



## Alterations associated in dendritic cells sub-types in smokers and non-smokers

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### General Note

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### ABSTRACT

*Introduction:* Dendritic cells (DCs) consider as greatest significance cells of innate and adaptive immune system because they connect both types. These cells are necessary for controlling immunity and tolerance balance. *Objective:* Effect of cigarette smoking on immunological properties of DCs and their subtypes plasmacytoid DCs and myeloid dendritic cells. *Methods:* The study included 27 smokers (female and male) and 18 nonsmoker controls. Isolating peripheral blood mononuclear cells, later, counted the total white blood cells (WBCs/mm<sup>3</sup>) by hemocytometer. DCs population, maturation marker CD83, and intracellular cytokines (IL-12, IFN- $\alpha$ ) were detected using multiparametric flow cytometry analysis. *Results:* Increasing absolute number of pDCs was noted among smokers in general and among male smokers specifically. Therefore, mDCs/pDCs ratio was decreased in same populations. A comparison of male and female smokers reported that, increasing in the absolute number of pDCs in males. No detectable changes recorded for the expression of CD83 or the intracellular cytokines. *Conclusions:* Our results support that smoking alters the ratio for

mDCs/pDCs and could lead to the suppression of the immune system through increasing tolerance response, thus increasing the risk of cancer and infections in smokers.

**Keywords:** Cigarette smoking, immune system, dendritic cells, myeloid dendritic cells, plasmacytoid dendritic cells.

**Abbreviations:** CS: cigarette smoking; DCs: dendritic cells; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; COPD: chronic obstructive pulmonary disease; APCs: antigen-presenting cells.

## 1. INTRODUCTION

Cigarette smoking is an epidemic spreading around the world. It affects numerous organ systems and is a leading cause of major, even fatal, health issues (Chen et al., 2013). Inhalation of cigarette smoke is a risk agent that leads to developing cancer in human body, and breathing infections (Mehta et al. 2008). Numerous studies have confirmed that the immune system is affected by tar and nicotine because these substances are highly toxic. The effect varies by substances doses, and exposure duration (Nagorni-Obradovic et al., 2006). Immune system functions are protecting from infection both by stimulating adaptive and innate cells and sent multiple signals in response. Cigarette smoker body could given the function and subsequently, production of cytokines (Mehta et al., 2008).

DCs consider as very important cell for their protection roles against infectious pathogens and neoplasm and for their essential role in inducing and organizing immune responses. The first researcher to describe DCs was Professor Ralph Steinmann, who identified DCs as special hematopoietic lineage with uncommon morphology and movement and a strong capacity for stimulating a primary mixed leukocyte response (Liu & Cao, 2015). DCs play antigen-presenting cells and activate T cells by direct cell-cell interactions and/or cytokines production (Givi et al., 2015). DCs are classified into four major categories: conventional, Langerhans cells plasmacytoid, and monocyte-derived dendritic cells. According to the alterations in phenotype, another subset of DCs also exists, which is known as myeloid DCs (mDCs); this subset contains both cDCs and moDCs subtypes (Mbongue et al. 2014). The life cycle of DCs could be divided to 2 stage (immature and mature) depending on cell surface receptors expression such CD83, functional behavior, and cytokines production (Huang et al., 2018). After stimulation by different antigens, DCs travel from the blood, where they are in their immature state, to lymph nodes, where they reach their mature state, and activate T cells to become effector cells (Jensen & Gad, 2010). In addition, different pro-inflammatory cytokines can be produced by mature DCs of different types during inflammation. For example, type I interferons (IFNs) produced by pDCs are essential for viral prevention (Haniffa, Collin, & Ginhoux, 2013) while IL-12 produced by mDCs are importunate antigen-presenting cells (Blanco et al., 2008; Ma et al., 2015).

