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(54) Title: ANDROGEN RECEPTOR AGONISTS FOR TREATING TYPE 2 INFLAMMATIONS

(57) Abstract: The present invention relates to the treatment of type 2 inflammation. Based on the role of ILC2 in the induction of airway inflammation, the inventors investigate whether ILC2 could be influenced by sex hormones. In this study (effected on mice), they have unravelled a novel level of regulation of ILC2 responses. Analysis of multiple tissues from both sexes revealed a significantly increased presence of ILC2 in females compared with males. Interestingly, this difference was not due to an enhancing effect of estrogens in females, but rather to the inhibition of ILC2 development in males mediated by androgens through androgen receptor expression within the hematopoietic compartment. As a consequence, females developed exacerbated lung inflammation in response to house dust mite (HDM) extract or to IL-33 administration. These results demonstrate the interest of androgen receptor as a target on ILC2. Thus, the invention relates to an agonist of the androgen receptor expressed on group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.



NEW METHOD FOR TREATING TYPE 2 INFLAMMATION

FIELD OF THE INVENTION:

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The present invention relates to an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.

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BACKGROUND OF THE INVENTION:

Type 2 immune responses are characterized by the secretion of IL-4, IL-5, IL-9 and IL-13. This specific cytokine signature is crucial to control parasitic infection but is also associated with allergic reactions. These responses involve the coordinated action of the type 2 innate lymphoid cells (ILC2) and the T helper (Th) 2 lymphocytes. The ILC2 are rapid and potent producers of the type 2 cytokines representing a critical early source of mediators responsible for the initiation of Th2-dependent immune responses and the rapid elimination of intestinal parasites (Eberl et al., 2015). Indeed, they are widely distributed at mucosal surfaces where they can be activated by epithelial stress signals such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (Barlow et al., 2013; Bartemes et al., 2012; Imai et al., 2013; Kim et al., 2013; Salimi et al., 2013; Schmitz et al., 2005). In response to these stimuli, ILC2 secrete large amounts of IL-5, IL-13 and IL-9. The secretion of IL-5 by ILC2 leads to the recruitment and activation of eosinophils and mast cells, while IL-13 activates goblet cells and mucus production by epithelial cells. Moreover, IL-13 production by ILC2 is critical to mount an effective Th2 cell response by instructing dendritic cells (DC) to prime Th2 cells in draining lymph nodes (Halim et al., 2014). ILC2 have therefore recently emerged as critical cells in the initiation of allergic inflammatory responses such as asthma or atopic dermatitis (Halim et al., 2012; Halim et al., 2014; Salimi et al., 2013). They also play a key role in protective immunity against parasitic helminth infection (Moro et al., 2010; Neill et al., 2010) and are associated with metabolic homeostasis (Molofsky et al., 2013). Besides cytokines, additional mediators including the arachidonic acid metabolites leukotriene D4 (LTD4) and prostaglandin D2 (PGD2) appear to be potent regulators of ILC2 function (Doherty et al., 2013; Wojno et al., 2015). However, our current understanding of the homeostatic regulation of ILC2 is limited

and whether other endogenous factors, such as sex steroid hormones, also influence ILC2 responses is currently unknown.

Asthma is a hallmark of type 2 immune response-mediated disease causing chronic inflammation of the airways (Lambrecht and Hammad, 2015). Both clinical observations and murine models revealed that asthma incidence, prevalence and severity differ according to gender (Townsend et al., 2012). While males are more susceptible to asthma than females in childhood, the onset of puberty reverses that trend for most allergic disorders (Almqvist et al., 2008; Carey et al., 2007). The drop in asthma incidence observed in and around the time of puberty in males is suggestive of a protective action of male sex hormones (Almqvist et al., 2008; Carey et al., 2007). Along the same line, hypogonadism have been associated with enhanced susceptibility to asthma when compared to men with normal testosterone levels (Mulligan et al., 2006). Although these observations support a protective role for androgens in the susceptibility to allergic asthma, the underlying mechanisms responsible for this effect are unknown.

SUMMARY OF THE INVENTION:

Based on the role of ILC2 in the induction of airway inflammation (Gold et al., 2014; Halim et al., 2012; Halim et al., 2014), the inventors investigate whether ILC2 could be influenced by sex hormones. In this study, they have unravelled a novel level of regulation of ILC2 responses. Analysis of multiple tissues from both sexes revealed a significantly increased presence of ILC2 in females compared with males. Interestingly, this difference was not due to an enhancing effect of estrogens in females, but rather to the inhibition of ILC2 development in males mediated by androgens through androgen receptor expression within the hematopoietic compartment. As a consequence, females developed exacerbated lung inflammation in response to house dust mite (HDM) extract or to IL-33 administration. These results demonstrate that androgen signalling exerts a critical cell-intrinsic role in regulating ILC2 differentiation and the development of ILC2-dependent type 2 inflammatory responses.

Thus, the present invention relates to an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof. Particularly, the invention is defined by its claims.

DETAILED DESCRIPTION OF THE INVENTION:

A first aspect of the invention relates to an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.

5 As used herein the term “group 2 innate lymphoid cells or ILC2” has his general meaning in the art and denotes a group of cells playing the crucial role of secreting type 2 cytokines in response to helminth infection. They have also been implicated in the development of allergic lung inflammation.

10 Thus, in a particular embodiment, the invention relates to an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of lung inflammation and allergic lung inflammation.

In a particular embodiment, type 2 inflammation, lung inflammation and allergic lung inflammation regroup all diseases characterised with chronic inflammation of the airways. These diseases can be selected in the group consisting in allergic asthma, eosinophilic
15 bronchitis, eosinophilic pneumopathy and Churg-Strauss syndrome.

Thus, in one embodiment, the invention relates to an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in allergic asthma.

According to the invention, agonist of the invention may be the testosterone or the 5-
20 alpha dihydrotestosterone (5a-DHT; also known as dihydrotestosterone). Others agonists can be selected in the group consisting in: DHEA, AKT1, BAG1, Beta-catenin, C-jun, Calmodulin 1, Caveolin 1, CDK9, COX5B, CREB-binding protein, Cyclin D1, Cyclin-dependent kinase 7, DACH1, Death associated protein 6, L-DOPA, EFCAB6, Epidermal growth factor receptor, FOXO1, GAPDH, Gelsolin, GNB2L1, GSK3B, HDAC1, HSP90AA1, HTATIP, MAGEA11,
25 MED1, MYST2, NCOA1, NCOA2, NCOA3, NCOA4, NCOA6, NCOR2, NONO, p300, PA2G4, PAK6, PATZ1, PIAS2, PRPF6, PTEN, RAD9A, RANBP9, RCHY1, Retinoblastoma protein, RNF14, RNF4, SART3, SIRT1, SMAD3, Small heterodimer partner, Src, SRY, STAT3, SVIL, Testicular receptor 2, Testicular receptor 4, TGFB1I1, TMF1, TRIM68, UBE2I, UXT, and ZMIZ1 (see [wikipedia.org/wiki/Androgen_receptor#AR_agonists](https://www.wikipedia.org/wiki/Androgen_receptor#AR_agonists), Päivi Pihlajamaa
30 et al., 2015 and Arthi Thirumalai et al., 2017).

In a particular embodiment, the agonist of the androgen receptor can be a SARM.

As used herein the term SARM for "Selective Androgen Receptor Modulators" denote a class of androgen receptor ligands. They are intended to have the same kind of effects as androgenic drugs like anabolic steroids but to be much more selective in their action, allowing

them to be used for many more clinical indications than the relatively limited legitimate uses that anabolic steroids are currently approved for.

In a particular embodiment, the SARM may be Enobosarm, BMS-564,929, LGD-4033 (ligandrol), AC-262,356, JNJ-28330835, LGD-2226, LGD-3303, S-40503, S-23, RAD140, Acetothiolutamide, Andarine (GTx-007 Selleckchem ref # S1140), LG-121071, TFM-4AS-1 (Sigma ref# SML0342), CI-4AS-1 (TOCRIS ref#3812), Ostarine, GTx-024, MK-2866 (Selleckchem ref # S1134) and YK-11 (see the website: wikipedia.org/wiki/Selective_androgen_receptor_modulator, Päivi Pihlajamaa et al., 2015 and Arthi Thirumalai et al., 2017).

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects at risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or

treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

As used herein, the term "subject" denotes a mammal. In a preferred embodiment of the invention, a subject according to the invention refers to any subject (preferably human) afflicted with lung inflammation.

In one embodiment, agonist of the invention may be a small chemical entity, e. g. a small organic molecule (natural or not).

The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, more preferably up to 2000 Da, and most preferably up to about 1000 Da.

In order to test the functionality of putative agonist of the androgen receptor expressed in group 2 innate lymphoid cells on inflammation, a test is necessary. For that purpose ILC2 can be generated in vitro from ILC2P isolated from the bone marrow of mice. ILC2P are then cultured in vitro on OP9-Delta-1 stromal cells in the presence of IL-7 and IL-33 as described in Laffont et al 2017 and in example 2 below (see for example Figure 7 and Figure 8). Putative agonist can thus be tested on the ILC2P for their capacity to inhibit the development of CD90^{hi} KLRG-1^{low} ILC2s.

Another object of the invention relates to a method for treating lung inflammation comprising administering to a subject in need thereof a therapeutically effective amount of an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2).

Therapeutic composition

Another object of the invention relates to a therapeutic composition comprising a agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently can be used.

Pharmaceutical compositions of the present invention may comprise a further therapeutic active agent. The present invention also relates to a kit comprising an agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) according to the invention and a further therapeutic active agent.

For example, further therapeutic agent useful for treating type 2 inflammation can be added to the pharmaceutical composition as described below.

These agents may be nonsteroidal anti-inflammatory drugs like aspirin, ibuprofen, and naproxen, β -agonistes, corticoids, anti-histaminiques, anti-leukotrienne, antibodies anti-IgE, anti-IL5 or anti-IL4Ra (see for example Akdis CA, 2012).

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

5 FIGURES:

Figure 1. Male mice develop less severe HDM-induced allergic asthma than female mice. (A) Quantification of leukocyte populations present in the bronchoalveolar lavage fluid of HDM-challenged male or female mice 17 days after treatment using May-Grünwald-Giemsa staining (MGG). The total number of each leukocyte subset is shown (n=5 mice/group). (B) Quantification of serum concentration of IgE and IgG1 by ELISA at the indicated time points (n = 5 mice/group). Comparison between groups was calculated using the unpaired Student's t tests. *p≤ 0.05; **, p<0.01; ***, p<0.001.

Figure 2. Male castration abolishes sex differences in IL-33-mediated lung inflammation. Male, female and castrated (Cx) male mice were injected i.p. with huIL-33 (4 µg/mouse/day) on day 0, 1, 4, 5 and 6 and analyzed on day 7. Control mice received PBS. (A) Frequency and absolute numbers of ILC2 in the lungs. Pulmonary ILC2 were identified by flow cytometry as (CD90+GATA-3+) pre-gated on Singlet, Live, CD45+, CD3-, CD19-, NK1.1-cells. (B) Total numbers of ILC2 producing IL-5 and IL-13 are shown. (C) Histological score of lung inflammation. Data from 5 mice/group are shown. Comparison between groups was calculated using the unpaired Student's t tests. Error bars indicate the mean and SEM. *p≤ 0.05; **, p<0.01; ns, not significant. Data are representative of 2 independent experiments.

Figure 3. ILC2p numbers are reduced in male mice while AR-blockade promoted ILC2 development in vitro. (A) Frequency and total number of ILC2p between male (black) and female (white) mice. (B) Expression (RPKM, reads per kilobase of exon model per million mapped read values) of sex hormone receptor mRNA in female ILC2p by RNA-seq. (C) In vitro differentiation of ILC2p on OP9-DL1 feeder cells in complete medium supplemented with mouse IL-33 in presence or absence of dihydrotestosterone (DHT, 10 nM) or the AR-antagonist Flutamide (100 nM). Quantification of ILC2 expansion expressed as the fold increase as compared to ILC2p input after 7 and 10 days of differentiation. Data representative of 3-4 experiments are shown. Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t tests. *p≤ 0.05; **, p<0.01; ***, p<0.001.

Figure 4. AR-deficiency abrogates sex differences in ILC2-dependent lung inflammation. Bone marrow chimeras reconstituted with cells either from ARKO male B6

mice or their littermate WT controls were injected with huIL-33 as in Fig. 2 at 8 weeks after reconstitution and the inflammatory response was analyzed. (A) Quantification of total leukocyte populations (MGG staining) (left) and eosinophils (right) present in the bronchoalveolar lavage fluid of IL-33 injected male or female chimeric mice as indicated. (B) Quantification of leukocytes (CD45+ cells) infiltrating the lungs of chimeric mice. (C) Total numbers of ILC2 (SingletLiveCD45+Lin-CD90+GATA3+). (D) Frequency of KLRG1-expressing ILC2 (left) and geometric mean fluorescence intensity of KLRG1 on ILC2. (E) Histological score of lung inflammation. Data from 5-6 mice/group are shown and are representative of 2 experiments performed. Comparison between groups was calculated using the unpaired Student's t test or ANOVA. Error bars indicate the mean and SEM. * $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Figure 5: 5a-DHT treatment limits in vivo lung ILC2 development at steady state.

