RELAZIONE ATTIVITA' SCIENTIFICA PREMIO NOVARTIS FARMACOLOGIA DI GENERE
Dott.ssa Rosalia Crupi
(Università di Messina)

Relazione di metà periodo della Dott.ssa Rosalia Crupi (Università ddi Messina) – Premio Novartis Farmacologia di genere. Innovazione nell'ambito del sistema respiratorio - Phosphodiesterase inhibitors as a drug target candidate for preventing chronic obstructive pulmonary disease: Gender difference

Background

Sex differences in the biology of different organ systems and the influence of sex hormones in modulating health and disease are increasingly relevant in clinical and research areas. Although work has focused on sex differences and sex hormones in cardiovascular, musculoskeletal, and neuronal systems, there is now increasing clinical evidence for sex differences in incidence, morbidity, and mortality of lung diseases including allergic diseases (such as asthma), chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, lung cancer, as well as pulmonary hypertension. Epidemiologic evidence points to gender-based differences in incidence, risk, histology, and pathogenesis of certain lung diseases in women as compared with men (1). Gender influences not only physiological differences, but also the social, economic, and cultural context in which men and women coexist (2). The prevalence, morbidity, and mortality of COPD in women are increasing in the United States. Statistics say in fact that there are more women than men with chronic obstructive pulmonary disease (COPD), a disease characterized by all-or-nothing progressive, presently irremediable, alveolar loss: primary or idiopathic pulmonary hypertension (PPH) is well known as a disease of women in the childbearing years, while asthma is increasing in prevalence and more rapidly in women: in the decade 1982-1992 its prevalence in the U.S. increased by 82% in women versus 29% in men. In 2000, for the first time, the number of women dying of COPD in the United States surpassed the number of men. The pathology of COPD involves small airways and lung parenchyma, with chronic inflammation leading to luminal obstruction, thickening of the airway wall by increased deposition of matrix molecules and proliferation of mesenchymal cells, and narrowing of the airway by fibrosis, causing a decrease in lung function (3,4). The aetiology of COPD is not completely clear. Smoke, environmental pollution, airway hyper-reactivity, age, and genetic predisposition are known risk factors. Development of COPD may be caused by various harmful gases and particulates that are inhaled into the airway, leading to an inflammatory response in the lungs. A number of inflammatory cells, including macrophages, neutrophils, T cells, B cells, eosinophils, and epithelial cells are involved in the disease process (5,6). These inflammatory cells may induce immune responses, resulting in changes in a large number of inflammatory mediators, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, the levels of which were found to be significantly increased in induced sputum samples from patients with COPD (7). COPD is a complex chronic inflammatory disease, and the long-term imbalances in immune regulation may result in lung tissue remodeling or damage, leading to decline in lung function. Oxidant-antioxidant imbalance also causes inflammation and destruction of lung tissue. Even more interesting is the possibility that the nature of biologic injury may difference between men and women. Women with COPD also report more dyspnea and lower self-reported health status compared with men after adjusting for smoking burden and lung function. Men and women with COPD may also differ in the intensity of symptoms and health-related quality of life associated with their disease (8). Neurobiologic studies demonstrate that women have a higher intrinsic sensitivity to noxious somatic sensations, including dyspnea. Neuroimaging studies also demonstrate gender differences in the laterality of prefrontal cortical processing of noxious stimuli. Because this region is involved in the cognitive modulation of emotion, it may be responsible for differences in the affective associations with dyspnea for women. Therefore, it is clear that have to exist a factor of susceptibility or protection, that goes beyond the natural history of the individual and the environment that surrounds it, and that explains this discrimination between men and women. This factor could be attributed to hormone production, which is the main discriminating in men physiology against that of women, and to the changes of this production that occur during the life. In a paper of the 2005, Gharaee-Kermani et al. (9) showed that female rats had higher mortality rates and more severe fibrosis than did male rats, suggesting that gender

