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Androgen and Androgen Receptor as Enhancers of M2 Macrophage Polarization in Allergic Lung Inflammation

Mireya Becerra-Díaz,* Ashley B. Strickland,* Aleksander Keselman,* and Nicola M. Heller*,†

Allergic asthma is a disease initiated by a breach of the lung mucosal barrier and an inappropriate Th2 inflammatory immune response that results in M2 polarization of alveolar macrophages (AM). The number of M2 macrophages in the airway correlates with asthma severity in humans. Sex differences in asthma suggest that sex hormones modify lung inflammation and macrophage polarization. Asthmatic women have more M2 macrophages than asthmatic men and androgens have been used as an experimental asthma treatment. In this study, we demonstrate that although androgen (dihydrotestosterone) reconstitution of castrated mice reduced lung inflammation in a mouse model of allergic lung inflammation, it enhanced M2 polarization of AM. This indicates a cell-specific role for androgens. Dihydrotestosterone also enhanced IL-4-stimulated M2 macrophage polarization in vitro. Using mice lacking androgen receptor (AR) in monocytes/macrophages (AR^{fllox}LysMCre), we found that male but not female mice exhibited less eosinophil recruitment and lung inflammation due to impaired M2 polarization. There was a reduction in eosinophil-recruiting chemokines and IL-5 in AR-deficient AM. These data reveal an unexpected and novel role for androgen/AR in promoting M2 macrophage polarization. Our findings are also important for understanding pathology in diseases promoted by M2 macrophages and androgens, such as asthma, eosinophilic esophagitis, and prostate cancer, and for designing new approaches to treatment. *The Journal of Immunology*, 2018, 201: 000–000.

Allergic asthma is a chronic inflammatory disease of the lungs characterized by an aberrant Th2 inflammatory immune response against an otherwise innocuous stimulus. Th2 cytokines, such as IL-4 and IL-13, and chemokines enhance inflammation, mucus production, airway constriction, and inflammatory immune cell recruitment in the lungs. This Th2 environment contributes to asthma symptoms in humans and in mouse models of allergic lung inflammation. In the noninflamed lung, alveolar macrophages (AM) are the most abundant immune cells in the alveolar space and one of the first cell types in contact with the allergenic stimulus. Th2 inflammation polarizes AM to an M2 phenotype (1), and accumulation of M2-polarized AM in the lung correlates with asthma severity (2). AM secrete eosinophil-recruiting chemokines and cytokines during allergic lung inflammation (3). Lung eosinophilia is a hallmark of allergic asthma, and eosinophils enhance airway hyperresponsiveness and mucus

production (4). Furthermore, human and mouse M2 AM in the asthmatic lung produce excess remodeling and inflammatory factors that decrease lung function and aggravate asthma severity (5).

Asthma outcome in humans and animal models is influenced by biological sex. The incidence and severity of asthma is greater in women than in men (6) and in female compared with male mice (7, 8). However, before puberty, boys are more likely to have asthma than girls (9). This change in the incidence of asthma suggests that sex hormones play a key role in affecting the disease. Previously, we had demonstrated that AM from female mice more highly expressed genes characteristic of M2 polarization than male macrophages. This was shown both in a mouse model of allergic lung in vivo and following IL-4 stimulation in vitro. We showed that estrogen (E2) augmented M2 gene expression, and this was dependent on E2 receptor α (ER α). The incidence of asthma between adult men and women suggests that the increase in male sex hormones after puberty may have the opposite effect to E2 and control asthma.

Testosterone and other androgens, such as dihydrotestosterone (DHT), have broad immunoregulatory effects that suppress immune responses (10). Male mice produce less Th2 cytokines and specific IgE and have fewer lung lymphocytes after OVA sensitization and challenge in the asthma model (8). Castrated mice have increased OVA-induced eosinophil and lymphocyte infiltration in the bronchoalveolar lavage fluid (BALF) (11). Experimental use of androgens improved asthma symptoms in up to 88% of women with premenstrual asthma (12). The mechanisms by which androgens improve asthma symptoms or severity are poorly understood. Androgens induce relaxation of airway smooth muscle (13), and Laffont et al. (14) showed recently that androgens decrease proliferation of group 2 innate lymphoid cells (ILC2) in the lung. However, the role of androgens in macrophage polarization and function in the lung is unknown. Given the positive correlation between M2 macrophages in the lung and asthma severity and the lower incidence of asthma in men than in women,

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Abbreviations used in this article: AM, alveolar macrophage; AR, androgen receptor; BALF, bronchoalveolar lavage fluid; BMM, bone marrow macrophage; DC, dendritic cell; DHT, dihydrotestosterone; E2, estrogen; ER α , E2 receptor α ; ILC2, group 2 innate lymphoid cell; MFI, mean fluorescence intensity; MHC-II, MHC class II; PAS, periodic acid–Schiff; PCa, prostate cancer; qPCR, quantitative PCR; TAM, tumor-associated macrophage.

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we hypothesized that androgens, unlike E2, suppress M2 polarization of macrophages. Therefore, in this study, we aimed to determine the role of androgens in macrophage polarization.

Materials and Methods

Animals

Six-week-old C57BL/6J wild-type (strain number 664) and LysMCre (strain number 004781 - B6.129P2 *Lyz2*^{tm1(cre)lfo}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Castrated male C57BL/6 mice (3-wk-old) were purchased from Charles River Laboratories (Germantown, MD). AR^{fllox} mice on a C57BL/6 background, generated by Karel De Gendt (15), were obtained from the European Mutant Mouse Archive (Rome, Italy), were rederived by the Johns Hopkins Transgenic Core, and bred in our facilities. All mice were housed in the Johns Hopkins University mouse facility, and all experiments were conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee.

Genotyping of AR^{fllox} and AR^{fllox}LysMCre mice

Genomic DNA from the AR^{fllox} and AR^{fllox}LysMCre mice was isolated from ear tissue using the REDExtract-N-Amp Tissue PCR Kit Protocol (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Primers used for genotyping were AR28: 5'-AGCCTGTACTACAG-TTGGGG-3' (16), AR29: 5'-AATGCATCACATTAAGTTGATACC-3' (16), LysMCre mutant: 5'-CCCAGAAATGCCAGATTACG-3', LysM wild-type: 5'-TTACAGTCGGCCAGGCTGAC-3', and LysM common: 5'-CTTGGGCTGCCAGAATTCTC-3'. PCR products were resolved on a 3% agarose gel, and DNA bands were visualized by ethidium bromide staining.

Mouse allergic sensitization and challenge

We used the protocol of Wang et al. (17) for OVA-induced allergic lung inflammation. Briefly, 8-wk-old mice were sensitized by i.p. injection with 100 µg OVA (Sigma-Aldrich) emulsified in 100 µl of Imject Alum (ThermoFisher Scientific, Grand Island, NY) on days 1 and 6. Next, mice were exposed to an airway challenge with nebulized OVA (1% in PBS) for 40 min on days 12 and 14. On day 16, mice were anesthetized with an i.p. injection of 2.5% Avertin (2,2,2-tribromoethanol; Sigma-Aldrich) to collect BALF, serum, and lung tissue for further analysis. For the DHT reconstitution experiments, we implanted DHT-releasing (Sigma-Aldrich) or placebo pellets s.c. into castrated mice on day 7 of the OVA protocol to avoid interfering with T cell priming.

Bone marrow macrophage and AM culture

Bone marrow macrophages (BMM) from 5–6-wk-old male and female C57BL/6 mice were grown as published previously (18). Briefly, bone marrow cells were seeded in phenol red-free MEM Alpha containing 40 ng/ml of recombinant mouse M-CSF (Gemini Bio-Products, West Sacramento, CA) and cultured for 10 d prior to experimentation. BMM grown in M-CSF were used for all experiments, except for Fig. 2B–D as noted below.

For DHT pretreatment experiments, cells were incubated with indicated concentrations of DHT overnight prior to stimulation for 48 h with 1 ng/ml IL-4 (R&D Systems, Minneapolis, MN) in phenol red-free MEM Alpha with 10% charcoal-stripped FBS and 40 ng/ml mouse M-CSF.

For development of macrophages with an “AM-like” phenotype (Fig. 2B–D), bone marrow cells were collected as described and seeded onto six-well plates and cultured in MEM Alpha containing 40 ng/ml of recombinant mouse M-CSF or GM-CSF (Gemini Bio-Products) for 10 d prior to experimentation.

For differentiation of dendritic cells (DC), bone marrow cells were collected as described, seeded onto 10-cm dishes, and cultured in MEM Alpha containing 20 ng/ml of recombinant mouse GM-CSF (Gemini Bio-Products) and 40 ng/ml of recombinant mouse IL-4 (R&D Systems). After 10 d of culture, nonadherent cells were collected for flow cytometric analysis.

AM were enriched from BALF as previously reported (18). Briefly, BALF cells were placed in MEM Alpha for 2 h at 37°C in 5% CO₂. AM were isolated by adhesion in culture, and eosinophils and lymphocytes were removed by washing with cold PBS.

For androgen receptor (AR) degradation, cells were incubated with ASC-J9 (10 µM; Advanced ChemBlocks, Burlingame, CA) for 24 h before surface and intracellular staining for flow cytometry.

For the flutamide inhibition of AR experiments, BMM cultured for 10 d were pretreated with 10 µM flutamide (Sigma-Aldrich) for 2 h before adding DHT (10 nM). After 18 h, cells were stimulated with 1 ng/ml IL-4 (R&D Systems) for 48 h in MEM Alpha with 40 ng/ml mouse M-CSF. After this time, cells were lysed for mRNA analysis by quantitative PCR (qPCR) as described below.

Proliferation of AM by BrdU incorporation

AR^{fllox} and AR^{fllox}LysMCre mice were injected i.p. with 1 mg of BrdU (BD Biosciences, San Jose, CA) on days 1, 3, and 5. Also, 500 ng of rmIL-4 was delivered intratracheally on day 5 to all mice to stimulate proliferation. On day 8, mice were anesthetized with an i.p. injection of 2.5% Avertin as previously described, and BALF was collected as previously described. AM were enriched from BALF as previously reported (18). BrdU incorporation was measured as an index of AM proliferation as indicated in the kit and analyzed by FACS.

qPCR

mRNA from AM or BMM was collected in RLT buffer and processed with the RNeasy Mini Kit (Qiagen, Valencia, CA). qPCR was carried out with the indicated primers in a 7500 Fast Real-Time PCR instrument from Applied Biosystems (Grand Island, NY). The primer sequences used for M2 genes have been published previously (19). For qPCR analysis, we obtained AM by 2-h adherence on six-well culture plates. Nonadherent cells were removed after this time by washing with PBS.