Female mice were ovariectomized before sexual maturity (4 week-old) and treated or not at 6 weeks of age with 5a-DHT for 2 weeks. Mice were then sacrificed and lung infiltrating cells isolated for analysis by flow cytometry. Age-matched control male mice were used. ILC2 were identified as: singlet, alive, CD45+, Lin-(CD3, CD4, CD8, TCRab, B220, CD19, CD11b, CD11c, GR-1, DX5) expressing CD90+ and GATA-3+. (A) Frequency and (B) number of lung ILC2 from male (M), OvX female (OvX F) treated or not with 5a-DHT. (C) Frequency and Geometric mean fluorescence intensity (GMFI) of KLRG1 on ILC2. (D) Representative CD90/KLRG1 expression profile on ILC2 from OvX female treated or not with 5a-DHT. Comparison between groups was calculated using the Mann and Whitney test. Error bars indicate the SEM from 5 mice/group.

Figure 6 : 5a-DHT inhibits ILC2P development in vivo. Female mice were ovariectomized before sexual maturity (4 week-old) and treated or not at 6 weeks of age with 5a-DHT for 2 weeks. (A) Flow cytometry analysis of ILC2p in the BM. ILC2p (CD25+Sca-1+) were gated on singlet, live, Lin-, CD117lowCD127+. (B) Frequency of ILC2p. (C) Geometric mean fluorescence intensity (GMFI) of CD25 on ILC2p. Comparison between groups was calculated using the Mann and Whitney test. Error bars indicate the SEM from 4-5 mice/group.

Figure 7: AR-blockade promotes optimal in vitro ILC2 development. In vitro differentiation of ILC2Ps on OP9-DL1 feeder cells in complete medium supplemented with mouse IL-33, IL-7 and SCF in the presence or absence of 1 nM DHT or 100 nM of the AR antagonist Futamide. (A) Flow cytometric analysis of ILC2s (Gata3+CD90+ cells) after 7 d of differentiation. (B) Frequency and (C) number of ILC2. Comparison between groups was

calculated using the Mann and Whitney test. Bars indicate the means from triplicate cultures pooled from 2 experiments.

Figure 8: The SARM CI-4AS-1 blocks ILC2P to ILC2 transition as efficiently as 5a-DHT. In vitro differentiation of ILC2p on OP9-DL1 feeder cells in complete medium supplemented with mouse IL-33 (1 ng/ml), IL-7 (5 ng/ml) and SCF (15 ng/ml) in presence or absence of AR-agonist 5a-DHT (1nM) or CI-4AS-1 (10nM) and the AR-antagonist Flutamide (100nM). (A-D) Flow cytometric analysis of ILC2 after 7 days differentiation. Pre-gated on live, Sca1+ and Gata-3+. (A) Representative dotplot and proportion of expression of CD90 and KLRG1 on ILC2. (B-D) Representative histograms of (B) frequency and (C) quantity of CD90-expressing ILC2. (D) Frequency of KLRG1 expression in triplicates culture. Error bars indicate the mean + SEM.

EXAMPLE:

Example 1:

Material & Methods

Mice.

Female and male C57BL/6J (B6) mice were purchased from the Centre d'Elevage R. Janvier (Le Genest St. Isle, France) or the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The congenic C57BL/6-Ly5.1 (CD45.1) mice were purchased from Charles River (Saint-Germain Nuelles, France). Mice were used at 8-12 weeks old unless otherwise stated. Bilateral orchiectomy and ovariectomy were performed in anesthetized 4-5 week-old mice. Mice were then rested for a period of 5-6 weeks before use. Mice selectively lacking ER α in the hematopoietic compartment (ER α fl/fl x Tie2-Cre) have been described elsewhere (Lelu et al., 2011). Mice lacking AR have been described previously (Sato et al., 2004). ARL-/+ females on a B6 background, bearing one AR-null allele, were bred with wild-type B6 males to produce ARKO (ARL-/y) and AR+/y male mice. For the generation of irradiation bone marrow chimeras, mice were γ -irradiated (11 Gy, 137Cs source) and engrafted with 5 to 10 $\times$ 10⁶ bone marrow cells/mouse. Mice were used 6-8 weeks after reconstitution. Mice were housed in specific pathogen-free conditions and handled in accordance with the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Research (study approval number #05187.01), the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines and were approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

House dust mite induced allergic asthma and IL-33 injection.

HDM (Dermatophagoides pteronyssinus extracts, Greer Laboratories, NC, USA) was used to induce allergic airway inflammation. Mice were sensitized intranasally with 1 µg HDM extracts on day 0 and were subsequently challenged with 10 µg/mouse on days 7-11. Six days after the last challenge, lungs, bronchoalveolar lavage fluid and serum were collected for flow cytometry, histological analysis and ELISA. Human recombinant (huR) IL-33 injections were performed as described with some modification (Lefrancais et al., 2014). Briefly, mice were treated intraperitoneally with 4 µg recombinant human IL-33₉₅₋₂₇₀ for two consecutive days, then left untreated for two days and injected again for 3 consecutive days. Twenty-four hours after the last injection, bronchoalveolar lavage fluid and lungs were collected for flow cytometry and histological analyses. Recombinant human IL-33₉₅₋₂₇₀ was produced as described in (Lefrancais et al., 2014).

Tissue Preparation.

Lungs were cut into small fragments and digested for 30 min at 37°C with Collagenase III (1 mg/ml; Worthington) and DNase I (200 µg/ml; Sigma). Red blood cells were lysed by treatment with hypotonic solution then filtered. Perigonadal adipose tissue was used as representative visceral adipose tissue. Adipose tissue was finely dissected with a scalpel blade and digested in 3 ml of phosphate buffered saline (PBS) containing Collagenase III (0.2 mg/ml; Worthington) and 4% BSA at 37°C for 45 min with gentle agitation. Digests were filtered through 70 µm sterile cell strainers and centrifuged at 800 g for 15 min to enrich for immune cells in stromal vascular fractions. Single cell suspensions were blocked with PBS containing 5 µg/ml anti-CD16/CD32 (2.4G2) and stained for 30 min on ice with fluorophore-conjugated antibodies. The following antibodies, purchased from BD Biosciences, were used for the identification and purification of ILC2 cells: CD19 (ID3), B220 (RA3-6B2), CD3 (145-2C110), CD4 (GK1.5), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), TCR (H57-597), NKp46 (29A1.4), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), CD117 (2B8), CD127 (A7R34), Sca1 (E13-161.7), KLRG1 (2F1), Thy1.2 (30H12) and ST2 (DJ8 or RMST2-2). Intracellular staining was performed using the Transcription Factor Staining Buffer Set (eBioscience) and monoclonal antibodies to Gata-3 (TWAJ), IL-13 (eBio13A) and IL-5 (TRFK5). Intracellular cytokine staining for IL-13 and IL-5 was performed following stimulation for 4 h with PMA (50 ng/ml) and ionomycin (100 ng/ml), in the presence of Brefeldin A (1 µg/ml). Ki67-staining analysis (BD Pharmingen) and Annexin V staining analysis (eBioscience) were performed

according to the manufacturer's protocol. Cells were analyzed using a Fortessa (BD Biosciences), and FlowJo software (Tree Star) was used for analysis. Flow cytometric sorting was performed with a FACS Aria (BD Biosciences).

ILC2 differentiation.

To induce ILC2 differentiation in vitro, cell-sorted ILC2p from female bone marrow cells were cultured for 7 days in α -MEM complete medium (10% heat-inactivated FCS) on OP9-DL1 with indicated amount of SCF, IL-7 and IL-33. 5-alpha dihydrotestosterone (DHT; Sigma-Aldrich) or the AR antagoniste Flutamide (Sigma-Aldrich) were diluted in methanol respectively at 10^{-2} M and 10^{-3} M to generate a stock solution and then added at the indicated concentration on day 0, +4, and +7 of culture.

Hormone receptor expression determined by RNA-Seq.

RNA was isolated from ex vivo ILC2p from female wild-type bone marrow using the Qiagen RNeasy Micro kit. Libraries were generated using the Illumina Truseq RNA sample preparation kit following the manufacturer's instructions and were subjected to a transcriptome 75-bp paired-end sequencing on an Illumina Next-Seq instrument. Sequence reads were aligned to the GRCm38/mm10 build of the Mus musculus genome using the Subread aligner (Liao et al., 2013). Only uniquely mapped reads were retained. Genewise counts were obtained using featureCounts (Liao et al., 2014).

Lung histology and MGG staining.

Lung tissue was fixed in 10% buffered formalin for 24 h and then placed in ethanol 70% before embedding in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin. Histological disease scores from 0 to 3 were attributed based on the severity of peribronchial, perivascular and interstitial immune cell infiltration, together with thickening of peri-bronchial epithelium, resulting in a maximum score of 12. May-Grünwald-Giemsa staining (MGG) of BAL cells were performed using a standard procedure. All scores were attributed on a blinded basis.

ELISA.

For serum IgG1 quantification, rat anti-mouse IgG1 (LO-MG1-13, Serotec) Ab was used for coating and rat anti-mouse/HRP (LO-MK-1) was used for the secondary detection step, all purchased from Serotec. For IgE, rat anti-mouse IgE (LO-ME-3, Serotec) Ab was used for

coating. Biotin-conjugated rat anti-mouse IgE mAb (BD Pharmingen) and streptavidin–HRP conjugate (Amersham) were used for detection. Quantification standards were established using mouse IgG1 and IgE mAb (Serotec).

Statistical analysis.

For all experiments, the difference between two groups was calculated with the Student t test using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). ANOVA and Bonferroni's test were used for multiple comparisons. All graphs show mean and SEM. *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

Results

Males are less susceptible than females to allergic airway inflammation

In an experimental model of ovalbumine-induced asthma, airway inflammation is more severe in female than in male mice (Melgert et al., 2005). We first investigated whether such a sex bias was also observed in the more physiological setting of house dust mite (HDM)-induced allergic airway inflammation, where the initial immunological challenge occurs in the lung in the absence of an adjuvant (Lambrecht and Hammad, 2015). Inflammatory leukocyte numbers found in bronchoalveolar lavage fluid, particularly eosinophils, were higher in female than in male mice (Fig. 1 A). This was correlated with enhanced inflammatory infiltrates in the lung of female mice (data not shown). A similar trend was observed for all the immunological parameters examined. Serum IgE and IgG1 concentrations were strongly up-regulated upon HDM challenge. This effect was more robust in female mice as compared to male, suggesting that exacerbated type 2 immunity preferentially developed in a female sex environment (Fig. 1 B). Indeed, the numbers of Th2 lymphocytes (data not shown) were markedly higher in the inflammatory lung tissues of female mice than in males. Thus, these data revealed a strong female sex bias for all the cardinal feature of HDM-induced airway inflammation.

Sex bias in steady-state ILC2 numbers is controlled by male androgen hormone

As ILC2 have been previously reported to play a critical role in priming Th2 responses in the HDM-induced asthma model (Gold et al., 2014), we next examined whether the ILC2 distribution in various organs was subjected to sex differences at steady-state. In the lungs, the frequencies and total numbers of ILC2 were 2-fold higher in females than in males (data not shown). These differences in ILC2 numbers were accompanied with changes in the expression

of phenotypic markers. ILC2 from male mice showed an increase in the expression of KLRG1 and IL33 receptor (ST2) (data not shown), whereas CD25, the high-affinity α -chain of the IL-2 receptor, was similarly expressed between both sexes (data not shown). KLRG1 interactions with E-cadherin expressed on epithelium have been shown to alter ILC2 function and act as a suppressive mechanism to dampen down the ILC2 responses (Salimi et al., 2013). Thus, our data suggest that both developmental and phenotypic differences occur between the different sexes. Furthermore, these differences in ILC2 extended to other tissues in which ILC2 dominate such as the visceral adipose tissues (VAT) (data not shown) and mesenteric lymph nodes (data not shown). In VAT, ILC2 are critical regulators of metabolic homeostasis and their loss leads to adiposity and insulin resistance when animals are placed on a high-fat diet (Molofsky et al., 2013). Our results showing reduced ILC2 numbers in male VAT are compatible with the observation that male are more vulnerable to high-fat diet-induced weight gains in terms of onset or magnitude (Hwang et al., 2010).

