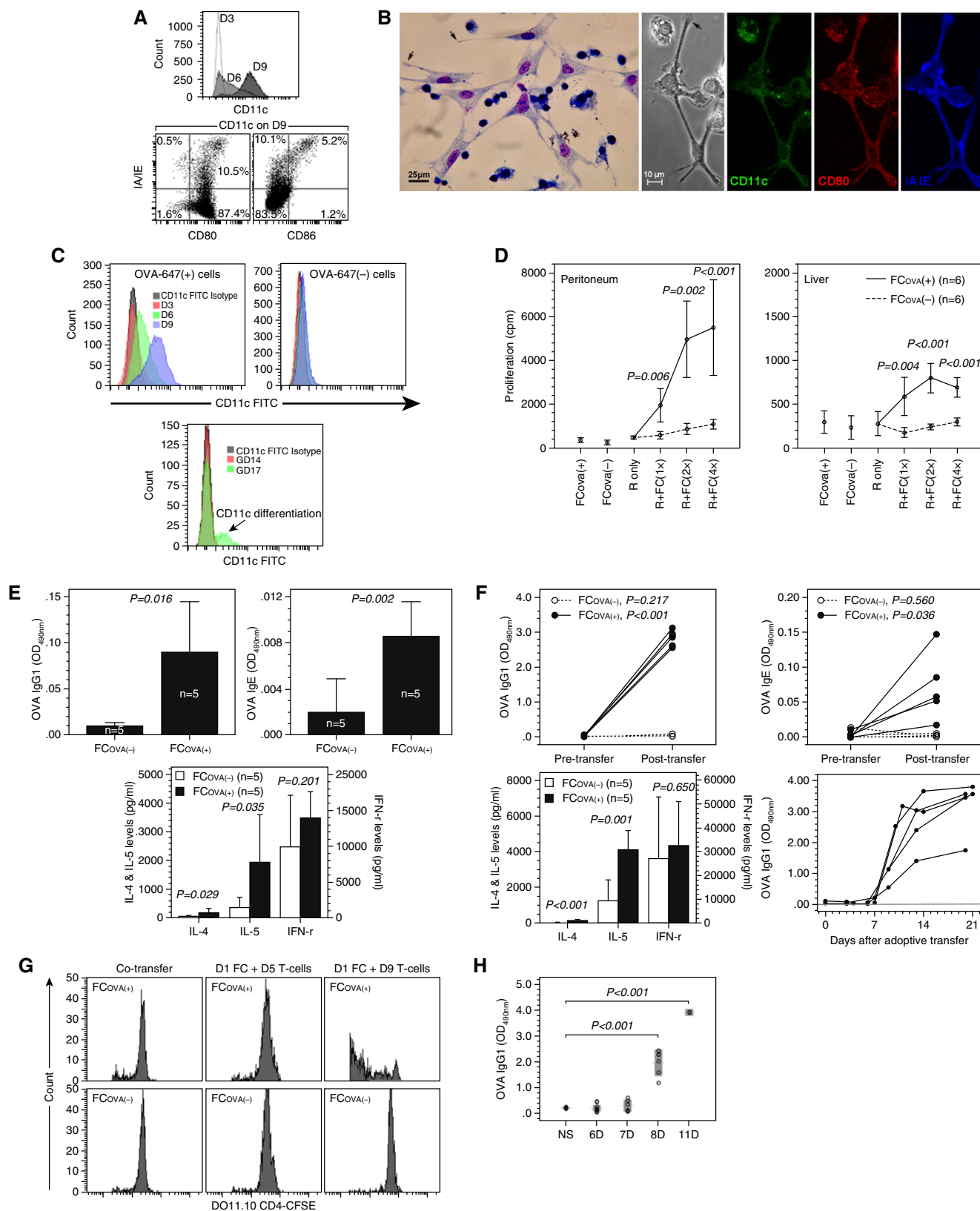


Figure 6. Dendritic cell (DC) differentiation potential of fetal phagocytes and their functional evaluation. (A) Gestational Day 14 (GD14) fetal peritoneal or liver cells were grown in granulocyte-macrophage colony-stimulating factor (GM-CSF). CD11c⁺ cells gradually appeared after 3-day culture (D3) (from D3 to D9) and simultaneously expressed major histocompatibility complex class II I-A/I-E (IA/IE) and costimulatory molecules of CD80 or CD86 (D9), phenotypically equivalent

exposure. OVA-laden phagocytes lacked characteristic phenotypes of professional antigen-presenting cells (APCs), except for a minority of them expressing F4/80 (Figure E7A). Notably, they lacked surface MHC class II, despite the possibility that they might express surface MHC class I (Figure 5C) or intracellular MHC class II (Figure 5D).