FACS

Cells collected from the BALF were stained as previously published (18) for LIVE/DEAD, CD11c-PECy7 (clone N418), Ly6G-APCCy7 (clone 1A8), Ly6C-PerCPy5.5 (clone HK1.4; BioLegend, San Diego, CA), and Siglec F-BV421 (clone E50-2440; BD Biosciences). For intracellular YM1 (STEMCELL Technologies, Vancouver, BC) or AR (Cell Signaling Technology, Danvers, MA) detection, cells were stained with a secondary PE-anti-rabbit Ab (BioLegend). For the DC stain, CD209a-PE (clone MMD3), CD80-PE (clone 16-10A1), and CD86-Alexa Fluor 700 (clone GL-1; BioLegend) were used. Stained cells were analyzed with a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA), courtesy of the Anesthesiology and Critical Care Medicine Flow Cytometry Core. CytExpert software v.2.0 (Beckman Coulter) was used for analysis.

DHT and placebo pellets

DHT-releasing and placebo pellets were implanted s.c. in the back of the necks of anesthetized mice on day 7 of the OVA protocol for allergic lung inflammation. The pellets, which consisted of 1-cm-long standard silicone tubing (Helix Medical, Carpinteria, CA), contained compressed DHT (Sigma-Aldrich) or placebo and were sealed with A-100 medical silicone adhesive (Factor II, Lakeside, AZ). Pellets were prepared in our laboratory and disinfected before implantation.

As an indicator of DHT release, mice were weighed before pellet implantation and at the end of the experiment at sacrifice. Seminal vesicles were also collected and weighed at the end of the experiment as a bioassay for the systemic release of DHT. Differences in weight among groups were calculated and plotted.

Western blotting

Equal volumes of denatured BALF from the different experimental groups were loaded onto 4–20% Criterion TGX polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) for electrophoresis under reducing conditions. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories) and probed with Abs against YM1 (STEMCELL Technologies) and FIZZ1 (Abcam, Cambridge, MA). Secondary Abs conjugated to HRP were used to detect target proteins, which were visualized with the ECL method (Bio-Rad Laboratories). Chemiluminescence from the blots was captured using a KwikQuant Imager (Kindle Biosciences), and target band intensities were measured using the Image Studio Lite software (LI-COR Biosciences, Lincoln, NE).

ELISA

We determined levels of CCL24, YM1, and CCL5 in BALF samples by ELISA (R&D Systems) using the manufacturer's protocols. For total IgE quantification, we collected blood by cardiac puncture and centrifuged it to obtain serum. Total serum IgE was measured with the OptEIA kit from BD Biosciences. We determined the concentration of testosterone

(Immuno-Biological Laboratories, Minneapolis, MN) in mouse serum according to the manufacturer's instructions.

Histology

Lung sections were obtained as previously reported (18, 20) and stained with periodic acid–Schiff (PAS), H&E, and Masson's Trichrome by the Molecular and Comparative Pathobiology Histology Laboratory at the Johns Hopkins University School of Medicine. Samples were number coded to reduce experimenter bias and were counted by an independent examiner blinded to the experimental groups. Cells that were positive for PAS were counted in five randomly chosen alveoli from each sample and normalized to the pixel circumference of each selected alveolus. The data were then represented as PAS⁺ cells per 1000 pixels. For the H&E analysis, multiple images of alveolar spaces were taken for each mouse. Cell infiltration was scored on a scale of zero to three. Examples of different inflammation grades and details can be seen in Supplemental Fig. 1C. The infiltration scores for each image were averaged per mouse.

Statistical analysis

GraphPad Prism software (La Jolla, CA) was used for statistical analysis and graph generation. Each experiment was carried out independently at least three times. Mouse experiments contained three to five mice per group and were repeated either two or three times, as indicated. Statistical significance was measured by using a parametric Student *t* test, and a *p* value <0.05 was considered statistically significant. Data are plotted as the mean ± SEM.

Online supplemental material

Supplemental Fig. 1 shows the weight of seminal vesicles, the concentration of serum testosterone after castration with and without DHT reconstitution, and the histologic analysis of cellular infiltration of the mouse lungs, which was scored on a scale of 0–3 with H&E stain. Supplemental Fig. 2 shows MHC class II (MHC-II) and CD86 expression in macrophages and DC. Supplemental Fig. 3 shows quantification of gene expression in IFN- γ -stimulated BMM pretreated with DHT, the effect of the AR antagonist flutamide on DHT enhancement of IL-4-stimulated M2 macrophage polarization in BMM, and the quantification of gene expression in BMM from AR^{fllox} and AR^{fllox}LysMCre male mice stimulated with the indicated concentrations of IL-4 for 48 h. Supplemental Fig. 4 shows a schematic summary of AR-mediated enhancement of M2 macrophage polarization during allergic lung inflammation.

Results

DHT reconstitution of castrated male mice reduces cell recruitment to the bronchoalveolar space but enhances M2 polarization of AM

To determine the role of androgens in lung inflammation, particularly on the polarization of AM, we evaluated lung inflammation in an OVA model of allergic lung inflammation in castrated male mice (Fig. 1A). Mice were castrated at 3-wk-old, prior to sexual maturation to avoid long-term remodeling effects of sex hormones, as has been demonstrated for E2, on chromatin (18, 21). At 7 wk of age before the allergen challenge with aerosolized OVA, mice were implanted with pellets that either released DHT, a potent and nonaromatizable metabolite of testosterone (22), or placebo. Pellets were implanted after the last sensitization to minimize hormone effects on the development of T cell responses, as the timing of ovariectomy/E2 withdrawal affects outcomes in allergic lung inflammation (23). To confirm the systemic activity of DHT, we compared the body mass of the placebo- and DHT-implanted groups (24). Both groups reconstituted with DHT exhibited significantly greater weight gain than the placebo groups (Fig. 1B). As another indicator of the systemic activity of DHT, we compared the weight of the seminal vesicles and quantified serum testosterone and DHT in the mice from the different experimental groups. As expected, DHT pellet implantation induced an increase in the weight of the seminal vesicles and an increase in serum testosterone compared with the placebo-implanted mice (Supplemental Fig. 1A, 1B).

Histological analysis of lung sections from these mice showed no differences in mucus production in airway epithelial cells after

DHT reconstitution (Fig. 1C, top). Although cell infiltration was observed in both OVA groups in the periphery of alveolar spaces by H&E staining, no difference was found with DHT treatment (Fig. 1C, bottom). Total IgE in serum was quantified to examine the effect of DHT on Ig production from B cells. The DHT–OVA group produced less total IgE than did the placebo–OVA group (Fig. 1D).

Next, we analyzed the cellularity, composition, and polarization status of AM in the BALF. We focused on AM in particular because only AM showed changes in macrophage polarization from our previous work in this model (18). Live CD45⁺ cells were identified as AM (CD11c⁺Siglec F⁺), eosinophils (CD11c⁺Siglec F⁺), monocytes (Ly6C⁺Ly6G⁺), and neutrophils (Ly6C^{low}Ly6G⁺) (Fig. 1E). AM were also highly autofluorescent CD64⁺, CD11b⁺, Ly6C⁺, Ly6G⁺, CD24⁺, and MHC-II^{low} cells (data not shown). As expected, the total number of cells in the BALF was significantly increased in the placebo–OVA group (Fig. 1F). DHT reconstitution reduced BALF cellularity in the OVA group, consistent with a regulatory role for androgen in suppressing inflammation in the lung [(13); Fig. 1F]. Eosinophils were the major cell type recruited into the BALF after challenge (Fig. 1G, left). DHT reconstitution reduced eosinophils in the BALF after OVA challenge (Fig. 1G, left), and there was no change in the number of AM (Fig. 1G, right).

To examine the effect of DHT on polarization of AM, we measured expression of intracellular YM1, a canonical M2 marker (25), in AM and of secreted YM1 in the BALF. YM1 is induced in AM (18) and in other cells, including lung epithelial cells (26), by allergic lung inflammation. Furthermore, we and others have reported previously that YM1 is differentially expressed in female and male mice (7, 8, 18). It is expressed more highly in AM from female compared with male mice during allergic lung inflammation (18). DHT reconstitution reduced YM1 in the BALF from OVA-challenged mice (Fig. 1H), consistent with suppression of allergic inflammation by DHT. The higher expression of YM1 in BALF from placebo-implanted mice suggests that other cell types, possibly epithelial cells (26), express more YM1 in the absence of systemic androgen. Previously, we have reported that E2 acting through ER α enhances M2 polarization in macrophages induced by IL-4. The increased production of YM1 observed in female macrophages and also induced by implantation with E2 indicated the importance of E2 in this process. Therefore, we analyzed how androgen reconstitution with DHT affected YM1 production in AM from castrated males and compared this with the YM1 production in intact male and female mice. We found that DHT reconstitution of castrated male mice resulted in the recovery of YM1 expression in AM (Fig. 1I, dark gray bar with diagonal stripes) to a similar degree as that measured in intact male mice (Fig. 1I, checkered bar). However, females from the OVA group had a higher intracellular expression of YM1 in AM (Fig. 1I, black bar) compared with both intact and DHT-implanted mice from the OVA groups. This observation suggests that androgen/AR in males plays a similar role to E2/ER α in females in inducing expression of YM1 and M2 polarization but that E2 has a stronger effect than androgen. Together, the YM1 data indicate that DHT may enhance the overall M2 macrophage polarization program and that DHT may have different effects on macrophages compared with other cell types.