and sex hormones must have a role in the host response to lung injury, inflammation, and fibrosis. Moreover, it was showed that 17β-estradiol (E2) produces anti-inflammatory effects after acute lung injury in ovariectomized female rats, so that it is clear that hormonal imbalances, most noticeable in the female that in male menopause, has substantial effects on the body, the estrogens have an important protective action and their absence is involved in the gender-related incidence of pulmonary pathologies. To date, the achievements of the treatment of idiopathic pulmonary fibrosis (IPF) has led to avoid the formation of new scars and can only relieve symptoms and improve quality of life. Several aspects of lung development, homeostasis, and physiopathology are regulated by estrogens. Sex differences related to lung maturation, such as alveolar type II cell activity in surfactant production or ion channel expression in the respiratory epithelium, have been extensively studied and reconciled with a direct effect of sex steroid hormones on the developing lung structures, with estrogens displaying stimulatory effects (10). Similarly, gender differences in the lung of sexually mature animals, including size and function of respiratory structures and their responsiveness to cholinergic stimulation, are controlled by estrogens (11). In line with the above-mentioned effects, interstitial and airway lung diseases were also reported to be modulated by estrogens, which either contribute or protect against disease pathogenesis, depending on the disease involved (12). These experimental data provide strong support to the evidence that human lung disorders are influenced by circulating levels of estrogens, which seem to affect the prevalence and severity of lung pathologies such as fibrosis, asthma, infection, and cancer (13). The physiological reduction in estrogens level that occurs at menopause is associated with a general increase in the inflammatory responsiveness and exposes women to a higher risk for pathologies, such as those affecting bone and cardiovascular or central nervous systems, which are associated with inflammation (14,15). Our previous observations showed the influence of 17β-estradiol (E2) on inflammatory injury of the lung induced by carrageenan (CAR) injection and the involvement of ERs in protective effects of hormone; similarly, other studies addressed the positive influence of estrogens on acute lung injury models (16). Moreover, in a recent study we have provided novel findings related to estrogens action in lung: 1) ER-alpha is the molecular mediator of E2 inhibitory activity on CAR-induced lung inflammation; 2) resident macrophages and endothelial cells are direct targets of this activity; 3) E2 activity is conserved among females and males; and 4) aging is associated with a high inflammatory reaction that is gender independent and E2 unresponsive. We believe that these observations give a deeper understanding of estrogens action in lung physiopathology. In fact, although previous studies we have showed that E2 reduces inflammation in lung, the molecular and cellular details of this action were mostly unknown. Whereas both ERs are required for pulmonary alveolar formation (17), lung cells were shown to express higher levels of ER-alpha than ER-alpha mRNAs (18) and the ER- alpha protein was detected by IHC in pulmonary airway structures, whereas ER- alpha protein expression could not be detected (19). Novel generation drugs in the treatment of lung injury, such as COPD disease, consist in the therapy with Phosphodiesterase 4 (PDE4) and Phosphodiesterase 7 (PDE7) Inhibitors. The PDE enzyme superfamily inactivates cyclic adenosine monophosphate and cyclic guanosine monophosphate. PDE4 is the main PDE isoenzyme occurring in cells involved in inflammatory airway diseases. Inhibitors of PDEs allow the elevation of cAMP and cGMP which lead to a variety of cellular effects including airway smooth muscle relaxation and inhibition of cellular inflammation or of immune responses. PDE4 inhibitors specifically prevent the hydrolysis of cAMP, and PDE4 isozymes are present in inflammatory cells. Selective PDE4 inhibitors have broad spectrum anti-inflammatory effects such as inhibition of cell trafficking, cytokine and chemokine release from inflammatory cells, such as neutrophils, eosinophils, macrophages and T cells. Moreover, PDE7 is involved in T cell activation, so that a dual PDE4-PDE7 inhibitor may be more effective in asthma and COPD.

