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Influence of cigarette smoking on immune response of PBMCs to inactivated bacteria and on wound healing

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Content

Index of tables and figures

List of tables

List of figures

Abbreviation

AP-1	activated protein 1
CAP	community acquired pneumonia
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CSE	cigarette smoke extract
CSIF	cytokine synthesis inhibitory factor
CTL	cytotoxicity T lymphocytes
E. f.	*Enterococcus Faecali*
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GFs	growth factors
GM-CSF	granulocyte/macrophage colony-stimulating factor
HmGB1	high-mobility group box 1 protein
HSP70	heat-shock protein 70
IFN	Interferone
IL	Interleukin
IRAK-1	IL-1R-associated kinase-1
JAK-STAT	Janus kinases, signal transducer and activator of transcription
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRSA	Methicillin-resistant Staphylococcus aureus
MSC	mesenchymal stem cells
MSSA	methicillin-sensitive Staphylococcus aureus
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor κB
NK	natural killer

P. a.	*Pseudomonas Aeruginosa*
PAMPs	pathogen-associated molecular patterns
PBMCs	Peripheral Blood Mononuclear Cells
RAGE	receptor for advanced glycation end-products
ROS	reactive oxidizing species
S. a.	*Staphylococcus Aureus*
SD	*Standard deviation*
S. e.	*Staphylococcus Epidermidis*
SEB	staphylococcal enterotoxin B
SEM	Standard error of the mean
SRB	Sulforhodamine B
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	Thymic Stromal Lymphopoietin
VEGF	vascular epidermal growth factor

1. Introduction

1.1. Smoking-related epidemiology

Smoking is the most preventable cause of mortality worldwide and is associated with numerous diseases, mainly chronic obstructive pulmonary disease (COPD), coronary heart disease, infection, cancer, stroke, gastroduodenal ulcers and peripheral vascular disease (Fagerström, 2002, Perez-Warnisher *et al*., 2019, Shaik *et al*., 2016, Ahluwalia *et al*., 2018). Meanwhile, there are still many non-smokers who are insidiously and passively exposed to second-hand smoke that could also cause various health problems (Tsai *et al*., 2018). The average life expectancy of smokers is at least 10 years shorter than that of non-smokers (Kalkhoran *et al*., 2018, Jamrozik, 2004, West, 2017, Lee *et al*., 2012, Jha *et al*., 2013). Furthermore, approximately 6 million people die from smoking, many of which are youngsters (Fagerström, 2002, Edwards, 2004, Perez-Warnisher *et al*., 2019, Shaik *et al*., 2016, West, 2017, Lee *et al*., 2012, Davis *et al*., 2007, Zarocostas, 2009). Contemporary forecasts imply that the number of smokers worldwide will reach up to 1.6 billion people in the next 25 years, which means an increase in the number of deaths due to smoking (Perez-Warnisher *et al*., 2019). As a result, the number of deaths due to smoking will exceed the sum of deaths caused by other diseases such as tuberculosis, maternal deaths, automobile accidents, AIDS, suicide and homicide (Jamal *et al*., 2015). However, in that period, developing countries will contribute nearly 70% of smoking deaths (Edwards, 2004, Jamrozik, 2004).

Nowadays, there is a declining trend in developed countries, while smoking rates in developing countries are rising (Galanti, 2008, Kalkhoran *et al*., 2018, Jha *et al*., 2006). Actually, smoking rates squint towards people with the lowest levels of income and education and concomitant mental disorder or substance abuse disorder (Kalkhoran *et al*., 2018, Perez-Warnisher *et al*., 2019). A total of 80% of the world's smokers are from middle-income countries (Health and Services, 2004, Control and Prevention, 1993). About 23% of adults globally, including more than 1 billion men and 250 million women, consume tobacco products today (Perez-Warnisher *et al*., 2019). Generally speaking, mortality in smokers is three to four times higher than in non-smokers for both males and females (Perez-Warnisher *et al*., 2018). In the region of the Western Pacific region, the daily smoking rate of males is fifteen times higher than in females, which is the largest

gender difference compared to the other places (Figure 1). However, as more and more women smoke, the difference in gender among these smokers will become smaller and smaller (Perez-Warnisher *et al*., 2019).

Cigarette smoking is so prevalent that it has created a heavy economic burden on society. The cost of medical care associated with smoking in the United States alone is as high as $170 billion. The early death and productivity decline caused by smoking directly led to a loss of $156 billion (Do and Maes, 2016, Galanti, 2008). In addition, the economic burden of smoking has already reached 130 billion euros in Europe since 2000 (Galanti, 2008), within which the cost has been estimated to range from £2.7 billion to £5.2 billion in the UK (Ekpu and Brown, 2015).

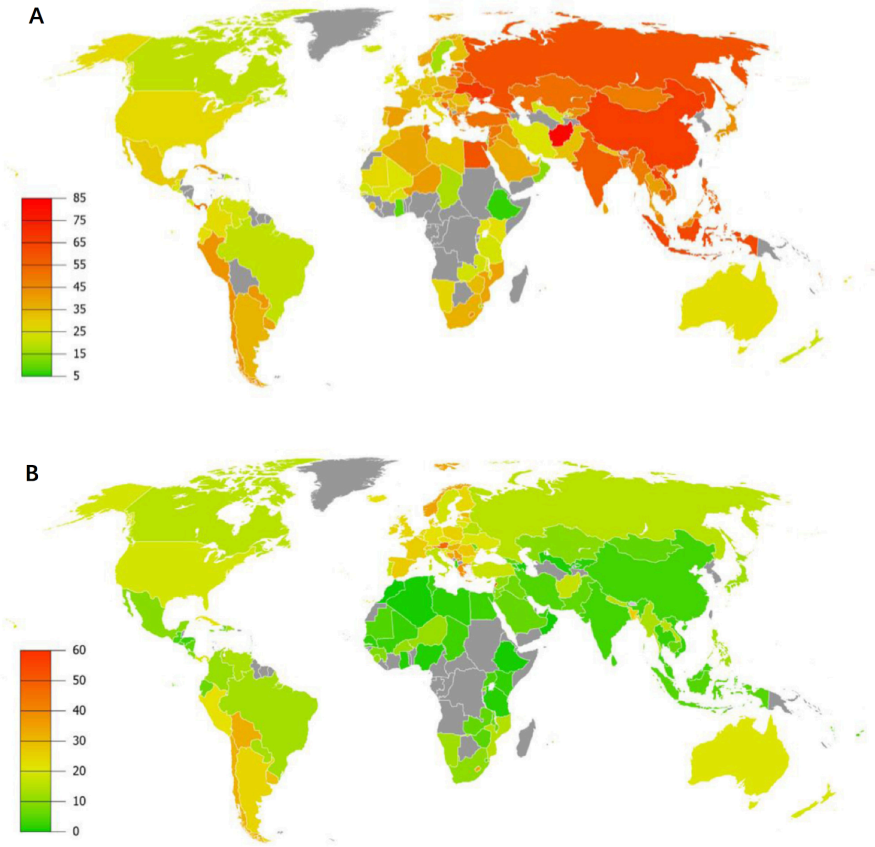


Figure 1. The prevalence of smoking is largely related to gender and geography.

Smoking distribution worldwide: A. Men smoking distribution. B. Women smoking distribution. The countries with the highest male smoking rates are mainly middle-income

countries, while the countries with the highest female smoking rates are mainly high-income countries [quoted from (Perez-Warnisher et al., 2019)].

1.2. Constituents of combustible tobacco products

Combustion is a critical procedure in producing the reactive oxygen species (ROS). As combustion products, cigarette smoke has at least 6,500 chemicals in its solid and gaseous phases including more than 70 known carcinogens that are created by burning at least 600 components in raw cigarettes (Stämpfli and Anderson, 2009, Talhout *et al*., 2011, Smith and Hansch, 2000, Hatsukami *et al.,* 2008). The products after burning tobacco can be divided into gas and solid microparticles (Figure. 2) (Lee *et al*., 2012). The toxic components of cigarette smoke are mainly found in solid micro-particles (Witschi, 2004, Lee *et al*., 2012), containing toxins (*e.g.;* acetone, carbon monoxide, ammonia, nicotine, hydroquinone, *etc.*); carcinogens (*e.g*.; methylcholanthrene, benzo-α-pyrenes, *etc.*); and ROS (*e.g.;* nitrogen oxides, superoxide *etc*.) (Stämpfli and Anderson, 2009). Solid microparticles in the smoke can directly activate macrophages and epithelial cells in the lungs and jeopardize proteins, lipids, DNA, intracellular matrices and organelles, thus producing pro-coagulant and pro-inflammatory effects (Valavanidis *et al*., 2009). However, cigarettes could also possess immunosuppressive and anti-inflammatory features that can alter the transcription process (Modestou *et al*., 2010). What's more, this condensate itself could indirectly produce secondary oxidation partitions and DNA adducts by inducing nitric oxide synthase and oxidative bursts within the body (Rahman *et al*., 2002). When smoke enters the body, it will cause oxidation, nitrosylation, acetylation and hydroxylation of the extracellular matrix, as well as interference with signal transduction, which will have a negative impact on cell activation and differentiation (Stämpfli and Anderson, 2009, Fowles and Dybing, 2003).

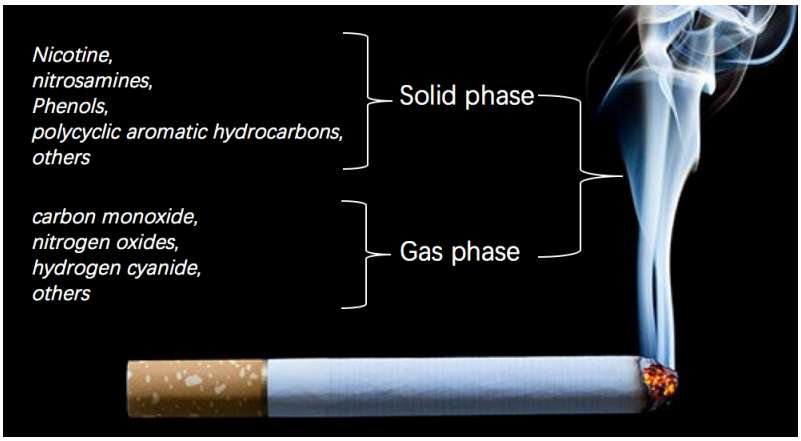


Figure 2. After burning, thousands of chemicals are produced from a cigarette.
Nicotine, nitrosamines, polycyclic aromatic hydrocarbons and phenols are submicron solid particles, while other ingredients, such as carbon monoxide, nitrogen oxides and hydrogen cyanide, are gases (Perez-Warnisher et al., 2019). Moreover, most of these chemicals are toxic, while some damage the immune system, some cause cancer, and some are addictive (Lee et al., 2012)

1.3. Smoking-related diseases

1.3.1. Smoking and COPD

Currently, the three main non-infectious causes of preventable death are COPD, cardiovascular disease, and lung cancer, all of which could be caused by smoking (Office of the Surgeon *et al*., 2004). Because of its high morbidity and mortality, COPD is a major public health issue that remains a challenge for clinicians in the 21st century (Fletcher and Peto, 1977). Exposure to cigarette smoke is a major risk factor for COPD, whether it is active or passive smoking, as passive or active exposure to CS leads to rapid dissolution and systemic uptake of toxins in the fluid of the oral/airway epithelial lining (Lee *et al*., 2012). Besides, contact with indoor and outdoor air pollution and industrial dust and chemicals are other risk factors (Scanlon *et al*., 2000). Epidemiology has indicated that smokers with lung cancer or COPD are easy to combine with other diseases, which suggests that they have the same munity determinants (including oxidative stress and disorders of the inflammatory response) (Alberg *et al*., 2005). It is logical to conclude that the immunological mechanisms related to COPD as discussed above (munity determinants) are more or less the cancer determinants. As exhaustion of associated B

cells and alveolar macrophages, inflammatory responses are an important shared determinant for the development of lung tumors. On balance, COPD is the fifth leading cause of years of loss due to early death or disability globally and the third leading cause of death just after heart disease and cancer (Mathers and Loncar, 2006, Association, 2013).

1.3.2. Smoking and cardiovascular disease

Smoking also has a significant impact on the morbidity and mortality of cardiovascular disease. According to epidemiological studies, smoking increases the incidence of fatal coronary artery disease and myocardial infarction, regardless of gender (Health and Services, 2004, Jha *et al*., 2006, Health and Services, 2014). A total of 20% of all deaths from ischemic heart disease and 33% of all cardiovascular deaths globally are caused by smoking (Perez-Warnisher *et al*., 2019). There are also causal relationships and possible synergistic interactions between smoking and other major risk factors for coronary heart disease, such as hypertension, hyperlipidemia, and hyperglycemia (Benowitz, 2003, Perez-Warnisher *et al*., 2019). It is worth noting that non-smokers exposed to passive smoking at home or at work are also 25-30% more likely to have heart disease (Peters *et al*., 2013, Rea *et al*., 2002, Huxley and Woodward, 2011).

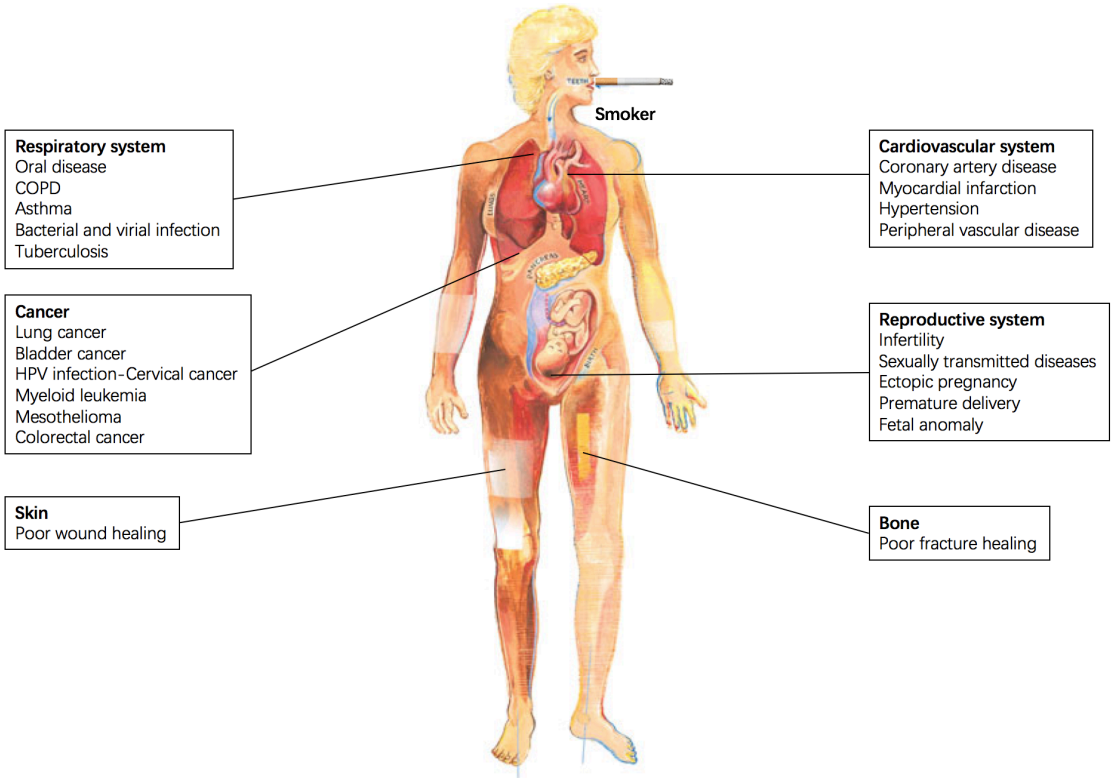


Figure 3 Smoking-related problems

Cigarette smoke has a negative impact on all aspects of human health. Cardiovascular disease, respiratory diseases, and cancer are the three leading causes of smoking-associated deaths. There are also other smoking-related health risks such as diabetes, weakened immune resistance, decreased fertility in males and females, wound healing disorders, and sexually transmitted diseases (Stämpfli and Anderson, 2009).

1.3.3. Smoking and asthma

The double function of smoking as an irritant and inhibitor is obviously performed in asthma. Atopy which is one of the important nonspecific risk factors for allergy can be suppressed by smoking, whereas the serum level of IgE is contradictorily raised by smoking. Smoking could significantly deteriorate the severity of the consequences of asthma via decreasing the sensitivity to corticosteroids (Chaudhuri *et al*., 2003), reducing lung function and directly damaging to the immune system. However, some inflammatory mediators are reduced in smokers with asthma, compared to non-smokers with asthma. In one study (Chalmers *et al*., 2001), the number eosinophilia in the sputum from asthmatic non-smokers, which is similar to the eosinophilia induced by antigens in the airway (Thatcher *et al*., 2008), was higher than in the sputum from asthmatic smokers (Robbins *et al*., 2005). As noted above, the deteriorating clinical manifestations of asthmatic smokers including impaired ability to clear pathogens and decreased steroid sensitivity, are closely related to smoking-induced macrophage dysfunction (Hodge *et al*., 2007). In addition, asthmatic smokers may have irreversible airflow restrictions similar to COPD, and the asthma symptoms can be exacerbated by the increased risk of infection in smokers. Of particular note, epidemiology suggests that smoking in the womb and perinatal period could increase the risk of asthma in newborns (Bouzigon *et al*., 2008).

1.3.4. Smoking and cancer

Lung cancer is a well-known negative result of cigarette smoking, which is mutagenic. About 85% of patients with lung cancer are associated with smoking (Walser *et al*., 2008). Although lung cancer has been previously thought to have occurred mainly in men, the incidence of women's lung cancer has increased by 6 times since the 1950s (Egleston *et al*., 2009). After the late 1980s, the incidence of women's lung cancer in has surpassed that of breast cancer, ranking first in cancer deaths among women (Patel, 2005, General,

2001). People who start smoking at a young age are at greater risk of developing lung cancer, which is also closely related to cumulative lifetime smoking exposure (Health and Services, 2014, Jamal *et al*., 2015, Stanley, 1986). Furthermore, the carcinogenic impact of smoking is not confined to the lungs. Smoking could produce circulating carcinogens in the body that increase the incidence of other malignancies. Tobacco is connected with 13 different kinds of other cancers such as liver, colorectal, pancreas and breast (Patel and Steinberg, 2016, Control and Prevention, 1993, Freedman *et al*., 2011). Worryingly, epidemiology suggests that exposure to cigarette smoke ingredient in the womb or perinatal period could increase the lifetime risk of tumors (Ng *et al*., 2006). Smoking may also reduce the activity of cytotoxic T cells which may take part in the process of tumor metastasis and recurrence after treatment (Ng *et al*., 2006).

Some tumor formation processes, such as increased lymphogenesis and angiogenesis, weakened ability of macrophages to eliminate dying or transformed cells efficiently, release of the regional growth factor and its own receptor abduction, and increased gene mutation (including translocations, truncations and deletions), could be promoted by related inflammation (Stämpfli and Anderson, 2009). Evidence also suggests that bacterial inflammatory burden is increased in the lung, while in the meantime, lung intraepithelial neoplasia develops (Ji *et al*., 2006). These observations may provide a link between host defense defects, increased inflammation, and increased cancer risk. The promotion of inflammation and deficiency of host immune defense could contribute to the high morbidity of tumors. For example, the pro-inflammatory nuclear factor κB (NF-κB) facilitates the growth and survival of tumors (Stämpfli and Anderson, 2009). In addition, matrix metalloproteinase 9 (MMP9) secreted by macrophages, mast cells or neutrophils could decompose the extracellular matrix, which has a closely relationship with tumor invasion and metastasis (Alberg *et al*., 2005). It has been emphasized that the airway epithelial cells bridge the development of COPD and the tumor formation and play a key role between them. Cancerous mutated epithelial cells have enhanced pro-inflammatory responses, while they have a decreased ability to resist pathogens (Anderson and Bozinovski, 2003).

Even though many lung cancer patients have undergone tumor resection at an early stage, their chances of developing tumor metastasis still remain very high, because primary

malignant tumors often release tumor cells that enter the blood circulation with the help of a disordered inflammatory reaction, resulting in metastasis (Nagrath *et al*., 2007, Maheswaran *et al*., 2008). Smoking can significantly raise the incidence of metastatic lung cancer, which is linked to impaired immune surveillance resulting from chronical cigarette exposure and repeated microbial infection (Lu *et al*., 2007).

In addition, new research indicates that tobacco is responsible for age-related macular degeneration, adult onset diabetes, impaired immune system and increased susceptibility to tracheobronchial infections (Health and Services, 2014). Smoking can increase the mortality rate of colorectal cancer in women (Kenfield *et al*., 2008). New evidence suggests that ectopic pregnancy are associated with smoking (Jha *et al*., 2013, Patel and Steinberg, 2016).

1.4 Smoking and fracture and wound healing

1.4.1 Smoking and fracture healing

Smoking causes delayed fracture healing not only by directly affecting osteogenesis but also by indirectly inducing smoking-related diseases that worsen the bone growth environment (Sloan *et al*., 2010). The more effective the treatment of smoking-related diseases, the better the musculoskeletal effect (Capewell *et al*., 2009). Among patients with spinal fusion or facet joint fusion, smoking has adverse effects on fracture healing, and the incidence of pseudoarthritis in smokers (40%) is usually higher than in non-smokers (8%) (Brown *et al*., 1986). Non-smokers undergoing lumbar fusion surgery are more likely than smokers to successfully have vertebral fusion and return to work on time (Carpenter *et al*., 1996). There are several explanations for how smoking affects fracture healing, such as reduced blood supply to damaged sites, nicotine- induced attenuation of endothelial nitric oxide synthase function, low levels of antioxidants and vitamins, and high circulating ROS levels (Sloan *et al*., 2010). Other studies have shown that nicotine is toxic to calcitonin and osteoblasts (Gaston and Simpson, 2007). Cigarette smoke can affect bone health and accelerate femoral osteonecrosis and osteoporosis in males and females (Wright, 2006). Nevertheless, it was found that smoking women entered menopause two years earlier than female non-smokers and had a higher risk of osteoporosis (Baron, 1984). Because of increased accident rates, more work-related

damages, more car crashes, and increased bone vulnerability, smokers' fracture rates are generally higher (Wen *et al*., 2005).