Pretreatment with DHT enhances M2 gene expression, YM1, and FIZZ1 production in BMM

We asked whether DHT enhancement of M2 polarization in AM was a direct effect on macrophages. To address this, we used an

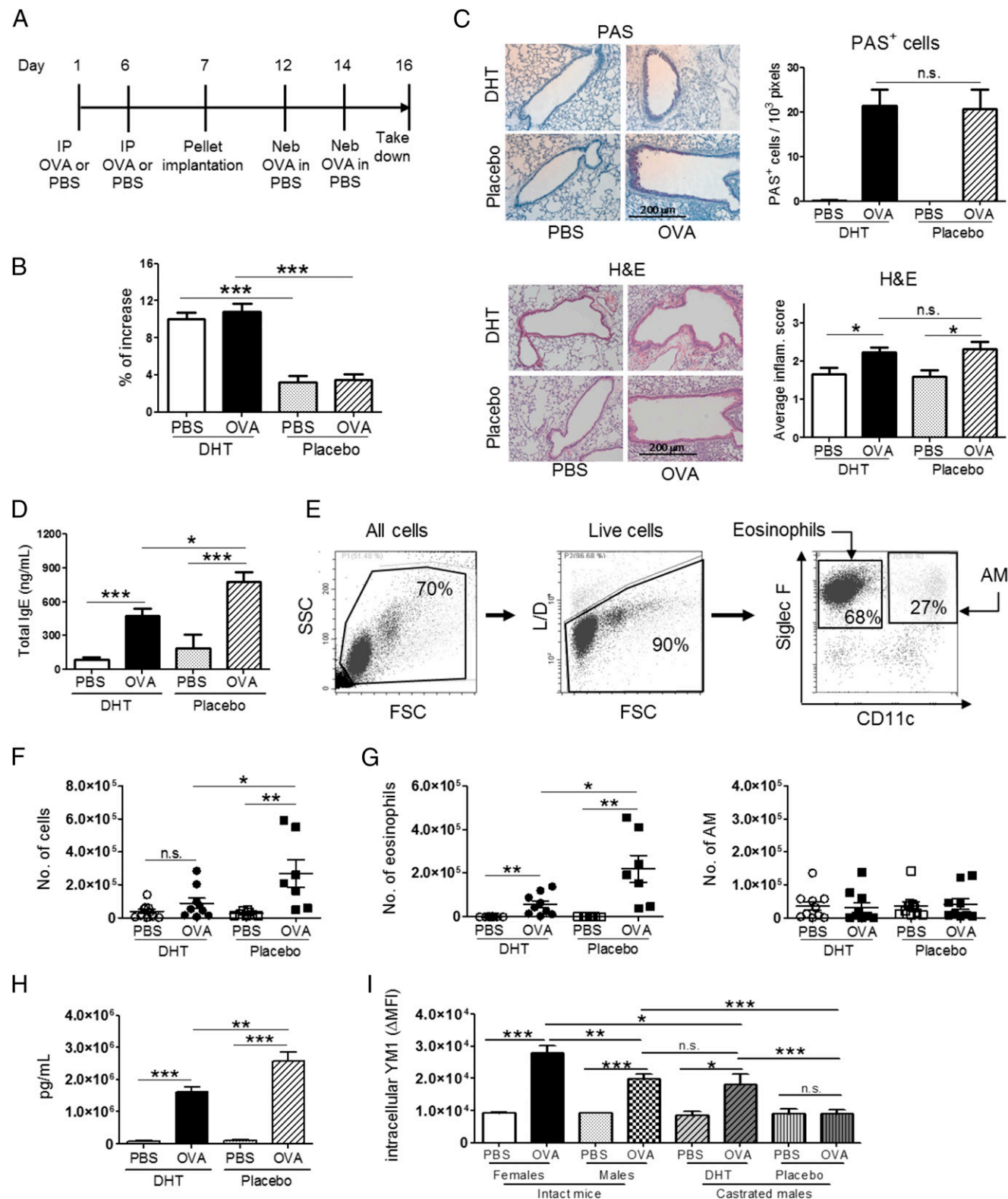


FIGURE 1. Androgen reconstitution of castrated C57BL/6 wild-type mice with DHT-releasing pellets decreases allergic lung inflammation. C57BL/6 male mice were castrated at 3 wk of age. At 7 wk, allergic lung inflammation was induced with the OVA protocol. **(A)** Schematic of the timeline for OVA-induced allergic lung inflammation and pellet implantation. On day 16, mice were anesthetized and BALF was collected. The left lobe from each mouse lung was inflated with 10% formalin and paraffin embedded for sectioning. **(B)** Percentage of change in mouse body weight on day 7 after OVA or PBS. **(C)** Immunohistochemical analysis of lung tissue from DHT- and placebo-implanted mice. Mucus production was measured by PAS staining. Cell infiltration was analyzed by H&E staining. Images were acquired with a 20× objective. PAS⁺ quantification is plotted as PAS⁺ cells per 1000 pixels. H&E graphic represents cell infiltration scored on a scale of 0 to 3 (0 = no inflammation, 1 = light infiltration in only a few areas, 2 = moderate cell infiltration encompassing <50% of the airways and vessels, and 3 = dense cell infiltration encompassing >50% of the airways and vessels). **(D)** ELISA quantification of total IgE in serum from the different implanted groups. **(E)** Gating strategy for BALF cells analyzed by FACS. Total cells (left), live cells (middle), eosinophils (LIVE/DEAD⁻CD11c⁺Siglec F⁺), and AM (LIVE/DEAD⁻CD11c⁺Siglec F⁺) (right). **(F)** Number of total live cells in BALF from (Figure legend continues)

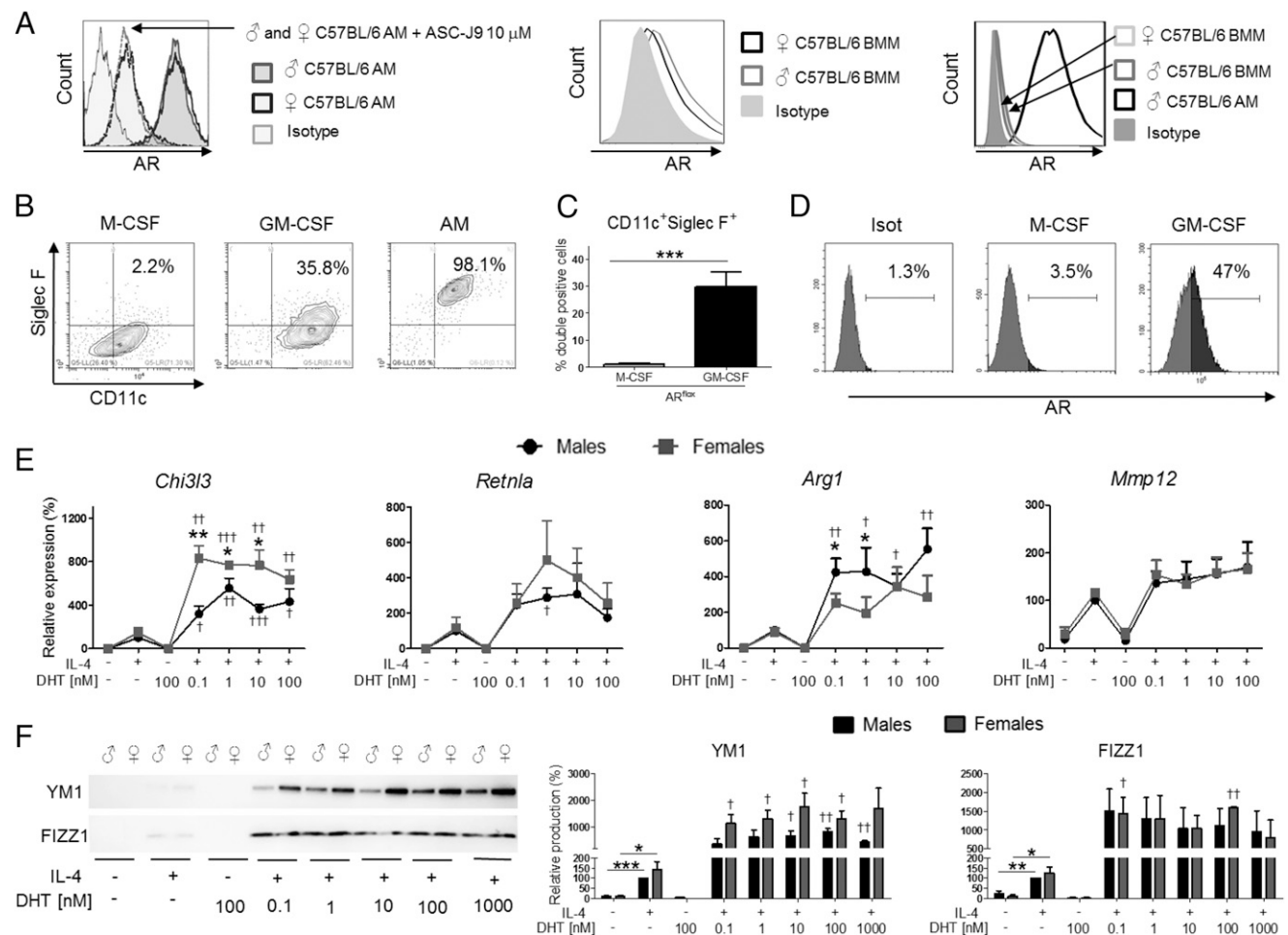


FIGURE 2. DHT enhances M2 gene expression, YM1 and FIZZ1 production in BMM. Bone marrow from female and male C57BL/6 mice was cultured for 10 d with M-CSF (40 ng/ml) to obtain BMM. **(A)** Validation of the specificity of anti-AR Ab. Left panel, AM from female and male mice were cultured for 24 h in the presence or absence of the AR-degrading drug, ASC-J9 (10 μ M). Middle panel, AR expression in BMM from C57BL/6 female and male mice. Right panel, Comparison of AR expression in BMM and AM. **(B)** BMM were cultured for 10 d with 40 ng/ml of GM-CSF or M-CSF in six-well plates, cells were collected, and viability determined. F4/80⁺ cells were selected in the live population. Dot plots of CD11c⁺Siglec F⁺ cells from F4/80⁺ cells cultured with GM-CSF or M-CSF. **(C)** Analysis of double-positive CD11c⁺Siglec F⁺ macrophages with GM-CSF or M-CSF. $n = 2$ experiments, two to three samples per treatment. **(D)** Analysis of AR⁺ cells from F4/80⁺ cells cultured with GM-CSF or M-CSF. **(E)** BMM were pretreated overnight with the indicated concentrations of DHT and then stimulated with IL-4 (1 ng/ml) for 48 h. Expression of the indicated M2 genes was determined by qPCR using the 2^{- $\Delta\Delta$} cycle threshold (CT) method and compared with the amount of mRNA in the male IL-4 sample (= 100%). **(F)** Expression of YM1 and FIZZ1 protein was determined by Western blot in the same BMM supernatants after 48 h of IL-4 stimulation. Equal volumes of supernatant were loaded in each lane. Densitometry of the specific bands was normalized to the amount of protein in male IL-4 (= 100%). Results are representative of four independent in vitro experiments. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ for the comparison between DHT pretreatment and IL-4 stimulation alone; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for the comparison between females and males for that specific treatment.