We next investigated the mechanisms that might underpin these sex differences in ILC2 and determined whether endogenous estrogens and estrogen receptor (ER) signaling were involved in lung ILC2 up-regulation. In ovariectomized mice, we could not detect significant changes in the frequency of lung ILC2 (data not shown). To exclude any potential role of estrogen signaling on ILC2 development or maturation, we analyzed the presence of ILC2 in mice that specifically lack ER α expression in the hematopoietic lineages. We found similar frequencies of lung ILC2 between ER $\alpha^{\text{flox/flox}} \times \text{Tie2-Cre}$ mice and their ER $\alpha^{\text{flox/flox}}$ control littermates (data not shown). By contrast, a significant up-regulation in ILC2 frequency was observed in the lungs of castrated males (Cx) (data not shown), indicating that male androgen hormones, rather than estrogens, were involved in the homeostatic regulation of ILC2 at mucosal surfaces.

Male sex hormones blunt IL-33-driven ILC2 expansion and lung inflammation in vivo

Systemic administration of IL-33 induces the proliferation of ILC2 and secretion of cytokines such as IL-5 and IL-13 resulting in lung inflammation (Neill et al., 2010). We therefore examined whether sex-differences exist in lung inflammation induced by administration of human IL-33₉₅₋₂₇₀, as described (Lefrancais et al., 2014). In this setting, IL-33 strongly increased the number and frequency of ILC2 in the lungs of treated mice compared with PBS-injected control mice (Fig. 2 A). ILC2 frequencies and numbers were however ~2-fold higher in female over male mice. The frequency of lung ILC2 which positively stained

with dead-cell-discrimination dye and/or annexin V was also determined in control and IL-33-treated mice (data not shown). As expected the numbers of dead cells and apoptotic cells were significantly increased in lung ILC2 from IL-33-treated mice as compared to untreated controls (data not shown). However, no differences in cell death and apoptosis were observed between lung ILC2 isolated from female or male mice, both at steady state or upon IL-33 administration (data not shown). Thus, enhanced apoptosis and/or impaired survival do not seem to account for the sex differences in ILC2 at steady state and during inflammation.

Interestingly, castrated males responded as intact females, indicating that endogenous male sex hormones act as critical regulators of IL-33-driven ILC2 expansion in vivo (Fig. 2 A). In IL-33-injected mice, there was a marked up-regulation (>10-fold) in the frequency of IL-5+IL-13+ ILC2 as compared to control mice (data not shown). Despite lack of significant differences in the proportion of cytokine producing-ILC2 between males, females and castrated males (data not shown), the absolute numbers of IL-5+IL-13+ ILC2 were dramatically increased in females and castrated males compared with intact male mice (Fig. 2 B). This exacerbated ILC2 response was correlated with a massive infiltration of inflammatory cells around the bronchi and pulmonary vessels (data not shown). The histological scores in females were significantly higher than in intact males. This difference was abrogated in castrated males, in which disease scores were similar to females (Fig. 2 C). Taken together, these results show that ILC2-mediated lung inflammation is more severe in female than male mice, and that orchietomy abolishes these differences.

Androgens restrain ILC2 development at steady state

As ILC2 develop from the ILC2 progenitor (ILC2p) in the bone marrow (Hoyler et al., 2012), we compared ILC2p frequency between both sexes. The absolute number and proportion of ILC2p (identified as Lin-CD117^{low}Sca-1⁺CD127⁺CD25⁺) were 2 to 3-fold higher in females than in males (Fig. 3 A), while the total cell numbers in the bone marrow were similar between both sex (not depicted). Thus, the increased number and frequency of ILC2 in peripheral tissues in female mice was correlated with a rise in ILC2p in the bone marrow. Contrasting with the lack of differential expression of CD25 between male and female tissue-resident ILC2, ILC2p from female mice significantly expressed higher level of IL2R α as compared to male ILC2p (not shown). To test whether sex-linked factors affect ILC2 proliferation at steady state, we examined intracellular expression of Ki67 in ILC2p and in lung ILC2 isolated from male or female mice. Whereas Ki67⁺ cells were detected at low frequency in ILC2p and ILC2 from male mice (ranging from 1-5 %), a significantly higher proportion of

ILC2p and lung ILC2 positively stained for Ki67 in female mice (7-15%) (data not shown). As male sex hormones, rather than estrogens, were shown to negatively control ILC2 (data not shown), we measured the expression profile of sex-steroid hormone receptors. Accordingly, ILC2p primarily expressed transcripts encoding for androgen receptor (AR), whereas those encoding for the ER (Esr-1 or Esr-2) genes were almost undetectable (Fig. 3 B). To test whether androgen signaling influences ILC2 development, ILC2p were cultured with IL-33 in absence or presence of dihydrotestosterone (DHT) or the AR-antagonist Flutamide (Fig. 3 C). Interestingly, addition of DHT to the culture inhibited ILC2 differentiation after 10 days (Fig. 3 C). On the contrary, Flutamide increased the numbers of ILC2 (~1.3-1.7 fold) that differentiated after 7 to 10 days of culture (Fig. 3 C). Of note, KLRG-1 expression on developing ILC2 was down-regulated in Flutamide-treated cultures (data not shown), indicating that AR-signaling controls KLRG-1 expression in male ILC2. Thus, androgen signaling actively represses ILC2 development through AR-signaling in ILC2p.

AR negatively controls IL-33-driven ILC2 expansion and lung inflammation

To evaluate the role of AR in hematopoietic cells on the sex bias in ILC2-mediated lung inflammation, we generated bone marrow chimeras. Lethally irradiated C57BL/6 mice, from either sex, were engrafted with bone marrow cells from wild-type or AR-deficient (ARKO) littermate male mice, and lung inflammation induced by IL-33 injection was monitored as described in Fig. 2. The absence of AR in the hematopoietic compartment abolished the sex differences in the total numbers of inflammatory cells, including eosinophils, in bronchoalveolar lavage fluid (Fig. 4 A). Lung-infiltrating cells were significantly reduced in wild type chimeric males compared with females, however this sex difference was absent in AR-deficient bone marrow chimeras (Fig. 4 B). ILC2 numbers were higher in the lungs of male mice engrafted with bone marrow cells from ARKO rather than wild-type mice (Fig. 4 C). KLRG1 expression was again significantly down-regulated on AR-deficient ILC2 confirming that the differences observed between males and females on ILC2 development and phenotype are dependent on AR signaling (Fig. 4 D), in agreement with the in vitro data in Fig. 3 C. This enhanced responsiveness to IL-33-driven lung inflammation in AR-deficient males was further confirmed by histological evaluation (Fig. 4 E). By contrast, hematopoietic expression of AR-deficiency had no significant impact on lung inflammation in female chimeric mice (Fig. 4 E). To determine whether the deficiency in ILC2-driven lung inflammation observed in ARKO male was cell intrinsic, mixed bone marrow chimera experiments were performed. Lethally irradiated CD45.1 male mice were transplanted with an equal mixture of bone marrow cells

from CD45.1 WT and CD45.2 ARKO mice. After 6 weeks of reconstitution, the ratio between CD45.1+ (WT) and CD45.1neg (ARKO) lung ILC2 were examined following IL-33 injection. We observed a significant deficiency in the proportion of WT ILC2 (CD45.1+) compared with ILC2 derived from ARKO progenitors (CD45.1neg) (data not shown), resulting in a WT/ARKO ILC2 ratio below 0.2 (data not shown). For comparison, the WT/ARKO ratios for total CD45+ lung infiltrating cells or lung eosinophils were above 0.7 (data not shown). Yet, the frequencies of Ki67+ cells and dead cells were similar between WT and AR-deficient ILC2 (data not shown). Interestingly, in agreement with results in Fig. 4 D, the reduced expression of KLRG1 on AR-deficient ILC2 was maintained in the mixed chimeras (data not shown). Altogether, these results indicate that endogenous androgens signal through hematopoietic AR in a cell-intrinsic manner to control the level of ILC2 responsiveness in the lung. As ILC2 survival and proliferation were not affected by AR-deficiency, these results suggest a major effect of androgen signaling on the regulation of tissue-resident ILC2 numbers at steady state.

The prevalence of asthma is consistently reported to be higher in boys than in girls (Almqvist et al., 2008; Carey et al., 2007). This pattern changes during adolescence where the onset of asthma becomes much less prevalent in male than in female. However, the mechanism underpinning this transition is unknown. Our results indicate that androgen-mediated regulation of ILC2-dependent Th2 responses to environmental allergens could contribute to this switch around puberty. At steady state, we observed a strong sex bias in ILC2 numbers, with an increased representation of ILC2 in various tissues, including lung, fat and mesenteric lymph nodes. This was associated with a strong up regulation of ILC2p in the bone marrow of female compared with male mice. Accumulating evidence indicate that male androgens are immunosuppressive, whereas female estrogens may promote innate and adaptive immunity (Markle and Fish, 2014). We excluded a positive impact of estrogens in this sex bias as ovariectomy or selective ablation of *Esr-1* gene in the hematopoietic compartment had no effect on the number and phenotype of lung ILC2 at steady state. By contrast, orchiectomy abolished this sex difference, indicating that endogenous androgens may negatively regulate ILC2 development and/or expansion in vivo. Analysis of sex hormone receptor expression in ILC2p demonstrated a selective expression of *Ar* gene mRNA, whereas those encoding for estrogen receptors *Esr-1* or *Esr-2* were barely detected. This observation is in agreement with recent reports showing that *Ar* is highly expressed in tissue-resident ILC2s, and has been classified as a prototypic ILC2 signature gene (Robinette et al., 2015), highly conserved during evolution (Vivier et al., 2016). In favor of a unique role for the male sex hormone androgen in the regulation of ILC2-dependent responses, we showed that orchiectomy or AR-deficiency in

hematopoietic cells, both abolished all phenotypic changes in IL-33-mediated lung inflammation. These results strongly suggest that ligand-induced activation of AR within ILC2 is the main signaling pathway contributing to the sex differences in this model. Indeed, we provided evidence for AR-dependent regulation of IL-33-driven ILC2 differentiation and expansion in vitro and demonstrated using mixed chimeras the cell-intrinsic requirement of AR for the inhibition of IL-33-mediated ILC2 expansion in vivo.

Although, previous studies suggested that female sex hormones might contribute to the sex differences in allergic asthma (Keselman and Heller, 2015), our results are the first to establish that male androgen hormones protect from ILC2-driven lung inflammation. Developing ILC2 themselves or their progenitors seem to represent the direct target of androgens. Cell-intrinsic AR signaling in ILC2p appears to promote the development of KLRG1hi ILC2, which are less frequent in female mice or castrated males. These cells, however, are unlikely to represent the KLRG1hi inflammatory ILC2 population, which is mobilized by IL-25 or infection, as these cells, unlike steady state ILC2, lacked ST2 and did not proliferate in response to IL-33 (Huang et al., 2015). Thus, we propose that androgen signaling may directly regulate the development of natural ILC2. However, as very few ILC2 develop from bone marrow progenitors in adult mice (Gasteiger et al., 2015), we believe that most of the sex differences observed in the IL-33-driven inflammation model are already imprinted by the pre-existing sex-bias in tissue-resident ILC2 reflected in the steady-state numbers. Enhanced CD25 expression in female ILC2p correlated with an increased frequency of proliferating (Ki67+) cells, suggesting that greater sensitivity to IL-2 in female ILC2p could promote ILC2 seeding in non-lymphoid tissues during ontogeny. This hypothesis is however not supported by recent findings demonstrating that CD25-deficient tissue-resident ILC2 proliferated to a similar extent as WT ILC2 in bone marrow chimeric mice, both at steady state and during acute helminth infection (Gasteiger et al., 2015). Although the environmental cues that orchestrate the sex bias in tissue-resident ILC2 at steady state still need to be identified, we cannot exclude that androgen signalling might also act by altering the IL-2/CD25-dependent regulation of ILC2 effector function during inflammation (Gasteiger et al., 2015).

AR is a ligand dependent transcription factor, which could directly or indirectly regulate the expression of key transcription factors or molecules involved in the maintenance of ILC2p or essential for their differentiation into ILC2 cells. Such specific downstream targets of AR in ILC2p remain to be identified. Alternatively, AR signaling may directly or indirectly regulate the IL-33 signaling machinery via the down-regulation of NF- κ B or AP-1 pathways, resulting in reduced cell proliferation. Studies of the T cell differentiation pathways suggested that

testosterone inhibited CD4 T-cell differentiation by up-regulating the phosphatase Ptpn1, which dephosphorylates Tyk2 the upstream kinase responsible for IL-12-induced Stat-4 phosphorylation thereby inhibiting Th1 polarization (Kissick et al., 2014). Whether altered IL-33 signaling occurs in androgen stimulated ILC2s will require further investigations.