1.4.2 Smoking and wound healing

Skin, the first line of defense for innate immunity, is a physical and chemical fence. Therefore, skin damage causes pathological microorganisms to enter into the body and leads to disorders of homeostasis (Takada *et al*., 2017). Although skin wounds usually heal very quickly, delayed wound healing often occurs in smokers, elderly people, infected patients, immunocompromised patients, and diabetic patients (Takada *et al*., 2017). Nicotine and related chemicals in tobacco could debilitate the regeneration of wound healing and soft tissue after fracture damage, thereby prolonging the healing time and reducing the quality of postoperative consequences (Pitts *et al.*, 1999). Fibroblasts, acute phase proteins, growth factors and mesenchymal stem cells (MSCs) are key moderators of wound healing and are in contact with circulating substance that exists in the bloodstream linked to smoking (Czernin and Waldherr, 2003). This adverse reaction is common in the skin of chronic smokers who suffer from loss of skin elasticity due to abnormal function of fibroblasts (Frances *et al*., 1991). Smoking cessation can not only improve their lung and cardiovascular function, but also optimize the healing process after surgery (Frances *et al*., 1991). MSCs and Fibroblasts are important generators of cytokines that help induce an initial inflammatory response and play an key role in the granulation tissue formation (Sorensen *et al*., 2002). For example, chemokines that are essential for attracting neutrophils and other white blood cells to the wound site are released by fibroblasts and MSCs (Wong and Martins-Green, 2004). The fibroblasts may also be differentiated into myofibroblasts that play a key role in wound closure and contraction. Furthermore, fibroblast-like progenitor cells separated from hematoma of the fracture can evolve into chondrocytes, osteoblasts and adipocytes in vitro (Oe *et al*., 2007). Smoking is supposed to affect and worsen the above-mentioned pathophysiological processes (Wong and Martins-Green, 2004, Gabbiani, 2003).

1.4.3 IL-4 and wound healing

The immune response to tissue damage plays an important role in the healing process of the skin, which determines the rate and outcome of healing (Julier *et al*., 2017). Furthermore, the role of cytokines in the wound healing is getting more and more

attention. However, the function of IL-4 within skin coalescence was reported contradictorily. For example, it was reported by Yang *et al.* that keratinocyte proliferation was promoted by IL-4 and was suppressed by anti-IL-4 antibodies in proportion to the dose. IL-4 stimulated the keratinocyte cycle to S phase from GO/G1 phase. The expression of c-myc which plays a very important role in the keratinocyte proliferation was induced by IL-4 (Yang *et al*., 1996). Junghans *et al.* reported that IL-4 enhances proliferation of keratinocytes and the expression of B7/BBl (Junghans *et al*., 1996). IL-4 treated keratinocytes demonstrated elevated ability to promote the proliferation of T-cells in the context of staphylococcal enterotoxin B (SEB). In addition, IL-4 was also reported to accelerate the healing of skin wounds in the mice and antagonists of IL-4 could delay the wound healing (Salmon-Ehr *et al*., 2000). Research completed by Elbe-BuÈrger *et al*. supported that IL-4 not only instigates the keratinocyte to propagate and motivates mast cells to reproduce, but also leads to deposition of collagen beneath the epidermal layer (Elbe-BuÈrger *et al*., 2002). However, other research showed that IL-4 resulted in the reduction of fibronectin leading to impaired keratinocyte wounding healing, which could be treated by local therapy with fibronectin (Serezani *et al*., 2017). This conclusion of IL-4 inhibiting the fibronectin was also endorsed by the viewpoint of IL-4 polarizing macrophages suppressing the fibrosis (Eming *et al*., 2017). Moreover, involucrin expression was reduced both in the skin of IL-4 transgenic mice in vivo and in HaCaT cells treated with IL-4 in vitro (Bao *et al*., 2016). The existence of IL-4 could also disturb the integrity of cell sheets by causing the keratinocyte HaCaT cells to express decreased levels of DSG1and DSC1, which encode the desmosomal cadherins in the keratinocytes (Omori-Miyake *et al*., 2014, Tsuchisaka *et al*., 2014).

1.5. Smoking and microbial infection

Smoking is one of the main causes of systemic infections, especially respiratory infections, whether active or passive smoking (Arcavi and Benowitz, 2004). The incidence of diseases such as community acquired pneumonia (CAP), tuberculosis, invasive pneumococcal disease, postoperative mediastinal influenza, meningococcal disease (Coen *et al*., 2006, Murray *et al*., 2012), periodontitis, and surgical wound infections is increased in smokers (Steingrimsson *et al.*, 2008, Cayci *et al.*, 2008, Bates

et al., 2007, Bergstrom, 2004, Nuorti *et al.*, 2000). The attributable risk ratios of smoking for the mortality of both pneumonia and influenza were 2.0 higher in males and 2.2 in females (Huttunen *et al*., 2011). A total of 6% of deaths from influenza and pneumonia are caused by smoking, which is more than deaths from other diseases caused by smoking, such as digestive system neoplasms (Correa *et al*., 2009, Huttunen *et al*., 2011). Studies have shown that smoke has a destructive effect on respiratory epithelial cells and mucosal immune systems. Smoke can promote the adhesion of bacteria on the surface of airway epithelial cells, impair the function of monocytes and macrophages, inhibit the production of immunoglobulins, destroy the ciliary activity of upper respiratory epithelial cells, and thus increase respiratory infections(Seaton *et al*., 2000, Dye and Adler, 1994). Parents smoking or other family members smoking can cause bronchitis, bronchiolitis or lower respiratory tract infection in newborns(Jones *et al*., 2011). Active and passive smoking is linked to primary tuberculosis, active tuberculosis, and risk of recurrent tuberculosis, while over 20% of global tuberculosis mortality is due to tobacco (Perez-Warnisher *et al*., 2019, Organization, 2007).

Smoking could aggravate the intensity of the pro-inflammatory response to pathogens without the reduction of pathogens-specific memory or pathogens clearance (Gualano *et al*., 2008). Therefore, the elementary immune protection has been compromised by smoking. However, the secondary inflammatory processes have remained unaffected (Robbins *et al*., 2006). Smoking could augment the numbers of goblet cells, decrease mucosal ciliary mobility and induce the hypersecretion, which reduces the removal of pathogens and increases their colonization (Patel *et al.*, 2002). The high level of inflammatory response is linked to the raised yield of pro-inflammatory mediators resulting from smoking (Stämpfli and Anderson, 2009). In addition, a study suggested that the intense inflammation resulted in stepped-up airway fibrosis and emphysema, giving evidence that the pathogenesis of emphysema could be on account of the changed response to pathogens (Kang *et al*., 2008). Likewise, inflammation response was found to be aggravated in smokers with the presence of *Haemophilus influenza* and *Pseudomonas aeruginosa,* which are linked to the deterioration of COPD (Gaschler *et al., 2009)*.

1.6. Four types of bacteria

1.6.1 Staphylococcus aureus

Staphylococcus aureus (*S. a.*) is a Gram-positive round bacterium that is commonly found in the upper respiratory tract and skin (Masalha *et al.*, 2001). *S. a.* can be of a "parasitic" nature, living in 30% of the normal population without being harmful to the host (Akmatov *et al.*, 2014, Rao *et al.*, 2015). As a main cause of community-associated and hospital-associated microbial infection (David and Daum, 2010), *S. a.* is often associated with severe pneumonia, suppurative osteomyelitis, infective endocarditis, bacteremia and sepsis. *Methicillin-resistant Staphylococcus aureus* (*MRSA*) and its resistance to multiple drugs is increasing, making infection control very tricky (Junie *et al.*, 2014). Hospital-associated-MRSA often infects individuals who have associated risk factors such as being in a post-operation, ventilator use, and prolonged hospitalization. However, those who do not have these risks are often infected with *methicillin sensitive Staphylococcus aureus* (*MSSA*) or community-associated-*MRSA* (Tokajian, 2014).

1.6.2. Staphylococcus epidermidis

Staphylococcus epidermidis (*S. e.*) is a Gram-positive and facultative anaerobic bacterium. It is also a part of the normal flora in human beings and is usually found on the skin (Fey and Olson, 2010). Although *S. e.* does not have pathogenicity under normal conditions, once the body's immunity declines, it is likely to cause infection. This infection usually occurs during hospitalization, especially happening to postoperative patients with implants such as catheters, tracheal tubes and chest tubes (Levinson, 2008, Natsis and Cohen, 2018). Actually, *S. e.* is now considered to be the most common cause of hospital sepsis (Nguyen *et al.*, 2017). *S. e.* can form biofilms that grow on those devices, which makes it very difficult for antibacterial drugs to be effect (Wilson *et al*., 2011, Xie *et al.*, 2019). This biofilm prevents immune responses by creating a physical barrier while producing anti-inflammatory effects to a certain extent (Spiliopoulou *et al.*, 2012). *S. e.* has also been reported as a pathogenic bacteria for skin and soft tissue infections (Akiyama *et al*., 1998, Mustafa *et al*., 2016).

1.6.3. Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. a.*) is a Gram-negative common rod-shaped bacterium with an encapsulated structure. It is an opportunistic pathogen that can bring about fatal

hospital-acquired infections, such as septic syndromes, ventilator-associated pneumonia and wound non-healing, when the immunity of humans is impaired (Ciszek-Lenda *et al.*, 2019, Lore *et al*., 2012, Watters *et al*., 2013). Interestingly, *P. a.* could increase the infection burden in smokers. However, when stimulated by *Haemophilus influenza*, the infection burden associated with exposure to *P. a.* is great (Drannik *et al*., 2004). *P. a.* which is the most common cause of chronic pneumonia is also the leading cause of the hereditary cystic fibrosis (Waters and Goldberg, 2019, Cigana *et al*., 2009). *P. a.* is also capable of forming biofilms; as a consequence, it is highly pathogenic and resistant to multiple antibiotic treatments (Alhede *et al*., 2014). Bacterial infections with biofilms are often dominated by chronic inflammation. Although the body has a large amount of neutrophil infiltration, these neutrophils destroy the body's immune system rather than the bacteria itself (Roilides *et al*., 2015, Marcinkiewicz *et al*., 2013). There are two explanations for the pathogenicity of bacterial biofilms: one is that the bacteria hide in the biofilm's matrix, avoiding contact with immune cells and antibodies; another is that the bactericidal effect of macrophages entering the biofilm's matrix is weakened (Hirschfeld, 2014).

1.6.4. Enterococcus faecalis

Enterococcus faecalis (E. f.), is a Gram-positive opportunistic pathogen that has come to existence as a leading cause of hospital-associated infections globally (Lossouarn *et al*., 2019, Lebreton *et al*., 2014). Normally, it belongs to the normal flora of the gastrointestinal tract as early as infancy (Gilmore *et al*., 2014) but can cause hospital-acquired pneumonia, gastrointestinal infections, urinary tract infections (30%), reproductive system infections, endocarditis, peritonitis (Agudelo Higuita and Huycke, 2014), bacteraemia (10%) and wound non-healing (70%) when the body's immunity is weakened (Tien et al., 2017, Wisplinghoff *et al*., 2004, Maki and Tambyah, 2001, Hidron *et al*., 2008, Gjødsbøl *et al*., 2006). Biofilm formation, avoidance of immune cell attack, and long-term survival in neutrophils and macrophages, allow *E. f.* to successfully colonize in the host (Baldassarri *et al*., 2005, Zou and Shankar, 2016, Zou and Shankar, 2014). *E. f.* can be found in 30%-90% of re-infected root canal treated teeth (Molander *et al*., 1998). Plasmid-encoded haemolysin of *E. f.*, known as cytolysin, is critical for the invasion pathogenesis in animal models, and its concomitant existence with high levels

of gentamicin resistance could increase the risk of death in patients with bacteremia by five times (Chow *et al*., 1993). Moreover, plasmid-encoded adhesion, known as aggregating substance, could augment its virulence in the infectious process (Hirt *et al*., 2002).

1.7. Smoking induces inflammation while suppressing immune function at the same time

Toxic substances in smoke and oxidative activators produced by combustion cannot be filtered out by cigarette holders (Huang MF, 2005). The particulate and gaseous components of cigarette smoking first interact with the immune system of the oral, nasal and tracheal mucosal surfaces (Lee *et al*., 2012) (Figure 4). Furthermore, the semiquinone radicals that exists in the particulate phase could continuously produce the ROS. Then, ROS induces DNA damage by sensitizing the oxidative cellular pathways and triggering peroxidation of lipids or other components of the airway epithelial cell membrane (Valavanidis *et al*., 2009, Kim *et al*., 2004). The ROS also induces the activation of inflammatory genes, by initiating the signaling cascades (Chung, 2005, Churg *et al*., 2002). The production of these inflammatory mediators can gear up the recruitment of chronic immune cells. The effects of smoking on the body's inflammatory system are not only stimulatory, but also inhibitory. The subsequent adaptive T lymphocytes can be classified as three T helper cells: Th1, Th2 and Th17, which have different priorities to activate the transcriptional factors to express special cytokines (Zhou *et al*., 2009). For instance, Th1 cells produce IFN-γ, which is a pro-inflammatory cytokine, whereas Th2 cells produce IL-4, which is an anti-inflammatory cytokine (Zhou et al., 2009). Smoking could weaken the function of Th1 cells while facilitating the response ability of Th2 cells, thus suppressing the development of Th1 inflammation and enhancing the Th2 responses (Nakamura *et al*., 2008). When stimulated by bacterial lipopolysaccharide (LPS), the reactive ability of dendritic cells that exposed to smoking was reduced, and was accompanied by the decreased secretion of IL-12 (Th1 cytokine) and IL-23 (Th17 cytokine) (Vassallo *et al*., 2005). Smoking could enhance the Th2 immune responses, such as eosinophilic recruitment in the airways (Phaybouth *et al*., 2006). Although there is no definitive evidence that smoking promotes the Th17 polarization immune response,

it has been reported that long-term chronic exposure to smoke can contribute to Th17 involvement in autoimmune responses (Shan *et al.*, 2009, Heliövaara *et al.*, 1993). Smoking can aggravate the extent of influenza virus and bacterial infection, and could also increase the prevalence of tuberculosis (Kolappan and Gopi, 2002, Nuorti *et al.*, 2000, Kark *et al.*, 1982). The underlying mechanisms include the regulation of signal transduction of airway epithelial cells and immune cells and suppression of congenital and adaptive immune responses (Gualano *et al.*, 2008).

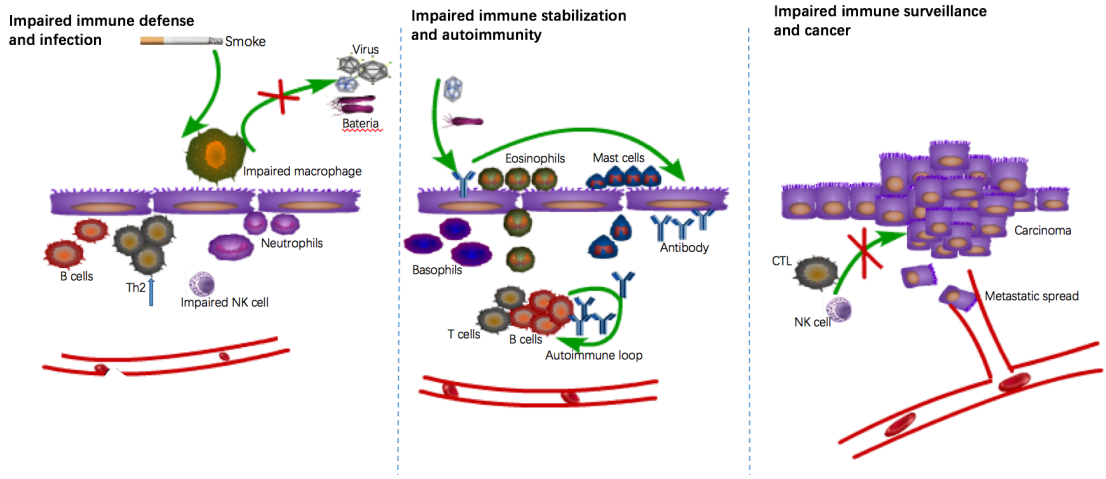


Figure 4. Sketch of respiratory immune defense, immune stabilization and immune surveillance impaired by smoking [modified according to (Stämpfli and Anderson, 2009)]. *Smoking attracts inflammatory cells to the lung parenchyma from the microcirculation, which sequentially affects epithelial cells and macrophages. While smoking promotes inflammation, congenital defenses, including NK cells, macrophages, dendritic cells, epithelial cells, are also damaged, which increases the risk, severity and duration of infection. Impaired immune stabilization was demonstrated in the following aspects: reduced ability to clear dead cells, increased abduction of IL-17-producing effector T cells and remains of amplified $CD8^+$ T cells, increased autoantibodies and related autoimmune loops produced by lymphoid gathering B cells and T cells together. Smoking induces tumor-associated macrophages to produce more inflammatory proteases, which facilitate the tumor metastasis by degenerating the extracellular matrix, destroying the tight link between the cells. Therefore, immune surveillance has been compromised (Stämpfli and Anderson, 2009).*

Smoking modulates the development of a Th2 allergy status by activating sensitizing factors. Smoking causes airway smooth muscle cells and epithelial cells to produce Thymic Stromal Lymphopoietin (TSLP), which induces dendritic cells to increase the Th2 polarization (Smelter *et al*., 2010). Investigation of single nucleotide polymorphisms in the TSLP gene uncovered the gene variants that were more vulnerable to inducement as a result of elevated attraction for AP-1 transcription factor binding (Harada *et al*., 2009). In addition, some TSLP gene polymorphisms would cause bronchial epithelial cells to produce more TSLP when influenza virus infections were present. TSLP activation is important mechanism for smoking to increase a permissive effect on hypersensitive inflammation in the respiratory tract. Smoking could decrease the production of IL-12 and IL23, while increasing the secretion of IL-8 and IL-10, in dendritic cells stimulated by CD40 ligand or LPS (Kroening *et al*., 2008). It has been shown that the ability of dendritic cells that were incubated with smoking to stimulate T cells was reduced (Mortaz *et al*., 2009). These consequences, intermediated by diversified inflammatory signaling factors, are induced by various smoking components, such as nicotine, ROS and other chemicals (Vassallo *et al*., 2008). It indicates that exposure to smoking in mice reduces the activation of dendritic cells in the lung, which decreases their ability to activate antigen-specific T cells propagation and to promote Th1 polarizing (Robbins *et al*., 2008). However, the ability of dendritic cells to move to the lymph nodes remains and is possibly even augmented (Robays et al., 2009). Except for dendritic cells, both B cells and macrophages have antigen presenting cell functions (Lee et al., 2012). The function of macrophages, which are increased and present in the sinuses and respiratory tract of smokers notwithstanding, is damaged by ROS in smoking (Kirkham *et al*., 2004). Smoking can reduce the ability of phagocytic cells to phagocytose bacteria and apoptotic cells, leading to the accumulation of debris of inflammatory cells and apoptotic cells and ultimately delayed wound healing (Kirkham *et al*., 2004). It is not clear whether smoking has an effect on the production of TNF-α in macrophages (Yang *et al*., 2006). For instance, one study (Pessina *et al*., 1993) reported that exposure to smoking could stimulate the macrophages to produce more TNF-α, whereas another study showed that the macrophages secreted less TNF-α when exposed to smoking (Gaschler *et al*., 2008).

Evidence (Mian *et al*., 2008) has revealed that NK cells secreted less TNF-α and IFN-γ and showed weaker cytotoxic effects in smokers than in nonsmokers.

1.8. Smoking related inflammatory cells

1.8.1. Airway epithelial cells

The respiratory ciliated columnar epithelium is the first line of defense against bacterial viruses. The overlying mucous gel layer can cleanse, phagocytose and kill pathogens, and the barrier function is obtained via tight junctions. However, smoking directly impairs the completeness of this barrier by increasing the permeability of the airway epithelial cells and compromising the clearing ability of mucus(Dye and Adler, 1994, Burns *et al*., 1989). Although smoking can stimulate the respiratory epithelium to produce pro-inflammatory factors, when there is a bacterial or viral infection, the pathogen-associated molecular patterns (PAMPs) (Pace *et al*., 2008) were attenuated, including LPS and pathogen double-stranded RNA which binds to TLR (Toll-like receptor) (Bauer *et al*., 2008). It has been confirmed that the epithelium can secrete significantly more inflammatory factors when incubated with pathogen double-stranded RNA and cigarette smoke extract (CSE). Although the inflammatory level is raised by the stimulus of CSE, the ability of this inflammatory status to kill the bacteria or viruses is decreased. One possible explanation is that smoking reduces the production of β-defensin-2 *(van der Toorn et al*., 2007). Besides, the β-defensin-2 was significantly decreased in the sputum and pharyngeal fluid of smokers with acute pneumonia, compared to nonsmokers acute pneumonia (Herr *et al*., 2009).

1.8.2. Alveolar macrophages

Smoking could augment the quantity of macrophages in pulmonary alveoli (Sopori, 2002) and stimulates them to generate more ROS, proteolytic enzymes, and pro-inflammatory mediators, further explaining the relationship between smoking and tissue damage and inflammation at the cellular level (Sopori, 2002, Russell *et al.*, 2002). Like airway epithelial cells, the ability of macrophages to swallow bacteria and dead cells is reduced by smoking, and so is the ability to respond to PAMPs (Chen *et al*., 2007, Drannik *et al*., 2004, Gaschler *et al.*, 2008). As a consequence, those necrotic or apoptotic cells that are not cleared can secrete related secondarily inflammatory chemicals, such as ligands of

the receptor for advanced glycation end-products (RAGE) and high-mobility group box 1 protein (HmGB1) (Liu *et al*., 2008). At the same time, related anti-inflammatory activities fail to function properly in this process, which is modified by TNF. From a mechanism perspective, it is imaginable that the production of a pro-inflammatory factors responsive to TLR activation is decreased due to the upregulated threshold that is changed by continuous stimulation of smoking. There is evidence that the smoking-associated pulmonary inflammation is prompted by the TLR4 agonist heat-shock protein (70HSP70), which induces the primary response gene 88 (MyD88) related signal route. It was reported that smoking could also trigger alveolar macrophages to display different inflammatory features with the stimulation of microbial infection, such as *Haemophilus influenza* (Gaschler *et al.*, 2009). Furthermore, with the presence of *Haemophilus influenza*, the smoking alveolar macrophages could produce significantly more CC-chemokine ligands, such as CCL2, CCL9 and CCL10, which are pro-inflammatory mediators, while secreting less TNF. The nature of this change may be one of the determining factors in the susceptibility to disease. There is an evidence that the alveolar macrophages in smokers have different conditions of activation, compared to the alveolar macrophages in non-smokers (Woodruff *et al*., 2005). Smoking may cause some M1-type macrophages to be inactivated, while some M2-type macrophages are activated (Woodruff *et al*., 2005). This role of smoking in the differentiation of macrophages has important significance for the development of the disease, as M1 alveolar macrophages are related to significant lung injury, such as emphysema, while M2 alveolar macrophages are associated with tumor development (Stämpfli and Anderson, 2009). The molecular mechanisms by which alveolar macrophage reactivity changes are not yet clear, but they can be repaired by exposure to the reduced state of glutathione, which means that the process involves oxidative stress (Woodruff *et al*., 2005). From subclinical inflammation to the outcome as cancer and COPD, in addition to the differential activation of alveolar macrophages, it is also related to the susceptibility of the body (Stämpfli and Anderson, 2009). The underlying mechanisms are not fully understood, but some host factors are suggested to increase the risk of developing related diseases. Host factors that include uncontrollable inflammatory responses, tendency to autoimmune diseases (Taraseviciene-Stewart *et al*., 2005), allergic constitution (Postma and Boezen, 2004), damaged oxidative defense,

connective tissue and matrix repair related genes mutations (Just *et al*., 2005), diminished detoxification enzyme (such as epoxide hydrolase and cytochrome p450 isozymes), blind elimination of effector cells leading to secondary necrosis (Hodge *et al.*, 2007), accelerated cellular aging process (Ito and Barnes, 2009), vascular repair defects (caused by decreased vascular endothelial growth factor) (Taraseviciene-Stewart *et al*., 2005), and mutated genes that control DNA damage response and DNA repair (Rangasamy *et al*., 2004), could contribute to the increased risk of smoking related diseases, such as emphysema, lung cancer. The risk of infection is further complicated by defects or polymorphisms in the host's congenital and acquired immune responses (Becker and O'Neill, 2007).