Bleomycin is an efficacious antitumor agent currently used in humans. Nevertheless, repeated administration of this drug may lead to lung inflammation and fibrosis as a side effect. Because this phenomenon is easily reproduced in different mammals, intratracheal administration of bleomycin has become the most widely used experimental model of lung fibrosis, although with certain limitations. This model is characterized by an early neutrophilic response, increased collagen deposition, and fibroblast proliferation (20). Bleomycin alters the balance between oxidants and antioxidant defense systems in the lung. In this particular organ, the selective absence of bleomycin hydrolase activity gives a high susceptibility to bleomycin-induced oxidative stress (21).

Aims

Based on experimental evidences, the idea of our study starts from sex-specific differences, which lie mainly in hormonal deficiency that occurs in women during climateric period or in a more advanced stages of life. With this aim in mind, in the first step of our study, we investigated the role of estrogen receptors (ERs) in inflammatory lung injury and assessed the role of gender and aging in estrogens action.

Experimental Methods and Procedures

Animals

Adult female and male, respectively young (4 months) and old (20 months) C57BL/6 mice (Harlan Nossan, Milan, Italy), weighing range 19–24 g, were used in all experiments. Mice were housed at a temperature of 20 ± 2°C, with free access to food and water (12-h light/ dark cycle). Animals were acclimated to these housing conditions for 1 week before any experimental manipulation. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J: of E.C.L 358/1 12/18/1986). The University of Melbourne Animal Experimentation Ethics Committee approved all surgical techniques, treatments, and experimental protocols.

Induction of lung injury by BLM

Mice received a single intratracheal instillation of saline (0.9%) or saline containing bleomycin sulfate (1 mg/kg body wt) in a volume of 100 µl. Animals were examined daily, and body weights and survival rates recorded. After 7 days of BLM injection mice were killed by pentobarbital sodium overdose.

Surgery-ovariectomy

To clarify the relationship between female sex hormone and lung fibrosis, female mice were ovariectomized. Animals were anesthetized with isofluorane/oxygen breathing mixture and placed on a heat pad maintained at 37°C. A longitudinal midline incision was then made through the skin, and blunt-tipped scissors were used to separate the connective tissue and to cut through the peritoneal wall. Ovaries were located and removed bilaterally, and the incision surgically stapled and closed. In sham-operated animals, all steps, except removal of ovaries, were carried out.

Exogenous estradiol treatment in ovariectomized mice

3 weeks after ovariectomy, mice were injected *sc* with vehicle (purified corn oil) or 50 µg/kg E_2 (Sigma, Milan, Italy) 2 h before BLM injection.

Histological examination

Lung biopsies were fixed for 1 wk in buffered formaldehyde solution (10% in PBS) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Serial sections (7 µm) in sagittal plane of the whole right lobe were cut using a microtome (Microtom HM 310; Zeiss, Milan, Italy); for each animal, two sections were analyzed that were about 25 µm distant from each other, and five fields of 700 x 900 µm each were scored in each section. Sections were deparaffinized with xylene, stained with hematoxylin and eosin, and studied using light microscopy Axiovision (Zeiss). The following morphological criteria were used for scoring lung histology under light microscopy: grade 0, normal lung; grade 1, minimal edema or inflammatory cell infiltration through alveolar or bronchiolar walls; grade 3, moderate edema and inflammatory cell infiltration without obvious damage to lung architecture; and grade 4, severe inflammatory cell infiltration with obvious damage to lung architecture. All histological studies were performed in a blinded fashion.

Myeloperoxidase (MPO) assay

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte accumulation was determined. At the specified time after BLEO injection, lung tissues were obtained and weighed, each piece homogenized in a solution containing 0.5% (wt/vol) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x*g* at 4 C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was mea- sured by spectrophotometry at 650 nm wavelength. MPO activity was defined as the quantity of enzyme degrading 1 µmol peroxide per minute at 37^{o} C and was expressed in milliunits per gram weight of wet tissue.