in vitro system with BMM from male and female C57BL/6 mice. Although BMM will never completely recreate the phenotype of a tissue macrophage, they have been successfully and widely used in vitro to interrogate macrophage biology. Thus, BMM were differentiated from bone marrow for 10 d with M-CSF. Intracellular flow cytometry–based staining confirmed expression of AR in BMM after we validated the specificity of the AR Ab in AM from C57BL/6 female and male mice with an AR-degrading drug ASC-J9 (Fig. 2A, left panel). Both male and female BMM expressed AR (Fig. 2A, middle panel), although male BMM expressed more (27). Interestingly, AM had higher expression (~10-fold more) of AR than did BMM (Fig. 2A, right panel). We speculated that this

might be due to differences in the environmental cues in the bone marrow and the lungs. It has been demonstrated that GM-CSF is involved in the development of AM in the lungs (28, 29); hence, we compared AR expression in BMM developed in either M-CSF or GM-CSF. As expected, GM-CSF not only induced higher expression of CD11c and Siglec F on F4/80⁺ BMM (Fig. 2B) but also induced ~25-fold more CD11c⁺ Siglec F⁺ double-positive AM-like macrophages than did M-CSF (Fig. 2C). The bone marrow cells that we grew in GM-CSF were not DC (30, 31). This was validated by comparing expression of CD86 and MHC-II on bone marrow grown in M-CSF and GM-CSF to cells grown in GM-CSF plus IL-4 [(32), Supplemental Fig. 2]. GM-CSF induced much higher AR

DHT- and placebo-implanted mice. Each data point represents one mouse. **(G)** Quantification of eosinophils (left) and AM (right) in BALF. **(H)** YM1 quantification in BALF by ELISA. **(I)** Analysis of intracellular YM1 in AM by FACS. The change in MFI of YM1 (MFI target – MFI isotype) is shown, with female data pooled from our other experiments in this study and previously. Results are representative of three different experiments. $n = 9$ –12 mice per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Neb, nebulization.

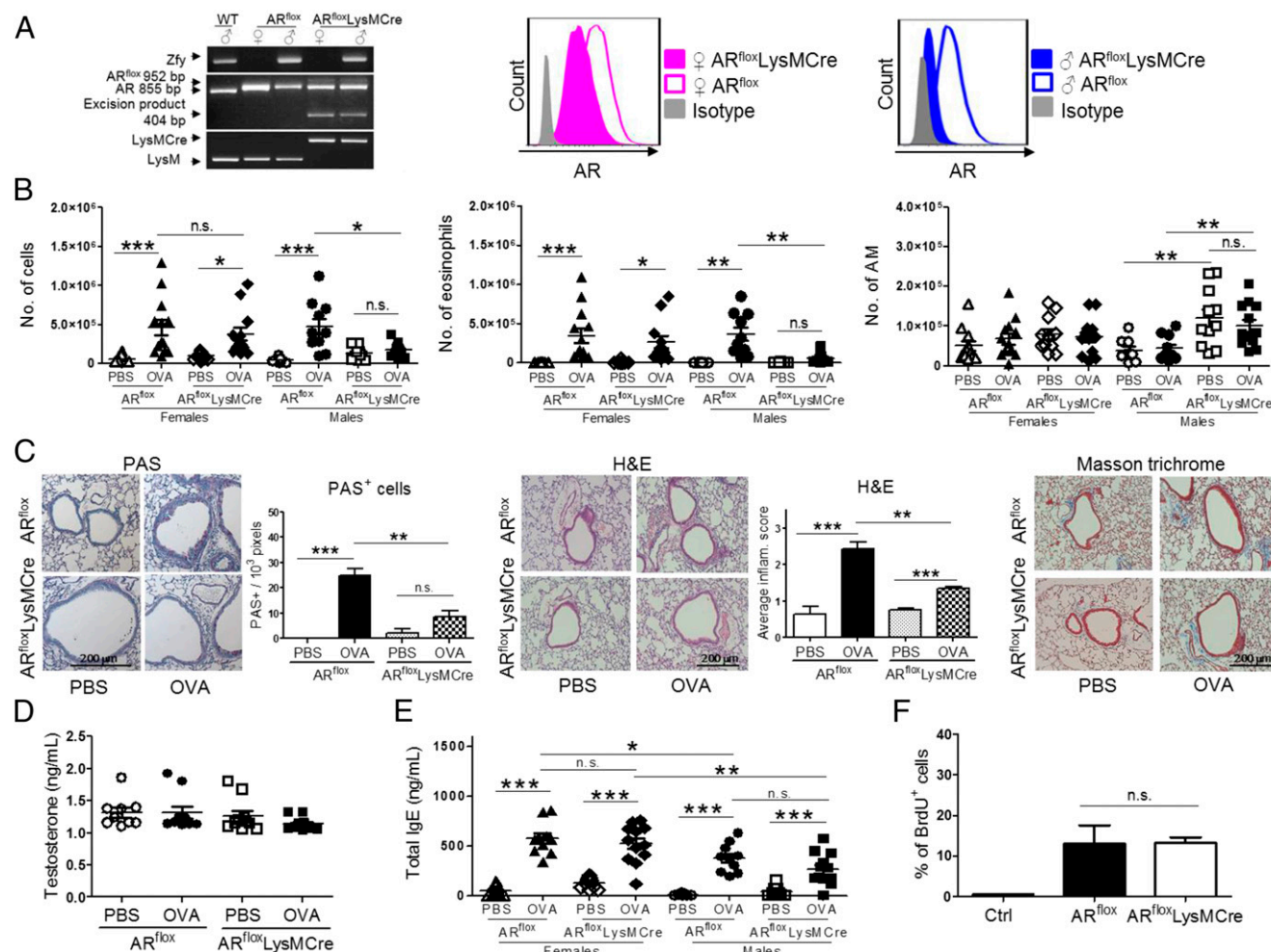


FIGURE 3. Bronchoalveolar cell recruitment is impaired in $AR^{fllox}LysMCre$ male mice in the OVA model of allergic lung inflammation. **(A)** Genotyping of AR^{fllox} and $AR^{fllox}LysMCre$ male and female mice. Zfy, AR (AR [855 bp], AR floxed gene [952 bp]), and the excision product [404 bp]), and *LysM* (*LysM* and *LysMCre*) genes were analyzed by electrophoresis on a 3% agarose gel with ethidium bromide (left panel). AR measurement by intracellular FACS stain of AM from AR^{fllox} and $AR^{fllox}LysMCre$ female (middle panel) and male (right panel) mice. **(B)** Total live cells (left panel), eosinophils (middle panel), and AM (right panel) recovered from the BALF of AR^{fllox} and $AR^{fllox}LysMCre$ female and male mice on day 16 of the OVA protocol. **(C)** Immunohistochemical analysis of lung tissue from AR^{fllox} and $AR^{fllox}LysMCre$ male mice. Mucus production was measured by PAS staining. Cell infiltration was analyzed by H&E staining. Images were acquired with a 20 $\times$ objective. PAS⁺ quantification is plotted as PAS⁺ cells per 1000 pixels. The H&E graph represents cell infiltration scored on a scale of 0–3, as in Fig. 1H. Collagen deposition was analyzed by Masson trichrome stain. $n = 3$ –5 mice per group. Concentration of **(D)** testosterone and **(E)** total IgE in serum were determined by ELISA. Results are representative of three independent experiments. **(F)** Proliferation of AM by BrdU incorporation is shown as percentage of BrdU⁺ cells in the total AM population ($n = 2$ independent experiments). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

expression in BMM ($38.86 \pm 4.26\%$ AR positive) than did M-CSF ($7.23 \pm 2.72\%$ AR positive, Fig. 2D). Hence, the tissue environment in which resident AM develop affects the expression of AR and, likely, the responsiveness of tissue macrophages to androgens.

Next, we treated BMM with DHT and then stimulated them with 1 ng/ml IL-4 for 48 h. Expression of mRNA for the M2 macrophage genes chitinase 3-like 3 (*Chi3l3*), resistin-like molecule α (*Retnla*), arginase1 (*Arg1*), and matrix metalloproteinase-12 (*Mmp12*) was determined by qPCR. DHT exposure increased IL-4-induced gene expression of *Chi3l3*, *Retnla*, and *Arg1* in both male and female BMM (Fig. 2E). DHT did not enhance IL-4-induced *Mmp12* gene expression (Fig. 2E), suggesting that many but not all IL-4-induced M2 genes are enhanced by DHT. Moreover, no differences in IFN- γ -induced M1 macrophage gene expression were observed following DHT pretreatment and IFN- γ stimulation of BMM (Supplemental Fig. 3A).

DHT pretreatment not only enhanced IL-4-mediated M2 gene expression but also increased IL-4-induced production of secreted YM1 and was found in inflammatory zone 1 [(FIZZ1); Fig. 2F]. Thus, the

enhancing effect of DHT on M2 polarization was present in both AM in vivo and BMM in vitro despite the differences in AR expression.

To demonstrate that DHT-induced augmentation of M2 gene expression was a result of AR activity in macrophages, we used flutamide, an AR antagonist, to block AR activity in BMM pretreated with DHT prior to IL-4 stimulation. We observed that pharmacological blockade of AR reduced the DHT-mediated increase in the IL-4-induced expression of *Chi3l3*, *Retnla*, and *Arg1* in BMM to the amount measured with IL-4 stimulation alone (Supplemental Fig. 3B). This suggests that the augmentation of IL-4-induced M2 gene expression by DHT is mediated by the AR in BMM.