1.8.3. Natural killer (NK) cells

NK cells are important for the innate immune system both in fighting against microbial invasion and preventing tumors through releasing the granzymes, perforin and chemokine and pro-inflammatory cytokines (Swann *et al*., 2007). Some studies have suggested that smokers have a lower number and cytotoxic activity of NK cells than in nonsmokers, which associates NK cells dysfunction with infections and tumors (Tollerud *et al*., 1989, Mian *et al.*, 2008, Lu *et al*., 2006). Studies have also shown that cancer metastasis is inseparable from the weakening of immune surveillance by NK cells(Ng and Travis, 2008). Furthermore, the recurrence of pulmonary infection is also associated with functional defects in NK cells.

1.8.4. Dendritic cells

As important antigen presenting cells, Dendritic cells are essential to activation of immune reaction (Mellman and Steinman, 2001). Because they are directly in the alveolar space or under the lung epithelium, they are extremely susceptible to smoke (McComb *et al*., 2008). Dendritic cells play an important role in the modulation of the immune system, owing to their influence on both innate and adaptive immunity. Related research has shown that the chemokine CX_3CL1, which is induced by dendritic cells, is increased in emphysema. Mature dendritic cells are decreased in the airways of smokers with COPD, and after smokers quit smoking, their mature dendritic cells returned to the level of non-smokers (Jahnsen *et al*., 2006). By comparison, smokers with COPD had higher level of naïve dendritic cells than smokers without COPD or nonsmokers (McComb *et al.*, 2008).

In other words, the maturity condition and the quantity of dendritic cells are highly sensitive to smoking, which causes an immune response disorder. The underlying mechanism may be that the Th17 cytokine IL-23 and Th1 cytokine IL-12 have been downregulated (Robbins *et al*., 2008).

1.8.5. B cells

Autoimmunity plays an important role during the process from smoking to lung disease, within which the B cells are substantially present. Smoking can stimulate the related tissue to generate significantly more granulocyte-macrophage colony-stimulating factor (GM-CSF), which secondarily induces the dendritic cells to present the antigens and promote Th2 immune responses (Trimble *et al.*, 2009). There is an evidence that all the immunoglobulin serum levels, except IgE, can be reduced by smoking. It is reported that smoking weakens the ability of antibodies to neutralize the extraneous antigens, and smoking can induce lymphoid follicles in lungs to sensitize the B cells to induce allergic reaction and to increase their response to autoantigens, which damages the tissues (Trimble *et al*., 2009). Autoimmune attacks take part in the development of COPD or emphysema caused by smoking. Currently known autoantibodies in lungs involved in the process are elastin antibodies, epithelial cell antibodies and endothelial cell antibodies (Lee *et al*., 2007, Feghali-Bostwick *et al*., 2008). As many inflammatory circumstances contain autoantibodies, but without pathogenesis, their etiological role is debatable.

1.8.6. T cells

Notably, there are significantly more cytotoxicity T lymphocytes (CTL) in the lungs of smokers with COPD or emphysema, compared to nonsmokers or smokers without emphysema or COPD. Actually, the $CD8^+$ T cells are increased through exposure to smoking and could remain for at least 6 months after stopping smoking (Motz *et al*., 2008). Accordingly, the T cells could promote alveolar macrophages to secrete more MMP, which is an important elastase that degrades fibrin or matrix, leading to emphysema (Grumelli *et al.*, 2004). Moreover, $CD8^+$ T cells could also meditate the inflammatory response and induce the destruction of tissue (Maeno *et al*., 2007). The defensive ability of $CD8^+$ T cells is reduced by smoking, whereas the specific memory to fight against pathogens is preserved (Gualano *et al*., 2008). Smoking could reduce the TCR repertoire breadth in lungs and continuously increase the susceptibility to the pathogens.

Interestingly, smoking activates the Th17 cells, while suppressing the regulatory T cells during the development of smoking related diseases (Harrison *et al*., 2008, Barcelo *et al.*, 2008).

1.9. Smoking related signal transduction pathways

Several important singling pathways have been interrupted by smoking, such as NF-κB, mitogen-activated protein kinases (MAPK), activated protein 1 (AP-1), and Janus kinases/signal transducer and activator of transcription (JAK-STAT) (Kroening *et al*., 2008). These pathways are involved in the inflammatory cells activation, regulation, and proliferation (Samet and Wipfli, 2010). Smoking influenced stimulation of the transcription factors of AP-1 and NF-κB is associated with changed responsiveness to acute invasion of pathogens, modulation of inflammatory chemokine production, changed adjustment of cell death, and changed resistance to corticosteroids (Modestou *et al*., 2010). It is also reported that NF-κB, a promoter for the expression of many inflammatory factor, which is essential for preventing cell death, can be activated by smoking, and the anti-apoptotic factors can be up-regulated by smoking (Liu *et al*., 2008). Smoking elevates the activity of Ras protein (cellular signal transduction) within the epithelial cells, which is reliant, at least in part, on the activation of the RAGE (Reynolds *et al*., 2011). It has been reported that increased Ras activity is a hallmark of lung cancer derived from epithelial cells and an important cellular juncture for the activation of RAGE induced by smoking, which can ultimately lead to NF-κB activation that is the downstream of RAGE (Bos, 1989). Furthermore, apart from directly activating NF-κB, smoking also cools down the high responses of NF-κB in the stressed epithelial cells stimulated by pathogens (Manzel *et al.*, 2011). There is evidence that when stimulated by *Haemophilus influenzae*, smoking mice have inhibited NF-κB and decreased the expression of defensive inflammatory factors, whereas the nonsmoking mice have promoted NF-κB and produced more relative inflammatory factors (Manzel *et al.*, 2011). Therefore, it could be concluded that smoking on one hand could activate NF-κB without infection, and on the other hand, it could also suppress NF-κB with the presence of infection. The AP-1 signaling pathway, important for the resistance to corticosteroids inflammation, also plays a pivotal role important in the production of IL-8 in

macrophages and monocytes, which are activated by ROS in smoking (Walters *et al.*, 2005). Smoking itself can induce AP-1 activation in bronchial epithelial cells independently, but in the case of pre-stimulation with LPS, the activation-effect of smoking on AP-1 is weakened (Laan *et al.*, 2004). These studies give strong backing to the viewpoint that smoking downregulates the mucosal defensive function and induces chronic inflammation in the airways, giving rise to reduced acute reactivity to infectious attacks (Lee *et al.*, 2012).

1.10. Aim of the project

Smoking causes many health problems, and smoking-induced infections are getting more and more attention. Since impaired immune function plays an important role in prerequisites for microbial infection, focus will be put on the effect of smoking on the immune function. *S. a., S. e., P. a., E. f.* are four types of pathogenic bacteria that are commonly seen in clinical infection. Human peripheral blood mononuclear cells (PBMCs) consist of lymphocytes (T cells, B cells and NK cells) that occupy the most part, monocytes that take up a small part, and only a small percentage of dendritic cells (Verhoeckx *et al.*, 2015). The following questions are addressed of this thesis:

1. How smoking affects the release of cytokines by PBMC?
2. How does the production of pro-inflammatory and anti-inflammatory factors changes when PBMCs from smokers and non-smokers are exposed inactivated bacteria?
3. Since some articles reported that IL-4 could facilitate the wound healing, whereas others showed that IL-4 inhibited the wound healing. Whether IL-4 promotes or inhibits wound healing?
4. How smoking affects wound healing by IL-4?

As for my own project, the hypothesis is that the levels of cytokines released by PBMCs must have been changed in smokers compared to nonsmokers. Based on this, different cytokine amounts released by the PBMCs of nonsmokers and smokers when stimulated by four types of inactivated bacteria (*S. a., S. e., P. a., E. f.*) will be investigated. In addition, the migration rate of HaCaT cells will be measured with or without the presence of IL-4. HaCaT cells are immortal human keratinocytes that have been widely used to simulate the pathophysiological activity of the skin in vitro (Seo *et al*., 2012). Meanwhile,

the migration rate of HaCaT cells treated by different IL-4 conditions that were produced by PBMCs when stimulated by the four types of inactivated bacteria will also be measured. The aim is to clarify the effects of smoking on cytokines and the effects of altered IL-4 induced by smoking on skin healing, thus providing a theoretical basis for the treatment of clinical smoking induced infections and delayed wound healing.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Table 1. List of solids, solutions and buffers used in the project

Article	Company	Article number
30% Hydrogen peroxide Solution	Roth	CP26.5
Acetic acid	Roth	3738.4
BSA	Roth	8076.4
DMEM high glucose (4.5 g/l) with L-Glutamine	Sigma	D5796
DPBS	Sigma	D8537
DPBS	Sigma	H15-002
Ethanol, 70%	Apotheke	MRI
Ethanol, 99%	Apotheke	UKT
FCS	Invitrogen	41G7141K
LSM 1077	PAA	J15-004
Penicillin/Streptomycin	Sigma	P0781
Resazurin sodium salt	Sigma	199303-1G
RPMI 1640	Sigma	R8758
SRB salt	Sigma	S1402-1G
TRIS	Roth	AE15.1
Trypan Blue 0.5% 100 ml	Biochrom	L6323
Trypsin/EDTA	Sigma	T3924
Tween 20	Roth	9127.1

Table 2. Buffers, Solutions and Mediums

Buffers/Solutions/Mediums	Compounds
ELISA Washing Buffer	0.05% Tween-20 in DPBS
ELISA Blocking Buffer	1% BSA in DPBS
Diluent	0.05% Tween-20 and 0.1% BSA in PBS
ABTS reaction buffer	0.1 M citric acid solution
ABTS stock solution	15 mg ABTS in 1 ml ddH2O
ABTS reaction solution	5 ml ABTS reaction buffer + 100 µl ABTS stock solution + 5 µl 30% H2O2
1% Acetic Acid Solution	1% acetic acid in ddH2O
10 mM TRIS Solution	1.2 g TRIS in 1 L ddH2O
SRB Solution	0.4% SRB in 1% acetic acid
Resazurin stock solution	0.025% in DPBS
Resazurin working solution	10% Resazurin stock solution in DPBS
HaCaT Cells Culture Medium	500 ml DMEM 5 ml Penicillin/Streptomycin 25 ml Fetal Bovine Serum

2.1.2. Equipment

Table 3. List of used apparatus

Equipment	Manufacturer	Type	Serial number
Agitator, magnetic stirrer	IKA-Werke GmbH	RH B2	06.050357
Agitator, magnetic stirrer	Heidolph Instruments GmbH	MR Hei-Mix L	040700340
Blood Needle	Multifly	0.8×19 mm	85.1638.235
Blood Tube	S-Monovette	9 ml	02.1726.001
Cell culture plate	Greiner bio-one	96-well, flat bottom	655180
Cell culture plate	Corning Inc.	48-well, flat bottom	3548
Cell culture plate	Greiner bio-one	24-well, flat bottom	662160
Cell culture plate	Corning Inc.	6-well, flat bottom	353046

Cell Star Tubes	Greiner bio-one	50 ml	227261
Cell Star Tubes	Greiner bio-one	15 ml	188271
Centrifuge	Dako Deutschland GmbH	Stat Spin	620E50000693
Centrifuge	Thermo Fisher Scientific	Megafuge 40 R	41307652
Centrifuge	Scientific Industries Inc.	SI DD 58	DD 58-1001
Centrifuge（Mirco）	Labnet International	BN 08060235	C 1301 B
Centrifuge（Mirco）	HERAEUS Med GmbH	Fresco 17	41250019
ELISA plate	Greiner bio-one	96-well, flat bottom	655061
Encapsulator	NISCO Engineering AG	UNIT VAR V1	LiN-0172
Eppendorf tube	SARSTEDT AG	0.5 ml, white	72.699
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, white	4182.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, blue	4190.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, green	4209.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, red	4189.1
Eppendorf tube	Carl Roth GmbH + Co.KG	1.5 ml, yellow	4204.1
Eppendorf tube	Eppendorf	2.0 ml, white	2549
Freezer -20 ℃	BSH	IQ500	GS51NYW41 (01)
Freezer -20 ℃	Liebherr	Med Line	LGex3410-21K 001
Freezer -80 ℃	Thermo Fisher Scientific	905	827860-2521
Freezer -86 ℃	Revco	ULT1386-9-V17	R10G-333095-RG
Fridge +4 ℃	Liebherr	Comfort	3523-21L
Fridge +4 ℃	Cool Compact Kühlgeräte G	HKMT 040-01	CC00412514
Gauze	Gazin	7.5×7.5 cm 2 stk/pcs	13621
Ice maker	Scotsmen	AF 80	DD 8837 11 X
Infusion syringe pump	B. Braun	Perfusor Secura FT	2313
Infusion syringe pump	B.Braun	Perfusor Space	137783
Infusion syringe pump	KD Scientific Inc.	KDS-100-CE	114297
Infusion syringe pump	Landgraf Laborsysteme	LEBC	280673
Incubator	Thermo Fisher Scientific	Heratherm OMS 60	41296334

Incubator	Binder GmbH	9040-0078	11-22649
Incubator	Binder GmbH	9040-0081	11-22190
Laboratory pump (Bench)	Carl Roth GmbH + Co.KG	Cyclo 2	1109-065
Liquid pump (Bench)	KNF Neuberger GmbH	N86KN.18	2.04119556
Microplate Reader	BMG Labtech GmbH	FLUOstar Omega	S/N415.1264
Microscope	Peqlab Biotechnologie GmbH	EVOS-fl	91-AF-4301
Mixer	Corning Inc.	Vortex Mixer	804995
Mixer	Labinco BV	LD-76	76000
Multichannel Pipettor	Corning Inc.	5-50 µl	151620022
Multichannel Pipettor	Corning Inc.	20-200 µl	551630277
Multichannel Pipettor	Thermo Electron Co.	0.5-10 µl	CH98998 4510
Multichannel Pipettor	Corning Inc.	50-300 µl	151640033
pH meter	Mettler-Toledo GmbH	Five Easy FE 20	1232315296
Pipette controller	Integra GmbH	Pipetboy acu	629619
Pipette controller	Heathrow Scientific LLC	Rota-Filler 3000	HSA05119
Pipette Tips	Sorenson BioScience, Inc.	0.1 - 10 µl	Colorless
Pipette Tips	Sarstedt AG & Co.	2 - 200 µl	Yellow
Pipette Tips	Ratiolab GmbH	100 - 1000 µl	Blue
Refrigerator	Cool Compact Kühlgeräte G	HKMT 040-01	CC 00412516
Refrigerator	Cool Compact Kühlgeräte G	HKMN 062-01	CC 00412513
Safety workbench	Thermo Fisher Scientific	Maxisave S2020 1.8	41293949
Safety workbench	Thermo Fisher Scientific	Maxisave S2020 1.8	41293948
Scale	Kern & Sohn GmbH	ABJ 120-4 M	WB 1140084
Shaker, laboratory	LTF Labortechnik GmbH	DRS 12	11 DE 243
Shaker, laboratory	Peqlab Biotechnologie GmbH	ES-20	010111-1107-0119
Shaker, laboratory	LTF Labortechnik GmbH	DRS 12	11 DE 090
Shaker, Laboratory	Corning Inc.	LSE Vortex Mixer	1101260
Single-channel Pipettor	Corning Inc.	0.5-10 µl	158220060
Single-channel Pipettor	Corning Inc.	2-20 µl	158230441

Single-channel Pipettor	Corning Inc.	10-100 µl	158240031
Single-channel Pipettor	Corning Inc.	20-200 µl	158250088
Single-channel Pipettor	Corning Inc.	100-1000 µl	058261237
Single-channel Pipettor	Eppendorf	0.1-2.5 µl	P35434B
Spectrophotometer	BMG Labtech GmbH	Fluostar Omega	415-1264
Water-bath	Lauder Dr. R. Wobser GmbH	Al 25	LCB 0727-11-0094
Water-bath	Lauder Dr. R. Wobser GmbH	ECO ET 20	LY 06.1

2.1.3. Software

Table 4. List of used Software

Software	**Company**
ImageJ	NIH
Excel, Powerpoint, Word	Microsoft
GraphPad Prism	GraphPad Software Inc.
TScratch	CSE-Lab
EndNote X8	Thomson ResearchSoft

2.2. Methods

2.2.1. Ethics statement

The study was performed according to the Declaration of Tübingen University and approved by the Regional Committee of Medical Research Ethics at Tübingen University (Ethical vote number, 538/2016BO2). Each donor was informed in advance of the purpose of the blood collection and the plan of our experiment, which obtained the agreement of all the participants.

2.2.2. Blood collection

The appropriate number and size of the test tubes were prepared for use. The donor was positioned in a chair, leaving the forearm horizontally placed on the table. When a vein was selected, the puncture point was cleansed in a circular motion from the inside out. After the puncture site was air-dried, it was not touched or palpated. If it was necessary to reassess the site by palpation, the area was re-cleaned prior to venipuncture. The donor was instructed to make a fist, making the veins slightly swell, and then the needle was

quickly inserted through the skin into the venous lumen. The needle was inserted at an angle 15-30 degrees to the surface of the arm and was not inserted too deeply. When the last tube was full, the tourniquet and needle were quickly removed from the donor's arm. Gauze was immediately placed the on the puncture site and sufficient pressure was maintained to avoid the formation of a hematoma. After maintaining a pressure for 1-2 minutes, a new gauze or Band-Aid was applied to the puncture site.

2.2.3. Isolation procedure of peripheral blood mononuclear cells (PBMCs)

Blood samples were taken from healthy nonsmokers and smokers (9 mL each donor) and then were transferred into 15 ml Falcon tubes with 6 ml lymphocyte separation medium 1077 (LSM) per each tube. The handling should be very careful to make sure the blood flows gently above the LSM. Density gradient centrifugation was performed at 1000 g for 20 min at 4°C without brake (Thermo Scientific). The PBMC layer was transferred to a new Falcon tube and washed twice with PBS (centrifugation at 600 g for 10 min at 4 °C). The blood plasma was transferred into a fresh Eppendorf tube to obtain a fetal calf serum (FCS) alternative, then centrifuged for 10 min at 10,000 g to get rid of pellet thrombocytes. The supernatant was discarded, and the pellet was re-suspended in 10 mL RPMI 1640 culture medium (Fuss *et al.*, 2009).

2.2.4. Cell counting and diluting

The coverslip on the hemocytometer was cleaned with ethanol 70%. The two bars of the hemocytometer were breathed on and the cover glass was put on. The cell suspension was thoroughly mixed, take out 10 µl from the PBMC solution and mix it with 10 µl trypan blue working solution. Afterwards, 10 µl of the mixture was transferred into each counting chamber. The cells in the four counting grids were counted and the average was calculated. Total number of cells = n * Vf * V * 10^4 (n: average of counted cells, Vf: dilution factor, V: volume of cell suspension (here it's 10 ml), and 10^4: factor of the cell count chamber). The PBMC suspension was then diluted into different volumes in order to make the concentration of each new suspension $5*10^5$ cell/ml.

2.2.5. Preparation of inactivated bacteria

The Institute of Medical Microbiology and Hygiene in Tübingen University has provided us with four inactivated bacteria (*S. a., S. e., P. a., E. f.*) that were prepared by heating method (Donkor *et al.*, 2012), also known as pasteurization (Taddese *et al.*, 2018). In the

food industry, pasteurization can preserve products for a long time while avoiding the destruction of essential nutrients. The mechanism of pasteurization inactivating bacteria is to raise the temperature up to 70 ° C for utmost 30 minutes to denature bacterial cell membranes and proteins (Plovier *et al.*, 2017).

2.2.6. Incubation

For incubation, 2% plasma or 2% FCS (Chen *et al*., 2014) was added to each PBMC suspension. After that, each PBMC suspension was divided into 5 treatment conditions as follows: the first condition had nothing added; the second condition had heat inactivated *S. a.* 4 µl/ml added; the third condition had heat inactivated *S. e.* 2 µl/ml added; the fourth condition had heat inactivated *P. a.* 2 µl/ml added; the fifth condition had heat inactivated *E. f.* 1 µl/ml added. Each treatment condition was put into incubator for 24 h (Donkor *et al.*, 2012).

2.2.7. Human Cytokine Antibody Array

Cytokine array kits were performed as described by the manufacture. Briefly, components were removed from storage and the components were equilibrated to room temperature. Antibody arrays were carefully removed from the plastic package and each membrane (printed side up) was placed into a well of the incubation tray (one membrane per well). A total of 2 ml of blocking buffer was pipetted into each well and was incubated for 30 minutes at room temperature. Blocking buffer was aspirated from each well with a pipette. A 1 ml sample was pipetted into each well and was incubated for 1.5 h at room temperature. Then, samples were aspirated from each well with a pipette. A total of 2 ml of wash buffer I was pipetted into each well and was incubated for 5 minutes at room temperature (washed 3 times). Then, 2 ml of wash buffer II was pipetted into each well and was incubated for 5 minutes at room temperature (washed 2 times). A total of 1 ml of the prepared biotinylated antibody cocktail was pipetted into each well and was incubated for 1.5 to 2 h at room temperature. Then, the biotinylated antibody cocktail was aspirated out from each well, and the membranes were washed wash buffer I and II as mentioned above. Then, a total of 2 ml of HRP-Streptavidin was pipetted into each well and was incubated for 2 h at room temperature. HRP-Streptavidin was aspirated out from each well, and the membranes were washed as above. Membranes were placed on the chromatography paper, and the surplus wash buffer was discarded from the membrane

with tissue paper. A total of 500 µl detection buffer mixture was pipetted into each membrane as was incubated for 2 minutes at room temperature. The membranes were covered with another plastic sheet and transferred to the chemiluminescence imaging system (ChemoCam, Intas) to be measured.