Measurement of cytokines

Portions of lung, collected at 7 d after bleomycin administration, were homogenized in PBS containing 2 mmol/L of phenyl-methyl sulfonyl fluoride (Sigma Chemical Co., Milan, Italy), and tissue levels of TNF-alfa and IL-1beta were evaluated. The assay was performed by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA) according to the manufacturer instructions. All cytokines determinations were performed in duplicate serial dilutions.

Statistical evaluation

All values are expressed as mean ± SEM of N observations. The results were analyzed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. $P < 0.05$ was considered significant.

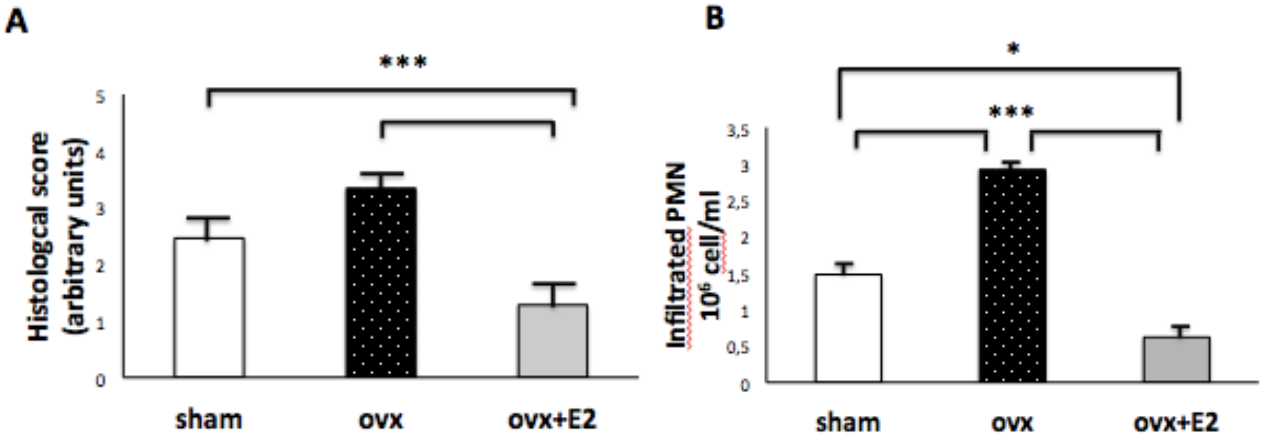
Results

Effects of gender on BLM-Induced morbidity and mortality

To determine the importance of gender in COPD, male and female mice were treated with endotracheal injections of BLM or saline on day 0. Animals were monitored daily for body weight and mortality rates were compared between male and female mice after BLM administration. Results showed that female mice had greater weight loss when compared to males (5 ± 1.0% versus 2 ± 1.1%) after BLM injection. Some of the female mice became very sick with severe respiratory distress as the disease progressed, resulting in respiratory failure with an high mortality rate. Thus, female gender appeared to enhance the effects of BLM treatment.

Endogenous estrogen and lung inflammation

Bleomycin injection is a well-accepted animal model of acute lung inflammation, which consists of a cellular exudate formation mainly made of PMN cells that invade lung parenchyma. To determine the role of endogenous estrogens, animals were ovariectomized. Sham-operated or ovariectomized (ovx) animals were injected with BLM in the pleural cavity. A histological score was used to grade the inflammatory reaction, which is shown in Fig. 1A. In parallel, PMN cells recovered from the pleural exudate are shown in Fig. 1B. No macroscopic differences were noted in the lungs of the two groups, whereas an increase in the number of PMN cells was observed in ovx mice, suggesting that ovary removal increases the inflammatory response in the lungs. Inflammation was absent in BLM-untreated sham and ovx mice, with a histological score of 0 and about 0.5 x 10 -6 PMN cells per milliliter in both groups (data not shown). To link more specifically the effect of ovariectomy with the lack of endogenous estrogens, a group of ovx mice received an injection of 50 µg/kg E2, 2 h before BLM injection. E2-treated animals showed both an improvement in the histological score (Fig. 1, A and B) and a lower number of infiltrated cells when compared with ovx mice; these parameters were even lower than those observed in the sham group (Fig. 1C). BLM-untreated ovx +E2 mice had no inflammatory signs (data not shown). Altogether these results suggest that deprivation of endogenous estrogens increases the inflammatory response in the mouse lung and that E2 is an effective pharmacological agent to reduce lung inflammation.