Our study suggests that androgen therapy could be useful to protect against allergic diseases through its inhibitory action on ILC2. Androgen replacement therapy is currently used for the treatment of subjects with deficient testicular function, and administration of dihydroepiandrosterone (DHEA), a weak androgen with less virilizing side effects, has shown some beneficial actions in the treatment of allergic asthma in human (Shah, 2004; Wenzel et al., 2010) and in mouse (Liou and Huang, 2011). Understanding further how AR signals in ILC2 or their progenitors may provide new therapeutics or downstream targets for the treatment of allergic diseases, while minimizing side effects of androgen therapy.

Example 2:

5a-DHT treatment limits ILC2 development in vivo at steady state

Our preliminary results show that short term treatment (2 weeks) of Cx female mice with DHT is sufficient to recapitulate the male phenotype at steady state, characterized by lower lung ILC2 frequency and numbers (Fig. 5 A and B), and with enhanced development of KLRG-1+ ILC2 (Fig. 5 C and D). In agreement with the inhibitory effect of androgen on lung ILC2 at steady state, we found that ILC2 expansion and ILC2-dependent lung inflammation in response to IL-33 or IL-2 administration were strongly inhibited by DHT administration (not shown).

We have also investigated whether ILC2P in the bone marrow could be amenable to pharmacological manipulation with androgen. As shown, in Fig. 6, treatment with DHT was associated with a significant reduction in ILC2P frequency (Fig. 6A) and a dramatic down-regulation of CD25 expression (Fig. 6B). We will use this model to investigate the impact of androgen signaling on the transcriptional signature of ILC2P.

Altogether these results strongly suggest that short-term treatment with AR agonist ligand inhibits the development of tissue-resident ILC2 (Fig. 5) and ILC2P in the bone marrow (Fig. 6).

AR-blockade promotes optimal in vitro ILC2 development

We set-up an optimal cytokine cocktail to promote ILC2 development from ILC2P in vitro. In this model, we assessed ILC2 development through the expression of the ILC2 marker CD90 (Fig. 7). By day 7-9 all Sca-1+ cells in the culture were also positive for GATA-3, whereas CD90 was differentially expressed depending on the culture condition (Fig. 7A).

As tissue-resident ILC2 homogenously express high level of CD90, we assumed that CD90 expression may identify truly differentiated mature ILC2 corresponding to their in vivo counterpart. Interestingly, blockade of AR-signaling with Flutamide enhanced CD90^{hi} ILC2 development, whereas increasing androgen levels through addition of 5a-DHT, dramatically inhibited the differentiation of CD90⁺ ILC2 (Fig. 7 A and B), in agreement with our recent work (Laffont et al, 2017). Moreover, AR-signaling was associated with enhanced differentiation of KLRG-1⁺ ILC2s (Fig. 7C and D), in agreement with our in vivo data (Fig. 5).

The SARM Cl-4AS-1 inhibits in vitro the development of CD90^{hi} ILC2 and promotes the differentiation of KLRG1^{hi} CD90^{lo} ILC2

Using the in vitro differentiation model described in Fig. 7, we then assessed the capacity of the SARM Cl-4AS-1 to modulate the development of ILC2 from ILC2P (**Fig. 8**). For the two doses tested (10^{-8} and 10^{-9} M), we observed a marked decrease in CD90⁺ ILC2 cell development, to level similar as the full agonist ligand 5a-DHT (10^{-9} M). The inhibitory action of Cl-4AS-1 or 5a-DHT were abrogated in the presence of Flutamide. Like 5a-DHT, activation of AR with Cl-4AS-1, not only inhibited the development of CD90^{hi} ILC2, but also promoted the expansion/differentiation of KLRG-1⁺ CD90^{dull} ILC2s. This latter population was already found in the control culture conditions, probably as a consequence of the presence of low amounts of androgens provided by the regular FCS used in the culture medium. Indeed, AR blockade with Flutamide abrogated the development of this cell population in FCS regular culture conditions without exogenous androgen supplementation (not shown).

Taken together, AR signaling induces a developmental switch in ILC2P to ILC2 transition in vitro, by favoring the differentiation of CD90^{dull} ILC2, from which 40% express high level of KLRG-1. AR-blockade favors in contrast the development of KLRG-1^{neg} CD90^{hi} ILC2, which resembles the dominant tissue-resident lung ILC2 population preferentially found in female mice (Laffont et al, 2017).

Our results provide the first demonstration that the SARM Cl-4AS-1 can fully substitute to the pure agonist ligand 5a-DHT to inhibit the transition of ILC2P to KLRG-1^{neg} CD90^{hi} ILC2.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

Akdis CA. Therapies for allergic inflammation: refining strategies to induce tolerance. Nat Med. 2012 May 4;18(5):736-49. doi: 10.1038/nm.2754.

Almqvist, C., M. Worm, B. Leynaert, and G.A.L.E.N.W.P.G. working group of. 2008.

5 Impact of gender on asthma in childhood and adolescence: a GA2LEN review. Allergy 63:47-57.

Arthi Thirumalai, Kathryn E. Berkseth , John K. Amory. Treatment of Hypogonadism: Current and Future Therapies. F1000Research 2017, 6(F1000 Faculty Rev):68 Last updated: 24 JAN 2017.

10 Barlow, J.L., S. Peel, J. Fox, V. Panova, C.S. Hardman, A. Camelo, C. Bucks, X. Wu, C.M. Kane, D.R. Neill, R.J. Flynn, I. Sayers, I.P. Hall, and A.N. McKenzie. 2013. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J Allergy Clin Immunol 132:933-941.

Bartemes, K.R., K. Iijima, T. Kobayashi, G.M. Kephart, A.N. McKenzie, and H. Kita. 15 2012. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. The Journal of Immunology 188:1503-1513.

Carey, M.A., J.W. Card, J.W. Voltz, S.J. Arbes, Jr., D.R. Germolec, K.S. Korach, and D.C. Zeldin. 2007. It's all about sex: gender, lung development and lung disease. Trends Endocrinol Metab 18:308-313.

20 Doherty, T.A., N. Khorram, S. Lund, A.K. Mehta, M. Croft, and D.H. Broide. 2013. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. J Allergy Clin Immunol 132:205-213.

Gold, M.J., F. Antignano, T.Y. Halim, J.A. Hirota, M.R. Blanchet, C. Zaph, F. Takei, and K.M. McNagny. 2014. Group 2 innate lymphoid cells facilitate sensitization to local, but 25 not systemic, TH2-inducing allergen exposures. J Allergy Clin Immunol 133:1142-1148.

Halim, T.Y.F., R.H. Krauß, A.C. Sun, and F. Takei. 2012. Lung Natural Helper Cells Are a Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced Airway Inflammation. Immunity 36:451-463.

Halim, T.Y.F., C.A. Steer, L. Mathä, M.J. Gold, I. Martinez-Gonzalez, K.M. McNagny, 30 A.N.J. Mckenzie, and F. Takei. 2014. Group 2 Innate Lymphoid Cells Are Critical for the Initiation of Adaptive T Helper 2 Cell-Mediated Allergic Lung Inflammation. Immunity 40:425-435.

Hoyler, T., C.S. Klose, A. Souabni, A. Turqueti-Neves, D. Pfeifer, E.L. Rawlins, D. Voehringer, M. Busslinger, and A. Diefenbach. 2012. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immunity* 37:634-648.

Huang, Y., L. Guo, J. Qiu, X. Chen, J. Hu-Li, U. Siebenlist, P.R. Williamson, J.F. Urban, Jr., and W.E. Paul. 2015. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol* 16:161-169.

Hwang, L.L., C.H. Wang, T.L. Li, S.D. Chang, L.C. Lin, C.P. Chen, C.T. Chen, K.C. Liang, I.K. Ho, W.S. Yang, and L.C. Chiou. 2010. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)* 18:463-469.

Imai, Y., K. Yasuda, Y. Sakaguchi, T. Haneda, H. Mizutani, T. Yoshimoto, K. Nakanishi, and K. Yamanishi. 2013. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proceedings of the National Academy of Sciences of the United States of America* 110:13921-13926.

Kim, B.S., M.C. Siracusa, S.A. Saenz, M. Noti, L.A. Monticelli, G.F. Sonnenberg, M.R. Hepworth, A.S. Van Voorhees, M.R. Comeau, and D. Artis. 2013. TSLP Elicits IL-33-Independent Innate Lymphoid Cell Responses to Promote Skin Inflammation. *Science translational medicine* 5:170ra116-170ra116.

Kissick, H.T., M.G. Sanda, L.K. Dunn, K.L. Pellegrini, S.T. On, J.K. Noel, and M.S. Arredouani. 2014. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. *Proc Natl Acad Sci U S A* 111:9887-9892.

Laffont Sophie, Eve Blanquart, Magali Savignac, Claire Cénac, Gilles Laverny, Daniel Metzger, Jean-Philippe Girard, Gabrielle T. Belz, Lucette Pelletier, Cyril Seillet, and Jean-Charles Guéry. Androgen signaling negatively controls group 2 innate lymphoid cells. *J. Exp. Med.* 2017 Vol. 214 No. 6.

Lambrecht, B.N., and H. Hammad. 2015. The immunology of asthma. *Nat Immunol* 16:45-56.

Lefrancais, E., A. Duval, E. Mirey, S. Roga, E. Espinosa, C. Cayrol, and J.P. Girard. 2014. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci U S A* 111:15502-15507.

Markle, J.G., and E.N. Fish. 2014. Sex matters in immunity. *Trends Immunol* 35:97-104.

Melgert, B.N., D.S. Postma, I. Kuipers, M. Geerlings, M.A. Luinge, B.W. van der Strate, H.A. Kerstjens, W. Timens, and M.N. Hylkema. 2005. Female mice are more susceptible

to the development of allergic airway inflammation than male mice. *Clin Exp Allergy* 35:1496-1503.

Molofsky, A.B., J.C. Nussbaum, H.-E. Liang, S.J. Van Dyken, L.E. Cheng, A. Mohapatra, A. Chawla, and R.M. Locksley. 2013. Innate lymphoid type 2 cells sustain visceral
5 adipose tissue eosinophils and alternatively activated macrophages. *Journal of Experimental Medicine* 210:535-549.

Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J.-I. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.

10 Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K.A. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, and A.N.J. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.

Päivi Pihlajamaa, Biswajyoti Sahu, and Olli A. Jänne. Determinants of Receptor- and Tissue-Specific Actions in Androgen Signaling. *Endocrine Reviews*, August 2015, 36(4):357–
15 384.

Salimi, M., J.L. Barlow, S.P. Saunders, L. Xue, D. Gutowska-Owsiak, X. Wang, L.C. Huang, D. Johnson, S.T. Scanlon, A.N.J. McKenzie, P.G. Fallon, and G.S. Ogg. 2013. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *Journal of Experimental Medicine* 210:2939-2950.

20 Sato, T., T. Matsumoto, H. Kawano, T. Watanabe, Y. Uematsu, K. Sekine, T. Fukuda, K. Aihara, A. Krust, T. Yamada, Y. Nakamichi, Y. Yamamoto, T. Nakamura, K. Yoshimura, T. Yoshizawa, D. Metzger, P. Chambon, and S. Kato. 2004. Brain masculinization requires androgen receptor function. *Proc Natl Acad Sci U S A* 101:1673-1678.

Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, and R.A. Kastelein. 2005. IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. *Immunity* 23:479-490.

Townsend, E.A., V.M. Miller, and Y.S. Prakash. 2012. Sex differences and sex steroids in lung health and disease. *Endocr Rev* 33:1-47.

30 Wojno, E.D., L.A. Monticelli, S.V. Tran, T. Alenghat, L.C. Osborne, J.J. Thome, C. Willis, A. Budelsky, D.L. Farber, and D. Artis. 2015. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol* 8:1313-1323.

CLAIMS:

1. An agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.
- 5 2. An agonist for use according to claim 1 wherein the type 2 inflammation is selected in the group consisting in allergic asthma, eosinophilic bronchitis, eosinophilic pneumopathy and Churg-Strauss syndrome.
3. An agonist for use according to claim 2 wherein the type 2 inflammation is allergic asthma.
- 10 4. An agonist for use according to claims 1 to 3 wherein said agonist is a SARM.
5. An agonist for use according to claim 4 wherein said agonist is the 5-alpha dihydrotestosterone (5a-DHT) or the SARM CI-4AS-1.
6. A therapeutic composition comprising a agonist of the androgen receptor express on group 2 innate lymphoid cells (ILC2) for use in the treatment of type 2 inflammation in a subject in need thereof.
- 15 7. A method for treating lung inflammation comprising administering to a subject in need thereof a therapeutically effective amount of a agonist of the androgen receptor expressed in group 2 innate lymphoid cells (ILC2).