Cell recruitment to the BALF increases in the absence of AR

To further validate the role of AR in regulating macrophage polarization during allergic inflammation, we compared lung inflammation and AM polarization in AR^{fllox} and $AR^{fllox}LysMCre$ male and female mice following OVA-induced allergic lung inflammation. Mice were genotyped for the AR gene 855 bp, AR floxed gene (AR^{fllox} 952 bp), and *Cre* (Fig. 3A, left). Expression of

the zinc finger protein, Y-linked (*Zfy*) gene, an indicator of genetic sex, was determined as inconsistencies between external and genetic sex in this strain were reported (16). Phenotypic females were also genetic females (Fig. 3A). A significant decrease in AR protein was determined in AM from male and female AR^{fllox} LysMCre mice (Fig. 3A, middle and right).

Based on our results from DHT delivery in vivo (Fig. 1), we hypothesized that the absence of a functional AR in macrophages would result in increased BALF cellularity in the AR^{fllox} LysMCre animals. Total cells in the BALF did not differ between AR^{fllox} males and females (Fig. 3B, left). Contrary to expectations, the total number of cells in the BALF was lower in the OVA AR^{fllox} LysMCre males (Fig. 3B, left), highlighting the importance of macrophages in allergic lung inflammation. The increased cellularity of the BALF in the wild-type mice was due to eosinophil recruitment (Fig. 3B) as before (Fig. 1G). However, no eosinophils were recruited in the AR^{fllox} LysMCre males challenged with OVA (Fig. 3B, middle panel, filled squares). Furthermore, AR^{fllox} LysMCre males had more AM than AR^{fllox} males (Fig. 3B, right). We observed no differences in AM in the females (Fig. 3B, right) nor monocyte or neutrophil recruitment in any group (data not shown). Hence, the increased number of AM in the BALF of AR^{fllox} LysMCre males was not due to recruitment of monocytes.

To determine if differences in eosinophils and AM in AR^{fllox} and AR^{fllox} LysMCre males affected pathology, we measured lung inflammation by histology. PAS staining showed significantly less mucus production in AR^{fllox} LysMCre males after OVA exposure (Fig. 3C, left). AR^{fllox} LysMCre males also had fewer inflammatory cells in the lung tissue, as shown by H&E staining (Fig. 3C, middle). We did not observe significant collagen deposition (Fig. 3C, right), typical of acute models of allergic inflammation (33).

The absence of AR in macrophages could potentially increase available testosterone as has been shown for the concentration of E2 in ER α -deficient mice (34–36). Serum testosterone in AR^{fllox} and AR^{fllox} LysMCre male mice was similar (1.315 ± 0.092 ng/ml and 1.14 ± 0.027 ng/ml, respectively; Fig. 3D). Thus, suppression of allergic lung inflammation in the AR^{fllox} LysMCre males was not due to differences in testosterone. No difference in total IgE concentration was measured caused by the absence of AR on macrophages (Fig. 3E). However, the female OVA groups had higher serum IgE than their male counterparts, as has been described previously (7).

AR triggers androgen-dependent proliferation of cancer cells (37), but its effects on proliferation of other cell types is poorly understood. The elevated number of AM in AR^{fllox} LysMCre mice

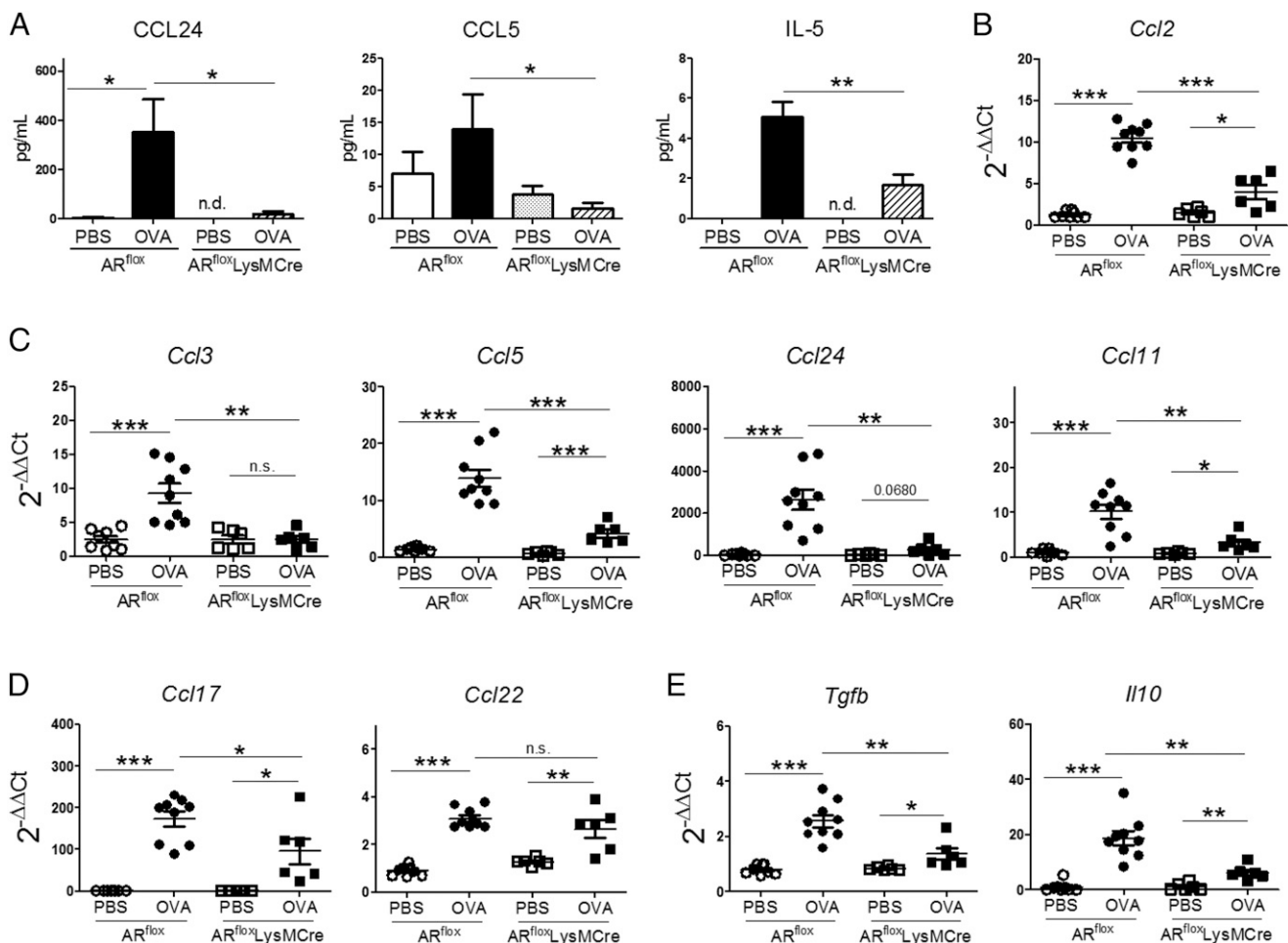


FIGURE 4. Absence of AR results in decreased expression of chemokines and cytokines in AM of male mice. BALF and AM were obtained from AR^{fllox} and AR^{fllox} LysMCre male mice on day 16 of the allergic lung inflammation model. (A) CCL24, CCL5, and IL-5 were measured in the BALF by ELISA. $n = 9$ –12 mice per group. (B–E) BALF cells were seeded for 2 h in MEM Alpha, and nonadherent cells were vigorously washed away. Relative gene expression in AM was analyzed by qPCR using the $2^{-\Delta\Delta CT}$ method and compared with the amount of mRNA in the AR^{fllox} male PBS sample (= 1). (B) *Ccl2* gene expression. (C) *Ccl3*, *Ccl5*, *Ccl24*, and *Ccl11* gene expression. (D) *Ccl17* and *Ccl22* gene expression. (E) *Tgfb* and *Il10* gene expression. Results are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

and the unexpected effect of androgens on M2 polarization prompted us to compare proliferation of AM from AR^{flx} and AR^{flx} LysMCre mice. Proliferation of AM was low ($\sim 13\%$), consistent with low AM turnover and the long-lived nature of these cells (38). Similar proliferation of AM from AR^{flx} LysMCre and AR^{flx} mice was measured by BrdU incorporation (Fig. 3F).

Impaired eosinophil recruitment in BALF of AR^{flx} LysMCre males correlates with decreased chemokine levels in AM and BALF

To discern whether the decreased recruitment of eosinophils in AR^{flx} LysMCre males was due to diminished eosinophil-recruiting chemokines and eosinophil survival cytokines, we measured CCL24, CCL5, and IL-5 in the BALF from AR^{flx} and AR^{flx} LysMCre males. Chemokines and IL-5 were significantly lower in the AR^{flx} LysMCre BALF than in AR^{flx} BALF (Fig. 4A). However, no differences were observed in TNF- α , IL-4, or IL-33, suggesting that AR regulates expression of some but not all cytokine and chemokine genes (data not shown).

Because BALF is a mixture of cytokines and chemokines from different cellular sources, we determined chemokine gene expression specifically in AM by qPCR. AM were enriched by adherence as previously reported [from ~ 10 up to 80% of the BALF cells from the allergic mice (18)]. *Ccl2*, a monocyte-recruiting chemokine, was significantly reduced in AR-deficient AM from the OVA group (Fig. 4B). We also found significantly reduced expression of *Ccl3*, *Ccl5*, and the eotaxins, *Ccl24* and *Ccl11* (eosinophil-recruiting chemokines), in AM lacking AR (Fig. 4C,

filled squares). A significant reduction in *Ccl17* but not *Ccl22* was found in AM from AR^{flx} LysMCre animals (Fig. 4D). However, the diminished M2 phenotype did not correlate with an increase in the “regulatory” cytokines, TGF- β and IL-10. In fact, expression of the mRNA for *Tgfb* and *Il10* was higher in the AR-sufficient AM after OVA challenge (Fig. 4E), possibly a macrophage-mediated mechanism to suppress allergic inflammation. Therefore, AR deficiency in AM resulted in decreased expression of the typical chemokines that recruit eosinophils to the allergically inflamed lung.