DC Differentiation Potential and Functional Evaluation of Fetal Phagocytes

We examined whether fetal phagocytes had DC differentiation potential. Under the aegis of granulocyte-macrophage colony-stimulating factor, fetal peritoneal or liver cells might differentiate into CD11c⁺I-A/I-E⁺CD80/86⁺ stellate-like cells (Figures 6A and 6B), morphologically and phenotypically compatible with DCs. Further pulse-chase analyses either *in vitro* or *in vivo* showed that OVA-laden phagocytes, negative for Flt3 but partly positive for FcγRIII/II/I (Figure E7C), gave rise to CD11c⁺ cells (Figure 6C), indicating a DC origin from Flt3-independent phagocytic progenitors after endocytosis. In addition to CD11b, F4/80, CD205, CD209, and CD16/32, fetal phagocyte-derived CD11c⁺ cells expressed langerin and E-cadherin (Figure E8), suggesting that they belonged to the family of epidermal Langerhans cells (10, 11).

To examine whether fetal phagocytes could function as APCs, we subjected fetal cells to OVA exposure and evaluated their capacity for OVA presentation. OVA-pulsed fetal cells elicited proliferation of CD4 lymphocytes with T-cell receptors specific to OVA peptide–MHC class II

complexes (Figure 6D). Furthermore, their adoptive transfer to fetal mice induced OVA-IgG1/IgE production and Th2-skewed lymphocyte differentiation (Figure 6E), indicating their pivotal role in hypersensitizing the fetus. In adult recipients, OVA-pulsed fetal cells triggered comparable responses and OVA-IgG1 steadily appeared about 9–10 days after cell transfer (Figure 6F). Antigen presentation activity evaluated on the basis of T-cell proliferation ensued 8 days later (Figure 6G). Consistently, OVA-IgG1 significantly emerged 8 days after GD14 injection of OVA (Figure 6H).

Attenuated OVA Proteolysis in Fetal DC Progenitors

Delayed antigen presentation might result from DC regulatory capacity for antigen processing, involving antigen intracellular transportation and proteolytic activity within lysosomes (12). In fetal phagocytes, internalized OVA were rapidly localized to lysosomes, some of which even contained MHC class II (Figure 7A). Lysosomal activity of OVA proteolysis was investigated by self-quenched DQ-OVA, which does not show bright green fluorescence until proteolytic degradation (13). We found that *in vivo* degradation of internalized DQ-OVA was not entirely an instant event in fetal phagocytes (Figure 7B). Pulse-chase analyses demonstrated delayed OVA degradation in fetal phagocytes, as opposed to rapid OVA degradation in CD11c⁺ DCs generated from fetal phagocytes (Figure 7C). Notably, OVA degradation could be facilitated when the milieu was acidified down to pH 4.5 (Figure 7D).

Discussion

It is not at all rare to see adult-level T-cell responses following fetal (14) or neonatal (15–17) exposure to foreign antigens. In a convenient way, researchers have usually ascribed this result to the misjudgment of an appropriate tolerization window for antigen exposure or stepwise expansion of an immunological repertoire during the gestational period and even after birth (14, 18). GD14 murine fetuses lacked CD3⁺T cells with their thymocytes at the CD3⁺CD4⁺CD8⁺ immature preselection stage. By GD17, the emergence of CD4⁺CD8⁺ thymocytes coincided with the beginning of thymocyte selection processes (19). Thus, murine GD14 should have been an immunologically privileged window in ontogeny for the establishment of self-tolerance to OVA, resembling allotolerance induction following allogeneic marrow injection (7, 8). However, murine fetuses did not tolerate OVA but rather developed adaptive immunity, evidenced by heightened recall responses upon postnatal OVA reexposure. Although deletional tolerance demands high-dose self-antigens (20), high-dose OVA (500 μg) remained immunogenic *in utero*. Again, our results challenged the paradigm of actively acquired tolerance.