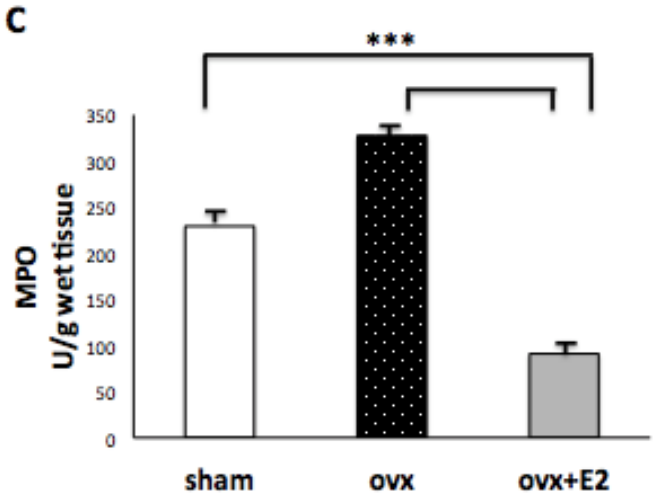


FIG. 1. Estrogen expression and lung inflammation in female mice. (A) histological score as described in Materials and Methods. (B) Pleural exudate was recovered and analyzed for infiltrated PMN cell number and (C) for MPO. Bars represent the average ± SEM of all the animals (n =10), each analyzed in triplicate. *, $P < 0.05$; ***, $P < 0.001$.

Cytokines levels

With the aim to demonstrate that ER alfa is active in lung inflammatory cells and mediates the observed antiinflammatory effect of E_2, we evaluated the expression levels of inflammatory mediators induced by BLM injection in control or E_2-treated mice. As shown in Fig. 2 A and B, the levels expression of both, TNF alfa and IL 1beta, are increased after ovariectomy. Further these data clarify the role of extrogens in lung inflammation.