AR promotes M2 polarization of AM

M2 polarization of AM during allergic lung inflammation induces eosinophil recruitment to the alveolar space (3) and expression of FIZZ1, ARGINASE 1, and YM1 (39). Given the diminished production of eosinophil-recruiting chemokines and cytokines by AM lacking AR, we tested the possibility that the AR deficiency diminishes polarization to the M2 phenotype.

First, we compared the amount of YM1 and FIZZ1 in BALF from AR^{flx} and AR^{flx} LysMCre males following allergic lung inflammation. YM1 and FIZZ1 in BALF were significantly induced by OVA challenge (Fig. 5A), but expression of both was lower in the AR^{flx} LysMCre males. The difference in YM1 protein in the BALF was confirmed by ELISA (AR^{flx} OVA group = 1496.24 ± 158.9 ng/ml; AR^{flx} LysMCre OVA group = 803.37 ± 113.9 ng/ml). We also quantified the AM production of MMP-12 and MMP-9, proteins involved in tissue remodeling and associated

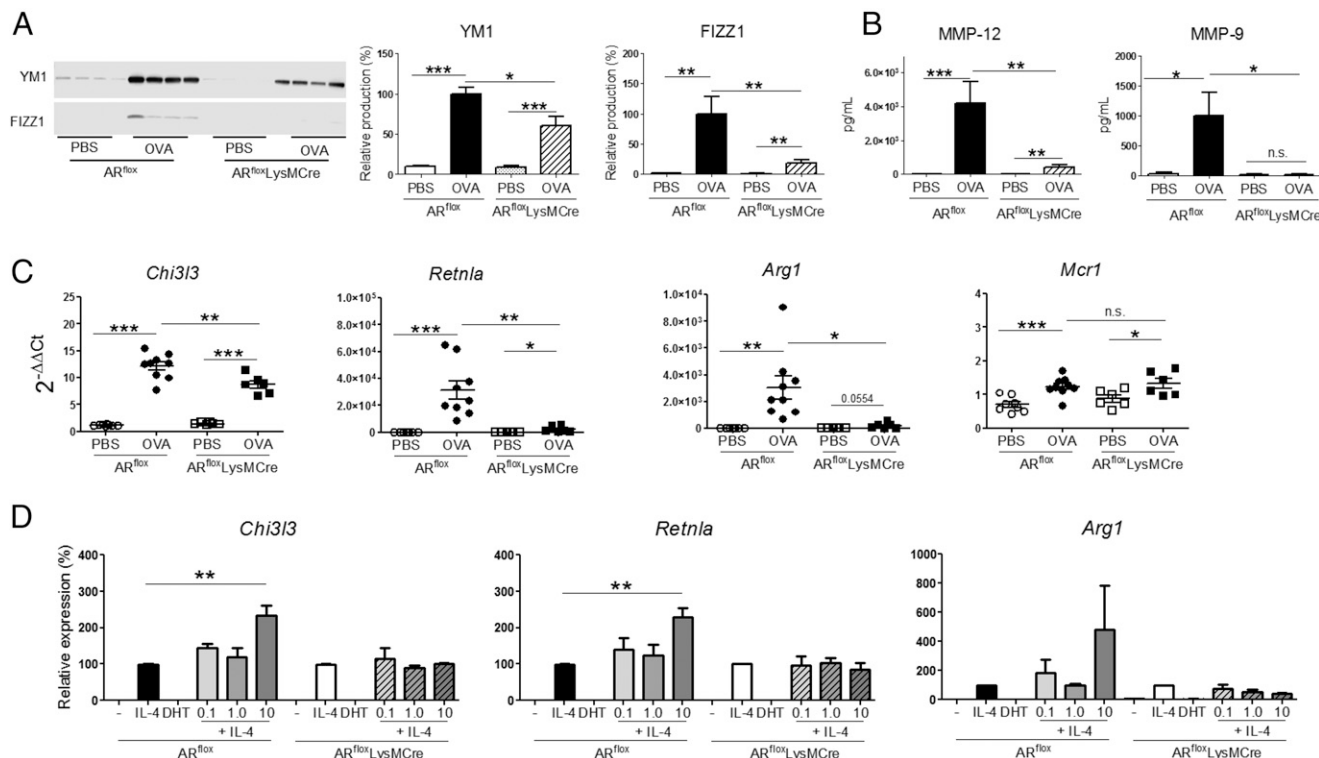


FIGURE 5. Impaired M2 polarization of AR-deficient AM. BALF and AM were obtained from AR^{flx} and AR^{flx} LysMCre male mice after induction of allergic lung inflammation with OVA. (A) The amount of YM1 and FIZZ1 protein in the BALF was determined by Western blot. Densitometry of the specific bands was normalized to the amount in the AR^{flx} OVA group (= 100%). (B) MMP-12 and MMP-9 were measured in BALF by ELISA. (C) AM from BALF were isolated as described in Fig. 4A and analyzed for expression of the indicated M2 genes by qPCR with the $2^{-\Delta\Delta CT}$ method. Amount of mRNA was compared with that in the AR^{flx} PBS sample (= 1). (D) BMM cultured from AR^{flx} and AR^{flx} LysMCre male mice as described in Fig. 2B were pretreated overnight with DHT at the concentrations shown and then stimulated with IL-4 (1 ng/ml) for 48 h. Relative gene expression was determined by qPCR with the $2^{-\Delta\Delta CT}$ method and compared with the amount of mRNA in the IL-4 sample (= 100%) for each genotype ($n = 3$ independent experiments). Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

with asthma exacerbations (40). OVA challenge induced significant MMP-12 in the BALF of both AR^{fllox} and AR^{fllox}LysMCre mice (Fig. 5B) but it was significantly less in AR^{fllox}LysMCre mice. MMP-9 production was induced only in the AR^{fllox} OVA-group (Fig. 5B). To determine if AM were responsible for these differences in BALF proteins, we analyzed M2 gene expression in AM. qPCR analysis revealed that expression of the M2 genes, *Chi3l3*, *Retnla*, and *Arg1*, was significantly lower in AM lacking AR (Fig. 5C). No differences were observed in *Mrc1* expression, although this gene was also less robustly induced.

Next, we measured M2 polarization in BMM from male mice treated with or without DHT prior to IL-4 stimulation. BMM from AR^{fllox} and AR^{fllox}LysMCre males exhibited no differences in M2 gene expression when stimulated with IL-4 alone (Supplemental Fig. 3C). However, treatment of BMM with increasing concentrations of DHT enhanced IL-4-induced gene expression of *Chi3l3*, *Retnla*, and *Arginase1* in the AR^{fllox} BMM but not in BMM lacking AR (Fig. 5D). Only the highest concentration of DHT (10 nM) induced a significant increase in the expression of *Chi3l3* and *Retnla* in AR^{fllox} BMM. This inability of DHT to enhance M2 gene expression in BMM lacking AR corroborated the decrease in M2 gene expression observed in AR^{fllox}LysMCre mice after the OVA protocol (Fig. 5A). Together, these results indicate that AM are a major source of YM1, FIZZ1, and ARGINASE 1 in allergic lung inflammation and that AR is responsible for DHT augmentation of IL-4-induced M2 gene expression.

In summary, we show genetically and pharmacologically that AR is important for full polarization of M2 macrophages in male animals. Rather than suppressing M2 polarization as we hypothesized, AR activation by androgens resulted in enhancement of the IL-4-activated M2 macrophage polarization in vitro, whereas lacking AR in AM impaired polarization to the M2 macrophage phenotype in vivo. Similar to that which we previously described for E2 and ER α , in this article, we describe a role for androgen and AR in enhancing M2 macrophage polarization in macrophages from male animals. Also, we demonstrate the importance of AR in macrophages in allergic lung inflammation in vivo. The diminished M2 profile of AM from male AR^{fllox}LysMCre mice during allergic lung inflammation dramatically reduced the overall inflammatory profile of the lungs.

Discussion

Allergic asthma is characterized by M2 polarization of AM that correlates with disease severity and eosinophil recruitment (2). Asthma affects more boys than girls before puberty and more women than men during adulthood (6, 41). Similar sex differences have been observed in mice (7, 8). Generally, this suggests male and female sex hormones suppress and enhance allergic inflammation, respectively. Previously, we showed that E2 and ER α increase M2 macrophage polarization in vivo and in vitro (18). Other work has shown a suppressive role for androgens in ILC2 (14) and airway smooth muscle cells (13) in allergic lung inflammation. From this work and our E2 study, we hypothesized that androgens would suppress M2 polarization of macrophages. Our current study has uncovered a novel, cell-specific role in macrophages for androgens acting through the AR. As expected, androgens (DHT) downmodulated Th2 inflammation (eosinophil recruitment, IgE production) in the OVA model of allergic lung inflammation, supporting the idea that androgens act as global immune suppressors. However, analysis of the M2 macrophage marker, YM1, in vivo revealed greater M2 polarization (more intracellular YM1) in the DHT-treated mice and in AR-sufficient AM (Supplemental Fig. 4). This unexpected finding suggests that

androgens may act differently in macrophages than in other immune cells in the setting of allergic inflammation.

Our in vitro analysis showed that DHT enhanced the expression of hallmark M2 genes (*Chi3l3*, *Retnla*, and *Arg1*) in BMM from both female and male mice, suggesting androgens are important immune modulators in both sexes. Taken with our previous work demonstrating a role for E2/ER α in M2 polarization, our findings in this study suggest both male and female sex hormones can polarize macrophages to the M2 phenotype. This is similar to earlier studies in B cells, which demonstrated that male and female sex hormones have the same effect on B cell lymphopoiesis (42–44). Other studies demonstrate that E2 can suppress the production of IL-2 by T cells from both women and men (45) and that DHT can increase IL-10 gene expression in T cells from male and female mice (46). However, physiologically available concentrations of male and female sex hormones in unmanipulated male and female animals would preclude M2 polarization by the opposite sex hormone and its receptor. Androgens are much lower in female animals than in males, and therefore, androgen-/AR-augmented M2 polarization would be less important in females compared with males. Thus, the mechanisms by which M2 macrophage polarization is enhanced in vivo are different between females (E2-driven) and males (androgen-mediated). As shown in Fig. 1I, androgen reconstitution of castrated male mice with DHT recovered YM1 expression to the degree observed in intact males, whereas the absence of androgens impaired YM1 induction. Altogether with our previous report demonstrating a stronger YM1 expression in females than males (18), this indicates that although both female and male sex hormones enhance M2 macrophage polarization, E2 acts as a more potent M2 macrophage enhancer than androgens. Our study reinforces the need for different, sex-dependent approaches to asthma therapy.

