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(54) **Title:** ESTROGEN RECEPTOR MODULATORS FOR THE TREATMENT OF DISEASES INVOLVING PLASMACYTOID DENDRITIC CELLS

(57) **Abstract:** The present invention relates to the use of an antagonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) for the treatment of autoimmune diseases. The invention also relates to the use of an agonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) for the treatment of cancers and infectious diseases or for the stimulation of immunity in a vaccine therapy.

ESTROGEN RECEPTOR MODULATORS FOR THE TREATMENT OF DISEASES INVOLVING PLASMACYTOID DENDRITIC CELLS

5   **FIELD OF THE INVENTION:**

The present invention relates to the general field of therapy of diseases involving plasmacytoid dendritic cells, namely autoimmune, infectious and cancerous diseases.

10   **BACKGROUND OF THE INVENTION:**

Autoimmune diseases are more prevalent in women than men. The most striking sex differences are observed in Sjögren's syndrome and systemic lupus erythematosus (SLE) which incidence is about 9 times higher in women [Whitacre, C. C. 2001].

15   Dendritic cells (DCs) are important in regulating both immunity and tolerance. In human, two main populations of DCs have been characterized in the blood. Conventional myeloid DCs (cDC) that express CD11c and MHC class II molecules which are considered as immature DCs migrating from the bone marrow to the peripheral tissue and plasmacytoid DC (pDC) which lack CD11c but express CD123 and BDCA2 [Dzionek, A., 2000]. pDCs are the

20   main cell population in the blood producing type-1 interferon following viral infection, and can exert potent paracrine effect on cDC or other cell types via the secretion of IFN-α. In addition pDCs are precursors of DCs (pre-DCs) which can aquire cDC morphology and functional properties upon activation in vitro [Wu, L. et Al., 2007 and Shortman, K. et Al., 2007].

25   It has been proposed that peripheral tolerance breakdown leading to SLE might be due to sustained production of type I IFN by pDCs. IFN-$\alpha\beta$ could activate immature myeloid DCs to become fully mature DCs able to activate autoreactive T cells that would help, together with pDCs, to expand autoreactive B cells in SLE. Indeed, it has been shown that prolonged expression of IFNα in preautoimmune (NZB x NZW) F1 mice accelerated disease

30   development leading to early lethality due to severe immune complex glomerulonephritis.

As of today, very few drugs are available for the treatment of autoimmune diseases that have an acceptable safety index.

Thus, there is a permanent need in the art for new molecules for the treatment of autoimmune diseases such as systemic lupus erythematosus.

SUMMARY OF THE INVENTION:

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In vivo assays conducted by the inventors showed that estrogens were able to modulate TLR-mediated type I IFNs production by pDCs.

Namely, the inventors evaluated the effect of an E2-treatment of post-menopausal women on the capacity of pDCs to produce IFN-α and TNF-α in response to TLR-9 and TLR-7

10   stimulation. Their data showed that in vivo E2-treatment enhanced TLR9-mediated IFN-α production by pDCs present in circulating peripheral blood mononuclear cells. This effect of E2 was not attributable to changes in pDCs numbers. Indeed, by intracellular cytokine staining by flow cytometry the inventors found that the frequency of pDCs producing IFN-α or TNF-α after TLR7-stimulation was significantly higher after estrogen-supplementation. In conclusion, their data point

15   to a direct causal link between estrogens and type I IFN-production by human pDCs upon TLR-activation.

Thus, the invention relates to the use of a compound which is a modulator of the estrogen receptor of plasmacytoid dendritic cells (pDCs) for the treatment of diseases involving plasmacytoid dendritic cells.

20   In one aspect, the invention relates to a compound which is an antagonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) for the treatment of an autoimmune disease.

In a second aspect, the invention relates to a compound which is an agonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) for the treatment of a cancer, for the

25   stimulation of the immune system in a vaccine therapy, to improve natural immune response against viral infections or to improve natural immunity in patients chronically infected with viruses such as hepatitis C virus.

DETAILED DESCRIPTION OF THE INVENTION:

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Definitions:

As used herein, the term "estrogens" denotes a group of steroidal compounds, known for their role in the oestrous cycle, and functioning as the primary female sex hormone. The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3).

As used herein, the term "estrogen receptor" denotes to a group of receptors which are activated by the hormone 17β-estradiol[1] (estrogen). There are two different estrogen receptors (ER), usually referred to as ERα and ERβ, each encoded by a separate gene (ESR1 and ESR2 respectively). Hormone activated estrogen receptors form dimers, and since the two forms are coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. Estrogen receptor alpha and beta show significant overall sequence homology, and both are composed of seven domains. Due to alternative RNA splicing, several ER isoforms are known to exist. At least three ERalpha and five ERbeta isoforms have been identified. The ERbeta isoforms receptor subtypes can only transactivate transcription when a heterodimer with the functional ERß1 receptor of 59 kDa is formed. The ERß3 receptor was detected at high levels in the testis. The two other ERalpha isoforms are 66 and 46kDa. Only in fish, but not in humans, an ERgamma receptor has been described.

As used herein, the term "autoimmune disease" denotes an overactive immune response of the body against a substances or tissue normally present in the body. The immune response may be restricted to certain organs (e.g. in thyroiditis) or involve a particular tissue in different places (e.g. Goodpasture's disease which may affect the basement membrane in both the lung and the kidney). The treatment of autoimmune diseases is typically through immunosuppressive drugs that decrease the immune response.

As used herein, the term "plasmacytoid dendritic cells (pDC)" denotes a rare subtype of circulating dendritic cells found in the blood as well as in peripheral lymphoid organs. These cells express the surface markers CD123, BDCA-2(CD303) and BDCA-4(CD304), but do not express CD11c or CD14, which distinguishes them over conventional dendritic cells or monocytes, respectively. As components of the innate immune system, these cells express intracellular Toll-like receptors 7 and 9, which enable the detection of viral and bacterial nucleic acids, such as ssRNA or CpG DNA motifs, respectively. Upon stimulation and subsequent activation, these cells produce large amounts of type I interferon (mainly IFN-α

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(alpha) and IFN-β (beta)), which are critical pleiotropic anti-viral compounds mediating a wide range of effects.

As used herein, the term a "modulator of the estrogen receptor of plasmacytoid dendritic cells" denotes a compound that is capable of binding ERs and that is either an agonist or an antagonist.

As used herein, the term "disease involving plasmacytoid dendritic cells" denotes diseases where plasmacytoid dendritic cells are involved such as autoimmune diseases, infectious diseases and cancer. Indeed, Type I IFNs produced by pDCs can act as adjuvant for a variety of vaccine strategies not only directed against infectious agents such as influenza virus [Bracci, L., et Al., 2006] but also against tumors such as melanoma [Sikora, A. G., et Al., 2009].

As used herein, the term "selective estrogen receptor modulator (SERM)" denotes a class of medications that act on the estrogen receptor. SERMs can be agonists or antagonists on the estrogen receptor. A characteristic feature that distinguishes SERMs from "pure receptor agonists and antagonists" is that their action is tissue specific thereby allowing to selective inhibition or stimulation of estrogen-like action in various tissues. Phytoestrogens are scientifically accepted SERMs from a botanical source. SERMs include but are not limited to afimoxifene (4-hydroxytamoxifen), arzoxifene, bazedoxifene, clomifene, femarelle (DT56a), lasofoxifene, ormeloxifene, raloxifene, tamoxifen, toremifene [Kuiper et Al., 2009].

As used herein, the term "pure antagonist of the estrogen receptor (or pure anti-estrogen)" denotes a molecule capable to bind the estrogen receptor without any estrogenic activity, either *in vitro* or *in vivo*, in any species or tissue studied, including all estrogen targeted tissues, e.g. uterus, mammary gland, ovaries or bone [Hermenegildo, C, 2000].

As used herein, the term "progestin" denotes a synthetic progestagen that has progestinic effects similar to progesterone. The two most frequent uses of progestins are for hormonal contraception (either alone or with an estrogen), and to prevent endometrial hyperplasia from unopposed estrogen in hormone replacement therapy. Progestins are also used to treat secondary amenorrhea, dysfunctional uterine bleeding and endometriosis, and as palliative treatment of endometrial cancer, renal cell carcinoma, breast cancer, and prostate

cancer. Progestin include but are not limited to norethynodrel (Enovid), norethindrone, norgestimate (Ortho Tricyclen, Ortho-Cyclen), norgestrel, levonorgestrel, medroxyprogesterone, desogestrel, and drospirenone.

5   As used herein, the terms "treating" or "treatment", denotes reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such a disorder or condition.

10   *Antagonists and agonists and uses thereof*

A first aspect of the invention relates to a compound which is a modulator of the estrogen receptor of plasmacytoid dendritic cells (pDCs) for the treatment of a disease involving plasmacytoid dendritic cells.

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In a particular embodiment, the estrogen receptor according to the invention is the estrogen receptor alpha.

In a first embodiment, the modulator is an antagonist of the estrogen receptor of

20 plasmacytoid dendritic cells (pDC) useful for the treatment of an autoimmune disease.