Different investigations were done to assessment smoking effects on DCs, although the results have been inconsistent. In one study by Robbins and colleagues, alveolar DCs of cigarette-exposed animals showed inhibition in number and maturation ability. This was confirmed by the low expression of certain cell surface receptors (Robbins, 2008). In 2013, a study compared the number of circulating DCs and their subtypes in smokers and nonsmokers and found a decrease in the ratio of mDCs/pDCs in smokers, indicating the inhibitory effect of cigarettes on mDCs known to stimulate the immune response (Chen et al., 2013). In contrast, other shown that, activating have effecting for cigarette smoking on DCs. For example, study done by Lommatzsch and colleagues examined the total number of mDCs and pDCs, and surface molecules expression. Increasing in DCs bronchoalveolar lavage samples and the expression of some cell surface receptors (Lommatzsch et al., 2010).

Cigarette and shisha (alnarjila) smoking are both increasing in Saudi Arabia, and smokers are starting from early ages in both male and, more recently, female generations. There is a shortage of studies in our country concentrate on studying cigarette smoking effects on immune system. Therefore, this study aimed to investigate changes associated in DC subtypes (myeloid DCs and plasmacytoid DCs) in terms of their number, maturation stages, and production of cytokines (IFN- $\alpha$  and IL-12) in smokers and nonsmokers. The study included both male and female smokers of young ages (ranging from 20 to 40 years) in the Kingdom of Saudi Arabia to understanding the mechanism of development of certain diseases in cigarette smokers.

## 2. MATERIALS AND METHODS

Experimental phase of our study was carried out at the Center of Excellence in Genomic Medicine Research, CEGMR, King Fahd Medical Research Center, King Abdul-Aziz University, Saudi Arabia. Samples were taken according to the guidelines of the CEGMR's Ethical Committee (approval code: 02-CEGMR-Bioeth-2019). Patients were written informed consent for our study.

### Human subjects

A total of 45 Saudi male and female smokers and nonsmokers enrolled in this study (October 2018–April 2019). Participants' ages ranged from 20 to 40 years. Each participant's body mass index was measured, and it was confirmed that they were not under any treatment course (Table 1).

### Peripheral blood mononuclear cells (PBMCs) isolation

A 5 ml blood sample was collected by venipuncture into Ethylenediaminetetraacetic acid (EDTA-coated) tubes. PBMCs were isolated using histopaque. In brief, diluted samples by phosphate-buffered saline pH 7.4 until it reached 8 ml and then added to the already aliquoted 5ml histopaque. After that, the centrifuging sample at 700g / 30 min. White layer of PBMCs was removed carefully using a pipette into a 15 ml tube and was washed with PBS by adding 13ml; the solution was then centrifuged on 1100 rpm for 10 min. the process repeated 2 time . The PBMCs were counted by mixing 10  $\mu$ l PBMCs with 10  $\mu$ l Trypan Blue Solution 0.4% and then loading the mixture into the hemocytometer to be counted by microscope. The average cell count from each of the sets of 16 corner squares was taken and the following equation used to determine the cell number:

$$\text{No. of cells counted} \times 10^4 \times \text{dilution factor (2)} = \text{number of cell / ml}$$

Then the mononuclear cells prepared with ( $1 \times 10^6$  cells /tube) for flow cytometry analysis.

**Table 1** Demographics and Clinical characteristics for the smoking and control subjects

	smokers	controls
Number	27	18
Age(years)	(20-40)	(20-40)
Sex (male/female)	18/9	9/9
Body mass index (BMI) (male-female)	27/26	24/24
Pack-year (male /female)	286/126	0/0
Total number of WBCs (cell $\times 10^3$ /mm <sup>3</sup> ) (male-female)	3.89/2.01	5.29/4.59

Pack-year and Total WBCs/mm<sup>3</sup> presented as means.