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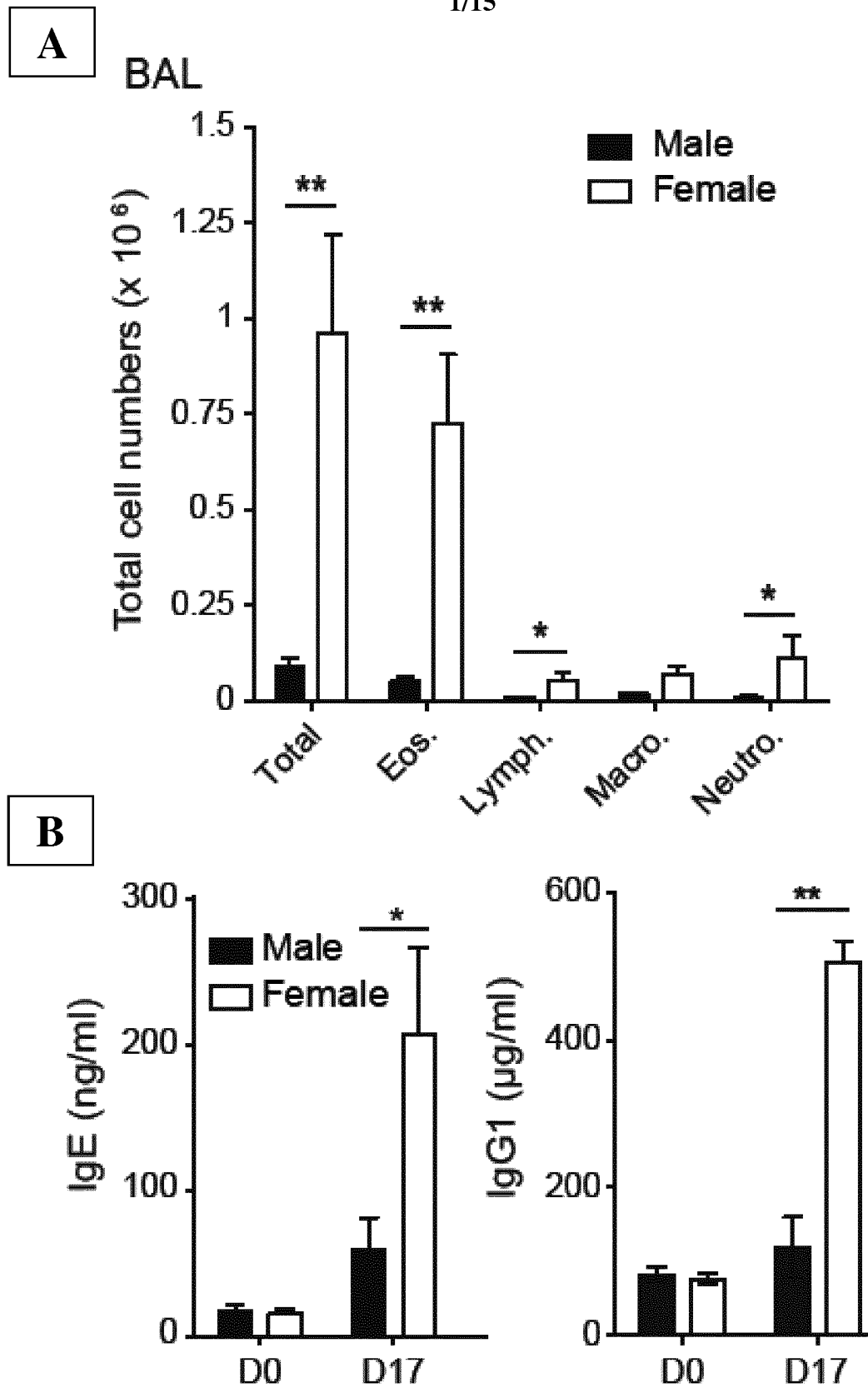


Figure 1 A and B

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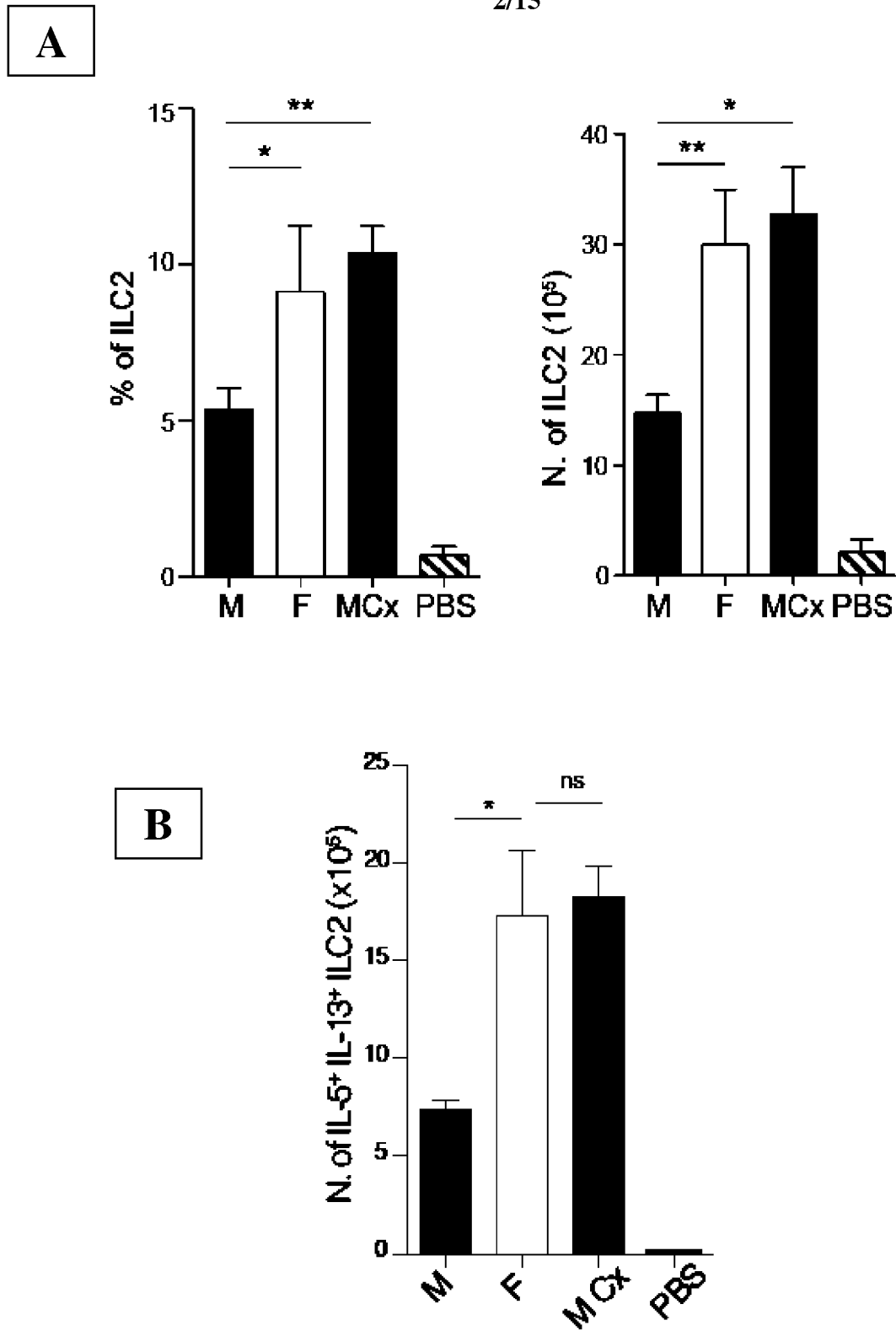
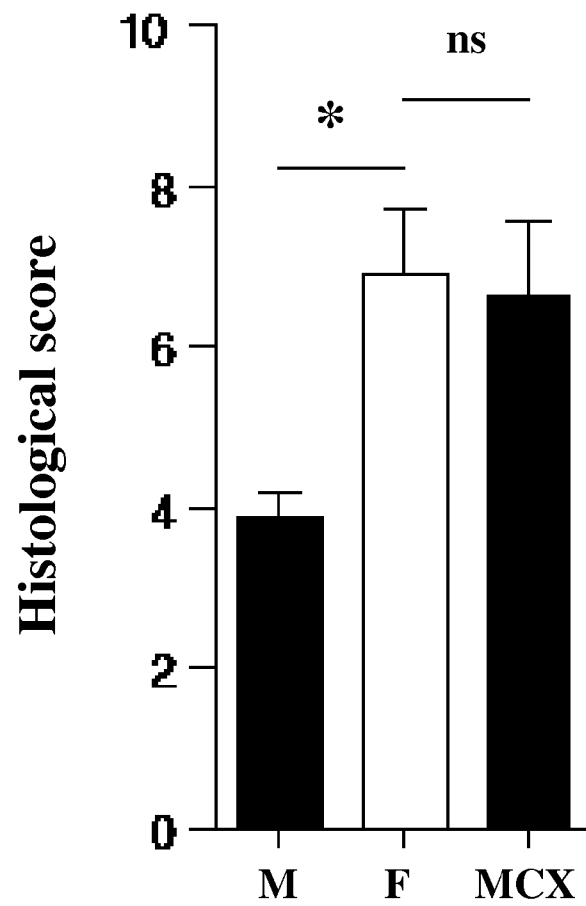


Figure 2 A and B

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C**Figure 2 C**

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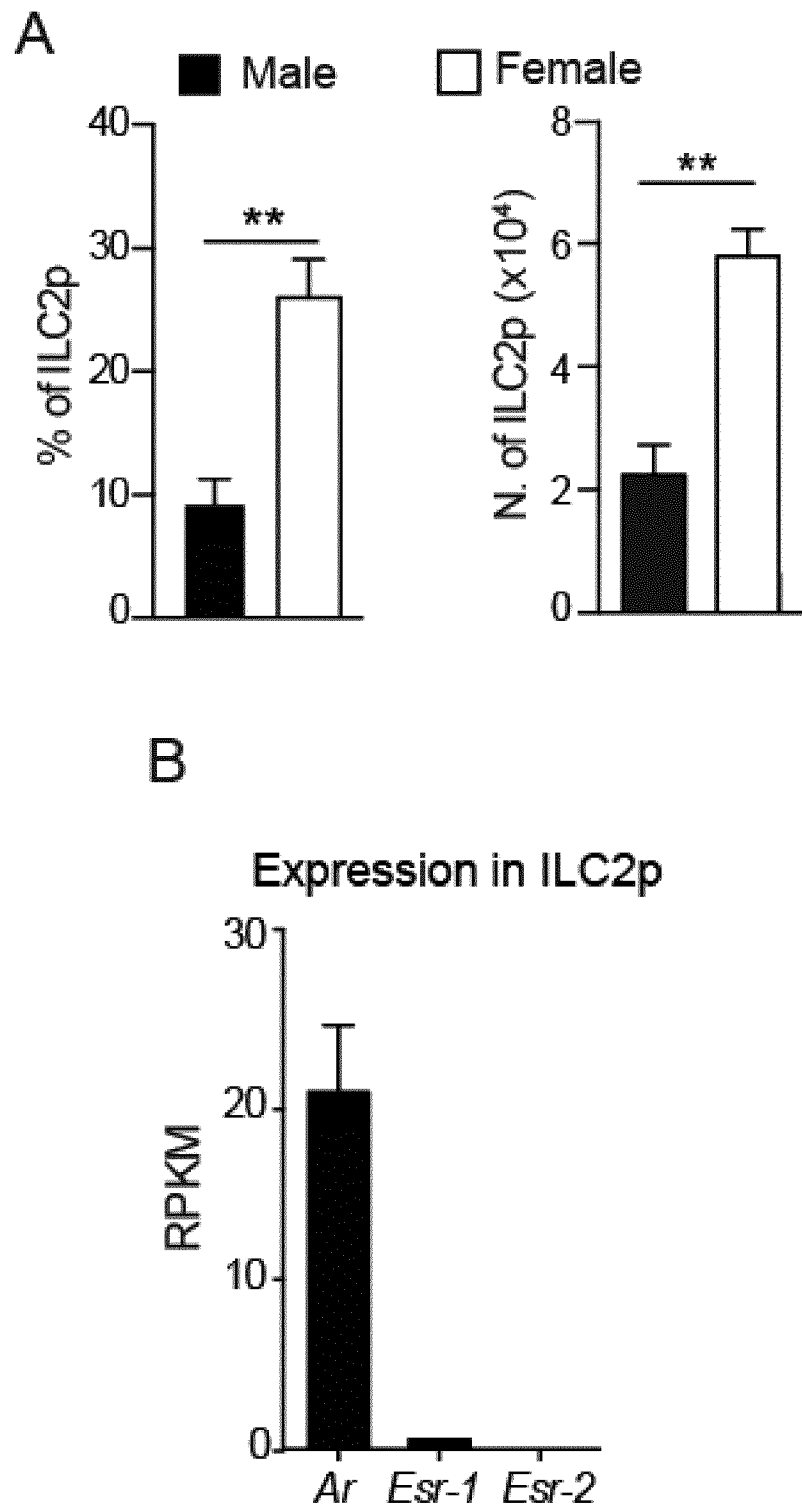