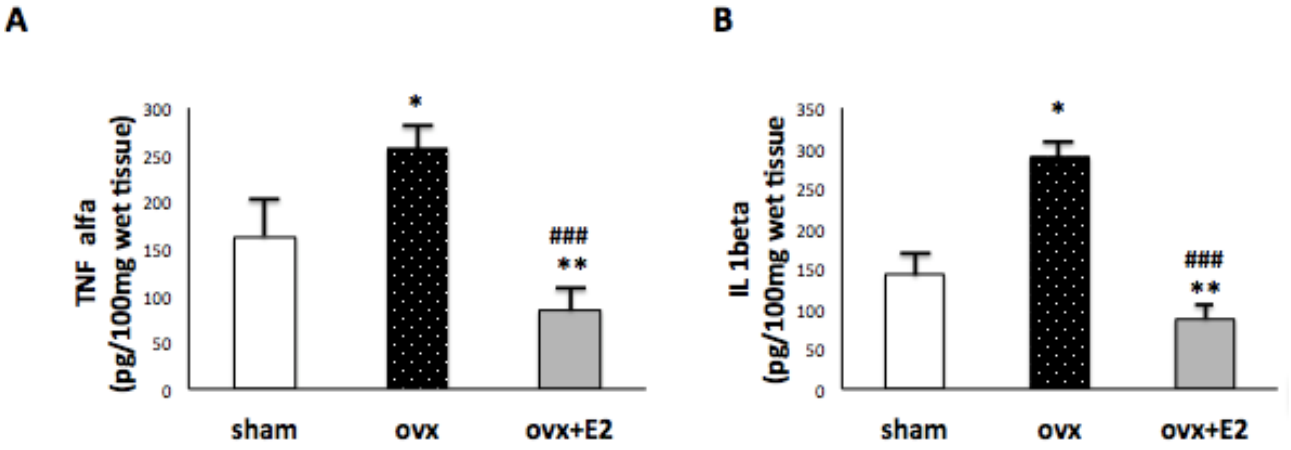


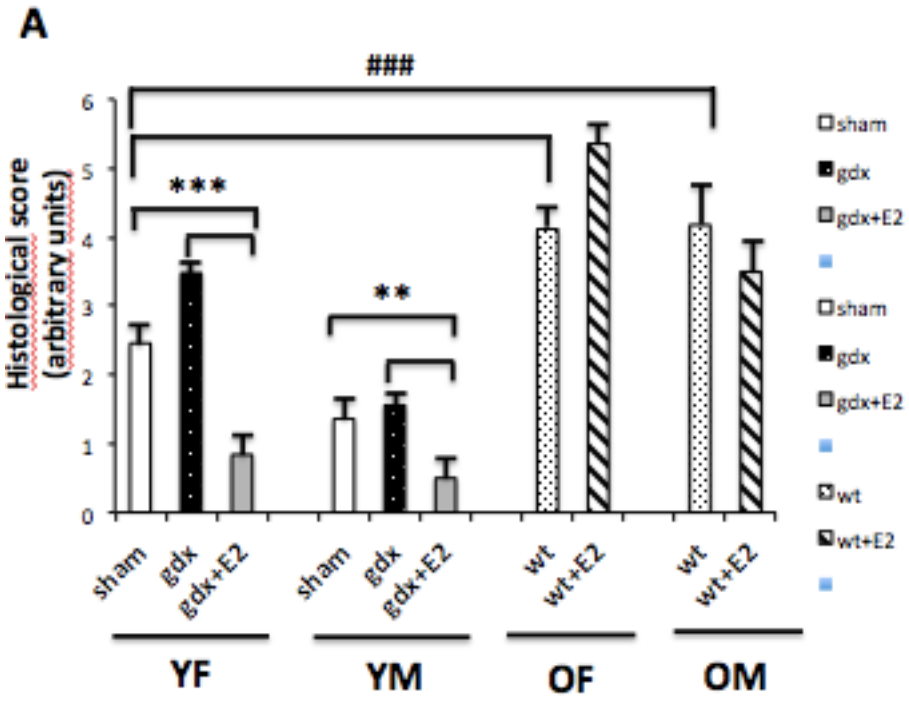
FIG. 2. Expression on TNF alfa and IL 1beta. TNF alfa and IL 1beta levels were analyzed by immunoenzymatic assay in the pleural exudate. Bars represent the average ± SEM of all the animals, each analyzed in triplicate. * vs. sham; **#** vs. ovx; * $P < 0.05$; **###** $P < 0.01$;

Effect of gender and aging in lung inflammation

With regard to the role of sex hormones on disease severity, much of the evidence suggests that the estrogens hormone, which is gender-specific, has a significant influence on disease progression. To determine whether E2 could act as a protective, antiinflammatory molecule also in male mice, we analyzed the effect of BLM-injection in male mice that underwent gonadectomy (gdx) to remove the endogenous source of estrogenic precursors. In parallel, a group of gdx animals received E2 administration before BLM

induction. As shown in Fig. 3 A and B, lower values of inflammatory parameters were observed in sham males as compared with females; this is shown by both a lower histological score and a reduced number of infiltrated cells in the lungs of 4-month-old male mice. gdx *per se* does not alter inflammatory parameters. E2 administration reduces lung inflammation, resulting in a lower grade of inflammation which is even smaller than intact animals.