Atopy characterized by allergen-specific Th2 immunity is an important risk factor for asthma (21). Evidence of transplacental allergen transfer (22, 23), together with allergen-specific IgE and T-cell memory in cord blood (21), strongly correlated atopy with fetal sensitization to allergens. Paradoxically, maternal allergen avoidance lacked protective effects on

Figure 6. (Continued). to DCs. A significant number of I-A/I-E-negative CD11c cells could be DCs that were in an immature state. (B) D9 cells on coverslips were stained with Diff-Quik (Sysmex, Kobe, Japan) or immunofluorescence antibodies. The adherent cells were stellate in shape and weakly stained basic. They had large nuclei with two to four nucleoli; exhibited long and fine cell processes with blunt terminations (arrows); and simultaneously expressed surface CD11c, I-A/I-E, and CD80. (C) Fetal peritoneal cells were fully pulse labeled with Alexa Fluor 647-labeled ovalbumin (OVA-647) for 3 days and grown in RPMI 1640 medium supplemented with GM-CSF. Antigen-laden phagocytes (D6 and D9) gave rise to CD11c⁺ cells (upper panels). CD11c isotype controls were D6 or D9 cells. GD14 fetal cells pulse labeled with OVA-647 were transferred intraperitoneally into fetuses with identical gestational age. In contrast to the supraoptimal *in vitro* conditions with GM-CSF, the *in vivo* milieu permitted CD11c differentiation only in 7.4–11.3% of OVA-laden phagocytes recovered from GD17 fetal peritoneum (lower panel; n = 3). CD11c isotype controls were GD17 cells. (D) In mixed lymphocyte reactions, BALB/c OVA-pulsed fetal peritoneal or liver cells (FC_{OVA(+)}) elicited proliferation of DO11.10 CD4 T cells (R) by tritiated thymidine incorporation. Controls were fetal cells maintained in OVA-free medium (FC_{OVA(-)}). Adoptive transfer of FVB/N FC_{OVA(+)} cells to (E) FVB/N fetuses and (F) FVB/N adults significantly elicited OVA-IgG1/IgE measured within 1–2 months after cell transfer and caused T-helper cell type 2 cytokine secretion in OVA-stimulated lymphocyte culture. A time-course analysis of OVA-IgG1 in adult recipients (lower right panel) disclosed that OVA-IgG1 did not readily appear until 9–10 days after cell transfer. (G) Proliferation of DO11.10 CD4 cells labeled with carboxyfluorescein succinimidyl ester (CFSE) was examined 3 days after their injection. DO11.10 CD4 cells notably proliferated when BALB/c FC_{OVA(+)} cells were transferred to BALB/c recipients 8 days in advance, but they hardly proliferated when BALB/c FC_{OVA(+)} cells were injected simultaneously with or 4 days before the DO11.10 CD4 cells. Nonreactivity of the latter condition negated the phenomenon of “cross-generation presentation,” wherein cytolytic OVA from dead fetal phagocytes was taken up again and presented by recipients’ antigen-presenting cells. (H) Serum OVA-IgG1 was measured 6–11 days (6–11D) after GD14 injection of OVA or normal saline (NS). NS controls were from 6–11D samples. FITC = fluorescein isothiocyanate; OD = optical density.