### PBMC staining

Extracellular staining was first prepared using 5 $\mu$ l from each Lineage Cocktail (Lin 1) FITC (Catalog No.340546) Contains CD3, CD14, CD16, CD19, CD20, and CD56. CD123 (9F5) PE\* (Catalog No.340545), CD11c (S-HCL-3) PE\* (Catalog No.333149), and BV510 Mouse Anti-Human CD83 (Catalog No.563223) added to PBMCs and incubated 30 min. in dark /20°C–25°C. To measure intracellular cytokine production, DCs intracellular staining done by adding 200 $\mu$ l of fixation/permeabilization solution kit (Catalog No.554714) to the cells and then mixing and incubating them on ice in dark /30 min. Cells centrifuged on 110 rpm for 10 minutes; 500 g, where supernatant, was aspirated and then washed with 2 ml Perm/Wash™ buffer by centrifuging it on 110 rpm for 10 minutes as the supernatant was discarded (this washing step was repeated twice). After that 5 $\mu$ l from each antibodies— V450 Mouse Anti-Human IFN- $\alpha$  [2b] (Catalog No.561382) and PE Mouse Anti-Human IL-12 (p40/p70) PE\* (Catalog No.554575)—was added to the cells, mixed, and incubated 30 minutes on ice in the dark. Finally, 1ml of PBS add, and the cells were fixed and analyzed by FACS fluorescence-activated cell sorting (FACS) flow cytometry.

### Absolute enumeration of blood DCs

Absolute number of DCs/mm<sup>3</sup>as of any DC subset, the equation used was the [Percent of a given DC subset x total number of white blood cells (WBCs) per mm<sup>3</sup>]/100.

### mDCs/pDCs ratio

mDCs/pDCs ratio as: an absolute number of mDCs /absolute number of pDCs.

### Statistical analysis

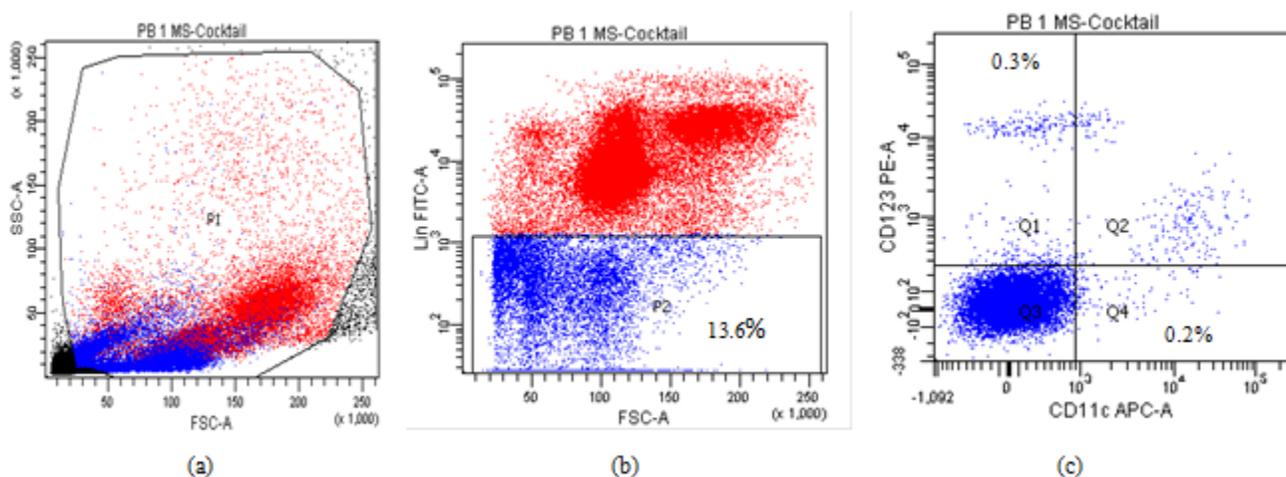
Data were summarized as mean  $\pm$  SD. For quantitative unpaired samples, T-test as well as the Mann-Whitney test were used to compare between two independent groups.  $P \leq 0.05$ .