The antagonist may be useful for the treatment of autoimmune diseases including , but not limited to systemic lupus erythematosus, Sjögren's syndrome, psoriasis, dermatitis herpetiformis, vitiligo, mycosis fungoides, allergic contact dermatitis, atopic dermatitis, lichen

25 planus, Pityriasis lichenoides and varioliforms acuta (PLEVA).

In a preferred embodiment, the antagonist has a dissociation constant between 10^{-11} M and 10^{-7} M, preferably between 10^{-10} M and 10^{-9} M.

30   The antagonist according to the invention is preferably a "SERM" and /or a "pure antagonist".

Preferably, the antagonist according to the invention is a pure antagonist and is selected from ICI 164384, ICI 182780, RU 58668, EM-139 and EM-800 [Hermenegildo, C, 2000].

5   In a preferred embodiment, the pure antagonist has a dissociation constant between 10^{-11} M and 10^{-7} M, preferably between 10^{-10} M and 10^{-9} M.

In a second embodiment, the modulator is an agonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) useful for the treatment of cancer, infectious diseases and

10   for the stimulation of the immune system in a vaccine therapy, to improve natural immune response against viral infections or to improve natural immunity in patients chronically infected with viruses such as hepatitis C virus.

The agonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) may be

15   useful for the treatment of a disease or a condition for which stimulation of the immune system is required.

In particular, the disease may be an infectious disease, whereby the agonist of the estrogen receptor acts to prevent an infection to occur or treats an existing infection.

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In a preferred embodiment, the infectious disease is a viral disease like HCV, herpes, influenza.

In another embodiment, the disease may be a cancer.

25   In a preferential embodiment, the cancer includes but is not limited to tissue specific cancers like colon cancer, hairy-cell leukemia, Kaposi's sarcoma, chronic myeloid leukemia and melanoma [Dunn et Al., 2006].

In another embodiment, the agonist may be used in a vaccine therapy.

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In a preferred embodiment, the agonist has a dissociation constant between 10^{-10} M and 10^{-8} M, preferably the dissociation constant is 10^{-9} M.

In one embodiment, antagonist or 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 another embodiment, antagonist or agonist of the invention may consist in an antibody which inhibits or activates the estrogen receptor of plasmacytoid dendritic cells or an antibody fragment which inhibits or activates the estrogen receptor of plasmacytoid dendritic cells.

Antibodies directed against the estrogen receptor of plasmacytoid dendritic cells can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against the estrogen receptor of plasmacytoid dendritic cells can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote et al., 1983); and the EBV-hybridoma technique (Cole et al. 1985). Alternatively, techniques described for the production of single chain antibodies (see, e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti-estrogen receptor single chain antibodies. Estrogen receptor antagonists and agonists useful in practicing the present invention also include anti-estrogen receptor antibody fragments including but not limited to F(ab')$_2$ fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')$_2$ fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to the estrogen receptor of plasmacytoid dendritic cells.

Humanized anti-estrogen receptor antibodies and antibody fragments thereof may also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human

immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

In still another embodiment, the estrogen receptor antagonists or agonists may be selected from aptamers.

Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

Therapeutic composition

Another object of the invention relates to a therapeutic composition comprising a compound according to the invention for the treatment of diseases according to the invention.

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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.

5   "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.

10   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

15   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,

20   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.

25   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.

In a preferred embodiment, the therapeutic composition comprising an agonist according to the invention comprises in addition a progestin substance.

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Alternatively, compounds of the invention which inhibit or activate the estrogen receptor signalling pathway can be further identified by screening methods as hereinafter described.

Screening methods:

Another object of the invention relates to a method for screening a compound which inhibits or activates the estrogen receptor signalling pathway of plasmacytoid dendritic cells.

In particular, the invention provides a method for screening an estrogen receptor of plasmacytoid dendritic cells antagonist or agonist for the treatment of different disorder.

For example, the screening method may measure the binding of a candidate compound to the estrogen receptor, or to cells or membranes bearing the estrogen receptor, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, a screening method may involve measuring or, qualitatively or quantitatively, detecting the competition of binding of a candidate compound to the receptor with a labelled competitor (e.g., antagonist).

Furthermore, screening methods may test whether the candidate compound results in a signal generated by an agonist of the estrogen receptor, using detection systems appropriate to cells bearing the receptor.

In a particular embodiment, the screening method of the invention comprises the step consisting of:

a) providing a plurality of cells expressing the estrogen receptor:

b) incubating said cells with a candidate compound ;

c) determining whether said candidate compound binds to and activates or not the estrogen receptor ; and

d) selecting the candidate compound that binds to and inhibits or activates the estrogen receptor.

The inventors have designed in vitro assays for the selection of ER-ligands that behave as agonist or antagonist regarding their capacity to modulate type I IFNs production by pDCs upon stimulation with TLR7 and TLR9 synthetic ligands. Two type of pDCs are used: i) ex vivo purified pDCs from PBMCs and ii) pDCs differentiated in vitro from CD34+ progenitors cultured in the presence of appropriate cytokines.

Agonist ER-ligands are added to the culture medium and selected for their capacity to enhance type I IFNs production by pDCs stimulation with various doses of TLR7 and TLR9 synthetic ligands. To select antagonist ligands, the compounds to be tested are added to E2-supplemented medium (E2 10^{-9} M) at the initiation of the culture. We then measure their

5   capacity to inhibit type I IFNs production by TLR7 and TLR9-stimulated pDCs.

In a particular embodiment, cells are selected from the group consisting of pDC isolated from PBMCs or pDC generated from cord blood CD34+ cells cultured with Flt3-L and IL-7 on OP9-Delta1 stroma cells (Olivier et al., Blood 2006 107:2694).

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The screening method of the invention may be employed for determining an agonist or an antagonist by contacting such cells with compounds to be screened and determining whether such compound activates or not the receptor through its capacity to modulate IFNa production by TLR-stimulated pDCs.

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According to a one embodiment of the invention, the candidate compound may be selected from a library of compounds previously synthesised, or a library of compounds for which the structure is determined in a database, or from a library of compounds that have been synthesised de novo or natural compounds.

20   The candidate compound may be selected from the group of (a) proteins or peptides, (b) nucleic acids and (c) organic or chemical compounds (natural or not). Illustratively, libraries of pre-selected candidate nucleic acids may be obtained by performing the SELEX method as described in documents US 5,475,096 and US 5,270,163. Further illustratively, the candidate compound may be selected from the group of antibodies directed against the

25   estrogen receptor.

Such the method may be used to screen estrogen receptor antagonists or agonists according to the invention.

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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.

FIGURES:

Figure 1: Enumeration of conventional and plasmacytoid dendritic cells in post-menopausal women after E2 treatment. (A) Protocol of the clinical trial, blood samples were collected at two time points before initiation of the treatment (S1 , S2). E2-treatment started at S2 and lasted for one month, sample S3 was collected at the end of this period. **(B)** PBMCs were gated on the side scatter (SSC) and CD45 expression. Among $CD45^+$ cells, DCs are identified as negative for lineage markers, CD3, CD14, CD16, CD19 and CD56 (Lin) and positive for HLA-DR. Conventional DCs are distinguished by the expression of CD11c from plasmacytoid DCs whose expressed CD123. **(C)** Absolute numbers of circulating cDC and pDC in whole blood was defined by TruCOUNT assay. The absolute number of DC subsets is expressed per µl of blood. **(D)** Percentage of cDCs and pDC among all PBMCs ($CD45^+$). Samples were collected in healthy donors at 3-4 weeks interval (S1 and S2). No significant difference between groups was found using repeated measures ANOVA or Friedman test.

Figure 2: IFN-α production by pDCs in PBMCs after stimulation with the TLR-9 ligand CpG. PBMCs purified from whole blood were cultured in X-Vivo synthetic medium at 5×10^6 cells/well in 96-well plates in the presence of the indicated concentrations of CpG 2216. Supernatants were collected at 24 hours and frozen. IFN-α concentration in culture supernatants was measured by ELISA. Data from the ELISA are normalized on the basis of the respective percentage of plasmacytoid DCs present in the PBMC fraction calculated from FACS analysis described in Figure 1B. **(A)** IFN-α production of PBMCs from 8 different postmenopausal women, before (dash line) or after E2 treatment (solid line). **(B,C)** IFN-α score before **(B)** and after **(C)** treatment was established by summing the concentration of IFN-α measured in response to the 3 different concentrations of CpG as shown in (A). **(D)** Fold increase of IFN-α production in S2 were calculated from S1 and the fold increase in S3

were calculated from the mean of IFN-α production of S1 and S2. Mean values are indicated

by the lines, and P values were determined by use of Wilcoxon signed rank test.

Figure 3: Effect of E2 administration on the frequency of pDC expressing IFN-α

and TNF-α in TLR7-stimulated PBMCs. Plasmacytoid DCs intracellular IFN-α and TNF-α

staining in unstimulated or stimulated (R-848 1 µg/ml) PBMCs fraction was measured by

flow-cytometric analysis. Plasmacytoid DCs were identified in the PBMC population by use

of the lineage-specific markers and BDCA-2 surface expression. **(A)** A representative

experiment from one post-menopausal woman is shown. **(B)** Percentage of pDC positive for

IFN-α (B, n=12) and TNF-α (C, n=17) before (S1-S2) and after (S3) treatment. Mean values

are indicated by the lines on Scatter plots, and P values were determined by use of Wilcoxon

signed rank test.