2.2.8. Enzyme-linked immunosorbent assay (ELISA)

Standard ABTS ELISA development kits were purchased from PeproTech, Inc, USA and the ELISAs performed as described by the manufacture. Conditions are described in Table 1. Before being added to the plate wells, each of the captured antibodies, standard antigens, and detection antibodies were diluted 100 times with different dilute as shown in Table 2. Meanwhile, the Avidin-HRP conjugate was diluted 2,000 times. The specific information of the ABTS reaction solution is shown in Table 3.

Table 5. Overview of stock reagents of different cytokines

Cytokine	Captured antibody	Standard antigen	Detection antibody
IFN-γ	100 µg/ml	1 µg/µl	100 µg/ml
TNF-α	100 µg/ml	1 µg/µl	50 µg/ml
IL-1β	100 µg/ml	0.5 µg/µl	100 µg/ml
IL-4	100 µg/ml	1 µg/µl	100 µg/ml
IL-6	100 µg/ml	1 µg/µl	100 µg/ml
IL-10	100 µg/ml	1 µg/µl	50 µg/ml
IL-12	100 µg/ml	1 µg/µl	100 µg/ml

A total of 50 µl captured antibody dilution was pipetted to each well of the 96-well plate and was incubated at room temperature (20–25 °C) overnight. The plate was washed three times (each used well was filled with 120 µl washing buffer, and the washing buffer was removed by tapping the plate onto absorbent material). A total of 100 µl blocking solution was added to each well and was incubated (20–25 ℃) for one h by shaking. The plate was washed one time (as described above). A total of 50 µl standard (S1–S8) or sample was pipetted to each well and was incubated (20–25 °C) for 2 h by shaking. The plate was washed 4 times (as described above). A total of 50 µl detection antibody dilution was added to each well and was incubated (20–25 °C) for 2 h by shaking. The plate was washed for 4 times (as described above). A total of 50 µl Avidin-HRP conjugate dilution

was pipetted to each well and was incubated at room temperature (20–25 °C) for 30 minutes by shaking. The plate was washed 4 times (as described above). Finally, 50 µl of ABTS reaction solution was added to each well and was incubated for 1 h. The absorbance was measured in an omega plate reader (Fluostar Omega, BMG Labtech GmbH) at 405 nm - 650 nm.

2.2.9. Test that cytokines are mainly released by PBMCs in the whole blood.

In most studies, PBMCs isolated from blood were used to investigate the production of cytokines *in vitro* (Le Meur *et al*., 1999), so it was speculated that cytokines are mainly produced by PBMC. The following test shall verify this hypothesis: whole blood was collected from 3 healthy nonsmokers and 2 healthy smokers, with 10 ml blood (one tube) per donor. Each 10 ml blood sample was divided into two parts: one part (9.7 ml) was used to isolate PBMCs and the other part (0.3 ml) was maintained as whole blood. The 0.3 ml whole blood sample was diluted to 1.5 ml after adding 1.2 ml PBS. PBMCs pellets were isolated from the 9.7 ml blood sample and then suspended in 1.5 ml RPMI, and 2% plasma was added to the new PBMC suspension. Both the whole blood solution and PBMCs solution of each donor were incubated for 24 h before being centrifuged. The supernatants were used as samples in ELISA measurements.

2.2.10. Find out the appropriate concentration of each of the four types of inactivated bacteria.

In the study conducted by Katial *et al.*, the concentration for *S. a.* was 10 µg/ml (Katial *et al*., 1998). However, with one Eppendorf tube per each type of bacterium, their amounts were not sufficient. Furthermore, their potency was not quite clear. On account of the frequent consuming of the four types of bacteria in the future experiments, the efficient concentration of each of the four types of bacteria was investigated. Two different concentration gradients, 2 µl/ml and 4 µl/ml, were set for each type of inactivated bacterium that was added to the PBMCs suspension.

Blood was taken from four new healthy nonsmoking donors, and the PBMCs were isolated. The PBMCs suspension was obtained according to the method referred to above. Each donor's PBMC suspension was mixed with 2% plasma and then was equally divided into ten parts: control, *S. a.* 2 µl/ml, *S.a.* 4 µl/ml, *S. e*. 2 µl/ml, *S. e.* 4 µl/ml, *P. a.* 2 µl/ml, *P. a.* 4 µl/ml, *E. f.* 2 µl/ml, and *E. f.* 4 µl/ml. All the conditions were incubated for 24 h

before they were centrifuged to acquire the supernatants that were used as samples in ELISA measurements.

2.2.11. Set the suitable concentration of PBMCs.

Since the concentration of inactivated bacteria had been determined, the next step was to determine the concentration of the PBMCs. The PBMCs concentrations used in previous publications were 5×10^5 cells/ml (Katial *et al*., 1998), 40×10^5 cells/ml *(Ngkelo et al*., 2012), 50×10^5 cells/ml (Friberg et al., 1994), 80×10^5 cells/ml (Corry *et al*., 1996) and 200×10^5 cells/ml (Jeurink *et al*., 2008). However, our blood donation is not enough to reach such a high concentration, so we tried to use a lower concentration. Two concentrations, 5×10^5 cells/ml and 2.5×10^5 cells/ml, were selected for the PBMCs. The amounts of cytokine (IFN-γ) secreted by the PBMCs were measured within these two selected concentration groups.

Blood was taken from four new healthy nonsmoking donors, and the PBMCs were isolated. After counting the PBMCs under the microscopy and re-diluting them to 5×10^5 cells/ml and 2.5×10^5 cells/ml for each donor, the final PBMCs suspensions were obtained. Each PBMCs suspension was mixed with 2% plasma. Each donor's new PBMCs suspension was equally divided into five parts: control, *S. a.* 4 μl/ml, *S. e.* 2 μl/ml, *P. a.* 2 μl/ml, and *E. f.* 1 μl/ml. All the ten conditions were incubated for 24 h before they were centrifuged to separate the supernatants that were used as samples in ELISA measurements.

2.2.12. Cytokine array of various cytokine levels released by PBMCs between nonsmokers and smokers stimulated by four types of inactivated bacteria.

Blood was taken from four new healthy non-smoking donors, and the PBMCs were isolated. The final PBMCs suspension was obtained after counting the cells in the microscopy and re-diluting them to 5×10^5 cells/ml for each donor. Each donor's PBMCs suspension was mixed with 2% plasma and then was equally divided into five parts: control, *S. a.* 4 μl/ml, *S. e.* 2 μl/ml, *P. a.* 2 μl/ml, and *E. f.* 1 μl/ml. All 40 conditions (5 conditions per donor) were incubated for 24 h before they were centrifuged to acquire the supernatants that were used as samples in cytokine arrays.

2.2.13. ELISA of anti-inflammatory and pro-inflammatory cytokine levels released by PBMCs between nonsmokers and smokers stimulated by four types of inactivated bacteria were measured and the effect of plasma was checked.

The cytokine array demonstrated that IL-10 and IL-4 (both of which have anti-inflammatory properties) were higher in PBMCs of smokers than in nonsmokers whether or not bacteria are present. Since these anti-inflammatory cytokines have been promoted by smoking, we postulated that pro-inflammatory cytokines should be reduced. ELISA was performed to investigate this change. Blood was taken from new healthy nonsmoking donors and healthy smoking donors (4 smokers and 4 nonsmokers every time), and the PBMCs were isolated. The final PBMCs suspension was obtained after counting the cells in the microscopy and re-diluting them to 5×10^5 cells/ml for each donor. Each donor's PBMCs suspension was divided into two parts, one mixed with 2% plasma (the donor's own plasma) and the other mixed with 2% FCS. Then, each new PBMCs suspension was continuously equally divided into five parts: control, *S. a.* 4 µl/ml, *S. e.* 2 µl/ml, *P. a.* 2 µl/ml, and *E. f.* 1 µl/ml. Thus, each donor had ten conditions: control with plasma, control with FCS, *S.a.* with plasma, *S. a.* with FCS, *S. e.* with plasma, *S. e.* with FCS, *P. a.* with plasma, *P. a.* with FCS, *E. f.* with plasma, and *E. f.* with FCS. All the PBMCs suspension conditions were incubated for 24 h before they were centrifuged; supernatants that were used as samples in ELISA measurements (Figure 5).

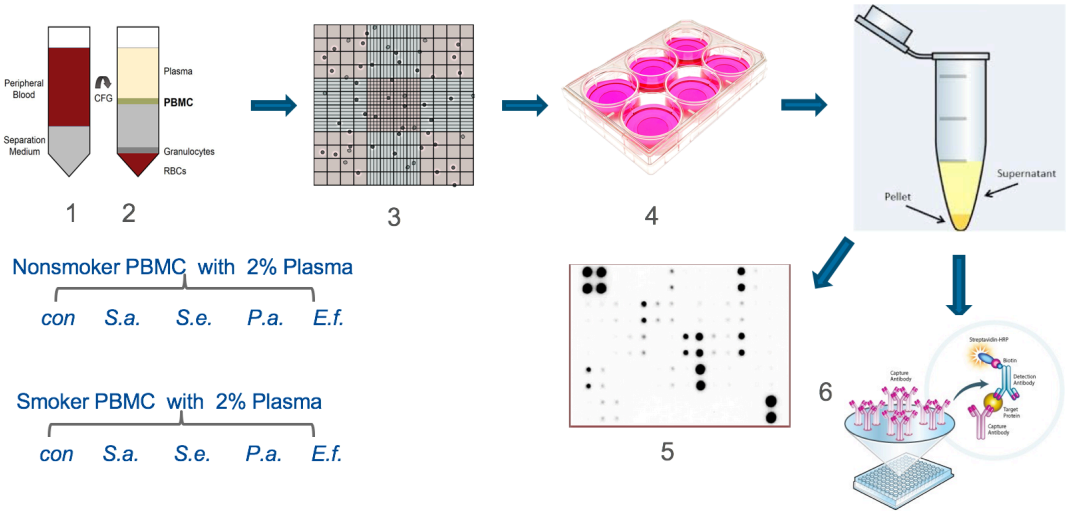


Figure 5. Schematic diagram of measurement of cytokines secreted by PBMCs stimulated with four types of inactivated bacteria.

Each smoking and nonsmoking donor's PBMCs suspension was divided into two parts, one mixed with plasma and the other mixed with FCS, which aimed at eliminating the effect of plasma. The PBMCs suspensions were continuously stimulated with different types of inactivated bacteria and were incubated for 24 h before they were centrifuged to separate the supernatants. ELISA and cytokine array were performed using the supernatants.

2.2.14. Determine the appropriate concentration of HaCaT cells that have just reached cell confluence.

In order to grow HaCaT cells into 100% confluence in 24 h, Wang *et al.* used a concentration of 6×10^5 cells/mL in his study (Wang *et al*., 2016). This concentration is confirmed again before use in our experiment. After culture and sub-culture, the HaCaT cells were seeded into the 96-well plate with concentration gradients as follows: 0/100 µl, 10 000 cells/100 µl, 20 000 cells/100 µl, 30 000 cells/100 µl, 40 000 cells/100 µl, 50 000 cells/100 µl, 60 000 cells/100 µl, and 80 000 cells/100 µl. After incubating the plate in the incubator (37 °C, 5% CO_2) for 24 h, the confluence of each concentration condition was examined by microscope and the Resazurin assay and SRB assay were performed to test the viability.

2.2.15. Resazurin assay and Sulforhodamine B (SRB) staining

Resazurin that is a redox-sensitive dye that can be transformed by mitochondria to become resorufin, both of which can be measured via the fluorescence (McMillian *et al.*, 2002). Resazurin conversion could reflect the viability, cytotoxicity, proliferation in cultured cells (Anoopkumar-Dukie *et al.*, 2005). The mechanism of SRB staining is that SRB can bind to surface proteins in acidic environment, so it could be used to measure the cell density (Skehan *et al*., 1990). SRB staining is independent of metabolic activity in cells (Vichai and Kirtikara, 2006), therefore the combined use of SRB staining and resazurin assay in our experiment. After removal of the culture medium from cells, 10 µl Resazurin working solution was added to each well and was incubated in an incubator (37 °C, 5% CO_2) for 30 minutes, followed by complete fluorescence measurement and calculation.

After removal of the culture medium from cells, the cells were covered with 99% ethanol (100 µl per well) and frozen at -20 °C for 1 h. Then, the cells were removed from the

ethanol. The cells were washed once with tap water and left to air- dry on plates. The cells were covered with SRB Solution (50 µl per well) and incubated for 30 min at room temperature by shaking (to protect from light). Then, the cells were removed from the SRB solution. To remove unbound SRB, the cells were washed 3–4 times with 1% acetic acid solution (stop when no pink stain resolves anymore). For quantification, SRB was resolved with 10 mM unbuffered TRIS solution (100 µl per well), followed by complete absorbance measurement and calculation.

2.2.16. HaCaT cells Migration Assay

HaCaT cells migration assay was performed to mimic the wound healing process *in vitro* (Kramer *et al.*, 2013). What role the IL-4 played in HaCaT cells migration was topic in our experiment. The treatment groups were stimulated by a supernatant of PBMCs (with FCS) stimulated by four types of inactivated bacteria (*S. a., S. e., P. a.*, and *E. f.*) from smokers and nonsmokers, which was used in ELISAs. The positive control group was stimulated by FCS with IL-4, while the negative control group was stimulated by FCS without IL-4.

A 96-well plate was taken out from refrigeration to the lab bench. Stoppers were seeded in the plate to ensure that stoppers were securely sealed against the bottom of the plate. A total of 100 µl suspended cells 60,000 cells/well (100 µl) were pipetted into each test well through one of the side ports of the stopper and gently tapped to evenly distribute the content of each well. The stoppers were removed after incubating for 24 h. The medium was removed with a pipette, and the wells were gently washed with 100 µl PBS to remove any unattached cells. A total of 50 µl HaCaT medium was added into each of the wells. Then, 50 µl ELISA supernatant was added into each different treated well, 50 µl RPMI + 2%FCS was added into each negative control well, and 50 µl RPMI + 2% FCS plus IL-4 (5 ng/ml) was added into each positive control well. A picture was taken under the microscope to get the baseline picture, and the contents were allowed to continue to incubate for 24 h. At which time, another picture was taken under the microscope to get the 24 h point picture (Figure 6).

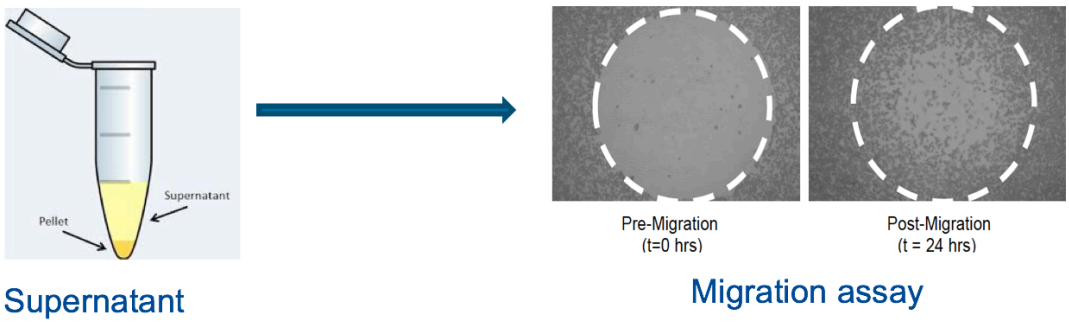


Figure 6. HaCaT cells were stimulated with supernatant of PBMCs.

HaCaT cells of 0 point were stimulated by PBMCs with FCS supernatant stimulated by four types inactivated bacteria (S. a., S. e., P. a., and E. f.), which were used in ELISAs. The 0 point area and 24 h point area were measured.

After collection of images, the image analysis was performed by using the software TScratch. For each condition well, the migration rate was calculated by the function (Figure 8) as follows:

$$\text{Migration Rate} = \frac{\text{Area}_{0\ \text{point}} - \text{Area}_{24\text{h}}}{\text{Area}_{0\ \text{point}}} \times 100\%$$

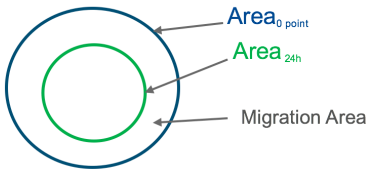


Figure 6. The sketch of migration function by which the migration rate of each group was calculated.

Function: (0 point area- 24 h point area)/ 0 point area ×100%.

2.3 Statistical Analysis

Statistical analysis was performed with GraphPad Prism Version 7 with one-way ANOVA (nonparametric) multiple comparisons and mean ranks of preselected pairs of columns. Based on the choices, Prism performed the Kruskal-Wallis test or Dunn's multiple comparisons test. Differences in comparison were considered statistically significant at values of *P < 0.05; ***P < 0.0005. After each data analysis was completed, it was discussed with PD Dr. Sabrina Ehnert.

3. Result

3.1. Cytokines were mainly secreted by PBMCs rather than the other cells in the blood.

The result demonstrated (as below in Figure 7) that the PBMCs mainly secrete the cytokines (IFN-γ). Isolated PBMCs secreted significantly more cytokines (IFN-γ) than whole blood.

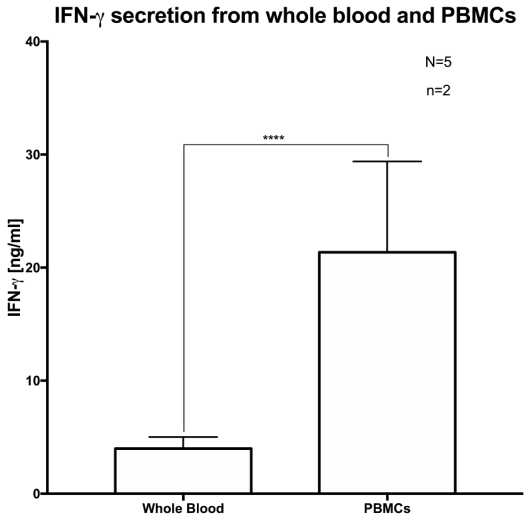


Figure 7. ELISA experiment showed that cytokines (IFN-γ) were mainly secreted by PBMCs rather than other cells in the whole blood.

*Both whole blood and PBMCs solution of each sample were incubated for 24 h before the supernatant was acquired by centrifugation. The cells number in whole blood group is 300 times that of the cells number in PBMC group. However, the IFN- γ released in PBMC group was significantly higher than in whole blood cells. Each point represents the mean ± standard deviation (SD) error bars of five donors (three nonsmokers and two smokers) from duplicate experiments. ***P < 0.0005 (PBMCs versus Whole blood).*

3.2. The appropriate concentration the four types of inactivated bacteria are *S.a.* 4 µl/ml, S.e. 2 µl/ml, P.a. 2 µl/ml, and *E.f.* 1 µl/ml.

The result demonstrated (as below in figure 8) that we decided to use *S. a.* 4 µl/ml, *S. e.* 2 µl/ml, *P.a.* 2 µl/ml, and *E. f.* 1 µl/ml during the following research. The normalized result of the INF-γ ELISA experiment shows that *S. a.* 4 µl/ml could stimulate PBMCs to secrete insignificantly more INF-γ than *S. a.* 2 µl/ml. Besides, *S. e.* 2 µl/ml could stimulate PBMCs to secrete insignificantly more INF-γ than *S. e.* 4 µl/ml. Furthermore, both *E. f.* 2

μl/ml and *E. f.* 4 μl/ml could stimulate PBMCs to secrete much more INF-γ. However, there was no significant difference between the two concentration gradients of *P.a.*

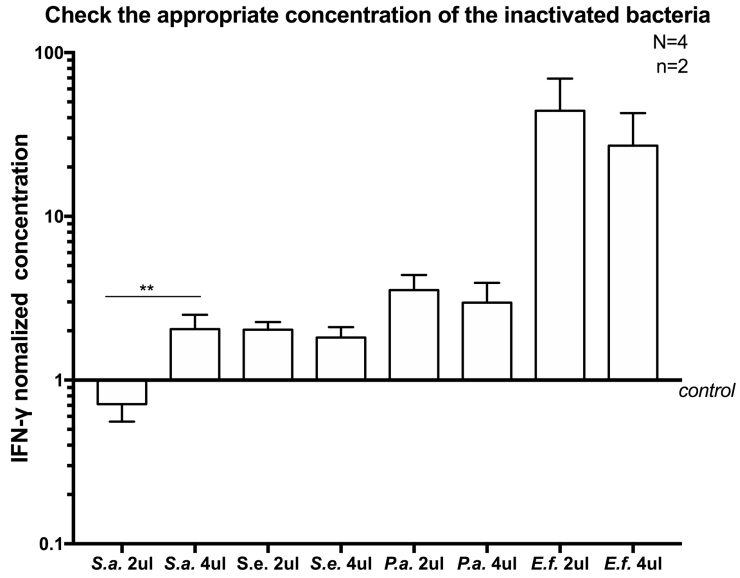


Figure 8. Two ELISA experiments have been done in order to check the suitable concentration of the four kinds of inactivated bacteria.

*ELISA showed that PBMCs stimulated with S. a. 4 μl/ml produced significant more IFN-γ than PBMCs stimulated with S. a. 2 μl/ml. However, there were no significant difference calculated among the three comparisons: S. e. 2 μl/ml versus S. e. 4 μl/ml, P.a. 2 μl/ml versus P.a. 4 μl/ml, and E. f. 2 μl/ml versus E. f. 4 μl/ml. Each point represents the mean ± SD error bars of four healthy nonsmoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05; ***P < 0.0005 (S. a. 4 ul/ml versus S. a. 2 ul/ml).*

3.3. The suitable concentration of PBMCs is 5×10^5 cells/ml

After the ELISA experiments, the result demonstrated (as below in Figure 9) that the 5×10^5 cells/ml PBMCs could produce more cytokines (IFN-γ) than the 2.5×10^5 cells/ml PBMCs in each of the four conditions. Therefore, 5×10^5 cells/ml will be fixed as the concentration of PBMCs in the following experiment.