Compensatory mechanisms have been described when either receptors or ligands are decreased (47, 48). Lack of a sex hormone receptor can cause supraphysiological elevations in that sex hormone as has been demonstrated for E2 in ER α -deficient mice (34–36). However, the concentration of testosterone in the serum of the AR^{fllox}LysMCre and AR^{fllox} mice was the same, indicating that the differences we saw were due solely to absence of the canonical AR not to increased concentration of testosterone.

Another important difference observed between in AR^{fllox}LysMCre and AR^{fllox} male mice was more resident AM in the AR^{fllox}LysMCre mice at steady-state. These data suggest that AR negatively regulates the number of AM in the lung, possibly by regulating proliferation or survival. Because BrdU incorporation into AM was similar, proliferation of AR-deficient AM was not higher than wild-type AM. Either increased survival or increased differentiation of monocytes to AM may explain the net difference in the number of AM. This contrasts with the role for androgens in driving cell proliferation in prostate cancer (PCa) (49) and with suppression of IL-33-driven proliferation/survival of ILC2 in allergic lung inflammation (14). Thus, AR deficiency could increase cell survival or differentiation of AM from lung resident self-renewing or blood-derived monocytes, resulting in more AM in the AR^{fllox}LysMCre male mice. We will address these questions in future work.

Recognizing the ability of androgens to enhance M2 polarization of macrophages could lead to new therapies for diseases, such as asthma and PCa, thought to be worsened by M2 macrophage polarization and sex hormones. For example, tumor-associated macrophages (TAM) are M2-like macrophages (50) that are protumorigenic in PCa (51). Because high concentrations of testosterone are associated with PCa development (52), hormone therapy and androgen-deprivation

therapy have been used to control this disease (53). Our work suggests that androgen-deprivation therapy is beneficial not only in reducing the proliferation of PCa cells but also in reducing M2 TAM polarization. The GM-CSF-induced enhancement of AR expression we observed in AM is also relevant to tumor immunology, as GM-CSF is used as an anticancer therapy (54). However, GM-CSF may also promote tumor progression and metastasis of colon adenocarcinoma (55), head and neck cancer cells (56), and colon cancer (57). Our data suggest that these results could stem from increased AR expression and polarization of TAM to the M2 phenotype.

In summary, this work expands our understanding of macrophage polarization, revealing an unexpected role for androgens and AR. Androgens enhanced, rather than suppressed, M2 macrophage polarization. The M2-promoting role of DHT was, however, specific for macrophages, as DHT suppressed allergic inflammation overall. The increased M2 macrophage polarization induced by androgens was mediated by AR (Supplemental Fig. 4), as this effect was absent in macrophages from AR^{lox}LysMCre male mice. These results suggest possible mechanisms to counteract diseases promoted by M2 macrophages and affecting mainly men, such as eosinophilic esophagitis (58) and PCa (59). A deeper understanding of the mechanisms by which androgen/AR enhances M2 macrophage polarization will help us to develop effective therapies against diseases in which M2 macrophages are important mediators.

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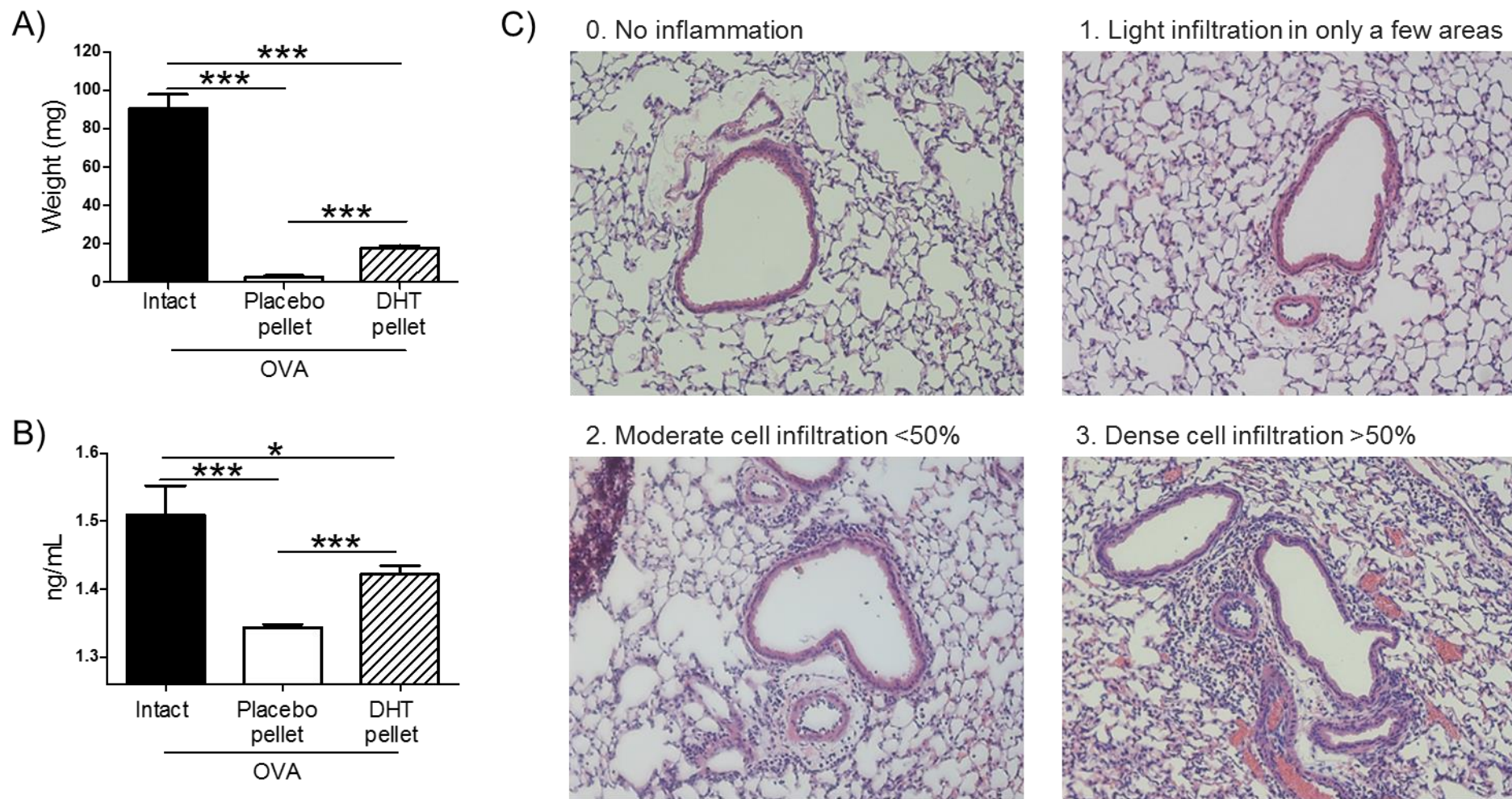
Disclosures

The authors have no financial conflicts of interest.