Figure 4 : Estrogen thought ERα increase the maturation of pDC. Bone Marrow

cells from WT or ERα$^{-/-}$ were differentiated in steroid-free medium (RPMI without phenol red

supplemented with 10% charcoal-dextran treated FCS) in the presence of Flt3L (200 ng/ml)

during 9 days in presence or absence of E2 (10^{-9}M). **(A)** The percentage of pDC (B220^{+}

CD11c^{+}) is indicated for the different culture condition. **(B-D)** pDC differentiated in presence

of E2 (black line) or not (filled grey) were stained for MHC II **(B)**, CD40 **(C)** and CD86 **(D)**.

Column bar indicate GMFI of CD40 and CD86 expression calculated from the gated

population indicated in **(A)**. The value represent mean ± SEM from 4 independent cultures

and *P* values were determined by use of Mann-Whitney U test.

Figure 5. Intrinsic expression of ERα is required to promote pDC maturation in

the presence of E2. CD45.2 bone marrow cells from WT or ERα$^{-/-}$ were mixed with CD45.1

bone marrow cells at a 1:1 ratio in steroid-free medium with Flt3L during 9 days in presence

or absence of E2 (10^{-9}M) as indicated. **(A)** The percentage of MHCII$^+$ pDC (B220$^+$ CD11c$^+$)

developed from CD45.1 (R2) or CD45.2 (R1) bone marrow is indicated for the different

culture conditions. **(B)** Column bar indicates the ratio between CD45.1 and CD45.2 MHC II$^+$

5   pDC. The value represent mean ± SEM from 4 independent cultures and P values were

determined by use of Mann-Whitney U test (*, $p < 0.05$).

Figure 6: Estrogen thought ERα increase the capacity of pDC to produce

IL12p40 after TLR engagement. Bone Marrow cells from WT or ERα$^{-/-}$ were differentiated

10   in steroid-free medium supplemented with Flt3L during 9 days in presence or absence of E2

(10^{-9}M). **(A)** pDC (B220$^+$ CD11c$^+$) were purified and stimulated with R848 (TLR-7)

overnight in presence of Brefeldin A. After stimulation pDC were stained for CD11c and

IL12p40. Dot plot shows pDC stained for IL12p40. **(B)** The percentage of IL12p40 positive

pDC from four independant experiments. The value represent mean ± SEM and P values were

15   determined by use of Mann-Whitney U test.

EXAMPLE:

20   <u>**Example 1: Effect of E2 administration on TLR-mediated responses of pDCs in**</u>
<u>**post-menopausal women**</u>

<u>**Materials & Methods**</u>

25   **Subjects.** Healthy post-menopausal women volunteers (46-59 years old, n=28) with

menopause diagnosed since at least 12 months, but less than 5 years were included in the

study after their informed consent. Three blood samples were collected. The two first samples

were collected at three to six weeks of interval before estrogen treatment. The subjects were

then randomized into two groups, one receiving transdermal E2 administration (Estrapatch,

60 μg/24h) and the other receiving E2 through the oral route (Estrofem, 2 mg/day). After 30 days of treatment the last blood sample was collected. The study has been reviewed and approved by an appropriate institutional review committee and the ethical committee of Toulouse Hospital.

Flow cytometric analysis of blood DCs. The following flurorochrome conjugated monoconal antibodies (mAb) were used for whole blood stainings: FITC-conjugated lineage cocktail (Lin) containing anti-CD3, -CD14, -CD16, CD56, -CD19, and -CD20, anti-HLA-DR-PerCp, anti-CD11c-allophycocyanin (APC), anti-CD123-phycoerythrin (PE) and anti-CD45-PE-Cyanin7 from BD Biosciences. Briefly, 100 μl of freshly isolated whole blood and appropriate amounts of mAbs were added to a TruCOUNT tube containing fluorescent beads (BD Biosciences). After 15 min incubation the blood was lyzed and the samples were acquired on LSRII flow cytometer (BD Biosciences) and analyzed using the FloJo software (Tree Star, San Carlos, CA). We collected 500 000 events by gating white blood cells defined by SSC characteristic and CD45-staining. Among $CD45^+$ mononuclear cells, conventional DC (cDC) and pDC subsets were defined by simultaneous HLA-DR expression, lack of lineage specific markers, and by CD11c or CD123 positive staining, respectively. The absolute number of circulating cDCs and pDCs (cells/μl of blood) was calculated by comparing cellular events to bead events following manufacturer instructions (BD Biosciences) as determined by the analysis of flow cytometry. DC subsets were also expressed as a pourcentage of peripheral blood $CD45^+$ monuclear cells.

Effect of E2-treatment on TLR-9-mediated IFN-α production by PBMCs. PBMCs (5×10^6 cells/well) isolated from whole blood by sedimentation over Ficoll/Hypaque were resuspended in phenol red-free serum-free X-Vivo 15 medium (Cambrex), and stimulated with titrated amounts of CpG-DNA oligodesoxynucleotide (ODN) 2216 (CpG-2216), ranging from 1 to 0.1 μg/ml, to activate TLR-9-bearing pDCs in a dose-dependent manner [Kadowaki, 2001 #1203]. Human IFN-α was measured by ELISA (Human IFN-α ELISA kit, PBL Biomedical Laboratories) in 24 hr culture supernatants. In accordance with the known propensity of pDC to produce large amount of IFN-α, CpG-2216 efficiently stimulate IFN-α production in pDCs. Data from the ELISA were normalized on the basis of the respective percentage of pDCs present in the PBMC fraction to show IFN-α production on a per-pDC basis as described [Longman, R. S., 2005]. These results were then used to establish at each

time points an IFN-α/CpG score by summing the data obtained for 3 different doses of CpG-2216 tested (from 1μg/ml to 0.1 μg/ml) excepted for the first patients (up to P#5) for which the titration started at 3 μg/ml. The data obtained for the two time points at pretreatment (S1 and S2) served at evaluating the intra-individual variability and the basal level of TLR9

5   responsiveness in post-menopausal women. We arbitrary decided to exclude from the analysis patients exhibiting high variability between S1 and S2 values ($0.5 \leq S1/S2 \leq 2$; 4 patients out 28) or when S1-2 values were exceeding by 2 x SD the mean basal responsiveness of all patients (1/28). The mean basal IFN-α/CpG score (±SD) was 0.78 ± 0.73 pg/ml per pDC. Among the 28 patients included in this study 23 (82%) were retained for further analysis of

10   E2 effect on the biological parameters analyzed.

Intracellular cytokine staining of pDCs. The functional activity of circulating pDC subset was assessed ex vivo by intracellular cytokine staining in response to short term TLR7-stimulation of freshly isolated PBMCs. Cytokine production was analyzed on the Alexa700 or

15   PE-channel on pDC subset defined as BDCA-2^{+} lineage negative cells. PBMCs (2.5 x 10^{6} cells/ml) were stimulated in X-Vivo 15 medium for 5 hrs in vitro in the presence of R-848 (3 or 1 μg/ml) a potent ligand of TLR-7 expressed on pDCs. Brefeldin A (5 μg/ml) was added for the last 2 hrs of culture. Cells were surface labeled with BDCA-2-APC (Miltenyi Biotec) and Lin-FITC antibodies, fixed, permeabilized, and stained for intracellular cytokine

20   production using anti-IFN-α-PE (Miltenyi Biotec) or anti-TNF-α-Alex700 (DB Bioscience) antibodies. Data were acquired on a BD LSR II cytometer and analyzed with FlowJo software. As expected, specific staining for IFN-α or TNF-α was observed in R-848-stimulated pDCs, but not in pDCs cultured in the absence of TLR-7 ligand. IFN-α producing cells were only detected in the pDC population (data not shown).

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Results

Estrogen administration in post-menopausal women does not modify the
30   **numbers of circulating cDCs and pDCs.**

We have performed longitudinal analysis in 23 post-menopausal women to evaluate the effect of E2-treatment on the frequency of circulating DC subsets. Blood samples were collected at three time points over a period of up to two months. The two first samples

collected at two to three weeks of interval before estrogen treatment served to evaluate the intra-individual variability of the biological parameters assessed. After randomization, subjects were treated with E2 either through the transdermal or oral route. After 30 days of treatment the last blood sample was collected. cDCs and pDCs were identified as lineage negative MHC class II positive cells, expressing either CD11c or CD123, respectively. The absolute numbers and frequency of cDCs and pDCs were measured during the course of the clinical trial (Fig. 1). No significant differences were observed with respect to the absolute numbers or the percentage among PBMCs of cDCs and pDCs at the two time points tested before initiation of the treatment. Although they were some variability among individuals, circulating DC numbers were constant among each individual during this 2-3 weeks interval before estrogen therapy and were not modified after one month of E2-treatment (Fig. 1). Similar results were obtained by analyzing the percentage of DC subsets among purified PBMCs. Thus, administration of E2 in post-menopausal women does not affect homeostatic numbers of circulating DC subsets, including pDCs.