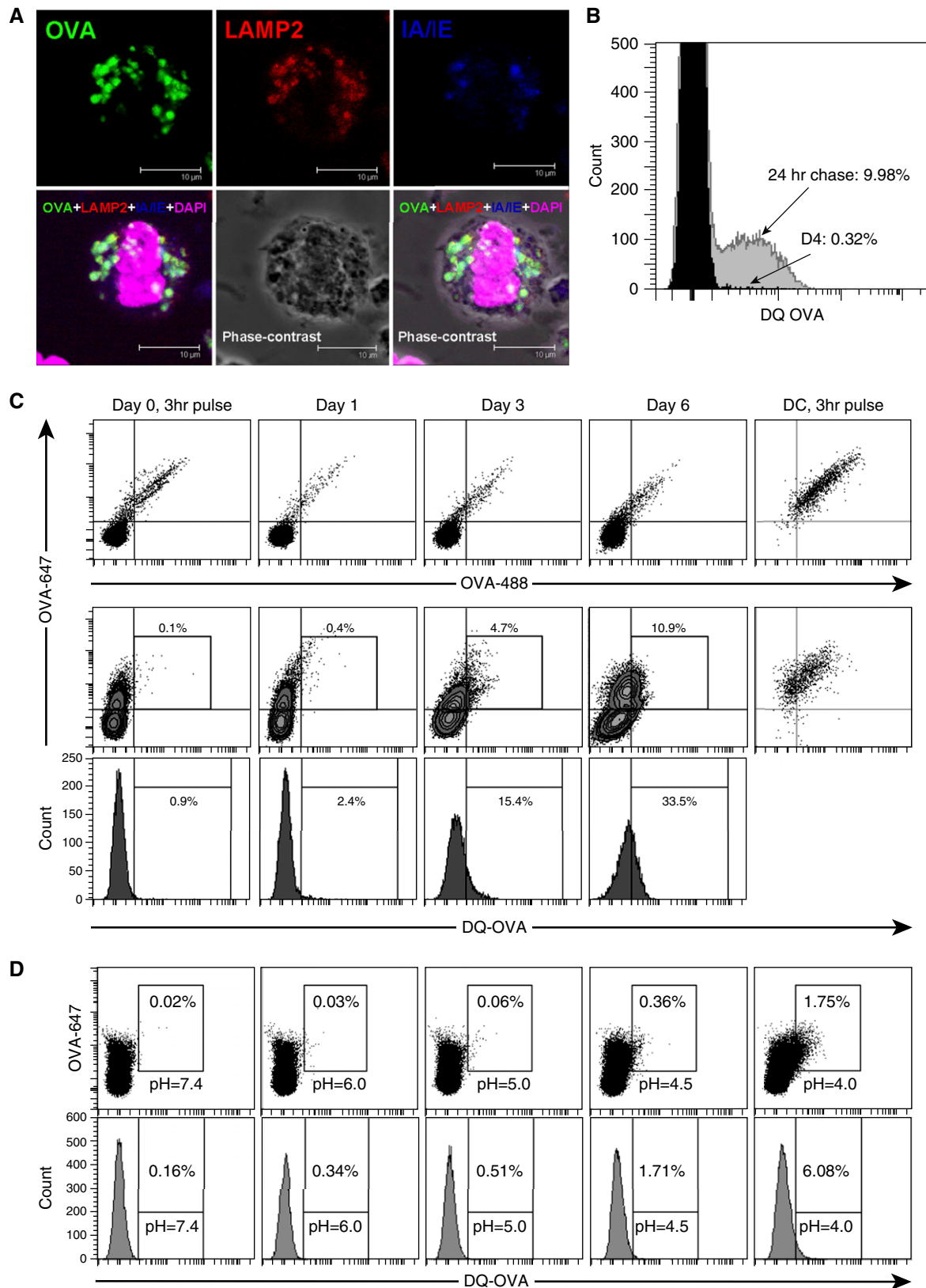


Figure 7. Studies for delayed ovalbumin (OVA) presentation. (A) Confocal images of fetal liver or peritoneal cells pulsed with OVA–fluorescein isothiocyanate for 4 hours and intracellularly stained for lysosome-associated membrane protein 2 (LAMP2)–phycoerythrin and major histocompatibility complex class II I-A/I-E (IA/IE) allophycocyanin showed colocalization of endocytosed OVA and LAMP2. Only parts of LAMP2⁺ lysosomes colocalized with intracellular I-A/I-E.

sensitization in humans (24). To definitively conclude the causal relationship of fetal allergen exposure and atopy necessitates a cohort study with subjects prospectively exposed to allergens. However, it is not an ethically sound approach in humans. Our animal studies provide solid support for this causality, showing that allergic airway diseases might develop as *in utero* imprinting of Th2-skewed atopy followed by repeated aerosolized allergen stress in postnatal life. This process triggered allergic lungs with methacholine-induced increment of airway resistance and bronchoconstriction, as well as airway structural alterations pathognomonic of human asthma (25, 26). Importantly, the prenatal sensitization was limited neither to OVA nor within an anticipated “sensitization window” beyond full T-cell development (27, 28), but it relied upon exceeding a mass concentration threshold of allergens. It fits with the observation that perennial house dust mite allergen of Derp I is more apt than seasonal allergen of *Lolium perenne* I to elicit a proliferative response of cord blood (29). Thus, the failure to trigger offspring’s allergic responses through maternal allergen exposure (30) might result from insufficient transplacental transfer of allergens to which the mother is exposed.