Figure 3 A and B

5/15

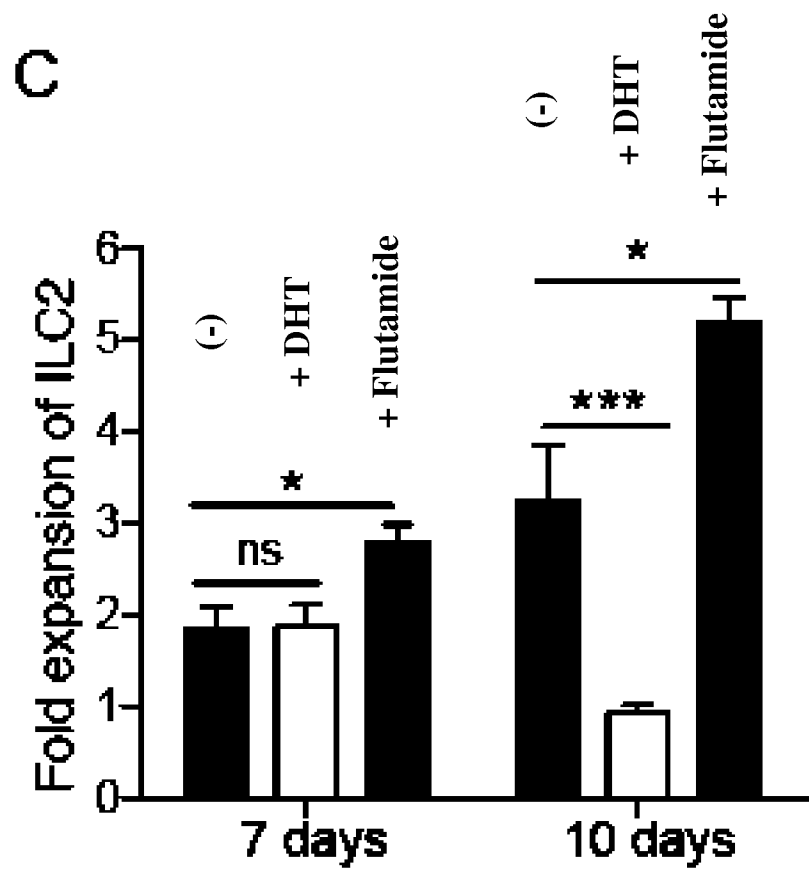


Figure 3 C

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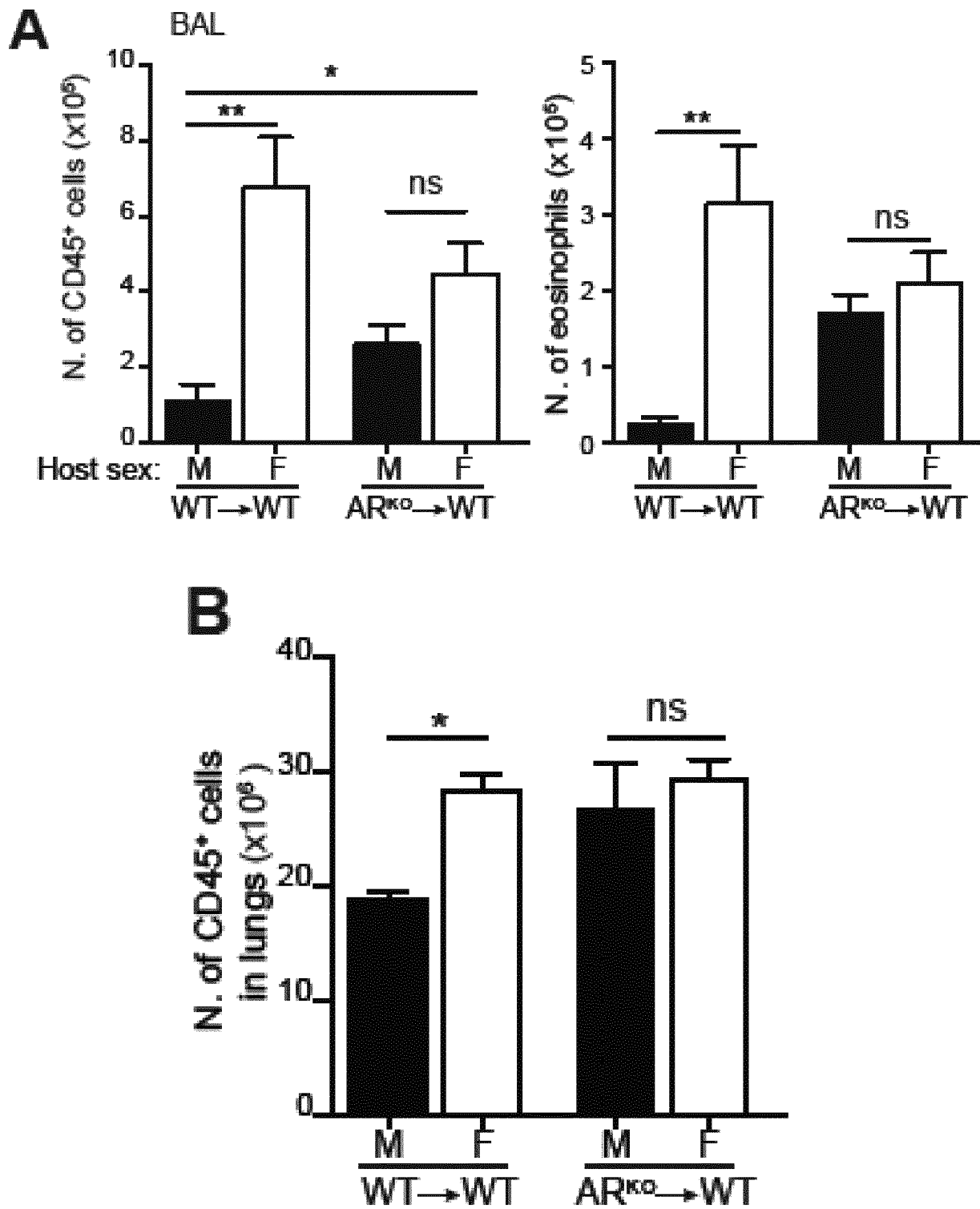


Figure 4 A and B

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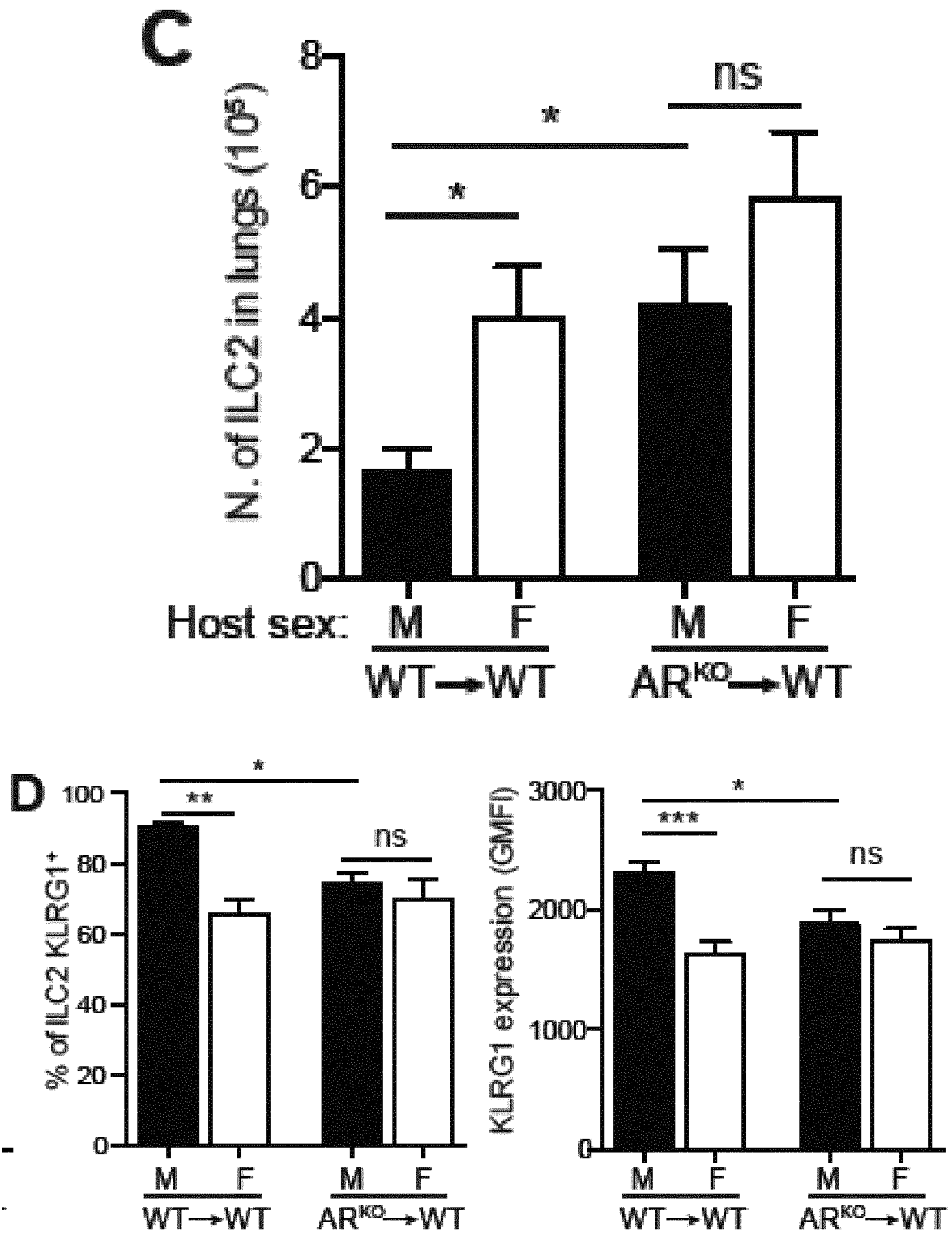


Figure 4 C and D

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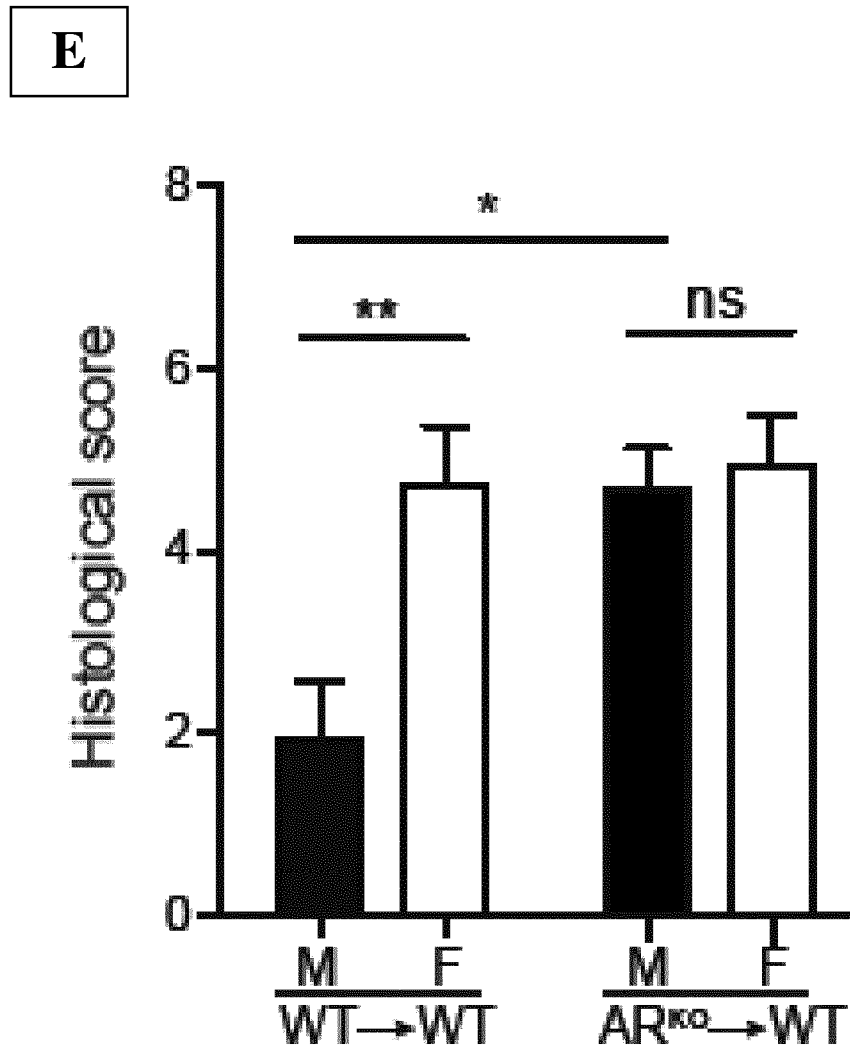