## 3. RESULTS

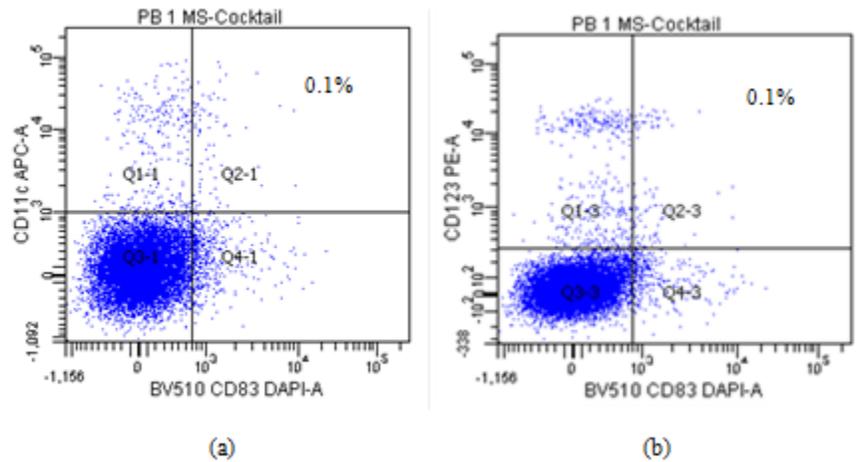
At first, we determined the proportion of total DCs, as well as mDC and pDC subsets, from total PBMCs. For this purpose, we used flow cytometry after staining the samples. We first gated total PBMCs in P1 to exclude cell debris and platelets. In Figure 1a, negatively selected cell population gated into P2 to exclude B cells, monocytes, and dead cells (positive for Lineage Cocktail 1 [Lin 1] FITC marker; see Figure 1b). mDCs and pDCs were next and gated into Q4 (positive for CD11c marker) and Q1 (positive for CD123 marker), respectively (see Figure 1c). We also determined the percentage of mDCs and pDCs that were positive for the CD83 marker indicating any alteration in the maturation state, as shown in Figures 2a and 2b. Finally, intracellular cytokines were determined IL-12 gated into Q1 (positive for PE Mouse Anti-Human IL-12 marker) and IFN- $\alpha$  gated into Q4-1 (positive for V450 Mouse Anti-Human IFN- $\alpha$  marker; see Figures 3a and 3b).

Our results showed no significant differences in the total number of WBCs in either group (controls or smokers), although they did indicate a tendency for the percentage of mDCs and pDCs to increase in smokers; the increase did not reach a significant level. However, the results showed a highly significant increase of an absolute number of pDCs in general smokers ( $27.52 \pm 28.22$  vs.  $10.13 \pm 14.41$ ,  $p$ -value = 0.008\*\*) and a significant increase in male smokers ( $37.98 \pm 29.18$  vs.  $15.21 \pm 18.85$ ,  $p$ -value = 0.017\*) compared with the controls (see Figure 4). This led to an imbalance in the mDC/pDC ratio where the results showed a highly significant reduction in general smokers ( $0.55 \pm 0.73$  vs.  $1.12 \pm 0.62$ ,  $p$ -value = 0.004\*\*) and in male smokers ( $0.11 \pm 0.13$  vs.  $1.14 \pm 0.62$ ,  $p$ -value = 0.000\*\*\*; see Figure 5). CD83 is the well-known maturation marker in DCs. Although no significant differences in the maturation of mDCs and pDCs between smokers and controls were observed in any group, the results indicated a possibility of increasing the expression of CD83 in smokers for mDCs but not pDCs (results are not shown). Finally, intracellular cytokines was assessed for the well-recognized cytokines for both DC subtypes as IL-12 produced by mDCs and IFN- $\alpha$  produced by pDCs. The results showed that there were no significant differences in any group (results are not shown).