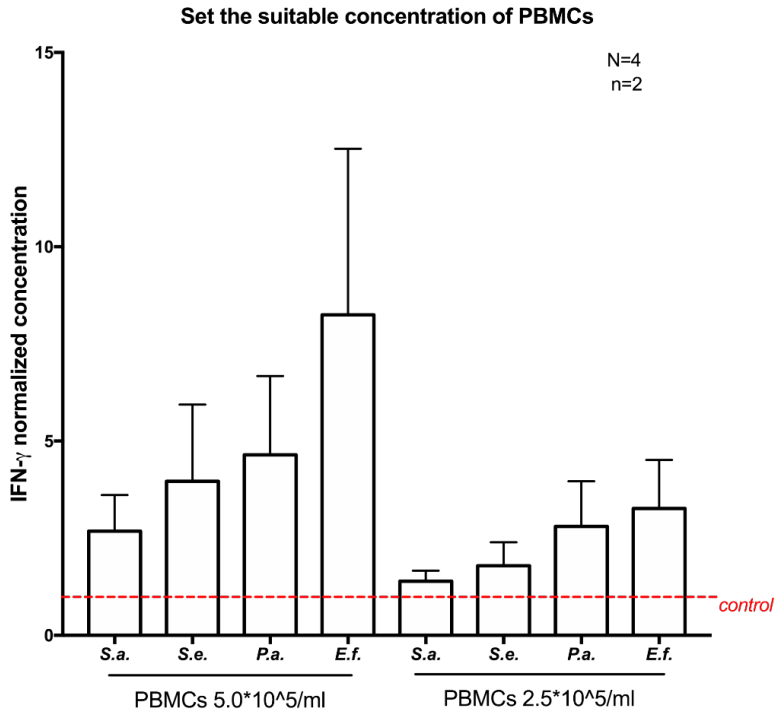


Figure 9. ELISA experiment shows that the 5×10^5 cells/ml PBMCs could produce more IFN-γ than the 2.5×10^5 cells/ml PBMCs for each condition, with however insignificant differences.

In both 5×10^5 cells/ml PBMCs group and 2.5×10^5 cells/ml PBMCs group, there were five sub-groups: the control sub-group with nothing added and four types of inactivated bacteria sub-groups were respectively set as S. a. 4 μl/ml, S. e. 2 μl/ml, P. a. 2 μl/ml, and E. f. 1 μl/ml. ELISA showed that *5×10^5 cells/ml PBMCs could produce in significantly more cytokines (IFN-γ) than the 2.5×10^5 cells/ml PBMCs could for each condition. Each point represents the mean ± SD error bars of four healthy nonsmoking donors from duplicate experiments.*

3.4. Cytokine arrays: anti-inflammatory cytokines secreted by PBMCs from smokers were higher than from nonsmokers

The result of cytokine arrays of the various cytokine levels released by the PBMCs between nonsmokers and smokers stimulated by four types of inactivated bacteria are shown in Figure 10.

The basal anti-inflammatory cytokines IL-10 and IL-4, were higher in smokers than in nonsmokers.

A.	Nonsmokers (4 donors)					Smokers (4 donors)				
	Control	*S.a.*	*S.e.*	*P.a.*	*E.f.*	*Control*	*S.a.*	*S.e.*	*P.a.*	*Ef.*
ENA-78	1.71765472			1.70073475	1.96105152	0.28234528	1.62399054	1.50197985	2.51421667	3.37154853
G-CSF	2	0.81463898	2.31265219	1.92198467	1.94244218	0		2.43085106	2.64872475	5.10409528
GM-CSF	1.60076874	0.50844863				0.39923126				2.9291805
GRO		0.2328901	0.39640043	0.74034292			0.16208465	0.74137023	0.2214383	3.42583396
GRO alpha	2		0.6833898		1.80515453	0	0	0.86778191		5.37367085
I-309	0.43666223	0.0300642	0.4158636	0.7453898	0.40653131					0
IL-1 alpha	0.87009714	0.14227215	0.46044026	0.39471264	0.38618631			0.56586231		0
IL-1 beta	0.95571248	0.37821518	0.75624775					0.50395342		0.50176322
IL-2	0.88321178	0.6156706	0.55127131		0.61832739			0.1628236		0.20559541
IL-3	1.60292848		0.73885811		0.11645801	0.39707152	2.13964212	0.10646766	3.32183029	0.79187338
IL-4	2	1.52614219	1.61353251	0	0	0	2.9528794	0	4.04355984	2.39861026
IL-5	2	0.80795983	2.3155029	1.72350816	1.69632616	0	1.75800758	2.3313889	3.08892832	3.99814951
IL-6		13.8170677	60.3550629	55.531321	38.6611667	0.85982904	18.8749285	134.261617	66.2513574	120.399334
IL-7	2		2.15041903	3.32230944	8.50179252	0	7.26872935	5.7668236	4.95921608	9.51328872
IL-8	0.83767055	0.20622274	0.25134329	0.57361372			0.55994238	0.55794761	0.43675885	3.06854357
IL-10			2.02253283	7.9944638		0.4186215		6.07927553	22.5248693	7.88130008
IL-12, p40/7	0.52849669	0.21346485	0.47496377	0.57530981	0.39389038			0.29742417		0
IL-13	0.75689453	0.26487666	0.33435781	0.39793233	0.54075122		2.20927789	0	2.90720545	0
IL-15	0.1379381	0.5537043	0.81622743	0.64954163		1.8620619	2.27648219	0.66358056	2.76004545	0.91361879
IFN gamma	0.55971919		0.80802212	0.72173096				0.22718664	2.89983786	0.65958059
MCP-1	0.75065955	0.30416374	0.1933613	0.15531998	0.50667625		0	0.26341988		
MCP-2	2			0	0	0	0.11498402	0	2.32341523	0.07295094
MCP-3	0.84379844	0.44243016	0.6322983	0.26285825	0.66915381		0.65232062	0.69328154	2.20969036	
M-CSF			2.74751618		6.51035838	0.46590503	5.33921556	4.33659525	6.10477211	4.4203897
MDC	0.58513068	2.12795774	2.41334778	2.77746347	13.1779023		11.335483	4.38901341	7.8711693	6.67379376
MIG	0.64147119	0.80757785							1.64856006	4.50655412
MIP-1 beta		0.90957621	0.817641				0.08869991	0.3767043		2.87693225
MIP-1 delta	0.44820891	0.18913354	0.14115127	0.09978957	0.11171473		0.42124967	0.31959216		0.15112955
RANTES	0.86027598	0.50367249	0.42700634	0.56669119	0.55897016		0.53611442	0.56131171		
SCF		0.58251931	0.42443086	0.36587477	0.62072135		0.42286969	0.30745083	0.2722828	0
SDF-1			0.72117473	0.61719137	0.4797532		0.2185629	0.53327833	0.47012666	0
TARC				0.86088515	0.27636004	0.43559191	0.00887278		2.75239353	0
TGF beta 1	2	0.55253678		0	0	0	0	0.08703289	3.42221633	0
TNF alpha	2			0	2.10429044	0	3.48059661	0.06977136	4.64865419	0
TNF beta					3.56776399	0.5238592	4.81954882		4.66549822	0
EGF					1.91350936				2.44339507	
IGF-1	0.71542253	0.83516928	0.84218797				0.89876453	0.64173842	2.03370101	0.26215625

B.	Nonsmokers (4 donors)					Smokers (4 donors)				
	Control	*S.a.*	*S.e.*	*P.a.*	*E.f.*	*Control*	*S.a.*	*S.e.*	*P.a.*	*Ef.*
ANG		0.54586723	0.64930025		0.78369758		0.10896788	0.53568176		0.9107759
OSM	0.31823493	0.1445977	0	0	0	1.68176507	0	0.08477497	0.60500954	
THPO	0.82046529	0.81800875	0.27901713	0	0		0.06254824	0.02077528	1.69111926	1.81870061
VEGF		0.82155761	0.66368393	0	0.2217423	0.89684459	0.02213209	0.25920904	0.4034554	0
PDGF BB	0.888176	0.64729867	0.76225893	0.5327948	0.5243965		0.28921179	0.83082566	0.8217078	
Leptin		2.32953034	2.71515497	0	0	0.80416821	0	1.68205762	3.89762527	0.26759708
BDNF	2	3.70344647	3.43915639	0	0.33598018	0	0.6832986		7.58594852	0
BLC	2	24.3542902	24.743544	0	15.8367718	0	34.2306544	0	73.922602	0
CCL23	1.43850509	1.83799052	1.60155678	0	2.87508747	0.56149491	2.68166176	0.57684922	4.13955195	0
Eotaxin 1		1.35476705	0.81659937	0.46943354	4.4101638	0.81610165	2.71529481	0.20683268	3.56187917	0.74988402
Eotaxin 2		1.3865331	1.4763291		2.6054033	0.78704512	2.05692847	0.6259036	2.49008847	0
Eotaxin 3					0.8797512			0.67061723	1.67038749	
FGF 4	1.24955812	0.14919624	0	0	0	0.75044188		0	0	4.20865713
FGF 6	1.72598621	0.75531275	0.25257002	0	0	0.27401379	0	0.19390116	0.77050205	3.06631971
FGF 7	0.91123173	0.91845505	0.89108523	0	0		0	0	0	0.5579955
FGF 9	0.59294146			0	0	1.40705854	0	0.29813322		1.79849194
Flt-3 Ligand	0	2.35057213	2.98018237	0	0	2	0	1.78484363	1.69517476	0
GDNF	0.79689766	1.74828108					1.80313021		4.1870376	0
HGF	0.35954572		0.31774477	1.69749306	3.75373392	1.64045428	2.13847977	0.4359969	3.98883557	0.52699432
IGFBP 1			0.6585376	0.74574735	2.15066644		0.5792117	0.50661159		
IGFBP 2				0.66834401	0.82762202	0.81602775	0.63336579	0.66358735	0.65173633	1.76248772
IGFBP 3	2	0.19782985	0.49726385	0	0	0		0.16751531	0	6.29640247
IGFBP 4	1.85108691		0.45243676	0	0	0.14891309	0	0.58984375	0	3.67776596
IL-16	1.68582019	2.10267231	2.18484757	0	0	0.31417981	0		0	0.65404908
IP-10	0.32449121	2.9116563	3.10019318	0	0		0	2.01042103	2.7980784	6.54255256
MIF	0	3.06337044		4.90468173		2	3.23823684	2.94874209	7.04493998	0
MIP-3 alpha	0	1.89772422	0.23221458	2.61004719	2.3119133	2	2.18255732	0	1.96243644	0
NAP-2	0.7263907		0.72658423	0.42405194	2.73925515		0.65001341	0.45688562	0.6698835	3.57311475
NT-3		0.79947602	0.47090871	0		0.79281703	0.02484909	0.02075037	0	2.39623252
NT-4	2	0.35119422		0	0	0	0	0.27920609	0	8.65353177
OPN		3.3067823	2.85085785	0	0	0.82204523	0	2.64068446	0	11.7735667
OPG				0	0		0		0.73274038	0.70494882
PARC	2	92.8225832	99.640969	0	0	0	0	80.7173709	0	130.6866
TGF beta 2	0.69165058	3.38461361	2.22162175	2.23783179	0		0	3.48929708	3.25198466	
TGF beta 3	0			3.03674208	0	2	2.06674767	2.59826776		0
TIMP-1	0.07587119	0.41642978		2.00108269	0.12379397	1.92412881	1.56519732	0.82663124	0	0
TIMP-2	0.59397313						0.75736539	0.54041878	0.01385173	0.56574938

Figure 10. Cytokine arrays were applied to verify the cytokines secreted by PBMCs between nonsmokers and smokers stimulated by the four types of inactivated bacteria. *There were 74 kinds of cytokines that have been checked (**A.** 37 kinds of cytokines and **B.** 37 kinds of cytokines). IL-10 and IL-4 were higher expressed in smokers than in nonsmokers. The concentration of PBMCs among each condition is 5×10^5 cells/ml (2% plasma). The control group with nothing added and four types of inactivated bacteria groups were respectively set as S. a. 4 μl/ml, S. e. 2 μl/ml, P.a. 2 μl/ml, and E. f. 1 μl/ml. Each colour unit represents the condition sample pool combined by four donors. Three colour gradation: green set as 0.2, black set as 1, and red set as 5.*

3.5. Cytokine levels released by PBMCs between nonsmokers and smokers among four types of inactivated bacteria condition were measured and the effect of plasma was checked.

3.5.1. Anti-inflammatory cytokines

3.5.1.1. IL-4

The result of the IL-4 ELISA experiment is demonstrated below in Figure 11. The graph **A** and graph **B** were drawn from the same data according to different grouping methods (graph **A** was grouped by different inactivated bacteria, while graph **B** was grouped by smoking or not). In figure 11-**A**, among the four control groups (nonsmokers Plasma-control, nonsmokers FCS-control, smokers Plasma-control and smokers FCS-control groups), the IL-4 level in the smokers FCS-control group was significantly higher than in the nonsmokers FCS-control group. When stimulated by *S. a.*, the PBMCs (incubated with FCS) from smokers produced significantly more IL-4 than the PBMCs (both with Plasma and FCS) from nonsmokers. Similarly, the PBMCs (incubated with FCS) that were stimulated by *P. a.* from smokers produced significantly more IL-4 than the PBMCs (both with Plasma and FCS) from nonsmokers. In addition, when stimulated by *E. f.*, the PBMCs (incubated with FCS) from smokers produced significantly more IL-4 than the PBMCs (with Plasma) from nonsmokers. Nevertheless, when treated with *S. e.*, he PBMCs (both plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing IL-4.

In figure 11-**B**, within each of the nonsmokers Plasma group, nonsmokers FCS group, smokers Plasma group, and smokers FCS group, when treated with the four types of inactivated bacteria respectively; the PBMCs did not produce significantly more IL-4 compared their own control group. There was a trend that the reaction of PBMC from nonsmokers to inactivated bacteria was more intensive than that from smokers.

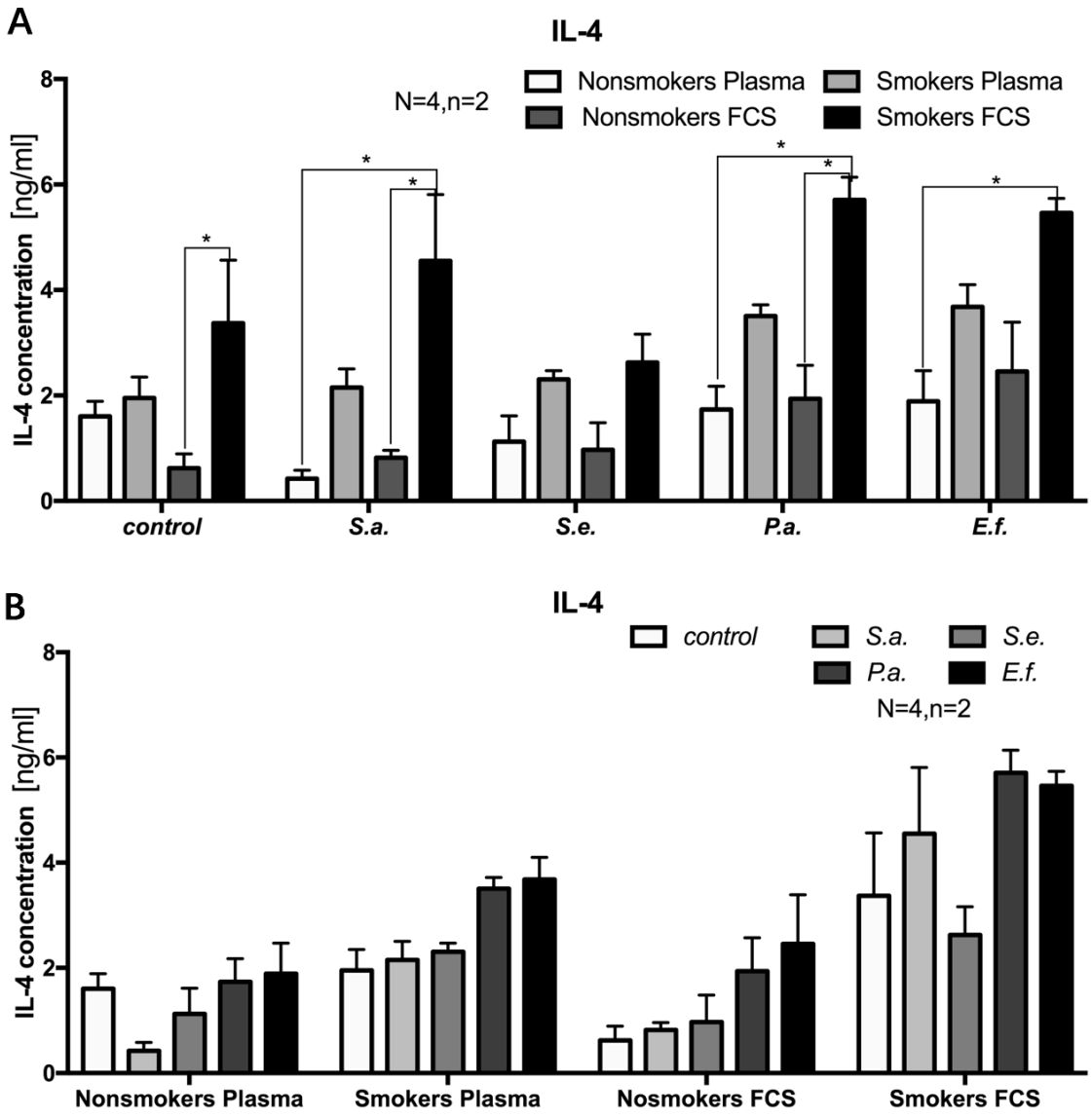


Figure 11. IL-4 released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

*The incubation time is 24 h. S. a. (Staphylococcus aureus 4 μl/ml), S. e. (Staphylococcus epidermidis 2 μl/ml), P. a. (Pseudomonas aeruginosa 2 μl/ml), and E. f. (Enterococcus faecalis 1 μl/ml). Each point represents the mean ± standard error of the mean (SEM) error bars of four healthy nonsmoking or smoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05; ***P < 0.0005 in one-way ANOVA (nonparametric) multiple comparisons.*

3.5.1.2. IL-10

The result of the IL-10 ELISA experiment is demonstrated below in Figure 12. In figure 12-**A**, without the stimulation of inactivated bacteria, the PBMCs from smokers produced higher levels of IL-10. Although insignificant, this trend was clear. There was no significant difference in IL-10 between the nonsmokers Plasma-*S. a.* group and the smokers Plasma-*S. a.* group. Likewise, when treated with the other three types of inactivated bacteria (*S. e., P. a.* and *E. f.),* respectively, the PBMCs from smokers and PBMCs from nonsmokers did not display significant difference in producing IL-10. There is a trend that the PBMCs could produce relatively more IL-10 when stimulated by smoking.

In figure 12-**B**, as for the nonsmokers Plasma group, all the nonsmokers Plasma treatment groups' PBMCs produced insignificantly more IL-10 than their own control group. Besides, when treated with the four types of inactivated bacteria, the PBMCs from smokers produced significantly less IL-10 than their own control group.

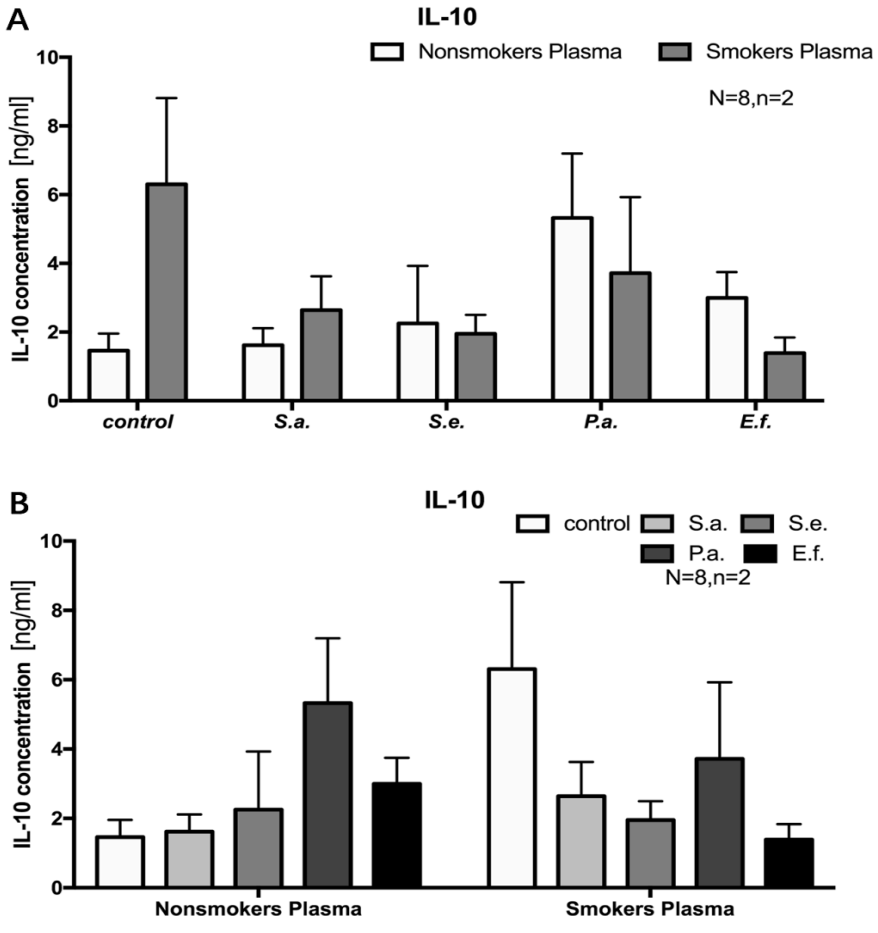


Figure 12. IL-10 released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

*The concentration of PBMCs among each condition is 5×10^5 cells/ml. The incubation time is 24 h. S. a. (Staphylococcus aureus 4 µl/ml), S. e. (Staphylococcus epidermidis 2 µl/ml), P. a. (Pseudomonas aeruginosa 2 µl/ml), and E. f. (Enterococcus faecalis 1 µl/ml). **A** and **B** were drawn from the same data according to different grouping methods (**A** was grouped by different inactivated bacteria, while **B** was grouped by smoking or not). Each point represents the mean ± SEM error bars of 8 healthy nonsmoking or smoking donors from duplicate experiments. There is no significant difference calculated in one-way ANOVA (nonparametric) multiple comparisons.*

3.5.1.3. IL-12

For the IL-12 ELISA experiment, the result is shown in Figure 13. In figure 13-A, among the four control groups: nonsmokers Plasma-control group, nonsmokers FCS-control group, smokers Plasma-control group and smokers FCS-control group, there was no significant difference in IL-12 levels. When treated with the four types of inactivated bacteria (S. a., S. e., P.a. and E. f.), respectively, the PBMCs (both Plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing IL-12.