E2-treatment enhances type I-IFN production by TLR9-stimulated PBMCs.

We next addressed the function of pDCs in the course of follow-up by measuring the production of IFN-α by PBMCs stimulated by titrated amounts of CpG-2216. We choose to stimulate PBMCs by CpG as it is primarily acting on the pDCs to induce IFN-α through TLR9-signalling [Kadowaki, N., S. Et al., 2001]. No cytokine secretion was observed in unstimulated cultures or when PBMCs were stimulated with control GpC DNA ODNs.

Data were normalized to the respective percentage of pDCs in each PBMC samples (Fig. 2A). In agreement with data in Fig. 1, the numbers of pDCs in PBMCs were stable in each patient and were not significantly affected by the treatment. To compare the dose-response curves of IFN-α secretion by TLR9-stimulated PBMCs at each time points of the longitudinal analysis, we calculated an IFN-α/CpG score by summing the concentration of IFNα produced on a per-cell basis in response to each amount of CpG (Fig. 2B and C). As expected, CpG-2216 triggered the production of IFN-α in a dose dependent-manner, with low intra-individual variability in estrogen-deprived post-menopausal women for the two time points analyzed (Fig. 2A and B). Interestingly, after one month of E2 treatment there was a significant rise in IFN-α production by CpG-stimulated PBMCs in many patients (Fig. 2A). Despite this variability, longitudinal analysis of all post-menopausal women demonstrated a highly significant enhancing effect of E2-treatment on TLR9-responsiveness ($p < 0.01$, Fig.

18

2C). In some patients, the enhancing effect of E2-treatment on IFNα production was observed for all doses of CpG used, including the lowest concentration that normally induced barely detectable levels of IFN-α in most PBMCs from untreated donors (Fig. 2A). When data obtained from S2 and S3 samples were normalized to S1 values (Fig. 2D), the mean fold increase of IFNα/CpG score was close to 1 for S2 values, indicating low intra-individual variability in agreement with data in Fig. 2C. By contrast, the mean fold increase of IFNα/CpG score was above three after E2-treatment for S3 values and significantly different from S2 ($P = 0.0047$, Fig. 2D). To test the respective roles of E2 treatment and the mode of E2 administration, a 2-way ANOVA was performed. No significant interaction was observed between these two factors ($P_{interaction} = 0.16$; $P_{mode\ of\ treatment} = 0.20$; $P_{E2} = 0.016$). Taken together, our data show that E2 enhances CpG-mediated type I-IFN production by human blood pDCs in vivo. Since the enhancing effect of E2-treatment was not attributable to an increase of circulating pDCs (**Fig. 1**), our data show that in vivo E2-treatment had enhanced TLR9-dependent responsiveness of pDCs.

Estrogens turn on human plasmacytoid dendritic cells in vivo to produce type I IFN and TNF in response to TLR7- activation.

The rise in TLR9-mediated type I IFN-production by PBMCs from E2-treated patients could result from an increased numbers of IFN-α-producing cells or an enhanced intrinsic capacity of pDCs to produce IFN-α on a per cell basis. To discriminate between these different possibilities and to firmly establish that E2 effect on IFN-α-production was due to pDCs and could also be observed upon TLR7 activation, intracellular cytokine analysis was performed on R-848-stimulated PBMCs. We analyzed the production of IFN-α and TNF-α in BDCA-2$^+$ cells from PBMCs cultured for 5 hrs with the TLR7 ligand R-848. Whereas TNF-α was produced by other cells types, only pDCs produced measurable IFN-α under these condition. By gating on this population we assessed the effect of E2-treatment on the percentage of IFN-α and TNF-α-producing pDCs. At pretreatment the average frequency (% mean ± SD) of IFN-α- and TNF-α-producing pDCs were 8.45 ± SD and 2 ± SD, respectively. There was no significant difference in the percentage of cytokine-producing pDCs at the two time points tested. In contrast, after one month of E2 administration there was a significant increase in the frequency of cells expressing either IFN-α or TNF-α among TLR7-stimulated pDCs (Fig. 3 A and B). Cytokine-production in pDCs was dependent on *ex vivo* TLR activation since the frequency of IFN-α- and TNF-α-producing pDCs was usually below 0.1

% in unstimulated PBMCs at all time points tested (not shown). Furthermore, the mean fluorescence intensity of IFN-α^+ or TNF-α^+ pDCs in TLR7-stimulated cultures was not affected by treatment (not shown). Altogether these data demonstrate that administration of E2 in post-menopausal women, modulate the functional state of circulating pDCs by increasing the frequency cells able to produce IFN-α and TNF-α upon TLR7 activation. Since the assessment of TLR-stimulation was performed in steroid free synthetic medium, we evaluated whether enhanced TLR-responsiveness was due to the absence of estrogen in vitro. The presence of E2 (10^{-9}M) during TLR7 stimulation did not modify the frequency of pDCs producing either IFN-α or TNF-α, whether PBMCs were obtained from estrogen-deprived or E2-treated post-menopausal women. Thus, the presence of E2 during the time of TLR activation did not affect cytokine production by pDCs. These data suggested that effect of E2 treatment on pDC functional properties was imprinted in vivo.

Example 2: Identification of the estrogen receptor alpha (Esr1 gene) as the receptor useful for this invention:

Materials and Methods

DC generation from murine bone marrow (BM).

BM-pDC were generated as previously described (16). Briefly, BM cells were flushed out from femurs and tibias. After lysis of red blood cells in ammonium chloride potassium (ACK), BM cells were cultured in complete medium (CM) or steroid-free medium (SFM) containing 200 ng/ml murine Flt3L (PeproTech, London, UK) at 2 x 105 cells/ml in bacteriological petri dish (Greiner Bio-One, Poitiers, France). On day 3, an equal volume of fresh medium with 200 ng/ml Flt3L was added to the culture and cells were collected on day 9 for further analysis. Complete medium (CM) was RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10 % FCS (ATGC Biotechnologie, Noisy Le Grand, France) supplemented with 1 mM sodium pyruvate, 1 % non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 50 μg/ml gentamicin (Sigma, Saint Quentin Fallavier, France). For experiments in hormone deficient conditions, steroid-free medium (SFM) containing phenol red-free RPMI 1640 (Eurobio) with 10 % dextran charcoal-treated FCS (Hyclone, Logan, Utah, USA) supplemented with 1 mM sodium pyruvate, 1 % non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 50 μg/ml

gentamicin (Sigma) was used during all the culture period. In some experiments cultures were supplemented with 1 nM 17ß-estradiol (Sigma), or with DMSO vehicle.

Maturation status was assessed by flow cytometry after staining for CD40, CD86 costimulatory molecules and MHC class II as described below. IL-12p40 intracellular staining

5   was achieved by incubation with 10 mg/ml brefeldin A (Sigma) for the last 4h of culture. After MHCII and CD40 surface staining, cells were permeabilized with 0.5 % saponin and incubated with PE conjugated anti-IL-12p40 mAb or isotype control mAb (BD Pharmingen).

Flow cytometry

10   Before staining, cells (5-10 x 105) were incubated 15 min at room temperature with blocking buffer (PBS with 1 % SVF, 3 % normal mouse serum, 3 % normal rat serum, 5 mM EDTA, 1 ‰ NaN3 and 5 μg/ml anti CD16/CD32 (2.4G2, ATCC). For cell staining, cells were incubated for 30 min on ice with FITC-, PE-, biotin- or APC-conjugated monoclonal antibodies diluted at the optimal concentration in FACS buffer (PBS 1 % SVF, 5 mM EDTA,

15   1 ‰ NaN3). pDC were stained for the DC marker CD11c (N418, BD Biosciences, San Jose, CA) and lineage markers B220. Maturation status was assessed by flow cytometry after staining for CD40, CD86 costimulatory molecules and MHC class II as described below. IL-12p40 intracellular staining was achieved by incubation with 10 mg/ml brefeldin A (Sigma) for the last 4h of culture in the presence of R-848 at 2 μg/ml. After CD11c surface staining,

20   cells were permeabilized with 0.5 % saponin and incubated with PE conjugated anti-IL-12p40 mAb or isotype control mAb (BD Pharmingen). Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, Mountain view, CA) and data analysed with FlowJo Software (Tree Star Inc., Standford, CA).

25   <u>Results</u>

E2 enhances the expression of maturation markers on pDCs through ERα-signaling.

Flt3L allows the development of CD11c$^+$ cDCs and pDCs from BM progenitors that can be distinguished by the expression of B220 marker. Only pDCs expressed B220 and

30   represent between 27% to 33% of the cells present in the culture after 9 days as shown in Fig. 4A. pDCs were generated from either WT or ERαKO BM cells in steroid-free medium supplemented or not with E2. After 9 days of culture the expression of maturation markers MHC class II and CD40 was analyzed by flow cytometry on CD11c$^+$ B220$^+$ pDCs. As shown in Fig. 4B, the frequency of pDCs expressing high level of MHC class II molecules was

increased by 15%-20% in E2 supplemented cultures of bone marrow cells (Fig. 4B). Likewise, we observed a 4-fold increase in the expression of CD40 molecules on pDCs from WT bone marrow cells generated in the presence of E2 (Fig. 4C). Similar results were obtained by analysing the expression of the co-stimulatory molecule CD86 (Fig. 4D). By

5   contrast, E2 supplementation had no effect on both MHC class II and CD40 expression on pDCs when bone marrow cells were obtained from ERαKO mice (Fig. 5 B-D). Altogether, these data demonstrate that E2 act on BM progenitors to enhance the expression of maturation markers on pDCs.