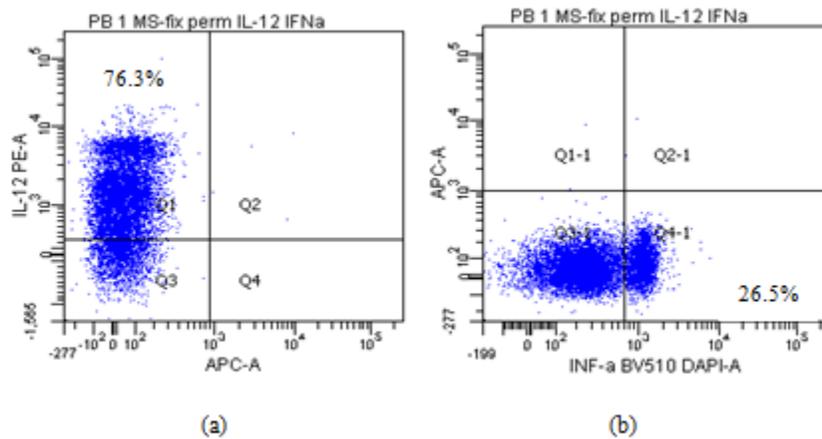
Comparing male and female smokers, results showed a significant increase in the total number of WBCs (cell $\times 10^3$ /mm<sup>3</sup>) in male smokers compared with females ( $3.89 \pm 2.87$  vs.  $2.01 \pm 1.78$ ,  $p$ -value = 0.027), as shown in Figure 6. There was a highly significant increase in the absolute number of pDCs among male smokers ( $37.98 \pm 29.18$  vs.  $6.60 \pm 6.29$ ,  $p$ -value = 0.000\*\*\*), as shown in Figure 7. Therefore, imbalance in the mDC/pDC ratio was recorded, as the results showed a highly significant reduction in the male mDC/pDC ratio ( $0.11 \pm 0.13$  vs.  $1.43 \pm 0.64$ ,  $p$ -value = 0.000\*\*\*; see Figure 8). Regarding CD83 expression, the results showed no detectable differences in either gender, although they indicated a possibility of increasing the expression of CD83 in male smokers for mDCs. Intracellular cytokines detection also did not show any significant changes in either gender, although IFN- $\alpha$  may tend to increase in male smokers (results are not shown).



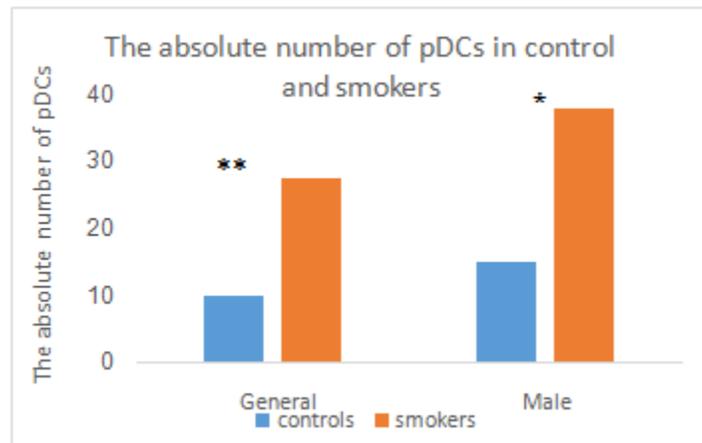
**Figure 1.** Steps for gating total DCs, mDCs, and pDCs from total PBMCs. (a). PBMCs gated into P1 to exclude debris and platelets. (b) DCs gated into P2 to exclude B cells, monocytes and other cells. (c) mDCs gated into Q4 and pDCs gated into Q1.