Figure 4 E

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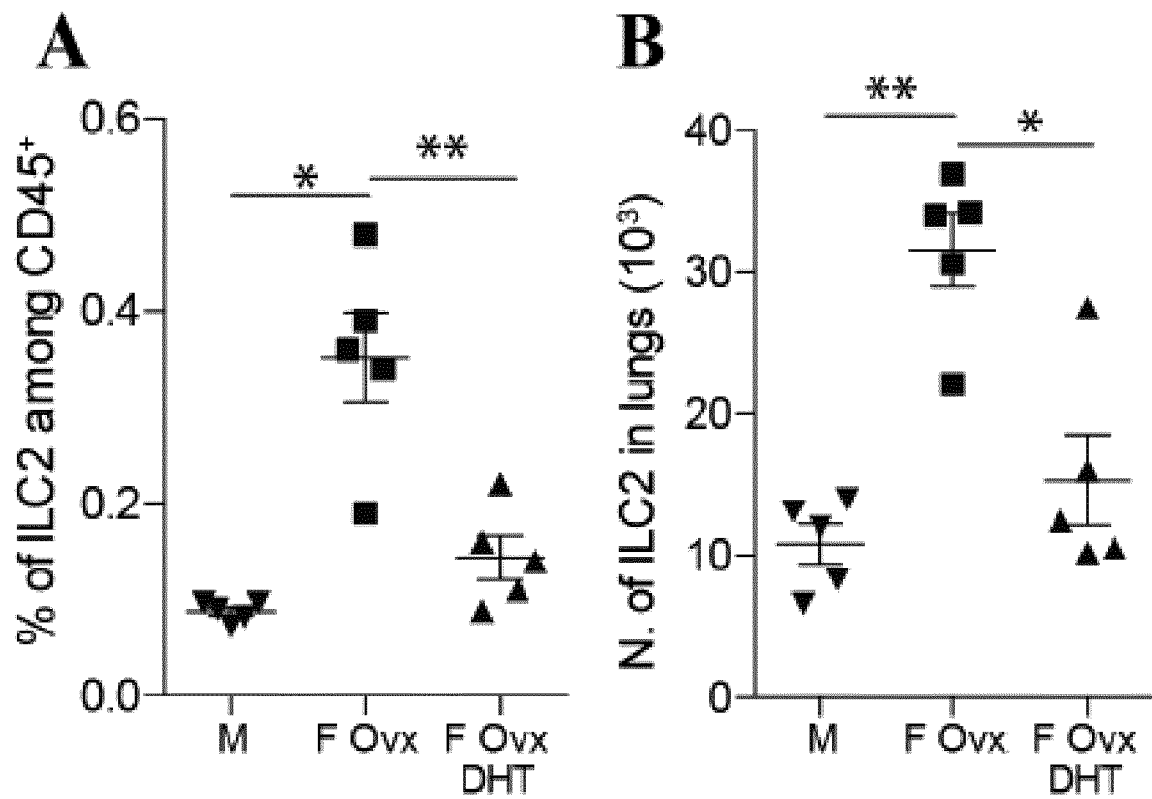


Figure 5 A and B

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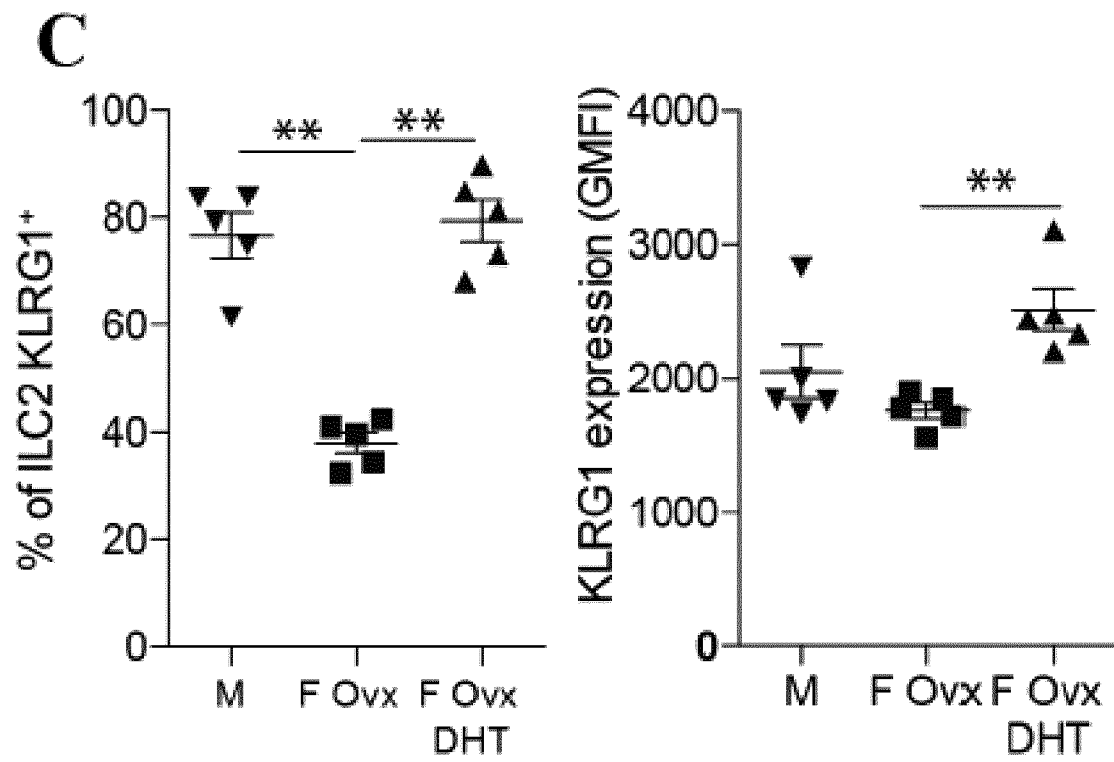


Figure 5 C

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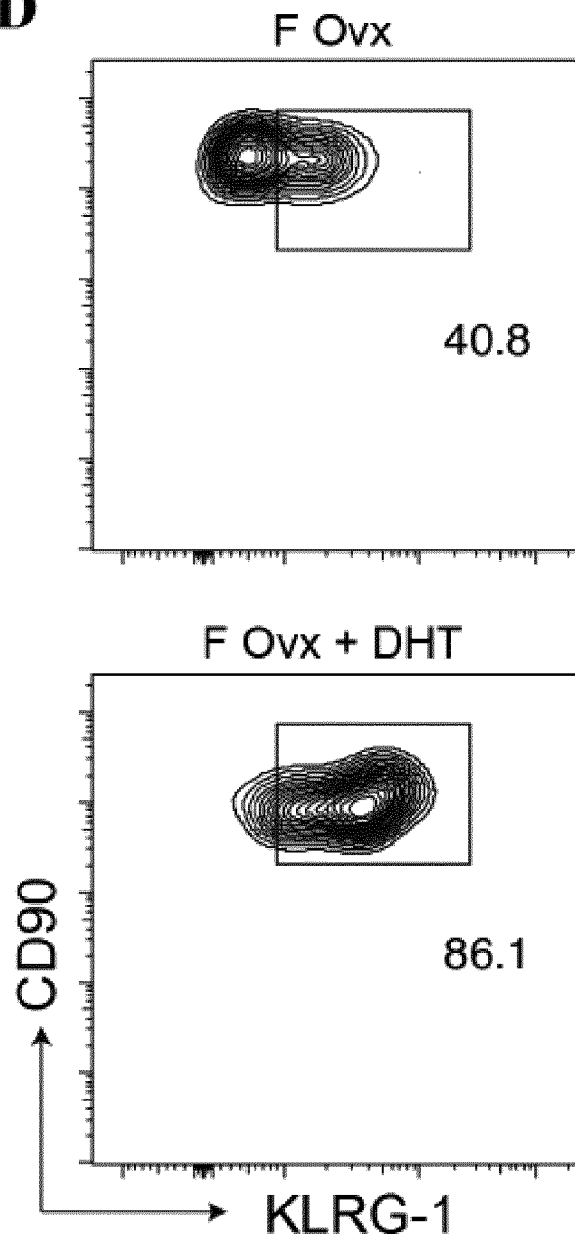
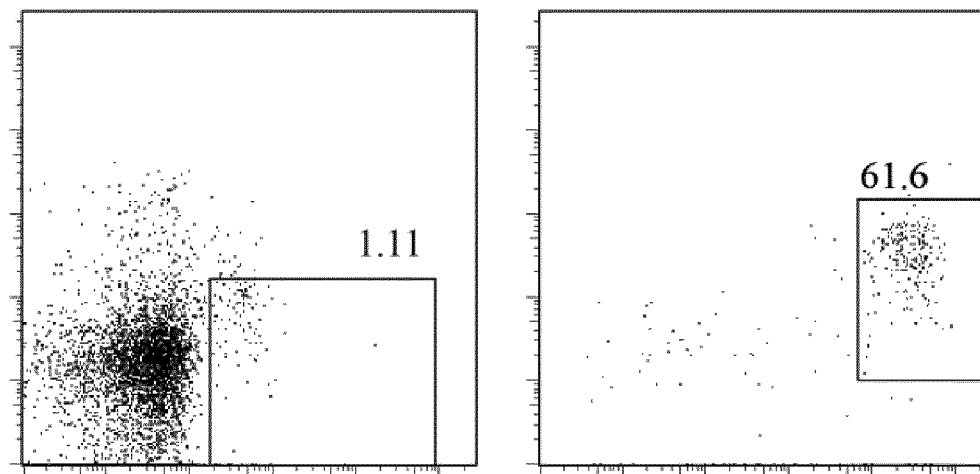
D

Figure 5D

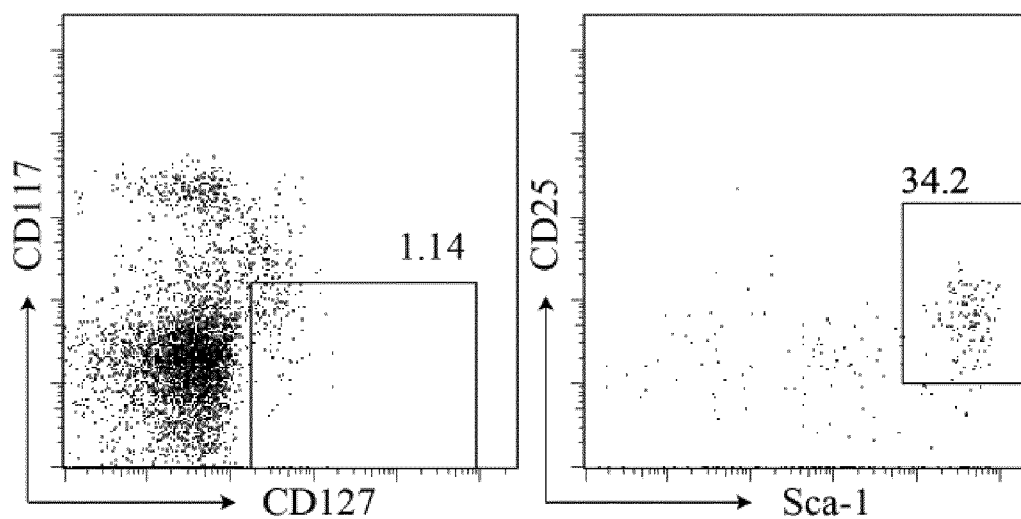
12/15

APre-gate : singlet, alive, Lin⁻

F Ovx



F Ovx + DHT

**Figure 6 A**

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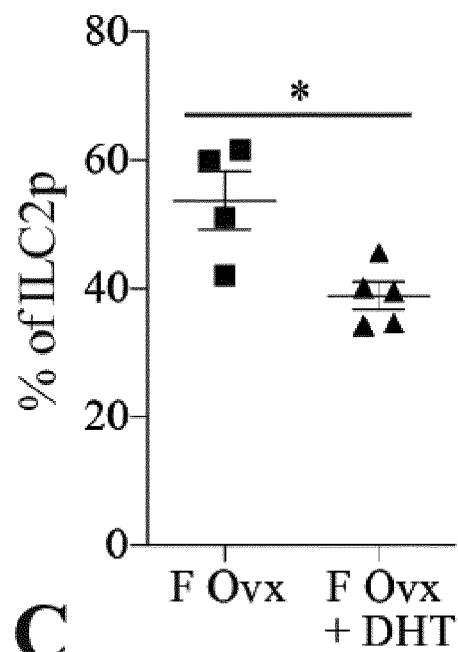
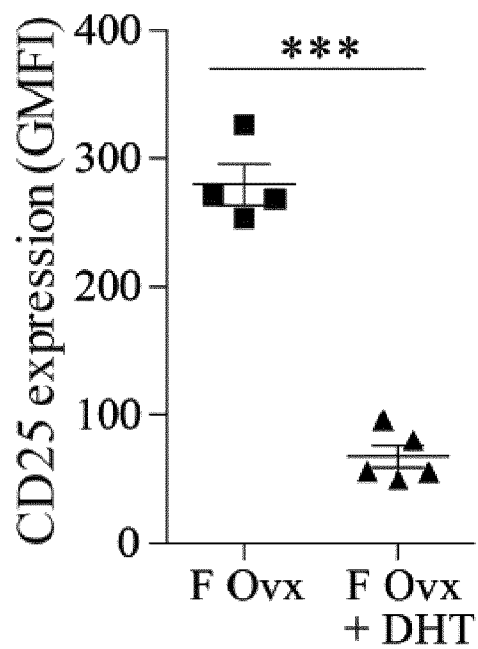
B**C**