In figure 13-B, within each of the three large groups (the nonsmokers Plasma group, smokers Plasma group, and smokers FCS group), when treated with the four kinds of inactivated bacteria, the PBMCs produced insignificantly more TNF-α than the PBMCs in their own control group. However, within the nonsmokers-FCS group, the PBMCs stimulated with P. a. produced a significantly higher level of IL-12 than in its own control group. There is also a trend that the reaction of PBMCs from nonsmokers to inactivated bacteria is significantly more strong than that from smokers.

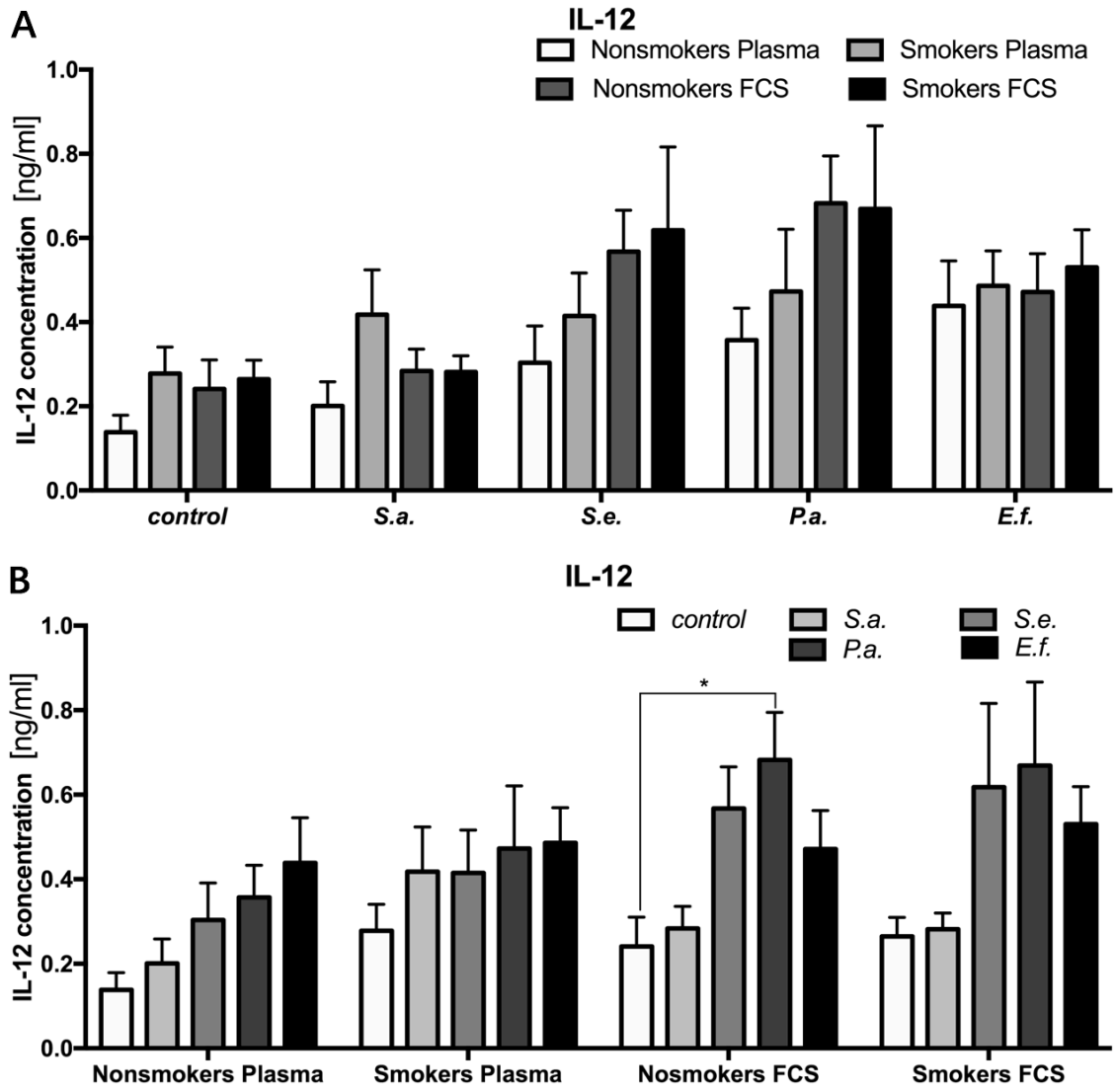


Figure 13. IL-12 released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

The incubation time is 24 h. *S. a.* (*Staphylococcus aureus* 4 μl/ml), S. e. (*Staphylococcus epidermidis* 2 μl/ml), *P. a. (Pseudomonas aeruginosa 2 μl/ml), and E. f. (Enterococcus faecalis 1 μl/ml). Each point represents the mean ± SEM error bars of 12 healthy nonsmoking or smoking donors from duplicate experiments.* ***A*** *and* ***B*** *were drawn from the same data according to different grouping methods (****A*** *was grouped by different inactivated bacteria, while* ***B*** *was grouped by smoking or not). Statistical significance is indicated by *P < 0.05(nonsmokers FCS-P. a. subgroup versus nonsmokers FCS-control subgroup).*

3.5.2. Pro-inflammatory cytokines

3.5.2.1. IL-1β

For the IL-1β ELISA experiment, the result is shown below in Figure 14. In figure 14-**A**, among the four control groups (nonsmokers Plasma-control group, nonsmokers FCS-control group, smokers Plasma-control group and smokers FCS-control group), there was also no significant difference in IL-1β levels. When treated with the four types of inactivated bacteria (*S. a., S. e., P.a.* and *E. f.)*, the PBMCs (both Plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing IL-1β.

In figure 14-**B**, within the nonsmokers Plasma group, the nonsmokers Plasma-*S. e.* group and nonsmokers Plasma-*P.a.* group PBMCs produced significantly more TNF-α compared to their own control group. Within the nonsmokers FCS group, when treated with the four kinds of inactivated bacteria, all the nonsmokers FCS treatment group PBMCs, except nonsmokers FCS-*S. a.* group, produced significantly more IL-1β than their own control group. However, within each of the two large smoking groups: the smokers Plasma group, and smokers FCS group, when treated with the four kinds of inactivated bacteria, the PBMCs produced insignificantly more IL-1β than in their own control group. There was a trend that the reaction of PBMCs from nonsmokers to inactivated bacteria was significantly more strong than that from smokers.

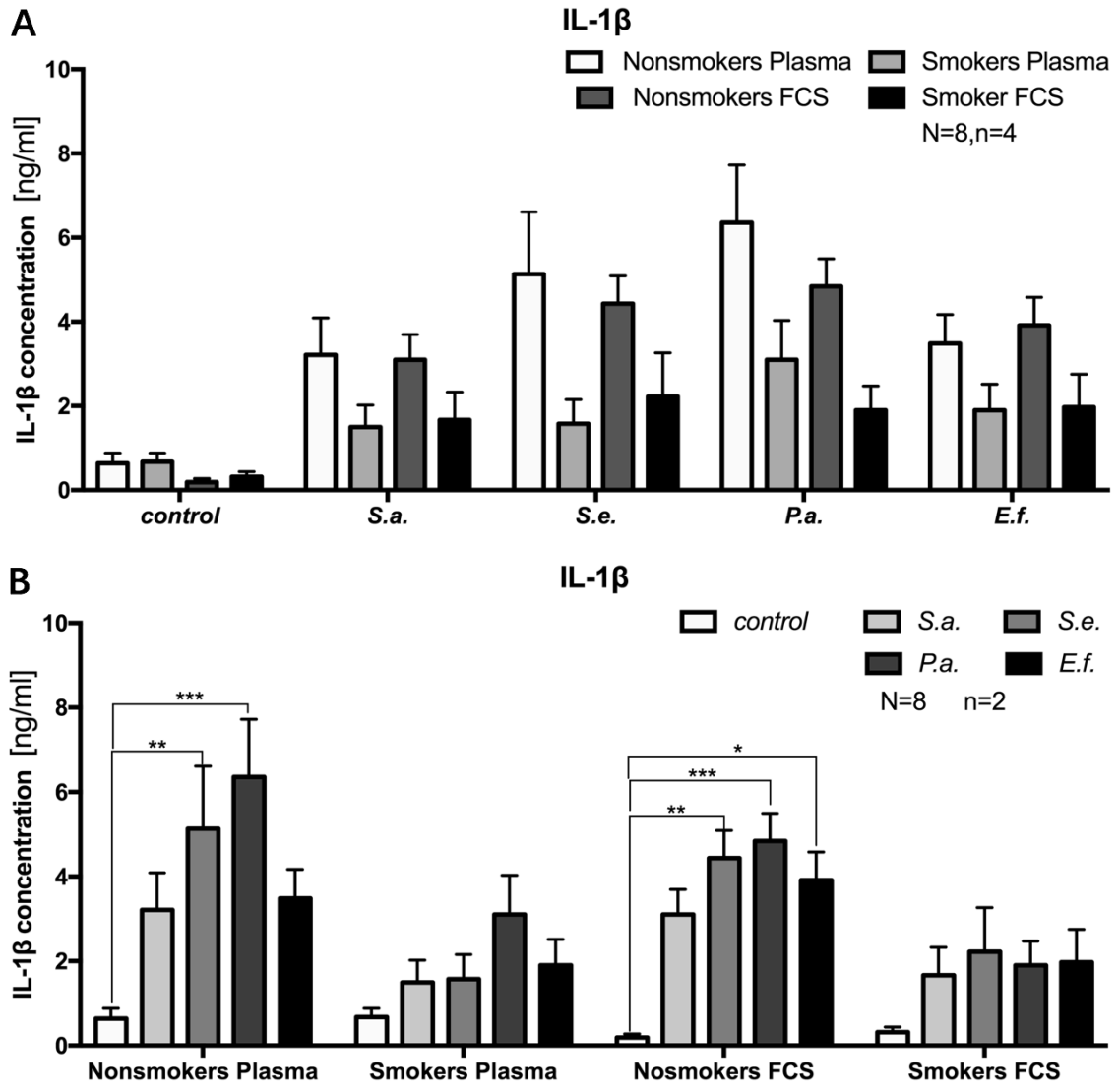


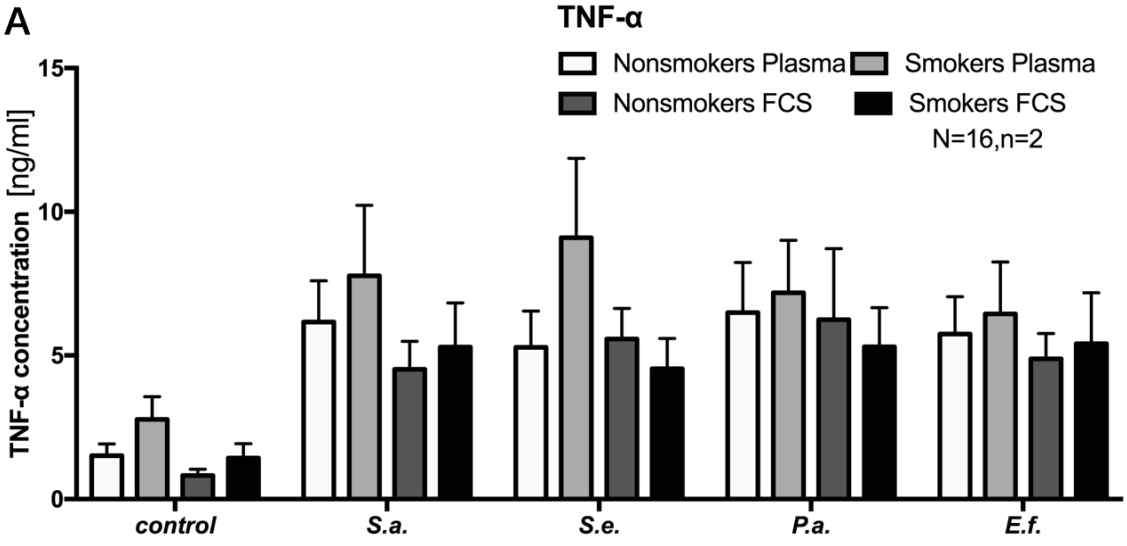
Figure 14. IL-1β released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

The incubation time is 24 h. S. a. (Staphylococcus aureus 4 μl/ml), S. e. (Staphylococcus epidermidis 2 μl/ml), P. a. (Pseudomonas aeruginosa 2 μl/ml), and E. f. (Enterococcus faecalis 1 μl/ml). ***A*** *and* ***B*** *were drawn from the same data according to different grouping methods (****A*** *was grouped by different inactivated bacteria, while* ***B*** *was grouped by smoking or not). Each point represents the mean ± SEM error bars of 8 healthy nonsmoking or smoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05; ***P < 0.0005 in one-way ANOVA (nonparametric) multiple comparisons.*

3.5.2.2. TNF-α

The result of the TNF-α ELISA experiment is demonstrated below in Figure 15. In figure 15-**A**, there was no significant difference in TNF-α levels among the following four control groups: nonsmokers Plasma-control, nonsmokers FCS-control, smokers Plasma-control and smokers FCS-control groups. When treated with the four types of inactivated bacteria (*S. a.*, *S. e., P.a.* and *E. f.),* respectively, the PBMCs (both Plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing TNF-α.

In figure 15-**B**, as for the nonsmokers Plasma group, when treated with the four types of inactivated bacteria, all the nonsmokers Plasma treatment group PBMCs, except nonsmokers Plasma-*S. e.* group, produced much more TNF-α than the nonsmokers Plasma-control group. However, in the smoker Plasma group, when treated with the four kinds of inactivated bacteria, all the smokers Plasma treatment group PBMCs did not produce significantly more TNF-α than the smokers Plasma-control group. As for the nonsmokers-FCS group, when treated with the four kinds of inactivated bacteria, all the nonsmokers FCS treatment group PBMCs produced much more TNF-α than the nonsmokers FCS-control group. However, in the smokers-FCS group, when treated with the four kinds of inactivated bacteria, all the smokers FCS treatment group PBMCs also did not produce significantly more TNF-α than the smokers FCS-control group. The trend that the reaction of PBMCs from nonsmokers to inactivated bacteria is significantly more intensive than that from smokers was clear.



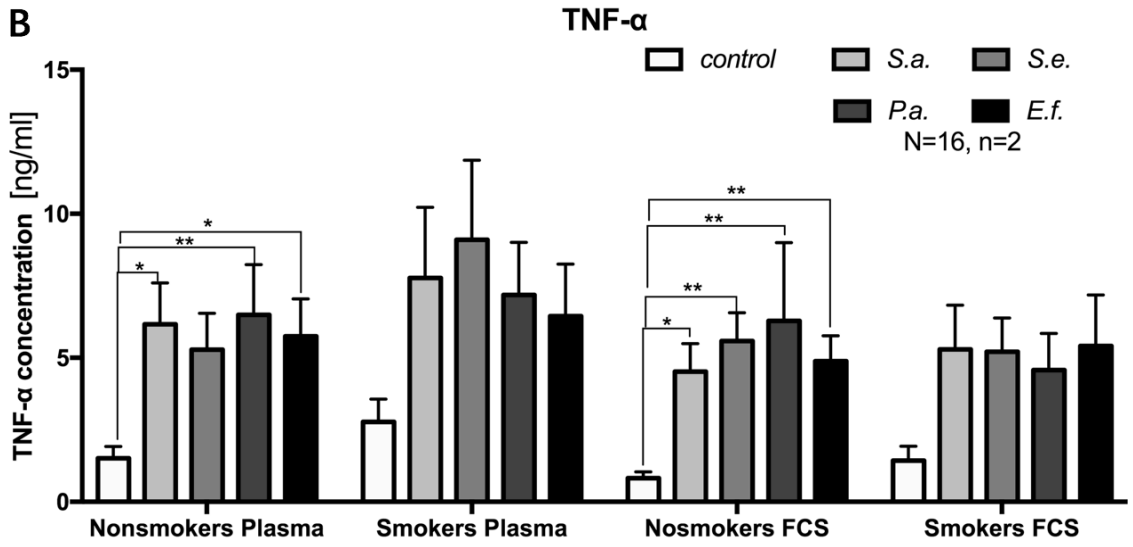


Figure 15. TNF-α released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

The incubation time is 24 h. S. a. (Staphylococcus aureus 4 µl/ml), S. e. (Staphylococcus epidermidis 2 µl/ml), P. a. (Pseudomonas aeruginosa 2 µl/ml), and E. f. (Enterococcus faecalis 1 µl/ml). ***A*** *and* ***B*** *were drawn from the same data according to different grouping methods (****A*** *was grouped by different inactivated bacteria, while* ***B*** *was grouped by smoking or not). Each point represents the mean ± SEM error bars of 16 healthy nonsmoking or smoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05; ***P < 0.0005 in one-way ANOVA (nonparametric) multiple comparisons.*

3.5.2.3. IFN-γ

For the IFN-γ ELISA experiment, the result is demonstrated below in Figure 16. In figure 16-**A**, among the nonsmokers Plasma-control group, nonsmokers FCS-control group, smokers Plasma-control group and smokers FCS-control group, the four control groups, there was no significant difference in IFN-γ levels. When stimulated with *S. a.,* PBMCs with FCS from nonsmokers produced higher level of IFN-γ than PBMCs with Plasma from nonsmokers. When treated with the other three types of inactivated bacteria (*S. e., P.a.* and *E. f.),* respectively, the PBMCs (both Plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing IFN-γ.

In figure 16-**B**, for the each of the four groups (nonsmokers Plasma, nonsmokers FCS, smokers Plasma, and smokers FCS), when treated with the four kinds of inactivated bacteria, all the treatment group PBMCs did not produce significantly different IFN-γ levels from its own control group.

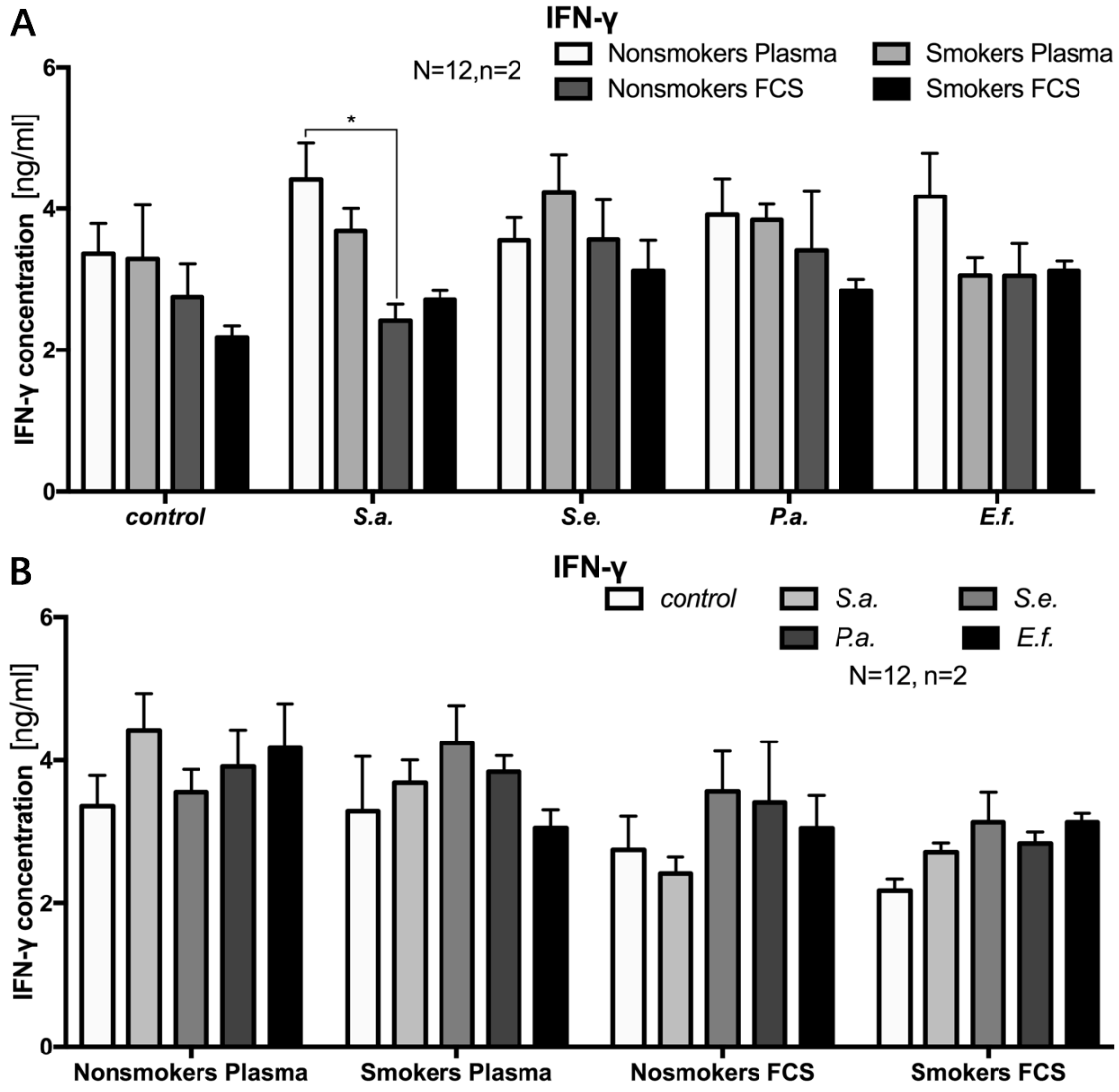


Figure 16. IFN-γ released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

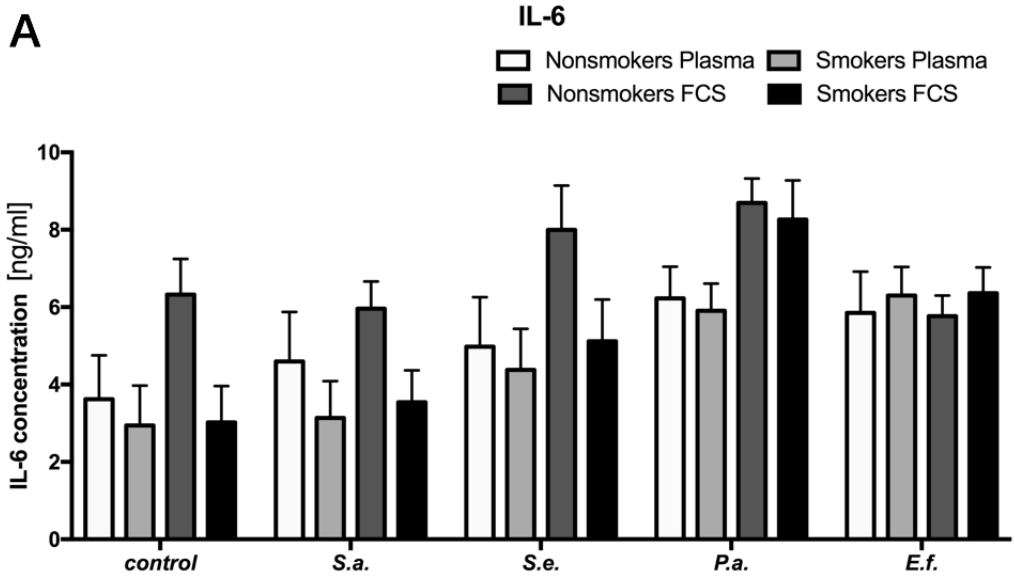
The incubation time is 24 h. S. a. (Staphylococcus aureus 4 μl/ml), S. e. (Staphylococcus epidermidis 2 μl/ml), P. a. (Pseudomonas aeruginosa 2 μl/ml), and E. f. (Enterococcus faecalis 1 μl/ml). ***A*** *and* ***B*** *were drawn from the same data according to different grouping methods (****A*** *was grouped by different inactivated bacteria, while* ***B*** *was grouped by*

*smoking or not). Each point represents the mean ± SEM error bars of 12 healthy nonsmoking or smoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05 (nonsmokers FCS-S. a. sub-group versus nonsmokers Plasma- S. a. sub-group).*

3.5.2.4. IL-6

For the IL-6 ELISA experiment, the result is demonstrated below in Figure 17. In figure 17-**A**, among the four control groups (nonsmokers Plasma-control, nonsmokers FCS-control, smokers Plasma-control and smokers FCS-control group), there was also no significant difference in IL-6 levels. When treated with the four types of inactivated bacteria (*S. a., S. e., P.a.* and *E. f.),* respectively, the PBMCs (both Plasma and FCS) from smokers and PBMCs (both Plasma and FCS) from nonsmokers did not display significant difference in producing IFN-γ.