Offspring’s immunoglobulin might be vertically transferred from inadvertently immunized dams due to OVA leakage during fetal injection. Amplification of existing Th2 immunity by postnatal OVA challenge, progressive elevation of offspring’s OVA-IgE, and the failure to elicit OVA-IgE in dams and pups by maternal exposure to high-dose adjuvant-free OVA refuted the maternal origin and supported the development of Th2 immunity in offspring. Th2 immunity has a negative effect on recruitment of CD8 cytotoxic lymphocyte precursors into mature effector cells (31), leading to the

notion that neonatal tolerization is a deviation toward nonprotective Th2 immunity rather than a result of immunological immaturity and inability to mount cytotoxic lymphocyte responses (15, 17). Thus, type II biasing of fetal or neonatal immunity may help tolerization. However, the question remains as to how fetuses mount Th2 adaptive immunity before full immune development. As we know, a full sensitization process necessitates highly specialized APCs, especially DCs for capturing, processing, and presenting of OVA to CD4 T cells and thereby activating the MHC-II-CD4 pathway to direct the immune responses toward adaptive immunity (32). However, the murine DC system required for adaptive immunity does not mature until mice are 5 weeks old, even though thymic CD11c⁺ DCs and CD11c⁺CD45R⁺ plasmacytoid pre-DCs are readily detected on GD17 (19). Our reappraisal of murine leukocyte development affirmed the immaturity of GD14 T-cell and DC systems, compatible with previous reports (19, 33). Thus, how antigen exposure in the absence of competent DCs and T cells triggered adaptive T-cell immunity was inexplicable in the immunological sense.

Murine fetal macrophages up to GD16.5 arise from yolk sac progenitors (34) and give rise to Langerhans cells (35) independently of Flt3 (10, 35). Thus, GD14 Flt3[−] phagocytes were highly compatible with the identity of yolk sac-derived macrophages. They took up prenatally injected OVA regardless of T-cell maturity, which has long been a matter of great concern for immunization. Their OVA endocytosis demanded a threshold dose of OVA, consistent with the threshold-dependent fashion of fetal sensitization to OVA. The dose-responsive relationship between OVA intensity and the proportion of OVA-laden phagocytes might reflect various OVA endocytic thresholds among fetal phagocytes at heterogeneous

developmental stages. Lacking surface MHC class II expression, OVA-laden fetal phagocytes were incompetent at antigen presentation to CD4 T cells. However, functional studies revealed their capacity to present OVA either *in vitro* or *in vivo*. Such a presentation activity by fetal phagocytes necessitated a time lag after OVA endocytosis. Thus, antigens endocytosed by fetal phagocytes could be belatedly but accurately presented to T cells. The ample evidence presented enables us to conclude that fetal phagocytes prenatally internalize antigens, differentiate into DCs, and function properly as APCs responsible for bridging toward adaptive T-cell immunity later in postnatal life.

In transfer studies, DCs and T cells rapidly localize to peripheral lymphoid organs within 20 hours and then initiate T-cell proliferation within 1 day after homing (36, 37). We showed that OVA-laden fetal phagocytes migrated systemically within hours and swiftly inhabited in the spleen once it developed. Thus, this lengthy presentation process in our transfer studies could not be ascribed to cell homing to peripheral lymphoid organs plus the latent period from DC-T-cell interaction to the beginning of T cell proliferation, but rather most likely is related to prolonged antigen processing of DC progenitors. Delayed presentation of internalized antigens was not unique to fetal DC progenitors. Immature DCs sequester endocytosed antigens for extended periods (hours to days) and postpone antigen presentation until their maturation (12, 38–41). Conceivably, DC progenitors would hold endocytosed antigens longer than immature DCs, with an estimated duration of 8–10 days in our studies. It is consistent with the timing of competent T-cell emergence around neonatal stage (15–17) to complete the MHC class II-CD4 pathway following GD14 OVA injection. Like early immature DCs, fetal DC progenitors rapidly delivered