Figure 6 B and C

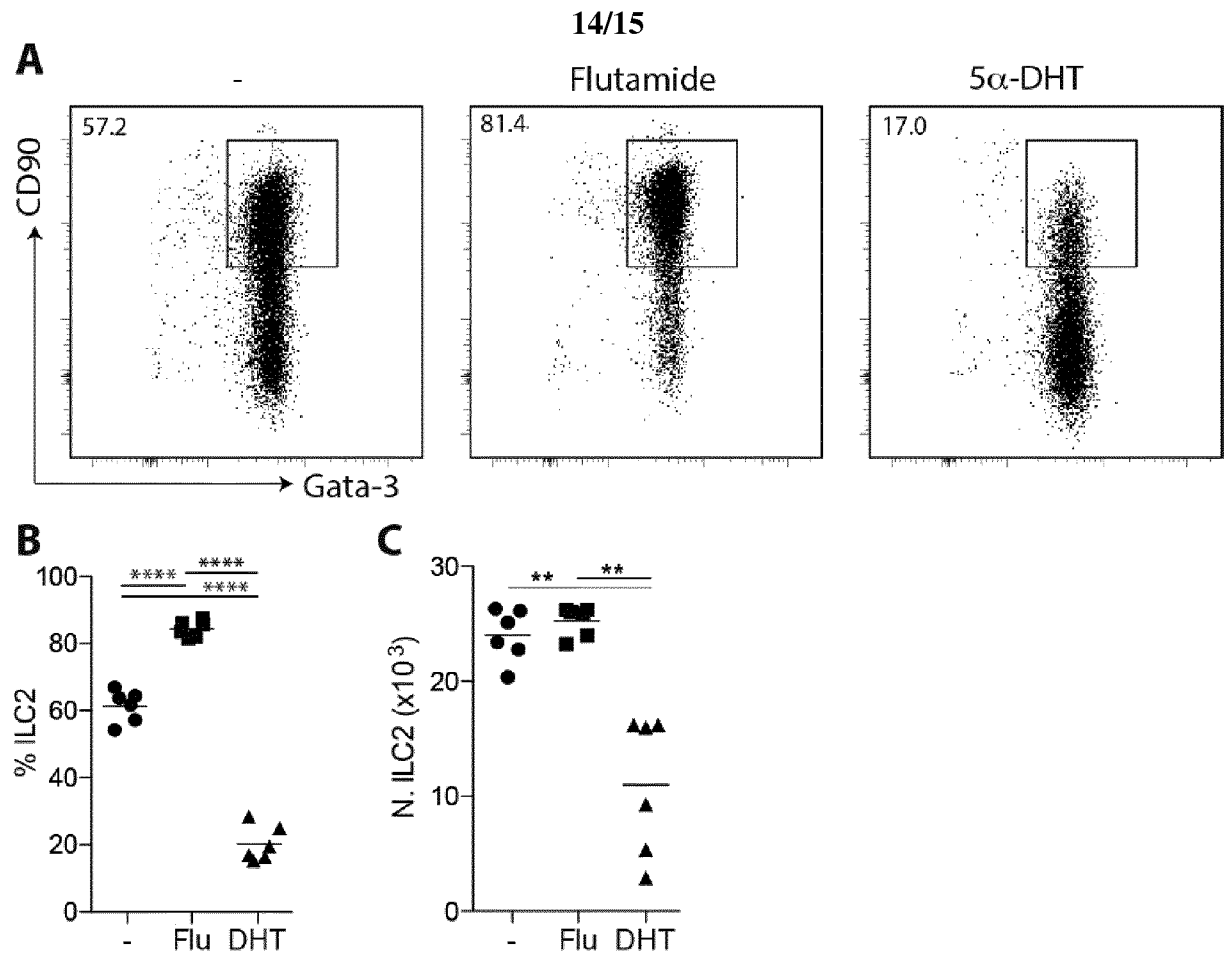


Figure 7

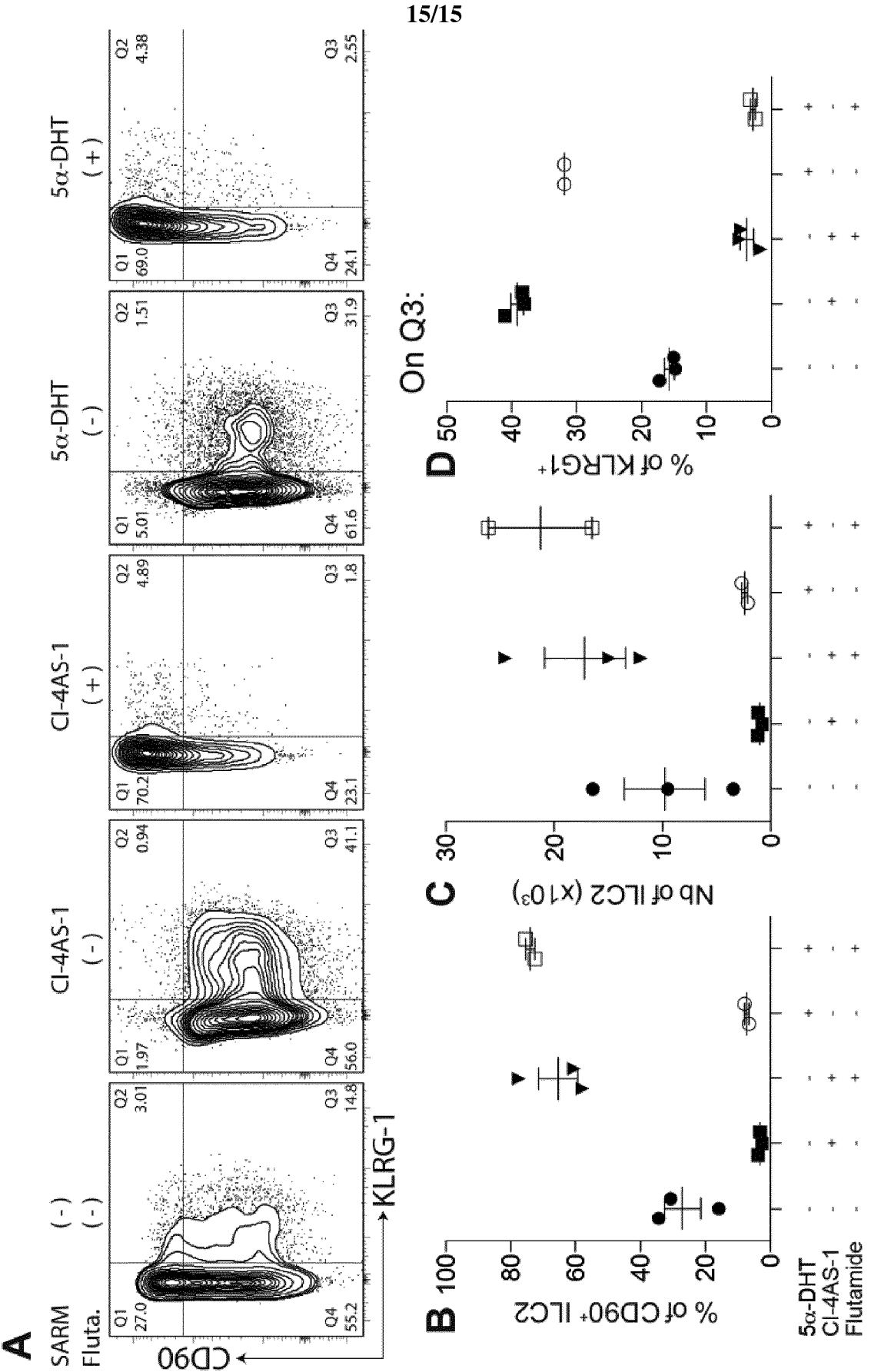


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/058309

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/568 A61K31/5685 A61K45/06 A61P11/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHUN-KEUNG YU ET AL: "Dehydroepiandrosterone attenuates allergic airway inflammation in Dermatophagoides farinae-sensitized mice", JOURNAL OF MICROBIOLOGY, IMMUNOLOGY AND INFECTION, vol. 35, no. 3, 1 January 2002 (2002-01-01), pages 199-202, XP055403500, abstract the whole document ----- -/--	1-4,6,7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 May 2018

Date of mailing of the international search report

04/06/2018

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Authorized officer

Langer, Oliver

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058309

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YU C K ET AL: "ATTENUATION OF HOUSE DUST MITE DERMATOPHAGOIDES FARINAE-INDUCED AIRWAY ALLERGIC RESPONSES IN MICE BY DEHYDROEPIANDROSTERONE IS CORRELATED WITH DOWN-REGULATION OF TH2 RESPONSE", CLINICAL & EXPERIMENTAL ALLERGY : JOURNAL OF THE BRITISH SOCIETY FOR ALLERGY AND CLINICAL IMMUNOLOGY, WILEY INTERSCIENCE, UK, vol. 29, no. 3, 1 January 1999 (1999-01-01), pages 414-422, XP000923037, ISSN: 0954-7894, DOI: 10.1046/J.1365-2222.1999.00484.X abstract the whole document	1-4,6,7
X	INSEON S. CHOI ET AL: "Effects of dehydroepiandrosterone on Th2 cytokine production in peripheral blood mononuclear cells from asthmatics", KOREAN JOURNAL OF INTERNAL MEDICINE KOREA (SOUTH), vol. 23, no. 4, 1 January 2008 (2008-01-01), page 176, XP055403492, ISSN: 1226-3303, DOI: 10.3904/kjim.2008.23.4.176 abstract the whole document	1-4,6,7
X	INSEON S CHOI: "Gender-Specific Asthma Treatment", ALLERGY, ASTHMA & IMMUNOLOGY RESEARCH : AAIR, vol. 3, no. 2, 1 January 2011 (2011-01-01), page 74, XP055403477, US ISSN: 2092-7355, DOI: 10.4168/aaair.2011.3.2.74 abstract the whole document	1-4,6,7
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058309

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHIAN-JIUN LIOU ET AL: "Dehydroepiandrosterone Suppresses Eosinophil Infiltration and Airway Hyperresponsiveness via Modulation of Chemokines and Th2 Cytokines in Ovalbumin-Sensitized Mice", JOURNAL OF CLINICAL IMMUNOLOGY, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 31, no. 4, 4 June 2011 (2011-06-04), pages 656-665, XP019939343, ISSN: 1573-2592, DOI: 10.1007/S10875-011-9529-3 abstract the whole document	1-4,6,7
X	L. M. MONTANO ET AL: "Androgens are bronchoactive drugs that act by relaxing airway smooth muscle and preventing bronchospasm", JOURNAL OF ENDOCRINOLOGY, vol. 222, no. 1, 11 June 2014 (2014-06-11) , pages 1-13, XP055403472, GB ISSN: 0022-0795, DOI: 10.1530/JOE-14-0074 abstract the whole document	1-7
X	ALICJA KASPERSKA-ZAJAC ET AL: "Dehydroepiandrosterone in Therapy of Allergic Diseases", RECENT PATENTS ON INFLAMMATION & ALLERGY DRUG DISCOVERY, vol. 3, no. 3, 1 November 2009 (2009-11-01), pages 211-213, XP055403478, NL ISSN: 1872-213X, DOI: 10.2174/187221309789257360 abstract the whole document	1-4,6,7