As ERα-signaling has been shown to regulate cytokine production in myeloid cells *in*

10   *vitro*, it was important to distinguish if the E2-effect on pDC development was caused by a cell-intrinsic mechanism or by an indirect effect due to autocrine or paracrine factors which could positively or negatively regulate pDC development. We examined the generation of $CD11c^+$ $B220^+$ pDCs from $ER\alpha^{-/-}$ CD45.2 BM cells when cocultured with equal numbers of CD45.1 BM progenitors in the presence of E2. We analyzed the proportion of pDCs

15   expressing high level of MHC class II molecules in each populations. As expected MHC class II expression was down-regulated on pDCs generated from WT CD45.1 or CD45.2 pDCs in steroid-free medium as compared to E2-suplemented cultures (Fig. 5A, left lower panels). By contrast, addition of E2 in cultures of $ER\alpha^{-/-}$ pDCs (CD45.2) generated in the presence of WT CD45.1 progenitors was able to selectively increase the frequency of MHC class II^{hi} pDCs on

20   WT CD45.1 cells but had no effect on $ER\alpha^{-/-}$ CD45.2 pDCs. Indeed, the MHC II expression profile of $ER\alpha^{-/-}$ pDCs (CD45.2) showed a 25-30 % reduction in the frequency of pDCs expressing high levels of MHC class II as compared to ERα-sufficient CD45.1 cells (Fig. 5B). This reduced frequency of MHC class II^{hi} pDCs was not influenced by the presence of WT progenitors since a similar phenotype was observed in $ER\alpha^{-/-}$ pDCs generated alone (see

25   Fig. 4).

Thus, these data demonstrate that E2 act through ERα-signaling in a cell-autonomous manner to regulate the expression of maturation markers on pDCs during their differentiation from BM precursors.

30   **The presence of E2 during Flt3-induced pDCs differentiation enhances TLR response of pDCs.**

We next examined the effect of E2 on the capacity of pDCs to produce pro-inflammatory cytokines upon stimulation through their TLR7. As a read-out to measure pDC

activation we analyzed the frequency of IL-12-producing pDCs upon stimulation with resiquimod (R-848) a ligand for TLR7. IL-12 is an important polarizing cytokines that drive expansion of naive $CD4^+$ T cells to the Th1 and is readily produced by mouse pDCs. pDCs were generated from either WT or ERαKO BM cells in steroid free medium supplemented or

5   not with E2. After 9 days of culture pDCs were purified using B220-specific magnetic beads and then stimulated with R-848 in the presence of brefeldin A for intracellular analysis of IL-12p40 production by flow cytometry. Data in Fig. 5 show that the frequency of IL-12p40$^+$ pDCs was increased by two-fold in E2-suplemented cultures from WT BM progenitors. This enhancing effect of E2 on TLR7-mediated IL-12 production was not observed in pDCs

10   generated form ERα$^{-/-}$ BM precursors (Fig. 5). By contrast, we were unable to document any significant effect of E2 on TLR-dependent cytokine production by pDCs when the hormone was added at the end of the Flt3L-mediated differentiation process. Thus differential cytokine production between pDCs that developed in the absence or presence of E2-signaling is imprinted during differentiation and therefore reflects an E2 effect on precursors or

15   developing pDCs rather than on already differentiated cells.

Conclusions:

        In conclusion, these data provide the first genetic evidence that activation of ERα by E2 can act in a cell autonomous manner during Flt3L-mediated pDC differentiation to

20   modulate the expression of maturation markers on their cell surface as well as their capacity to produce pro-inflammatory cytokines upon engagement of their TLRs. This potent regulatory effect of estrogens on TLR-mediated IFN-α production by human pDCs could account for the substantial gender bias observed not only in autoimmune diseases, such as SLE, but also in HIV-1- or HCV-associated pathogenesis or other infectious diseases for

25   which sex-based differences have been observed. This could also explain the observation that hormonal replacement therapy (HRT) is beneficial in post-menopausal women with chronic hepatitis C [Di Martino VP et al., 2004; Codes LT et al. 2007]. Although the precise mechanisms of HRT mediated protection of live fibrosis in HCV patients is not known [Di Martino VP et al., 2004; Codes LT et al. 2007], we believe that it could be mediated through

30   the enhancing effect of E2 on type I IFN-production by pDC. Indeed, pDCs are known to infiltrate the liver during HCV infection and it has been recently shown that they can sense HCV-infected hepatocytes through TLR7 resulting in the secretion of IFN-α which in turn inhibit infection [Takahashi KS et al., 2010]. Therefore, we propose that agonists of ERα may

be used to stimulate the TLR-mediated production of type I-IFNs by pDCs for the treatment of infectious diseases like HCV.

Thus the in vivo effect of E2 on human pDC observed in the clinical trial is more likely due to a direct effect of the hormone on pDC themselves rather than through an indirect action on other tissues.

The model system described in the present report may therefore be useful to screen SERMs (selective estrogen receptor modulators) with agonist properties that could mimick the enhancing effect of E2 on TLR-responsiveness of pDCs.

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.

Bracci, L., I. Canini, M. Venditti, M. Spada, S. Puzelli, I. Donatelli, F. Belardelli, and E. Proietti. 2006. Type I IFN as a vaccine adjuvant for both systemic and mucosal vaccination against influenza virus. Vaccine 24 Suppl 2:S2-56-57.

Codes, L., T. Asselah, D. Cazals-Hatem, F. Tubach, D. Vidaud, R. Parana, P. Bedossa, D. Valla, and P. Marcellin. 2007. Liver fibrosis in women with chronic hepatitis C: evidence for the negative role of the menopause and steatosis and the potential benefit of hormone replacement therapy. Gut 56:390-395.

Di Martino, V., P. Lebray, R. P. Myers, E. Pannier, V. Paradis, F. Charlotte, J. Moussalli, D. Thabut, C. Buffet, and T. Poynard. 2004. Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. Hepatology 40:1426-1433.

Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol. 2006 Nov;6(11):836-48.

Dzionek, A., A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, D. W. Buck, and J. Schmitz. 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J. Immunol. 165:6037-6046.

Hermenegildo, C., and and Cano. A., 2000. Pure anti-oestrogens. Human reproduction Update 200, Vol. 6 No. 3 pp. 237-243.

24

Ishimaru, N., R. Arakaki, S. Yoshida, A. Yamada, S. Noji, and Y. Hayashi. 2008. Expression of the retinoblastoma protein RbAp48 in exocrine glands leads to Sjogren's syndrome-like autoimmune exocrinopathy. *J. Exp. Med.* 205:2915-2927.

Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194:863-869.

Komi, J., and O. Lassila. 2000. Nonsteroidal anti-estrogens inhibit the functional differentiation of human monocyte-derived dendritic cells. Blood 95:2875-2882.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997 Mar;138(3):863-70.

Longman, R. S., A. H. Talal, I. M. Jacobson, C. M. Rice, and M. L. Albert. 2005. Normal functional capacity in circulating myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis C. *J Infect Dis* 192:497-503.

Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7:19-30.

Sikora, A. G., N. Jaffarzad, Y. Hailemichael, A. Gelbard, S. W. Stonier, K. S. Schluns, L. Frasca, Y. Lou, C. Liu, H. A. Andersson, P. Hwu, and W. W. Overwijk. 2009. IFN-alpha enhances peptide vaccine-induced CD8+ T cell numbers, effector function, and antitumor activity. J Immunol 182:7398-7407.

Takahashi, K., S. Asabe, S. Wieland, U. Garaigorta, P. Gastaminza, M. Isogawa, and F. V. Chisari. 2010. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. Proc Natl Acad Sci U S A 107:7431-7436.

Whitacre, C. C. 2001. Sex differences in autoimmune diseases. Nat. Immunol. 2:777-780.

Wu, L., and Y. J. Liu. 2007. Development of dendritic-cell lineages. Immunity 26:741-750.

CLAIMS:

1. A compound which is a modulator of the estrogen receptor of plasmacytoid dendritic cells (pDCs) for the treatment of diseases involving plasmacytoid dendritic cells.

2. A compound according to claim 1 wherein the estrogen receptor is the estrogen receptor alpha.

3. A compound according to claims 1 or 2 which is an agonist of the estrogen receptor of plasmacytoid dendritic cells (pDCs) for the treatment of a disease or a condition for which stimulation of the immune system is required.

4. A compound according to claim 3 for the treatment of an infectious disease.

5. A compound according to claim 3 for the treatment of a cancer.

6. A compound according to claim 5 wherein the cancer is selected from colon cancer, hairy-cell leukemia, Kaposi's sarcoma, chronic myeloid leukemia and melanoma.