Figure 7. (Continued). (B) Four days (D4) after GD14 injection of 50 μ g of DQ-OVA, peritoneal cells were collected and analyzed by flow cytometry immediately and after 24-hour chase *in vitro*. Cells that degraded DQ-OVA increased from 0.32% (black) to 9.98% (gray), in support of OVA sequestration. (C) Fetal peritoneal cells were pulsed with an equal amount of Alexa Fluor 647-labeled ovalbumin (OVA-647)/Alexa Fluor 488-labeled ovalbumin (OVA-488) or OVA-647/DQ-OVA for 3 hours and chased in granulocyte-macrophage colony-stimulating factor (GM-CSF)-containing RPMI 1640 medium at pH 7.4. Fetal phagocytes simultaneously and persistently expressed both OVA-647 and OVA-488, but slowly degraded and expressed DQ-OVA, in sharp contrast to fetal phagocyte-derived CD11c⁺ dendritic cells generated with GM-CSF, which rapidly degraded and expressed DQ-OVA. (D) OVA-647/DQ-OVA-laden fetal cells were chased in RPMI 1640 medium at the indicated pH values for 30 minutes. Acidification down to pH 4.5 initiated OVA degradation. Degradation was more efficient at pH 4.0. Fetal phagocytes gated as OVA-647⁺ are displayed in histograms (C and D) that give the phagocytes’ fraction to degrade DQ-OVA. All data shown were representative of three independent experiments. DAPI = 4’,6-diamidino-2-phenylindole.

internalized OVA to degradative compartments where MHC class II could be stored (40). Therefore, the access of internalized antigens to lysosomes was not the barrier to antigen processing observed in immature DCs (12, 39, 41). We further showed that OVA-laden DC progenitors did not degrade OVA until their environment was acidified down to pH 4.5, consistent with the strict requirement of lysosomal acidification at pH at or below 4.5 for OVA degradation in immature DCs (12). Apparently, the restricted capacity for lysosomal acidification rather than immaturity of lysosomal enzyme system was responsible for attenuation of OVA proteolysis, endowing fetal DC progenitors with the ability to sequester the antigens endocytosed and thereby effectively retaining the memory of antigens internalized in prenatal life to avoid premature presentation before full T-cell development.

Macrophage-like cells and DCs first appear at 5 weeks (42) and 9 weeks (43) estimated gestational age in humans but at GD10.5 (34) and GD17 (19) in mice. Considering macrophage-DC ontogeny, GD14 murine fetuses correspond approximately to human embryos of 5–9 weeks estimated gestational age. Thus, human fetuses might remain at risk of sensitization to transplacental allergens before mature T cells emerge at the end of the first trimester (33). In this study,

prenatal sensitization by a single shot of adjuvant-free OVA was much less demanding than adulthood sensitization, which necessitated repeated injections of aluminum-emulsified OVA. It strengthened the notion that antigen-laden DC progenitors were more immunogenic than well-differentiated DCs pulsed with antigens (44). This phenomenon might be attributed to limited proteolytic potential of DC progenitors, which favors antigen accumulation, processing, and then dissemination throughout the immune system to enhance antigen presentation and immunogenic T-cell responses (45).

In humans, absorbed OVA exists in free or immune-complex form (46). Both can cross the placenta (47, 48) and initiate fetal sensitization (33, 48, 49). *In utero* injection enables quantitative study of fetal OVA responses across placental barriers but bypasses maternal immune interactions with OVA. We showed that sera of allergen-sensitized mice might protect fetuses from allergic sensitization by raising the allergen threshold required for initiating sensitization. Although the exact humoral factor responsible for this protection remains obscure and awaits further experimental elucidation, FcγRIII/II/I expression on some GD14 phagocytes might have functional implications for binding to transplacental maternal IgG before the emergence of immunoglobulin on GD15 B cells (50).

Conceivably, it might endow fetal phagocytes with the ability to focus maternal IgG–antigen complexes to facilitate antigen clearance or downstream immune regulation.

Contrary to actively acquired tolerance, immunologically immature murine fetuses could not be “tricked” into tolerating OVA, but rather developed OVA-specific Th2 immunity. Such an event of *in utero* sensitization was initiated by fetal phagocytes that sequestered internalized OVA for delayed presentation in postnatal life. This specialty might result from their restricted capacity for lysosomal acidification to attenuate proteolysis of endocytosed OVA. Although allergens could be actively or selectively transported across the placenta (22) and variably detected in cord blood or amniotic fluid (23), we further proved that fetal sensitization necessitated a threshold of allergen exposure. Our results point the way to new mechanistic insights into prenatal imprinting of atopy and shed light on the way the immune system deals with antigens before its full maturation. The importance of macrophage-like fetal phagocytes in shaping the developing immune system and initiating allergic airway diseases cannot be overstated. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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