Ageing is associated with a progressive decline in both immune and pulmonary functions, with a higher level of inflammatory response, in that increased levels of inflammatory mediators, such as TNF alfa which contribute to increased impact and severity of infections among the elderly. For this reason we analyzed lung inflammation after BLM injection in 20- month-old mice and evaluated the effect of E2 on the inflammatory response; this group of old mice was not gonadectomize because we could not exclude that ablation of endocrine hormones in old mice would affect lung homeostasis and response to BLM injection. As shown in Fig. 3 A and B, both male and female mice at 20 months of age show a robust inflammatory reaction, as shown by a dramatic worsening of the histological score and massive infiltration of PMN cells after BLM injection; however, E2 administration does not cause inflammation in either gender. These data show that the dramatic changes in the inflammatory response that occur in the lung of aged animals are gender independent



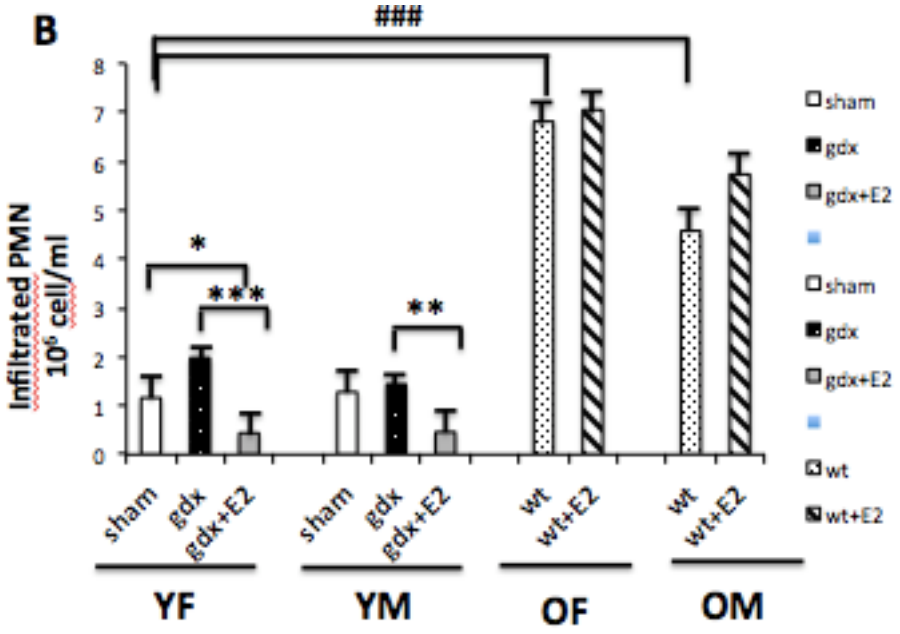


FIG.3 Effect of gender and aging on E2 signaling in lung inflammation. Lung inflammation was induced by BLM injection in young female and male mice (YF and YM in bar legend) or old female and male (Of and Om in bar legend; wt or wtE2), as indicated. Histological score (A) or infiltrated PMN cells numbers (B) are shown; bars represent the average ± SEM of all the animals (n =10), each analyzed in triplicate.* vs. sham; # vs. 4-month sham; *, P , 0.05; **, P , 0.01; ***, **##**, P < 0.001.

Conclusion

In the first step of our project we have shown that activation of ER in lung inflammatory cells might provide beneficial effects in adult, but not in aged mice of both genders, suggesting a key role of the estrogens signaling pathway in the physiology and therapeutic opportunities of lung inflammation.

In the second step we will investigate the effects of the treatment with PDE4 and PDE7 inhibitors on ovariectomized and not-ovariectomized female mice in the same experimental model of chronic obstructive pulmonary disease to assess the role of gender and aging under this treatment.