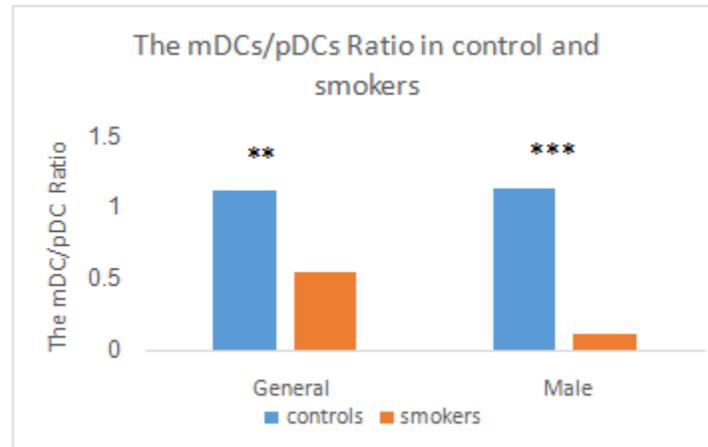
**Figure 2.** The percentage of DCs expression to CD83. (a) mDCs mature gated into Q2-1. (b) pDCs mature gated into Q2-3.



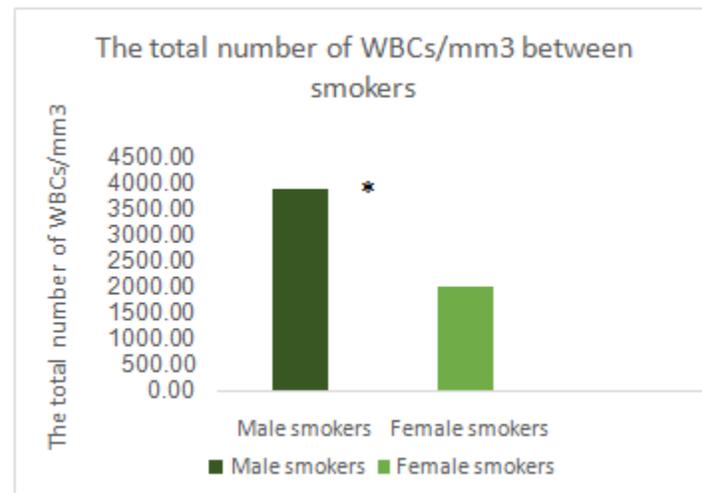
**Figure 3.** The percentage of DCs in intracellular cytokines IL-12 and IFN- $\alpha$ . (a) DCs expression to Anti IL-12 gated into Q1. (b) DCs expression to Anti IFN- $\alpha$  gated into Q4-1.



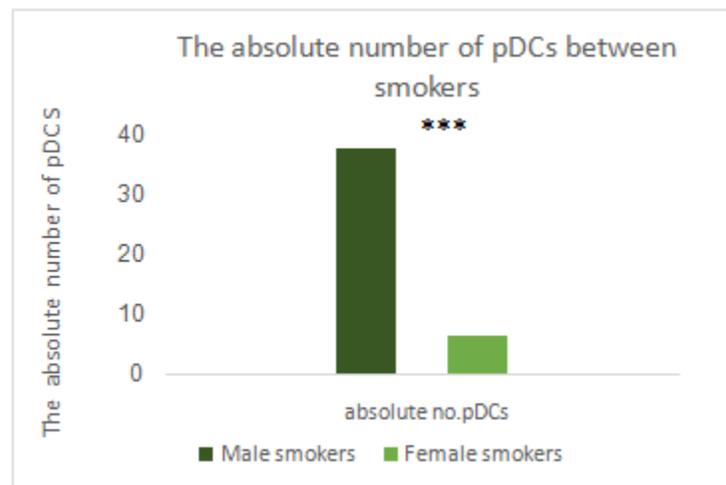
**Figure 4.** Comparison of absolute number of pDCs between control and smokers in general and male.