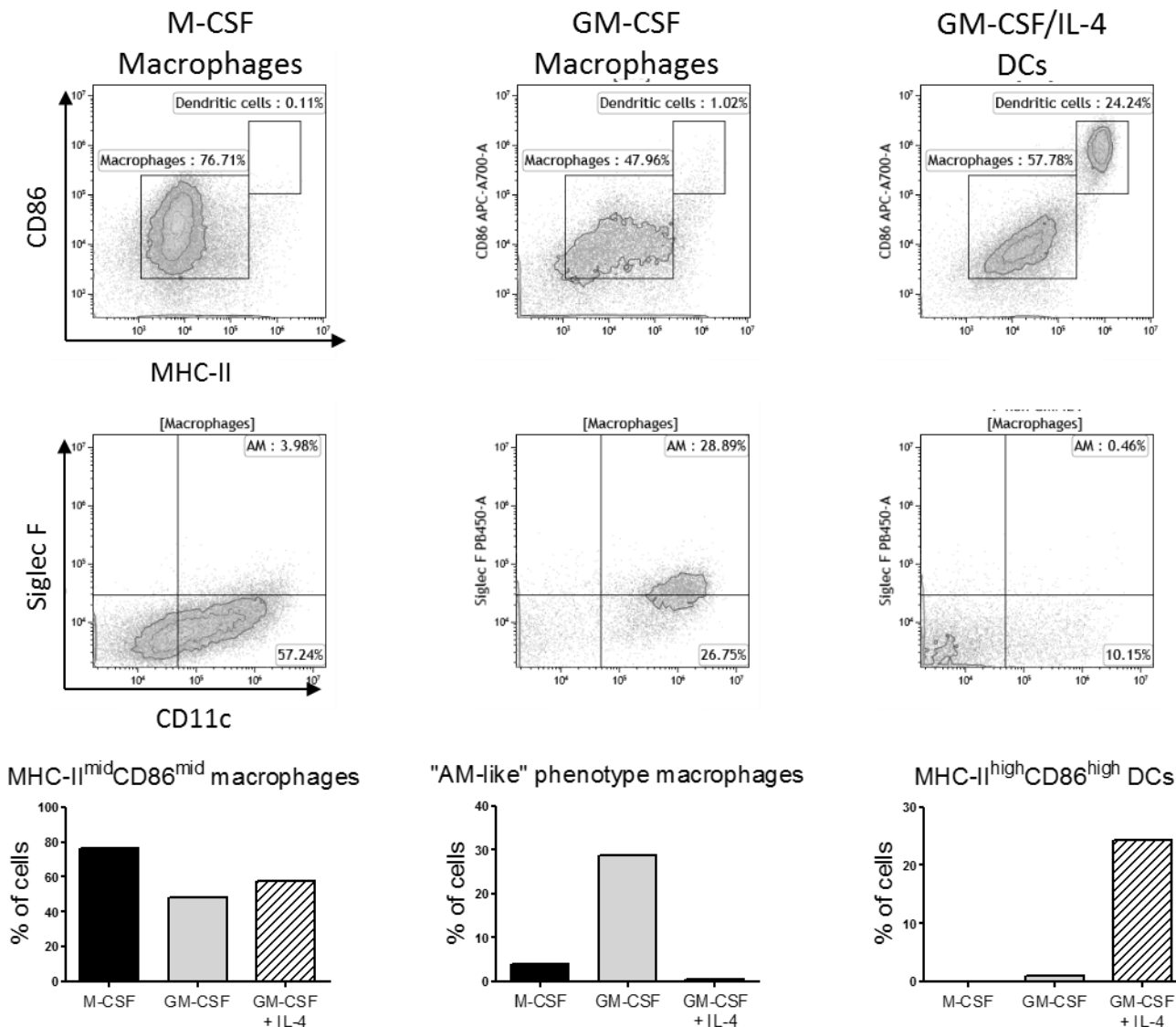
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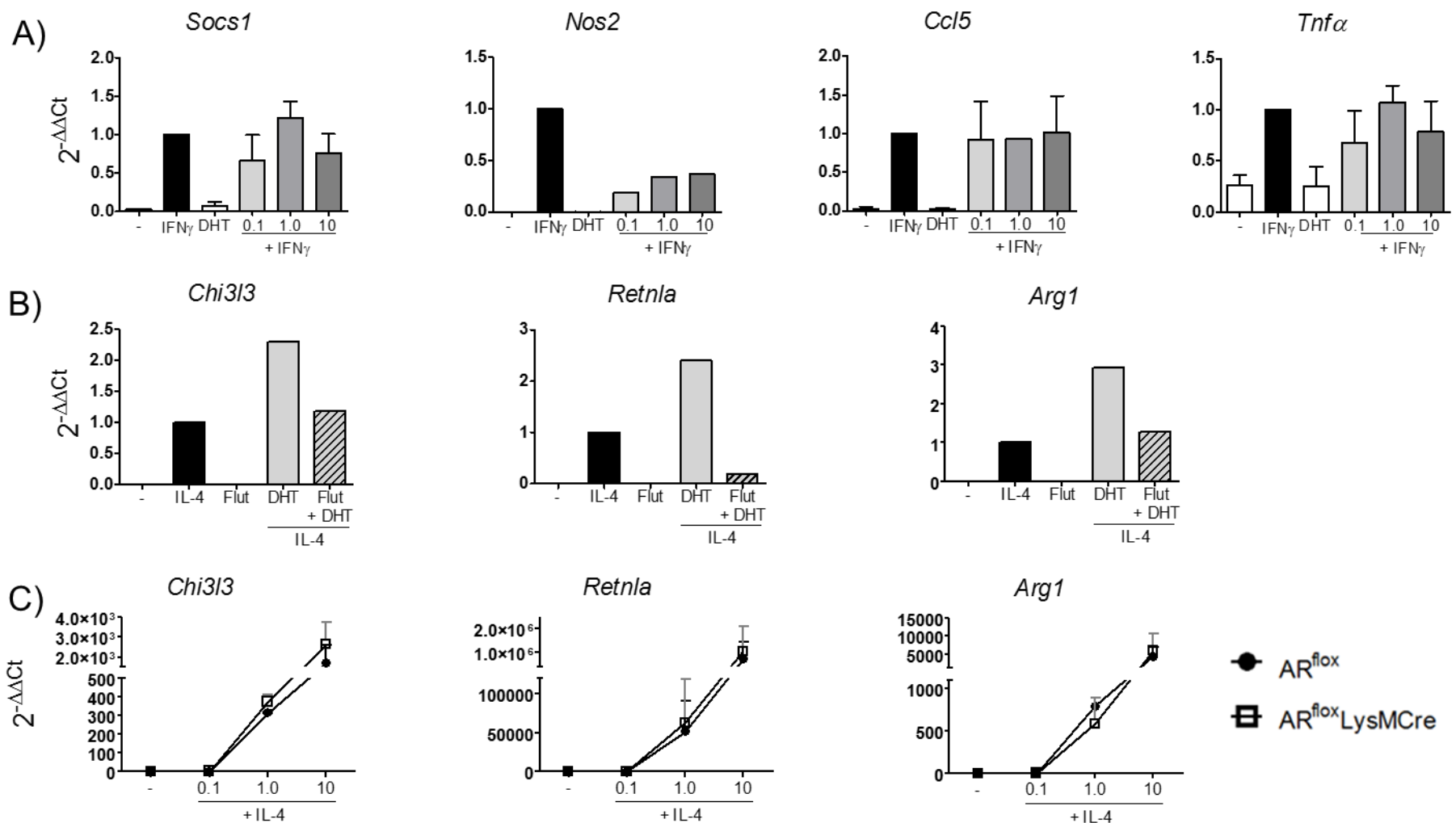
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Supplementary Figure 1. Increase in the weight of the seminal vesicles and in testosterone serum levels induced by DHT in castrated mice and scoring of cellular infiltration of the mouse lung. (A) Seminal vesicles were obtained from intact and castrated (DHT- and placebo-implanted) C57BL/6 male mice at the end of the OVA protocol and weighed. (B) Concentration of serum testosterone measured by ELISA. (C) Representative images of H&E-stained lung tissue from DHT- and placebo-implanted mice. Cell infiltration was scored on a scale of 1 to 3, with 0 representing no inflammation, 1 representing light infiltration in only a few areas, 2 representing moderate cell infiltration encompassing <50% of the airways and vessels, and 3 representing dense cell infiltration encompassing >50% of the airways and vessels. Data are plotted as the mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

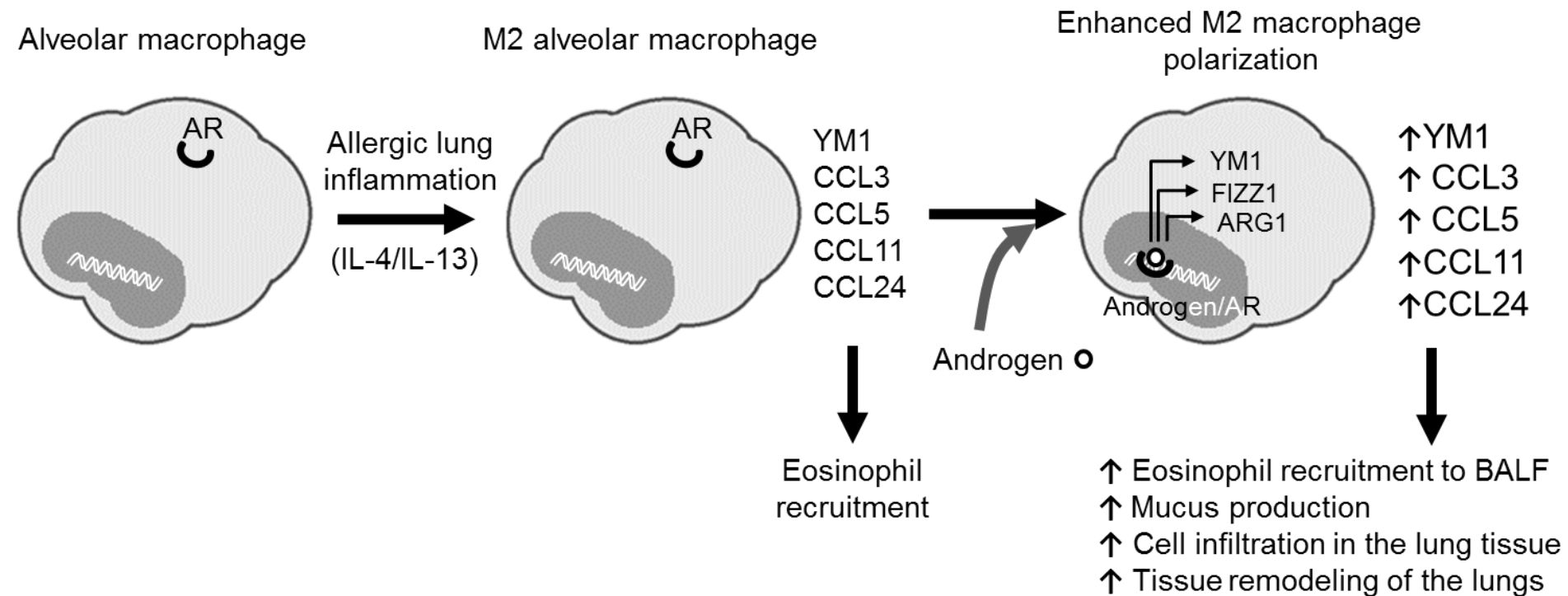


Supplementary figure 2. Differences in MHC-II and CD86 between BMM and DCs. Bone marrow from C67BL/6 male mice and cultured for 10 days with M-CSF (40 ng/mL), GM-CSF (40 ng/mL) or GM-CSF (20 ng/mL) + IL-4 (40 ng/mL) to obtain BMM and DCs, respectively. At day 10 cells were collected and stained for CD11c, Siglec F, MHC-II, and CD86. BMM were identified as MHC-II^{mid}CD86^{mid} cells and DCs as MHC-II^{high}CD86^{high} cells.



Supplementary Figure 3. Gene expression induced by IFN- γ in DHT-pretreated BMM, abrogation of the M2 macrophage enhancement due to DHT pretreatment in IL-4 stimulated BMM, and IL-4 induction of M2 gene expression in BMM from AR^{fllox} and $AR^{fllox}LysMCre$ male mice. Bone marrow from C57BL/6 male mice were cultured for 10 days with M-CSF (40 ng/mL) to obtain BMM. (A) BMM were stimulated with DHT (0.1, 1 or 10 nM, as indicated) for 18 h prior to stimulation with IFN- γ for 48 h. (B) BMM were pretreated with the AR antagonist, flutamide (10 μ M) for 2 h prior to adding DHT (10 nM) for 18 h. Then cells were stimulated with IL-4 (1 ng/mL) for 48 h. (C) BMM from AR^{fllox} and $AR^{fllox}LysMCre$ male mice were stimulated with IL-4 at the concentrations shown for 48 h. Messenger RNA was obtained from these cells and the indicated genes were analyzed by qPCR with the $2^{-\Delta\Delta CT}$ method. n= two independent experiments (A and C). Data are plotted as the mean $\pm$ SEM.

AR enhances M2 polarization of AM



Supplementary figure 4. AR enhances M2 polarization of macrophages. Schematic summary of AR-mediated enhancement of M2 macrophage polarization during allergic lung inflammation.