References

1. Martinez F, Curtis J, Sciurba F, Mumford J, Giardino N, Weinmann G, Kazerooni E, Murray S, Criner G, Sin D, et al.; NETT Research Group. Sex differences in severe pulmonary emphysema. Am J Respir Crit Care Med 2007;176:243–252.
2. Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM. Global burden of COPD: systematic review and meta-analysis. Eur Respir J 2006;28:523–532.
3. Moghaddam SJ, Clement CG, De la Garza MM, Zou X, Travis EL, Young HW, Evans CM, Tuvim MJ, Dickey BF. Haemophilus influenzae lysate induces aspects of the chronic obstructive pulmonary disease phenotype. Am J Respir Cell Mol Biol. 2008; 38(6): 629-638.
4. Barnes PJ. Small airways in COPD. N Engl J Med. 2004; 350(26): 2635-2637.
5. Sarir H, Henricks PA, van Houwelingen AH, Nijkamp FP, Folkerts G. Cells, mediators and Toll-like receptors in COPD. Eur J Pharmacol. 2008; 585(2-3): 346-353.
6. Jeffery PK. Comparison of the structural and inflammatory features of COPD and asthma. Giles F. Filley Lecture. Chest. 2000; 117(5 Suppl 1): 251S-260S.
7. Chung KF. Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl. 2001; 34: 50s-59s.
8. Mannino D, Homa D, Akinbami L, Ford E, Redd S. Chronic obstructive pulmonary disease surveillance: United States, 1971–2000. MMWR Surveill Summ 2002; 51:1–16.
9. Gharacee-Kermani M, Hatano K, Nozaki Y, Phan SH. Gender-based differences in bleomycin-induced pulmonary fibrosis. Am J Pathol. 2005; 166: 1593-1606.
10. Sweezey N, Tchepichev S, Gagnon S, Fertuck K, O'Brodovich H. Female gender hormones regulate

mRNA levels and function of the rat lung epithelial Na channel. Am J Physiol 1998; 274:C379 –C386.
11. MassaroGD,MortolaJP,MassaroD. Estrogenmodulatesthe dimensions of the lung's gas- exchange surface area and alveoli in female rats. Am J Physiol 1996; 270:L110 –L114.
12. Cuzzocrea S, Santagati S, Sautebin L, Mazzon E, Calabro` G, Serraino I, Caputi AP, Maggi A.17beta-estradiol antiinflammatory ac- tivity in CAR-induced pleurisy. Endocrinology 2000; 141:1455–1463.
13. Carey MA, Card JW, Voltz JW, Arbes Jr SJ, Germolec DR, Korach KS, Zeldin DC. It's all about sex: gender, lung development and lung disease. Trends Endocrinol Metab 2007; 18:308 –313.
14. Vegeto E,* Cuzzocrea S,* Crisafulli C, Mazzon E, Sala A, Krust A, and Maggi A. Estrogen Receptor- α as a Drug Target Candidate for Preventing Lung Inflammation. Endocrinology 2010; 151: 174-184.
15. Vegeto E, Ciana P, Maggi A. Estrogen and inflammation: hormone generous action spreads to the brain. Mol Psychiatry 2002; 7:236 –238.
16. Card JW, Carey MA, Bradbury JA, DeGraff LM, Morgan DL, Moorman MP, Flake GP, Zeldin DC. Gender differences in murine airway responsiveness and lipopolysaccharide- induced in- flammation. J Immunol 2006; 177:621–630.
17. Massaro D, Massaro GD Estrogen regulates pulmonary alveolar formation, loss, and regeneration in mice. Am J Physiol Lung Cell Mol Physiol 2004; 287:L1154–L1159.
18. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS.Tissue distribution and quantitative analysis of estrogen receptor- β (ER β) and estrogen receptor- β (ER β) messenger ribonucleic acid in the wild-type and ER β -knockout mouse. Endocrinology 1997; 138:4613–4621.
19. Patrone C, Cassel TN, Pettersson K, Piao YS, Cheng G, Ciana P, Maggi A, Warner M, Gustafsson JA, Nord M. Regulation of postnatal lung development and homeostasis by estrogen receptor β. Mol Cell Biol 2003; 23:8542–8552.
20. Chua F, Gauldie J, Laurent GJ. Pulmonary fibrosis: searching for model answers. Am J Respir Cell Mol Biol 33: 9–13, 2005.
21. Filderman AE, Genovese LA, Lazo J**S**. Alterations in pulmonary protective enzymes following systemic bleomycin treatment in mice. Biochem Pharmacol 37: 1111–1116, 1988.