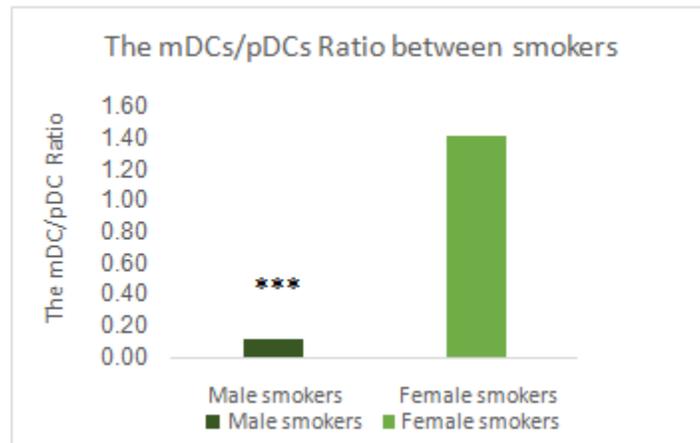
**Figure 5.** Comparison of mDC/pDC ratio between control and smokers in general and male



**Figure 6.** Comparison of total number of WBCs between smokers (male and female).



**Figure 7.** Comparison of absolute number of pDCs between smokers (male and female)



**Figure 8.** Comparison of mDC/pDC ratio between smokers (male and female)

#### 4. DISCUSSION

Cigarettes have above 45, 000 different chemicals and numerous toxic, mutagenic, and carcinogenic properties. With numerous available smoking products, differences in the content and concentration of chemical materials of cigarette smoke are important factors that might affect the results that could be obtained in this area (Fowles & Dybing, 2003; Richter et al., 2008; Rennard, 2004). The prevalence of cigarettes and shisha smoking in Saudi Arabia is increasing especially at an early age in males and females. However, there are not many studies in the region to study smoking effects of on immune system. Our aim was study the cigarette smoking on young generation's DCs and immune system activities.

Tobacco smoking have negative effects on immune system activities, several studies either *in vitro* or *in vivo* which showed there was immunosuppressive result of tobacco smoking on T and B-lymphocytes (Nagorni-Obradovic et al., 2006). Some studies have investigated correlation among cigarette smoking and irregularity of innate immune responses and confirmed there was positive affecting innate immune cells (Qiu et al., 2017).

DCs are necessary for controlling immunity and tolerance balance. Their effectiveness depends on a number of factors, including their number, subtype, and maturation status, as well as environmental signals and basic regulators (Audiger et al., 2017; Qian & Cao, 2018). Therefore, we designed to determine smoking effects on DCs number, their subtypes as mDC or pDCs, and the state of maturity in addition to the production of important cytokines.

Smoking causes recruitment of immune cells into the lunges and increase immune activation. This sustained inflammation is an important mechanism for the development of many disorders, including cancer of many organs, coronary artery disease, and respiratory infections such as COPD (Mehta et al., 2008). mDCs are considered to be as important a subtype as APCs while pDCs are the subtype responsible for tolerogenic response activation through the regulation of Treg cells (Martin et al., 2002; Ouabed et al., 2008; Wei et al., 2005). Our data showed a highly significant increase in absolute pDCs number in general smokers and more specifically in the male population. Our data is consistent with the study done by Chen, who recorded a significant increase in the absolute number of total DCs and pDCs in smokers comparing with control (Chen et al., 2013).

pDCs are well known for their high secretion of cytokines mainly IFN- $\alpha$  in response to virus infections that activate other immune cells including macrophages and T cells. In addition, pDCs are a very important regulator of tolerogenic response through the activation of Treg cells (Macri et al. 2017; Ouabed et al., 2008). Some studies have indicated an increase in the number of pDCs in samples of smokers diseases such as asthma and heart disease (Matsuda et al., 2002; McKenna et al., 2005).

The mDC/pDC ratio can be used to detect the possibility of developing certain diseases. This analysis may provide new treatment for patients with such disorders (Fukunaga et al., 2009). mDCs to pDCs were decreased among smokers in general and among male smokers in particular when compared with controls. When analyzing the absolute numbers of both subtypes, the absolute number of pDCs among smokers in general and male smokers in particular was higher than that of mDCs, and decline of mDC/pDC. These results agree with the findings from Chen et al.'s study, which also showed a significant decrease for the mDC/pDC as a result of an increase in pDCs. This points clear that, smoking in changing the balance and functionality of DC subtypes (Chen et al., 2013).