7. A compound according to claims 1 to 2 for the stimulation of the immune system in a vaccine therapy.

8. A compound according to the claim 1 which is an antagonist of the estrogen receptor of plasmacytoid dendritic cells (pDC) for the treatment of an autoimmune disease.

9. A compound according to claim 8 wherein the autoimmune disease is selected from Sjögren's syndrome, systemic lupus erythematosus, psoriasis, dermatitis herpetiformis, vitiligo, mycosis fungoides, allergic contact dermatitis, atopic dermatitis, lichen planus, Pityriasis lichenoides and varioliforms acuta (PLEVA).

10. A compound according to claims 8 or 9 wherein the antagonist is a SERM.

11. A compound according to claim 10 wherein the antagonist is a pure antagonist.

12. A compound according to claim 11 wherein the antagonist is selected from ICI 164384, ICI 182780, RU 58668, EM-139 et EM-800.

26

13. A pharmaceutical composition comprising an agonist according to claims 3 to 4 for the treatment of an infectious disease, and a pharmaceutical acceptable vehicle.

14. A pharmaceutical composition comprising a compound according to claims 8 to 12 for the treatment of an autoimmune disease, and a pharmaceutical acceptable vehicle.

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15. A pharmaceutical composition according to claim 14 further comprising a progestin substance.

16. A pharmaceutical composition comprising a compound according to claims 5 to 6 for the treatment of a cancer, and a pharmaceutical acceptable vehicle.