In figure 17-**B**, as for the smokers Plasma group, when treated with the four types of inactivated bacteria, PBMCs in smokers Plasma-*E. f.* group, produced much more IL-6 than its own control group. In the smoker FCS group, when treated with the four kinds of inactivated bacteria, PBMCs from smokers FCS-*P. a.* group and smokers FCS-*E. f.* group produce significantly more IL-6 than their own control group. However, as for nonsmoking groups (nonsmokers Plasma group, nonsmokers-FCS group), when treated with the four kinds of inactivated bacteria, PBMCs did not produce significantly more IL-6 than their own control group.



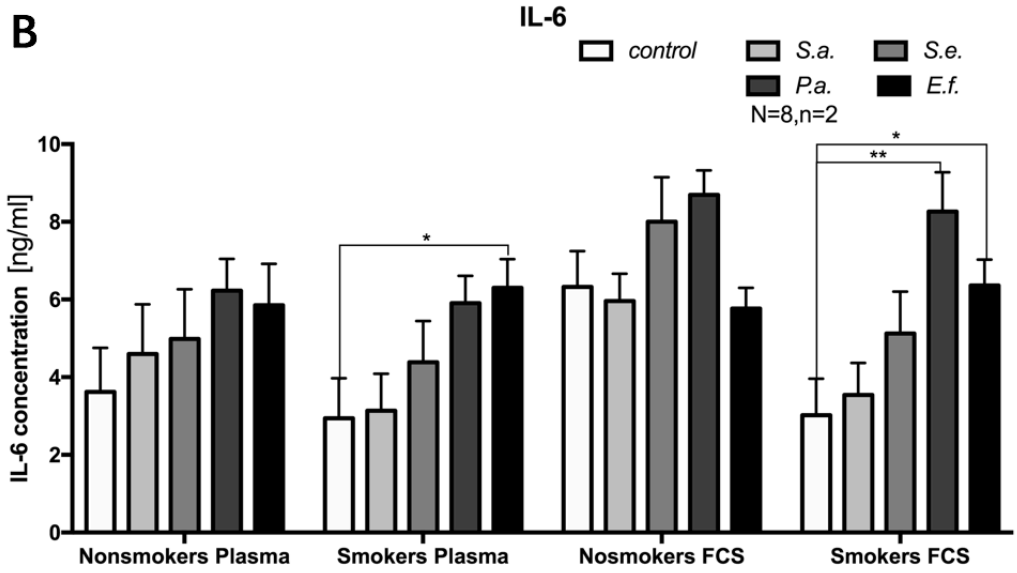


Figure 17. IL-6 released by PBMCs from nonsmokers and smokers stimulated by four types of inactivated bacteria.

The incubation time is 24 h. S. a. (Staphylococcus aureus 4 μl/ml), S. e. (Staphylococcus epidermidis 2 μl/ml), P. a. (Pseudomonas aeruginosa 2 μl/ml), E. f. (Enterococcus faecalis 1 μl/ml). ***A*** *and* ***B*** *were drawn from the same data according to different grouping methods (****A*** *was grouped by different inactivated bacteria, while* ***B*** *was grouped by smoking or not). Each point represents the mean ± SEM error bars of 8 healthy nonsmoking or smoking donors from duplicate experiments. Statistical significance is indicated by *P < 0.05; ***P < 0.0005 in one-way ANOVA (nonparametric) multiple comparisons.*

3.6. The appropriate concentration of HaCaT cells was 60,000 cells/well.

In order to ensure that the number of cells in each well in the following migration assay is the same and that at this number the cells were just enough to achieve confluence, we perform concentration-confluence experiment to determine this number. The result of the HaCaT cell concentration-confluence experiment showed that as the cell concentration is increased, the cells is grow to the confluence state. Only when the cells concentration reached 60,000 cells/well, could there be a confluence among the cells at the bottom of the well (Figure 18).

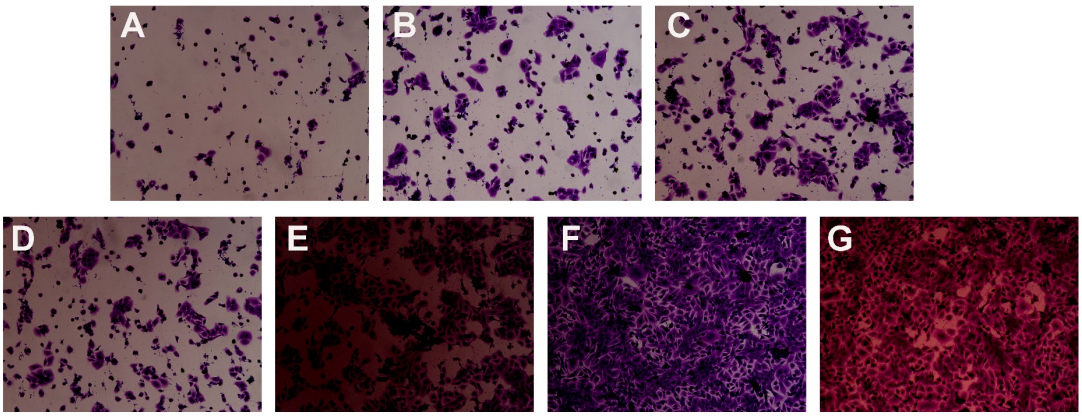


Figure 18. HaCaT cells cultured at different cell densities onto a 96-well plate to generate appropriate concentration that have just reached cell confluence.

The concentration gradient was set as follows: A. 10,000 cells/100 µl, B. 20,000 cells/100 µl, C. 30,000 cells/100 µl, D. 40,000 cells/100 µl, E. 50,000 cells/100 µl, F. 60,000 cells/100 µl, and G. 80,000 cells/100 µl.

3.7. IL-4 repressed the migration of HaCaT cells in Migration Assay

In order to determine whether IL-4 promotes or inhibits wound healing, the HaCaT cells migration assay had been performed. Each treatment group was used with PBMCs (FCS) supernatant, which were used in ELISAs. Seven nonsmoking supernatant samples and seven smoking supernatant samples were used in the migration assay with four times. Both the 0 point area and 24 h point area of the groups with nonsmokers (NS) PBMCs with FCS stimulated by four types inactivated bacteria (*S. a., S. e., P. a.*, and *E. f.*) and smokers (S) PBMCs with FCS stimulated by four types inactivated bacteria (*S. a., S. e., P. a.*, and *E. f.*) was measured (Figure 19).

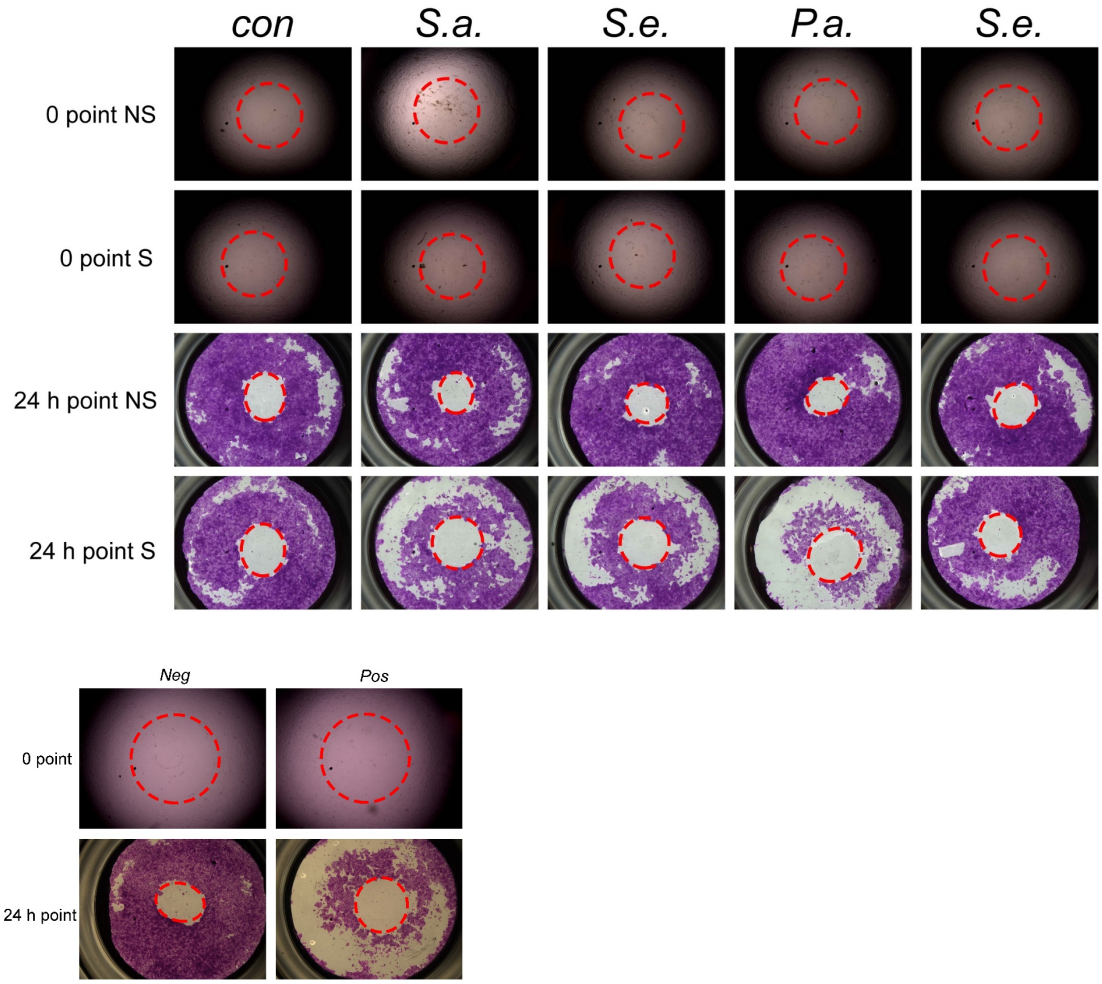


Figure 19. The baseline condition (0 point) and migrated condition (24 h point) of each group had been measured with T-scratch software.

NS: nonsmokers, S: smokers, Neg: negative, and Pos: positive.

The migration rate of each group was calculated using the migration function: (0 point area - 24 h point area)/0 point area ×100%. The result is shown below in Figure 20.

In Figure 20-**A**, the migration rate of the positive control group was the lowest among all the groups, and the migration rate of the negative control group, which was free from IL-4, was significantly higher than that of the positive group. It indicates that IL-4 had a negative effect on the movement of HaCaT cells and accordingly would inhibit the wound healing. All the inactivated bacteria stimulated groups nonsmokers large group had significantly higher migration rates than the positive control group. Furthermore, the migration rates in the nonsmokers inactivated bacteria stimulated groups were higher than

in smokers inactivated bacteria stimulated groups. Of particular note, the migration rate of the nonsmoking *P. a.* group was significantly higher than that of the smoking *P. a.* group (Figure 20-**C**). Besides, within the NS group, all the inactivated bacteria stimulated groups had insignificantly higher migration rates than their own control group (Figure 20-**B**). As the IL-4 level in smoking inactivated bacteria treated groups were higher than that in nonsmoking inactivated bacteria treated groups, which had been confirmed in the ELISA above, this can further explain to some extent why smoking is not conducive to wound healing.

Migration rate (%) (N=14, n=2)

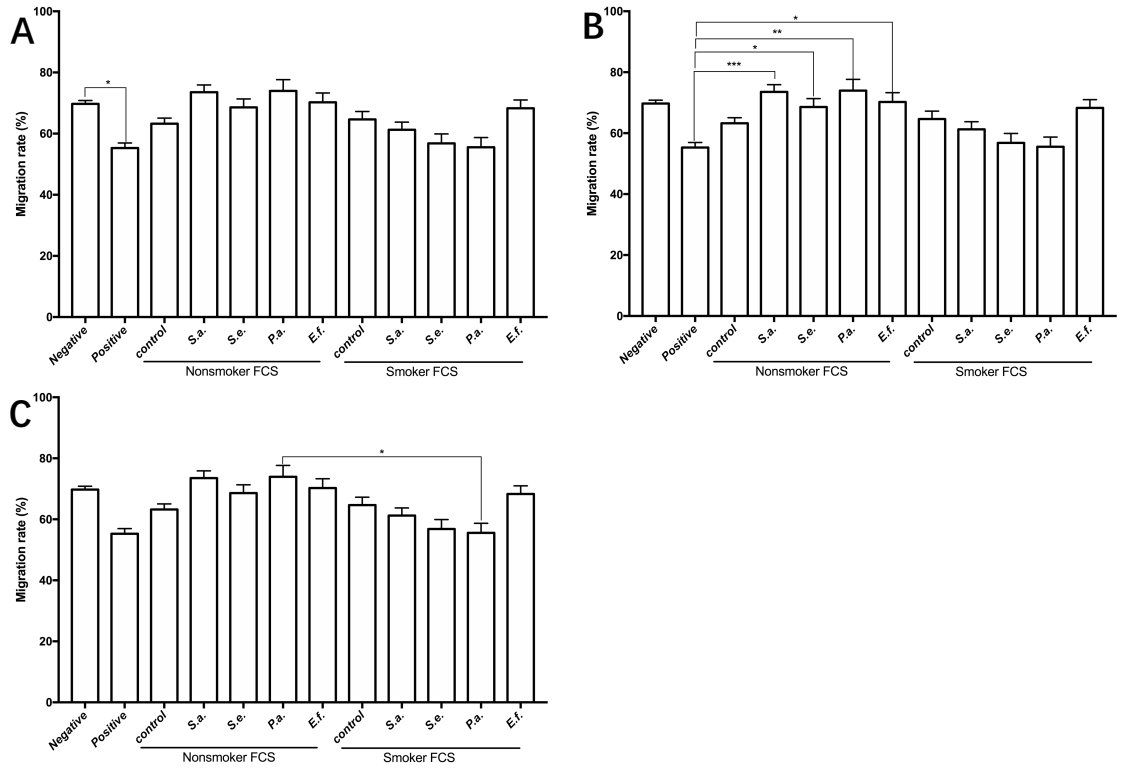


Figure 20. IL-4 decreased the migration rate and the migration rate in smoking group was lower than in nonsmoking group.

*The migration rate of each group was calculated. Each treatment group was used with PBMCs (FCS) supernatant, which were used in ELISAs. The data used in the three graphs above is the same. In order to avoid the all the significant difference markers being put together (it is not easy to interpret), the markers are separated in the three graphs (**A**, **B**, **C**). **A** showed that the positive control group (without IL-4) had lower migration rate than*

negative control group (with IL-4). ***B*** *exhibited that all the nonsmoking inactivated bacteria stimulated groups had significantly higher migration rate than positive control group.* ***C*** *demonstrated that migration rates in nonsmoking inactivated bacteria stimulated groups were higher than smoking inactivated bacteria stimulated groups especially the comparing P. a. subgroups. The PBMCs supernatant stimulated by four types of inactivated bacteria (S. a., S. e., P. a., and E. f.) from smokers and nonsmokers. The incubation time for migration is 24 h. Each point represents the mean ± SEM error bars of control group inactivated bacteria treated groups from 7 healthy nonsmoking and 7 smoking donors in duplicate experiments. Differences were regarded as statistically significant at values of *P < 0.05; ***P < 0.0005 in one-way ANOVA (nonparametric) multiple comparisons.*

4. Discussion

This pilot project aims to study the effects of smoking on inflammatory factors, in particularly the effects of IL-4 on wound healing. Due to the limited size of the study population, there was no attempt to balance other factors, such as gender and smoking status among the groups. The levels of various cytokines secreted by PBMCs have been used to estimate the true immune inflammatory response of the entire body, as this is also the method used in population biomonitoring studies (Le Meur *et al*., 1999, Arimilli *et al*., 2017, Prevost *et al*., 1990). In our ELISA experiments, we found that smokers show increased anti-inflammatory cytokines that have immunosuppressive effects. When stimulated by inactivated bacteria, PBMCs from smokers produce more both anti-inflammatory cytokines and pro-inflammatory cytokines. The experimental model about the influence of IL-4 on the HaCaT cell migration has been used to simulate the epidermal wound healing. In our HaCaT cell migration study, the migration of each group treated with different levels of IL-4 was measured. In general, IL-4 inhibited HaCaT cell migration, subsequently delaying wound healing. Smoking causes delayed wound healing (Czernin and Waldherr, 2003, Pitts *et al*., 1999), which can be explained by increased basal IL-4 levels in smokers.

4.1. Smoking increased anti-inflammatory cytokines.

First of all, it was detected by cytokine arrays that basal IL-4 and IL-10 was higher in the supernatant from PBMCs of smokers than those in supernatant from PBMCs of nonsmokers. This phenomenon is consistent with the conclusion that smoking could promote the production of more Th2 cytokines while suppressing the Th1 cytokines (de Heens *et al*., 2009, Lee *et al.*, 2012). IL-4 can induce the differentiation of Th0 cells to Th2 cells that will produce more IL-4, which is a positive feedback (Robays *et al*., 2009). As a key regulator in acquired immunity, IL-4 has the effect of stimulating the propagation of activated T cells, up-regulating production of major histocompatibility complex II (MHC II) and inducing proliferation of B cells that produce IgE, whereas reducing Th1 cells, macrophages (Hershey *et al*., 1997).

IL-10 that has multiple pleiotropic effects on inflammation and immune regulation is considered as an anti-inflammatory cytokine and is also known as cytokine synthesis inhibitory factor (CSIF) (Saxena *et al*., 2015). Although enhancing the antibody production, proliferation, maturation and survival of B cells, IL-10 down-regulates the MHC II antigens and expression of Th1 cytokines and stimulatory molecules on macrophages (Couper *et al*., 2008). IL-10 is able to repress the synthesis of pro-inflammatory cytokines, such as IL-1β, TNFα, IFN-γ, IL-2, IL-6 and GM-CSF, produced by Th1 T cells or macrophages (Opp *et al*., 1995, Aste-Amezaga *et al*., 1998, Varma *et al*., 2001). Furthermore, it also exhibits a potent capability to inhibit dendritic or other antigen presenting cells (de Waal Malefyt *et al*., 1991, Mittal and Roche, 2015).

IL-12, which is known as T cell stimulating factor, is a kind of anti-inflammatory cytokine (Hamza *et al*., 2010) and is mainly produced by activated antigen presenting cells, such as macrophages, dendritic cells, monocytes and B cells (Opstad *et al.*, 2017). It could induce the differentiation of Th0 cells into Th1 cells and activate the growth of T cells. Moreover, it promotes the T cells and NK cells to produce TNF-α and IFN-γ, and counteracts the suppression effect of IL-4 on production of IFN-γ. IL-12 intermediates the proliferation of $CD8^+$ CTL and elevated cytotoxic activity of NK cells. IL-12 secretion arises early in the innate and adaptive immune responses and appears to play a pivotal role in regulation of immunity and mediation of anti-tumor activity (Opstad *et al*., 2017). Low levels of IL-12 observed in smokers might have clinical implications regarding

inflammation and malignancy, which may partly explain why smokers are prone to COPD and lung cancer (Opstad *et al.*, 2017). It was reported by Lee *et al.* that cigarette smoking reduced the secretion of IL-12, which is a Th1 cytokine (Lee *et al.*, 2012).

4.2. Smoking decreased the pro-inflammatory cytokines

It was reported by Lee *et al.* that smoking reduced the Th1 cytokines (Lee *et al.*, 2012). Th1 cells could produce IFN-γ, whereas Th2 cells could produce IL-4, IL-6, and IL-10. However, Barbieri *et al.* referred to higher levels of IL-1β and of TNF-α in smokers (Barbieri *et al*., 2011). As for the results in our study, without the inactivated bacteria stimulation, the basal pro-inflammatory cytokines levels seem to have no significant difference between smokers and nonsmokers. However, the increase of pro-inflammatory cytokines in non-smokers is more obvious than that in smokers, when stimulated with inactivated bacteria, for the reaction of PBMCs to bacteria was reduced by smoking.