The main pDCs function is identify pathogen-associated molecular patterns via two types of Toll-like receptor (TLR) expressed on their surfaces; TLR7 for virus pathogens recognition and TLR9 for bacterial pathogens recognition. pDCs respond to infection by secreting large quantities of important inflammatory cytokines including IFN-type I, IL-6, and TNF- $\alpha$  (Rogers et al., 2013), thus

playing a role in the stimulation of T cells, differentiation of B cells (Shaw et al., 2010), activation of cDCs, and regulation of the T cell-mediated immune response against chronic infections (Cervantes-Barragan et al., 2012). On the other hand, some studies indicate the effect of pDCs on tolerance and their role in the suppression of the immune system. This is due to their limited ability to process endocytosis, as well as low levels of MHC class II and co-stimulatory molecules expressed which are necessary for antigen processing, compared to mDCs (Rogers et al., 2013). pDCs are shown to activate Treg cells that are important to developing tolerance and suppressing immunity in the event of increased activity required to eliminate the pathogen (Rogers et al., 2013) and in some types of cancer development (Johnson & Ohashi, 2013).

For that, pDCs have two opposite functions, one of which is to activate the immune system while the other is to support tolerance development and immune system suppression. The local microenvironment that affects and controls development of pDCs is an important factor governing their ultimate activity (Chistiakov, Orekhov, Sobenin, & Bobryshev, 2014). Some studies have shown that pDCs are involved in the immune response in several autoimmune pro-inflammatory diseases—for example, multiple sclerosis, inflammatory bowel disease, psoriasis, and systemic lupus erythematosus—confirming that their tolerance induction importance is more favorable (von Glehn et al., 2012). Our results are consistent with the study done by Chen, where they indicated that smoking increases the number of pDCs in healthy smokers and could stimulate Treg cells and thus the development of multiple diseases, including cancer (Chen et al., 2013).

When we compared male smokers to female smokers, in the same constant, we found the same results, as the absolute number of pDCs was highly significant in males compared to females. Therefore, the ratio of mDCs to pDCs decreased in male smokers. This may indicate that sex is an important factor that may affect the possible alterations associated with the effect of smoking on the immune system (Allen et al., 2014; Benson et al., 2010). The difference between males and females could be due to a number of reasons, such as hormones, number of packs smoked, and types of cigarettes smoked (Duskova et al., 2012).

## 5. CONCLUSION

This study showed that cigarette smoking leads to an increase in the number of DCs and specifically pDCs. This led to an imbalance in the mDC/pDC ratio and may lead to increased activation of pDCs compared with nonsmokers; as a result, a suppression of the immune system and thus an increased risk of cancer and infections could be a possible scenario. The study had a number of limitations, including the sample size that should be taken into account for future studies intended to confirm the results. Also, female smokers were hard to convince to participate in the study. Future studies could focus on different regions of the Kingdom, include a higher number of participants of both genders, and increase the age scale. In our study, the focus was on the young generation as we were interested in detecting the effect of cigarette smoking in the early stages; but the study could also be extended to the older generation who develop mostly chronic diseases. Our study could be the first step for future, larger studies on the level of the whole Kingdom and in cooperation with different universities, hospitals, and research centers. In addition, we need future studies to confirm that pDCs inhibit the immune system in smokers through analysis of Treg cells and the capacity of pDCs in the endocytosis process. We need future studies to explain the effect of sex on changes related to DC subtypes and cytokines in smokers as well.

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### Author Contributions

This work was carried out in collaboration of all authors. All authors read and approved final manuscript.

### Funding:

This study has not received any external funding.

### Conflicts of Interest

The authors declare that there are no conflicts of interests.

### Ethical clearance

The study was approved by the Medical Ethics Committee of King Abdulaziz University (ethical approval code: 02-CEGMR-Bioeth-

2019).

#### Data and materials availability:

All data associated with this study are present in the paper.

**Peer-review:** External peer-review was done through double-blind method.

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