17. A pharmaceutical composition comprising an agonist according to claim 7 for the stimulation of the immune system in a vaccine therapy, and a pharmaceutical acceptable vehicle.
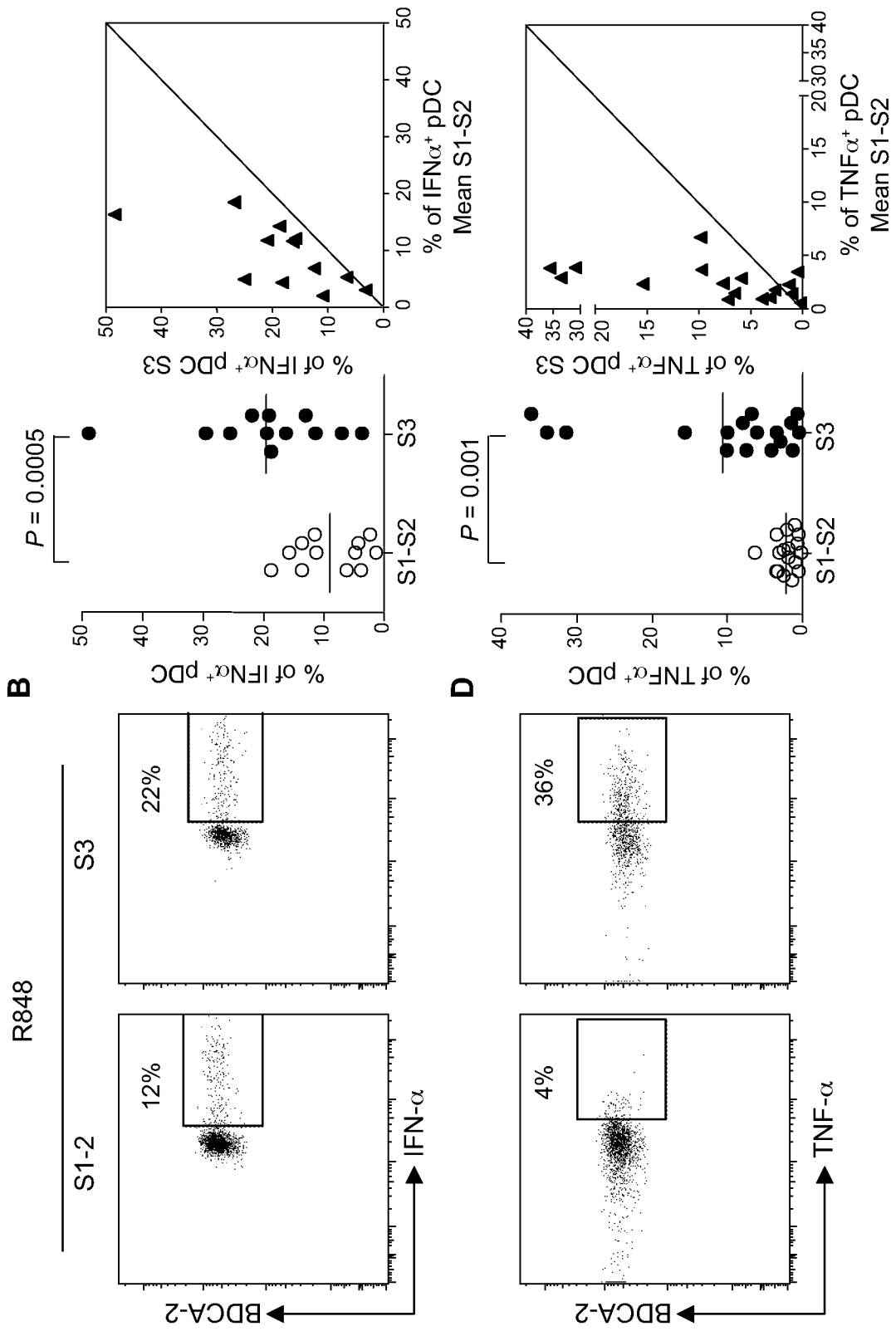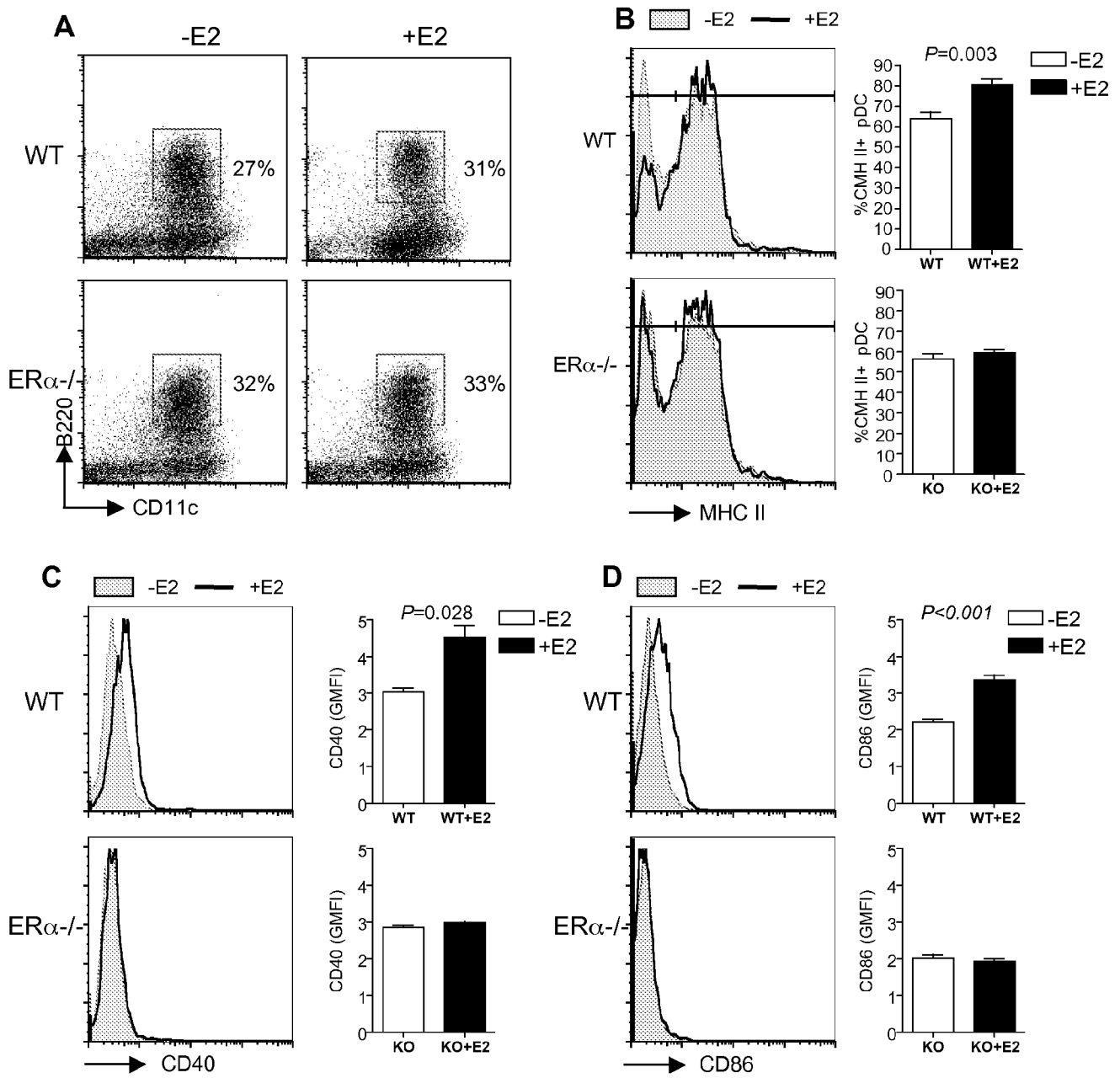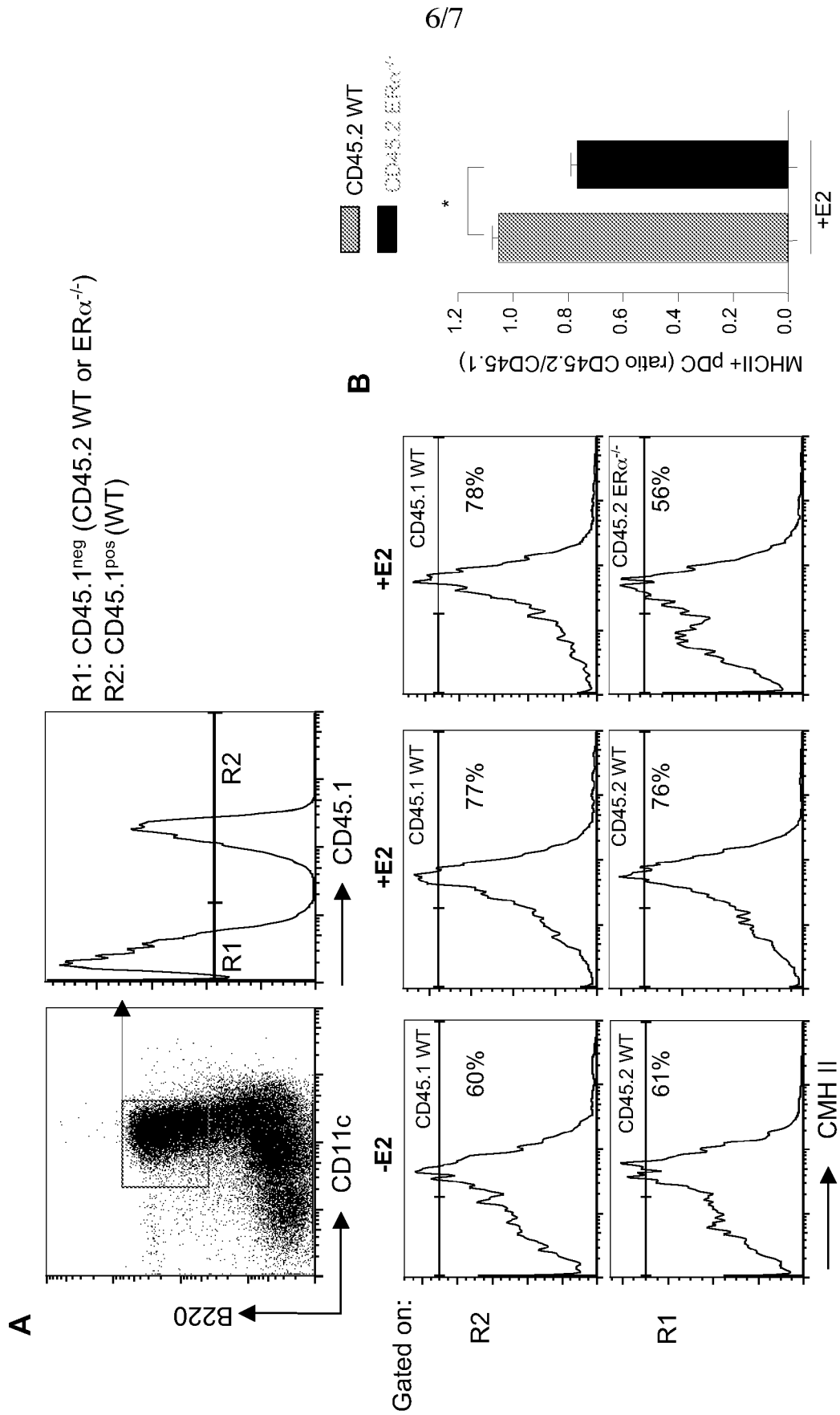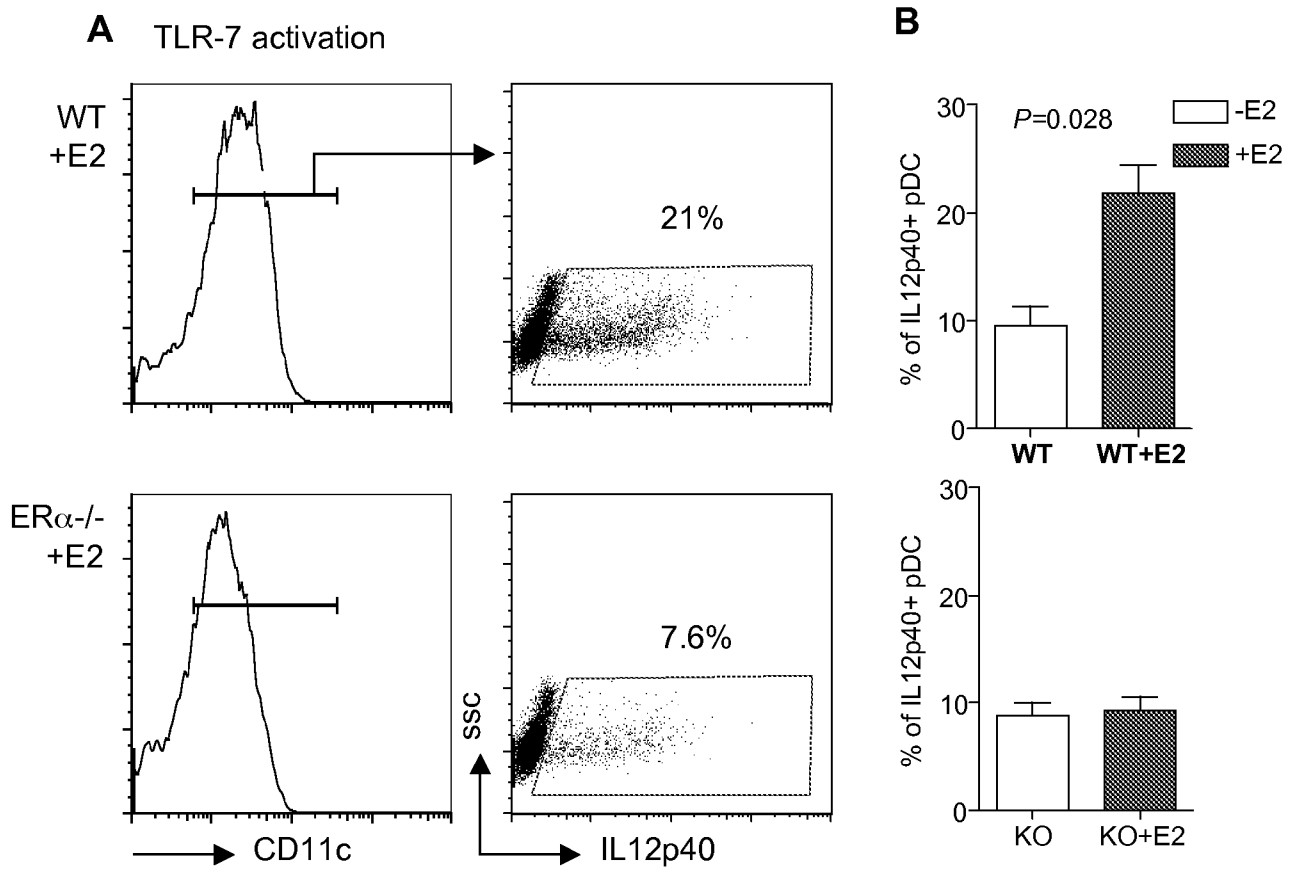