IL-1β (which is known as leukocytic pyrogen, lymphocyte activating factor, mononuclear cell factor or leukocytic endogenous mediator) (Bensi *et al*., 1987) is a member of the IL-1 super family of cytokines (Lopez-Castejon and Brough, 2011, Striz, 2017). Ouyang *et al.* has reported that smoking suppressed the production of IL-1β in PBMCs (Ouyang *et al.*, 2000). However, Moeintaghavi *et al.* reported that the IL-1β level was increased by smoking alone without other stimulating factors (Moeintaghavi *et al*., 2017). This was consistent with the result in our own IL-1β ELISA experiment, although the elevated IL-1β didn't increased much in the smokers compared to the nonsmokers. However, when stimulated with inactivated bacteria, the PBMCs from nonsmokers produced significantly much more IL-1β over the baseline, while the PBMCs from smokers didn't produce as much IL-1β. We concluded that smoking weakened the response of PBMCs to TLR-stimuli, which was supported by the result that TLR-stimulated inflammatory responses linked to the production of pro-inflammatory cytokines production were suppressed significantly by the combustible tobacco (Arimilli *et al*., 2017). TLR2/4 induced IL-1R-associated kinase-1 (IRAK-1), phosphorylated p38 and activated NF-κB was inhibited in smokers, while levels of TLR4 protein expression and TLR2, MD-2 mRNA and CD14 were not changed. The results indicate that the expression and activity of signaling intermediates were altered at the post-receptor level in relation to the immunosuppression caused by smoking (Chen *et al*., 2007).

TNF-α, that is also known as cachexia is one of the cytokines that initiates the acute phase response and also a cellular signaling cytokine involved in systemic inflammation. Although it can be produced by many cells such as NK cells, $CD4^+$ T cells, mast cells and neutrophils, it is mainly produced by activated macrophages (Olszewski *et al*., 2007). Substantial amounts of TNF-α are secreted in response to LPS, or other microbial products, and IL-1. In our TNF-α ELISA experiment, it was shown that smoking alone could stimulate PBMCs to release slightly more TNF-α, that is in agreement with data by Boström *et al.* (Boström *et al*., 1998). However, Ouyang *et al*. have reported that production of TNF-α by PBMCs was reduced in smokers (Ouyang *et al*., 2000). Nevertheless, there is no doubt that LPS or inactivated bacteria could stimulated PBMCs to secrete more TNF-α via TLR (Lin and Yeh, 2005, Cezário *et al*., 2011). The increased amount in nonsmokers was higher than in smokers, which could probably be explained by smoking having the ability to impaire the reaction of PBMCs to the TLR-stimuli (Sopori and Kozak, 1998).

IFN-γ, (also called type II interferon), which is predominantly produced by Th1 cells and NK cells, is essential for congenital and adaptive inmune responses against microbial infections. IFN-γ induces the macrophages to activate and could also promote the expression of MHC II molecules. Abnormal level of IFN-γ is linked to a variety of autoimmune diseases. It was believed that $CD4^+$ Th1 lymphocytes, $CD8^+$CTL, and NK cells predominantly produced IFN-γ (Schroder *et al*., 2004). Ouyang *et al*. reported that IFN-γ production was suppressed by smoking, whereas the basal IFN-γ level between smoking group and nonsmoking group didn't have much difference in our ELISA. LPS, which is the most abundant component within the cell wall of Gram-negative bacteria (Ngkelo *et al*., 2012), could stimulate PBMCs to release IFN-γ (Negishi *et al*., 2011). However, the response of releasing more IFN-γ which was made by PBMCs to inactivated bacteria in our study was, however, not obvious. This discrepancy may be due to our insufficient sample size. It is also possible that IFN-γ, which is a Th1 cytokine (Sopori and Kozak, 1998) could be inhibited by smoking (Ouyang *et al*., 2000). Besides, IFN-γ is also a pro-inflammatory factor (Poyraz *et al*., 2013) that could be induced by other cytokines such as IL-12 and IL-18 (Kannan *et al*., 2011). Perhaps it was the combined effect of these two factors that led to the change of IFN-γ being inconspicuous

in our study. As for this phenomenon that the IFN-γ production cannot be increased clearly by inactivated bacteria in our experiments, is probably due to smoking that is associated with the negative regulation effect on Th1 cytokines.

IL-6 is a pro-inflammatory cytokine that could be induced by LPS, TNF-α and IL-1 and serves as an indicator for systematized activation of proinflammatory cytokines (Frost *et al.*, 2003). However, IL-6 also has a anti-inflammatory property which downregulates the synthesis of proinflammatory cytokines such as TNF-α, IL-1, IFN-γ, MIP-2 and GM-CSF, whereas the effect of IL-6 on attenuating the synthesis of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) is faint (Xing *et al*., 1998). IL-6 is associated with generation of neutrophils in central blood and the production of the C-reactive protein (CRP) (Benedict *et al*., 2009). It induces the development of B cells and is antergic to the regulatory T cells (Srirangan and Choy, 2010). It was reported by Soliman *et al*. that smoking reduced the production of IL-6 (Soliman and Twigg, 1992). Within our ELISA experiments, the IL-6 was slightly decreased in smokers in the absence of stimulating inactivated bacteria, which was consistent in Soliman's conclusion. However, Koo and Han reported that smoking alone had stimulated bronchial epithelial cells to produce IL-6 (Koo and Han, 2016). IL-6 is mainly released by macrophages in reaction to specific bacterial molecules, which are known as PAMPs. Nevertheless, in our study, with the existence of inactivated bacteria, the response of PBMCs to release more IL-6 was not obvious. In the reasearch advocated by Arimilli *et al.*, TLR-stimulated LPS increased the production of IL-6, which was significantly decreased by smoking (Arimilli *et al*., 2017). This discrepancy could probably be associated with our insubstantial sample size since we performed a pilot study.

4.3. Implication of IL-4 in wound healing.

Wound healing is an crucial physiological procedure to sustain the completeness of the skin in animals or humans after trauma. It contains a complicated process that includes three successive yet overlapping stages: hemostasis and inflammation stage, propagation stage, and remolding stage (Wang *et al*., 2017). After skin damage the uncovered sub-endothelium, collagen and tissue factors will trigger platelet gathering, that induces degranulation and discharging chemokines and growth factors (GFs) to construct a clot, and all the aforementioned processes will contribute successfull hemostasis (Gauglitz *et*

al., 2011). Neutrophils, the first inflammatory cells to come to the location of the wound, purge bacteria or debris to supply a friendly milieu for wound healing (Wang *et al*., 2017). Next, macrophages aggregate and promote the phagocytosis of bacteria (Berman *et al*., 2017). The hemostasis and inflammatory stage often lasts 72 h. The subsequent propagation stage features an agggregation of substantial cells and aplenty conjunctional tissue. The wound encloses the keratinocytes, endothelial cells and fibroblasts. Extracellular matrix (ECM), which contains collagen, elastin, hyaluronic acid and proteoglycans, constructs a granulation tissue to substitute for the primarily formed clot (Su *et al*., 2010). Various cytokines are involved during wound healing, including the interleukin family (such as IL-4, IL-6), TGF family (such as TGF-β, which includes TGF-β1, TGF-β2, and TGF-β3), and angiogenic factors (such as vascular epidermal growth factor [VEGF]). This second stage takes place from days to weeks (Su *et al*., 2010). Any abnormality during this process may lead to delayed wound healing. The final procedure is the remodelling stage, which requires an accurate equivalence between generation of new cells and apoptosis of old cells (Wang *et al*., 2017). Progressive degeneration of profusive ECM and development of type I collagen and the premature type III collagen are essential in this stage, which endures from a few months to years (Plikus *et al*., 2017, Tsai *et al*., 2018).

The focus of our project was mainly on the second phase, within which many cytokines have critical effects. Among these cytokines, IL-4 is reported contradictorily about whether it promotes the wound healing. For example, in the study advocated by Salmon-Ehr *et al*., local administration of IL-4 on deliberate wounds in rats significantly speeded up the healing, whereas an IL-4 antagonist significantly delayed the healing (Salmon-Ehr *et al*., 2000). However, Serezani *et al*. reported that IL-4 could induce delayed re-epithelialization changes, and impair the response of keratinocyte to the wound by repression of fibronectin (Serezani *et al*., 2017). Thereby, we choose IL-4 as our research target and investigated the role of IL-4 in the activaty of keratinocytes. Actually, IL-4 was found to have a suppressive effect on HaCaT cells migration. The migration rate in the HaCaT cells groups that were stimulated with the nonsmokers' PBMCs supernatant was found to be higher than in the groups that were stimulated with smokers' PBMCs supernatant. It corresponded to the results of cytokine array and ELISA that IL-4 level

was higher in smokers compared to nonsmokers. Combining the above conclusions, we can infer that elevated IL-4 could probably be the one of important factors underlying the mechanism whereby smoking leads to delayed wound healing. Th2 cytokines, such as IL-4 and IL-13, were reported to downregulate the expression of genes encoding the structures of epidermal cells and their barrier function at the final phase of keratinocyte differentiation (Omori-Miyake *et al*., 2014). At the early phase of keratinocyte differentiation, IL-4 attenuates the expression levels of keratin, desmocollins, and desmogleins through the IL-4 receptor and STAT6 signalling pathway (Omori-Miyake *et al*., 2014). The p44/42 MAPK-dependent mechanism is a prerequisite for the IL-4-induced reduction of expression of keratin (Omori-Miyake *et al*., 2014). Besides, the diminished mRNA expression level of desmogleins and desmocollins in keratinocytes stimulated by IL-4 may be the consequence of a reduction in keratin (Wallace *et al*., 2012).

4.4. Limitation

Admittedly, the sample size used in our pilot study is not very large, for ELISA and the cytokine array. Furthermore, because the blood we used was collected from each person on the spot, not taken from the blood bank, the concentration of PBMCs in our study was less than that in previous researches (Ouyang *et al*., 2000). As a matter of fact, we have only investigated seven kinds of cytokines. We firstly planned to perform cytokine arrays to have a general idea about the situation of 74 different cytokines between smokers and nonsmokers. Then, we used ELISAs to specifically detect several cytokines of interest. Besides, the method of investigating the influence of smoking on PBMCs in our research was taking blood from smokers and nonsmokers. Although the smoking PBMCs taken from our smokers directly could truly reflect the PBMCs in smokers, the age, gender, smoking history and intake of donors might have influenced our results. In our migration assay experiment, we have connected smoking, IL-4 and wound healing. Even though the smoking-*E. f.* sub-group had the highest level of IL-4 production, however, the migration rate in this sub-group was not, as expected, attenuated. IL-4 contained in the supernatant of the PBMCs was not comprehensively negatively related to the migration rate. The reason may be because many other factors, such as growth factor and ROS, (Avezov *et al*., 2014) are involved in the supernatant of the PBMCs. Thus, some factors contained in the PBMCs supernatant also had an effect on the mobility of HaCaT cells. The complexity

regarding the comprehensive function of the multiple factors in wound healing, together with the single factor effect we probed, made our present experiment count as pilot study. In the light of the mechanism of the process of smoking affecting the inflammatory responses and immunity, the signalling pathways through which the smoking changes the mRNA expression of various cytokines needs to be further investigated. Seeing that our *in vitro* HaCaT cells migration assay couldn't adequately simulate and reproduce pathological changes in wound healing *in vivo*, the following schedules may include the establishment of animal models.

4.5. Conclusion

In this pilot project we aimed to clarify several questions as how cytokines were changed in smokers compared to nonsmoker. In addition, we had investigated how would smoking alter anti-inflammatory and pro-inflammatory cytokines, and how this alteration would be affected when the inactivated bacteria were present. Furthermore, we also determined whether IL-4 promotes or inhibits wound healing, and whether increased IL-4 in smokers contributed the delayed wound healing. In our research, it was PBMCs that mainly generated the cytokines. It was verified through cytokine arrays that smoking actually altered many of the 74 kinds of cytokines secreted by PBMCs stimulated by four types of inactivated bacteria. Above all, our cytokine arrays showed that IL-4 and IL-10 both of which are immune-regulatory cytokines was higher in smokers than in nonsmokers. As cytokines could be divided into pro-inflammatory cytokines (*e.g.,* TNF-α, IFN-γ, IL-6, and IL-1β) and immune-regulatory cytokines (*e.g.,* IL-4, IL-10, and IL-12), ELISA was to be performed to specifically investigate the abovementioned seven cytokines. Without inactivated bacteria stimulation, smoking could downregulate the pro-inflammatory cytokines and upregulate the immune-regulatory cytokines. Inactivated bacteria (*S. a., S. e., P. a.*, and *E. f.*) could stimulate PBMCs to produce more cytokines with elevations in both pro-inflammatory cytokines and immune-regulatory cytokines. However, smoking could repress the ability of PBMCs to react to inactivated bacteria to increase the expression of cytokines (both immune-regulatory cytokines and pro-inflammatory cytokines). IL-4, which was increased in the smokers' PBMCs supernatant as well as inactivated bacteria, was found to inhibit the migration of HaCaT cells. We

could speculate that IL-4 plays an important role in the mechanism underlying smoking-induced delayed wound healing. After all, our research just acts as a window for understanding the relationship among smoking, immunity and wound healing. In the following steps, the signaling pathway by which smoking induces the alteration of cytokines along with how IL-4 affects wound healing needs to be inquired. The animal models need to be established to investigate the in vivo wound healing mechanism, which could be transferred to patients. Certainly, several methods or materials are required to be updated or improved to design a guide to clinical treatment and prevention while improving the quality of life of patients.

5.1 Summary

Infection and delayed wound healing are two common complications in the clinic, which are more likely to happen due to smoking. Inflammation plays a key role in natural tissue repair, as well as being elemental to the host's defense against microbial infection. Smoking induces inflammation and changes the congenital and acquired immune responses, which contributes to the increased occurrence of infection and poor wound healing. Therefore, comprehensive understanding of how smoking influences immunity along with wound healing is clinically essential for prevention and treatment. The purpose of our study is to inquire as to the mechanism by which smoking affects immunity and wound repair. Blood was taken from healthy non-smoking and smoking donors, and the PBMCs were isolated. The supernatant which was used as samples in ELISA, cytokine array and migration assay, was acquired after the PBMCs were first isolated and then incubated with four types of inactivated bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Enterococcus faecalis*) for 24 h. The cytokine array showed that the levels of IL-4 and IL-10 were higher in the supernatant from smokers' PBMCs than those in the supernatant from non-smokers' PBMCs. Nonsmoking PBMCs with inactivated bacteria exposure produced significantly higher cytokines (*e.g.,* IL-1β and TNF-α) than nonsmoking PBMCs without inactivated bacteria exposure. Whereas, smoking PBMCs with inactivated bacteria exposure didn't exhibit this trend. As for the migration assay, the migration rate of the positive control group (with IL-4) was lowest among all the groups, and the migration rate in inactivated bacteria stimulated groups of smokers was lower than that of non-smokers. In conclusion, smoking alone seems to downregulate the pro-inflammatory cytokine response and upregulate the anti-inflammatory cytokine response. Though, inactivated bacteria could increase various cytokines released by PBMCs both in non-smokers and smokers, however, smoking seems to repress the reaction ability of PBMCs to inactivated bacteria to increase the expression of cytokines (both anti-inflammatory cytokines and pro-inflammatory cytokines). Moreover, our data suggest that IL-4 repressed the migration of the HaCaT cells and may, thus, impairing wound healing. Multiple mechanisms that may be responsible for the relationship among smoking, immunity and wound healing needs to be further investigated to guide clinical treatment and prevention.

5.2. Zusammenfassung

Es gilt als gesichert, dass Rauchen verschiedene destruktive Auswirkungen auf den menschlichen Körper hat. Infektionen und Wundheilungsstörungen sind zwei häufige klinische Komplikationen, die scheinbar durch das Rauchen begünstigt werden. Entzündungsprozesse spielen eine Schlüsselrolle in der physiologischen Gewebewiederherstellung. Des Weiteren sind sie elementar für die Immunabwehr gegenüber mikrobiellen Infektionen. Rauchen verändert diese Entzündungsprozesse und verändert somit die angeborenen und erworbenen Immunreaktion. Bei Rauchern treten erhöht Infektionen und Wundheilungsstörungen auf. Daher ist ein umfassendes Verständnis der Pathologie, wie das Rauchen das Immunsystem und die Wundheilung negativ beeinflusst, aus klinischer Sicht essentiell, um zukünftige Behandlungsstrategien zu definieren. Deshalb war das Ziel der vorliegenden Studie, zunächst Mechanismen mittels derer das Rauchen auf das Immunsystem und die Geweberegeneration Einfluss nimmt, zu untersuchen. Hierzu wurde Blut von ‚gesunden' Rauchern und Nichtrauchern entnommen und die PBMCs (=Peripheral blood mononuclear cells/ dt. mononukleäre Zellen des peripheren Blutes) isoliert und kultiviert. Diese PBMCs wurden dann mit hitzeinaktivierten typischen Krankenhauskeimen (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Enterococcus faecalis*) inkubiert. Anschließend wurde der Überstand mittels verschiedener ELISAs, Zytokin-Arrays und Migrationsarrays untersucht. Unsere Ergebnisse der Zytokin-Arrays zeigen eindeutig, dass PBMC-Kulturüberstande von Rauchern höhere Interleukin (IL)-4 und IL-10 Spiegel sezernieren, als die von Nichtrauchern. Nach Inkubation mit den vier inaktivierten Bakterienarten produzierten die PBMCs von Nichtrauchern darüber hinaus signifikant erhöhte Konzentrationen an IL-1β und Tumornekrosefaktor (TNF)-α als die der Raucher. PBMCs von Nichtrauchern die durch inaktivierte Bakterien stimuliert wurden, sezernierten deutlich höhere IL-1 β und TNF- α Spiegel als PBMCs von Nichtrauchern, die nicht von inaktivierten Bakterien stimuliert wurden. Allerdings produzierten PBMCs von Rauchern nicht signifikant mehr von diesen beiden Zytokinen, wenn sie von inaktivierten Bakterien stimuliert wurden, als PBMCs von Rauchern, die nicht von Bakterien stimuliert wurden.

Im Migrationsassay war die Migrationsrate der positiven Kontrollgruppe (mit IL-4) die niedrigste aller Gruppen und die Migrationsrate der durch inaktivierte Bakterien stimulierte Rauchergruppe niedriger als die der Nichtraucher. Schließlich kann Rauchen allein die proinflammatorischen Zytokine herunterregulieren und entzündungshemmende Zytokine aufheben. Obwohl inaktivierte Bakterien die Freisetzung von mehr Zytokinen von PBMCs sowohl bei Rauchern als auch bei Nichtrauchern fördern können, besteht die Reaktion von PBMCs auf inaktivierte Bakterien darin, den Ausdruck von Zytokinen (entzündungshemmende Zytokine und pro-inflammatorische Zytokine) zu erhöhen, Rauchen kann diese Reaktion hemmen. IL-4 kann die Migration von HaCaT-Zellen hemmen und die Wundheilung beeinträchtigen. Man sollte jedoch bedenken, dass es noch eine Vielzahl weiterer pathologischer Mechanismen gibt, die durch das Rauchen, wie die Immunität und die Geweberegeneration, beeinflussen. Diese müssen jedoch noch weiter identifiziert werden.

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7. Declaration

This work was conducted in the laboratory of Siegfried Weller Institute in Tübingen University for biochemistry under the direction and support of my supervisor Prof. Dr. Andreas Nüssler.

I declare that all the experiments were performed by myself and all the data were calculated by myself.

The MD thesis: “Influence of cigarette smoking on immune response of PBMCs to inactivated bacteria and on wound healing” is totally written by myself. Any sources from others were all quoted in the sentence and added with my own understanding, not a simple repetition.

Tübingen, 01.08.2019

Zi Li

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Finally, I thank my parents who have always encouraged and supported me throughout the overseas learning stage. I hope that I could accompany them at any time in the future and compensate them for things I have not done during the past several years.

10. Curriculum Vitae

Zi Li

Education

- Tübingen University — *Jul. 2018-Dec. 2019*
  Doctor in Medicine, Tübingen, Germany
- Huazhong University of Science and Technology — *Sept. 2014-Jun. 2017*
  Master in Orthopedic Surgery, Wuhan in Hubei, China
- Huazhong University of Science and Technology — *Sept. 2009-Jun. 2014*
  Bachelor in Clinical Medicine, Wuhan in Hubei, China
- Jianli First Senior High School — *Sept. 2006-Jun.2009*
  Senior high school student, Jingzhou in Hubei, China
- Xinjian Junior Middle School — *Sept. 2003-Jun.2006*
  Junior middle school student, Jingzhou in Hubei, China
- Xiaqiao Primary School — *Sept. 1997-Jun.2003*
  Elementary school student, Jingzhou in Hubei, China

Clinical Experience

Sept. 2014- Jul. 2018 Worked as an orthopedic resident
Tongji Hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology

Professional & Research Skills

PROFESSIONAL SKILLS

Percutaneous Endoscopic Lumbar Discectomy (PELD)
Debridement and Suturing
Vertebroplasty
Kyphoplasty

RESEARCH SKILLS

Immunohistochemistry
PCR
Western blot
ELISA

Research Experience

Silver-loaded nanotubular structures enhanced bactericidal efficiency of antibiotics with synergistic effect in vitro and in vivo. *01. 2015-06. 2016*

(Funded by the National Natural Science Foundation of China)

Supervisor: Prof. Dr. Xiong Wei, Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Objective: To determine the synergistic antibacterial effect of antibiotics and Ag nanoparticles against various bacterial strains in vitro and in vivo.

Methods: We combined the commonly used antibiotics and Ag nanoparticles with systemic test of the synergistic bactericidal effect against four bacteria species, including representative gram-negative Escherichia coli (ATCC25922), gram-positive S, aureus (ATCC25923), and MRSA strains (ATCC33591 and ATCC43300). Less quantity of Ag nanoparticles was incorporated into TiO 2-nanotubes (NTs; Ag-NTs), which minimized the risk of toxicity due to high concentrations of Ag ion. An implant infection model was built in a rat to demonstrate the enhanced antibacterial efficiency in vivo.

Main works: Cultivating and seeding bacterial cells, establishing animal models, immunohistochemical process.

Differentiating malignant from osteoporotic vertebral fractures scoring system based on MRI and CT *04. 2015-11. 2017*

***Supervisor*:** Prof. Dr. Xiong Wei, Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

***Objective*:** Our research was designed to obtain a scoring system by combining several MRI and CT signs to promote the differential diagnosis between MVFs (malignant vertebral fractures) and OVFs (osteoporotic vertebral fractures).

***Methods*:** A total of 150 MVFs and 150 OVFs in thoracolumbar vertebrae were analyzed. MRI and CT images were obtained within 2 months of the probable time of fracture. The sensitivity and specificity of 15 MRI and CT image findings were evaluated. A stepwise discriminant analysis using these signs as variables was used to create a scoring system to differentiate MVFs from OVFs.

***Main works*:** Computed tomography, Discriminant analysis, Magnetic resonance imaging, Malignant vertebral fracture, Osteoporotic vertebral fracture.

Publication

One original article has been published by me with the first author's identity as follows:

Title:

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A novel MRI- and CT-based scoring system to differentiate malignant from osteoporotic vertebral fractures in Chinese patients

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