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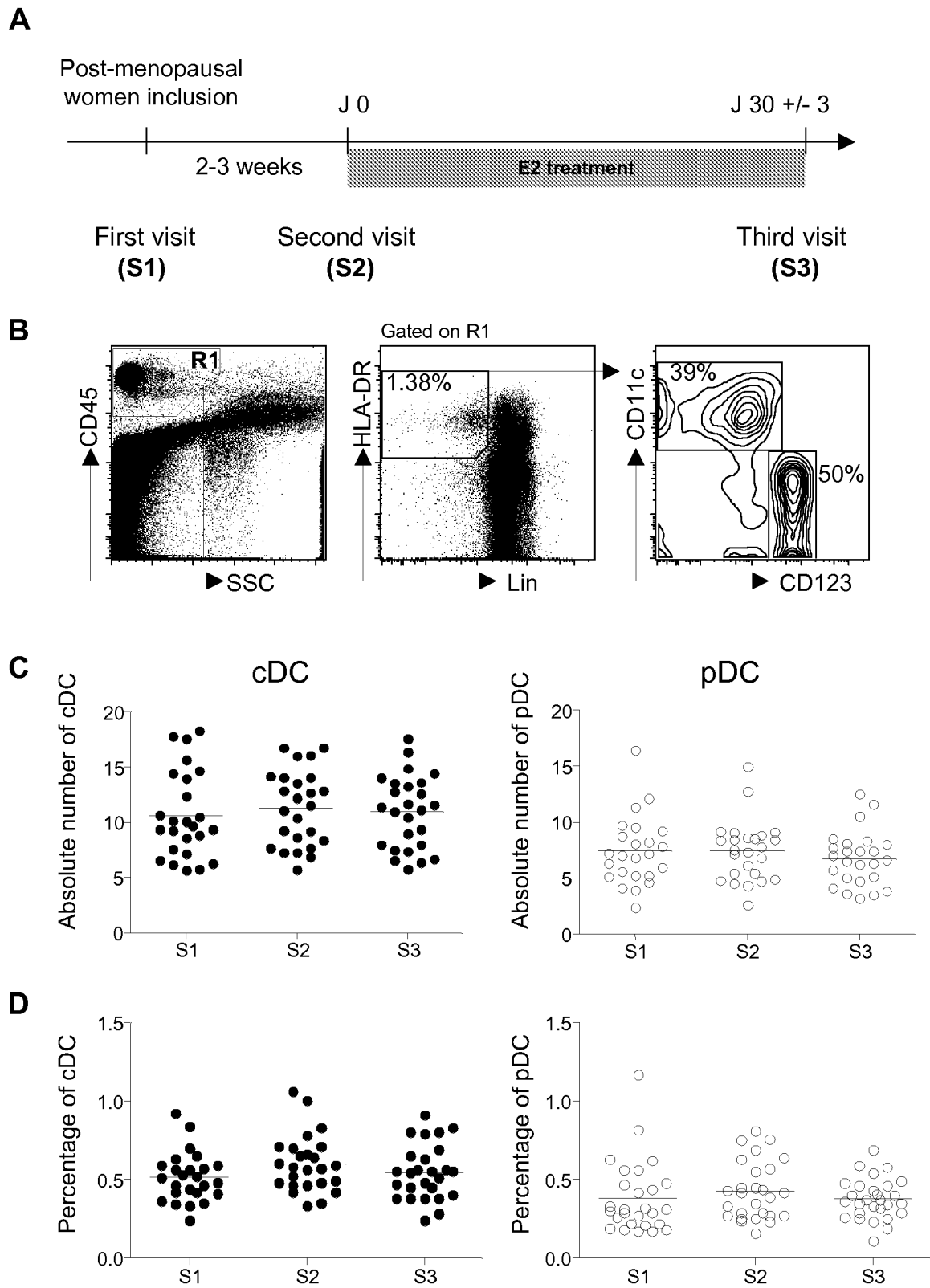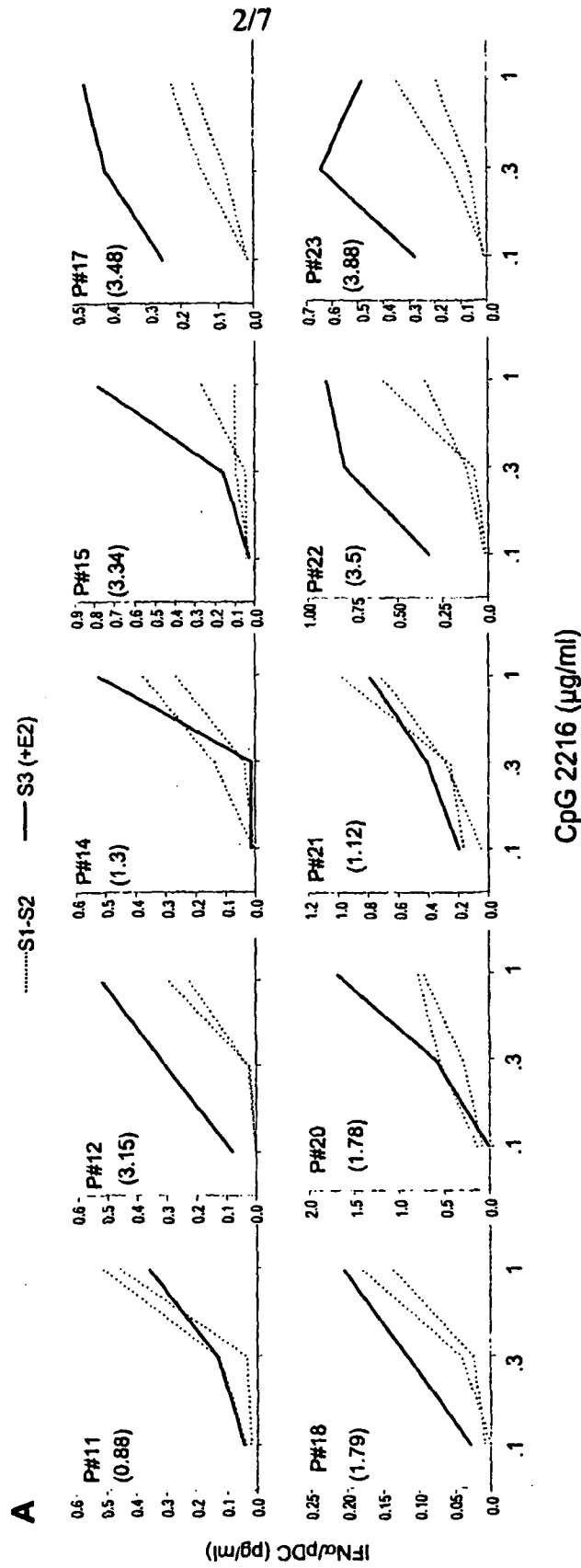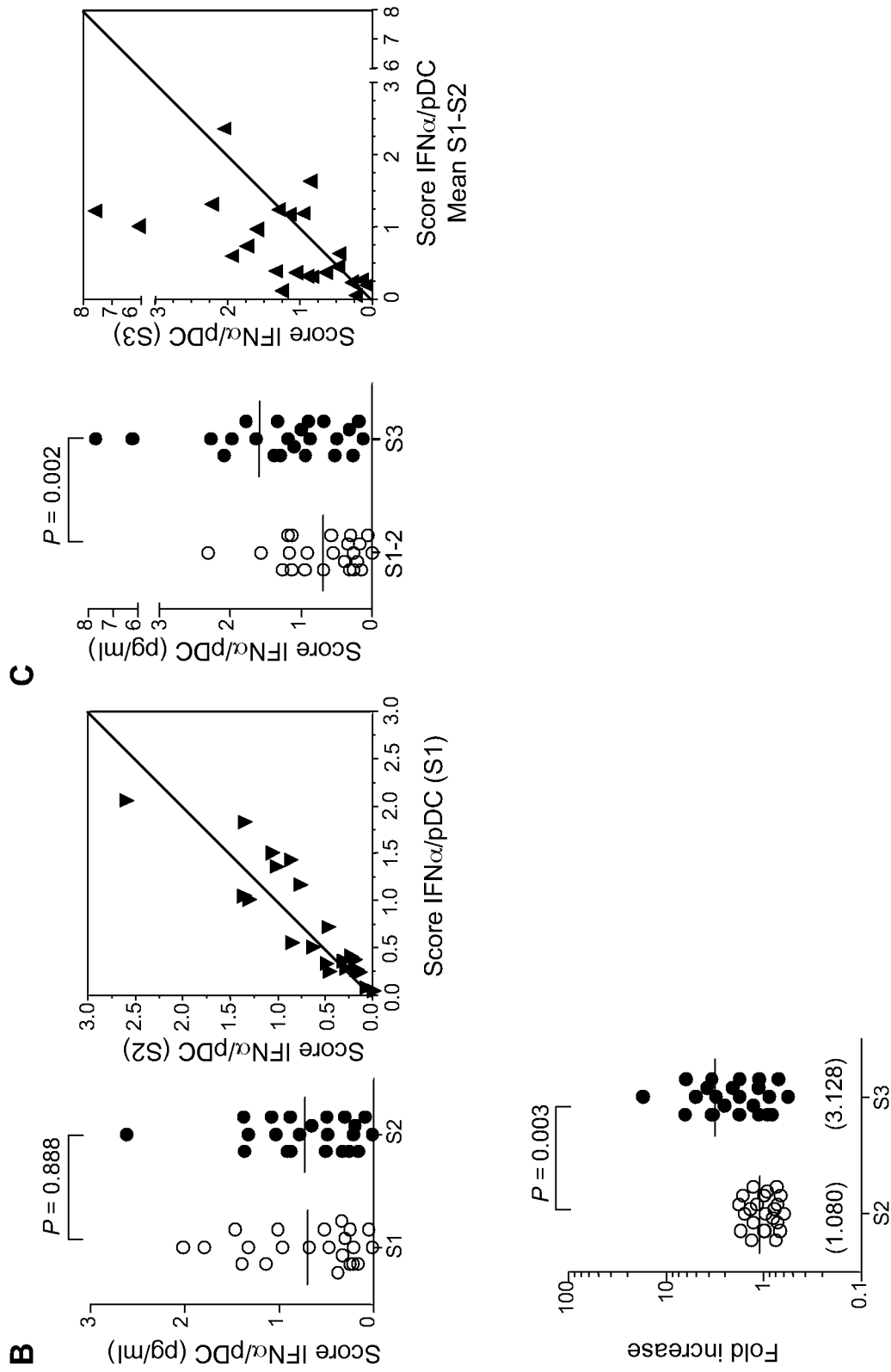
Figure 1

Figure 2A

Figure 2 B,C and D

Figure 3

Figure 4

Figure 5

Figure 6