CHAPTER II

LITERATURE REVIEW

2.1 Innate immunity

2.1.1 Introduction

It is tradition to divide the host immune responses to infection into 2 compartments, the innate and the adaptive immunity. The innate immunity is the first line of defense against infection and perceived as a primitive non-specific protective response. The mechanisms of the innate responses are surface defenses, cytokine elaboration, complement activation, and phagocytic responses. On the contrary, the adaptive immunity is a more slowly developing and highly evolved antigen-specific protective response. T and B lymphocytes are key cells to generate efficient clonal expansion for the adaptive immune response against microbes and this process takes 3 to 5 days. For this reason, containing the infection until the lymphocytes can begin to deal with it has long been thought as the main function of the innate immunity. During the past decades, researchers begin to realize that the innate immune response has a much more important and fundamental role in host defense. Activation of the innate immune system is a prerequisite for triggering and directing the type of the adaptive responses (Th1 or Th2). The greater degree of specificity in the activation of the innate immunity has now been well recognized than it was previously thought. This remarkable discrimination between the host and pathogens by the innate immune cells

relies on the powerful screening tools of a sophisticated family of evolutionarily conserved receptors known as the Toll-like receptors (Medzhitov and Janeway, 2000).

2.1.2 Toll-like receptor

The TLRs are a class of PRRs with unique functions in the innate and adaptive immune systems. The innate immune response has evolved as the immediate host defense system in response to foreign structures and it also serves to prime the adaptive immune response. *Toll* gene products were first discovered in 1985 as essential component of the pathway that establishes the dorsal-ventral axis of the early Drosophila embryo (Anderson et al., 1985a; 1985b) and plays an important role in the response of larval and adult Drosophila to microbial infections (Anderson, 2000). After that they described the Toll receptor as a type 1 transmembrane receptor that controls dorsal-ventral polarity during embryogenesis in *Drosophila melanogaster* flies (Stein et al., 1991). Further work revealed that these receptors, which are required for morphogenesis, also control the activity of antimicrobial peptide synthesis in these flies. Later it was found that Toll was part of the innate immune response of Drosophila to bacteria and fungal pathogens (Douglas et al.,2004). Toll-deficient flies became exquisitely susceptible to fungal infections but not Gram-negative bacterial infection (Hoffman et al., 1997). The homologues structures of Drosophila Toll were called Toll-like receptors that identified in mammals (Medzhitov et al.,1997).

Now it is clear that TLRs function as key pattern recognition-receptors of the innate immune system (Janeway and Medzhitov, 2002). They recognize

and distinguish highly conserved structures present in large groups of microorganisms. To date 10 TLRs in humans and 12 TLRs in mice have been described (Beutler, 2004). Each of the 10 known human TLRs is believed to respond to a distinctive aspect of microbial infection. As specificity for broad categories of PAMPs is provided by a relatively limited diversity of TLRs, in some cases combinations of TLRs are required for recognition of certain PAMPs (Ozinsky et al., 2000; Hajjar et al., 2001). For example, TLR2 forms heterophilic dimers with TLR1 and TLR6 to recognize diacyl and triacyl lipopeptides, respectively (Takeda and Akira, 2005). Many other human TLRs and their ligands are known as shown in table 1. For example TLR2 recognizes peptidoglycan, TLR3 recognizes viral double-stranded RNA, TLR4 recognizes LPS, TLR5 recognizes flagellin, TLR7 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial DNA. It should be noted that cell surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) seem to recognize microbial products whereas intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize nucleic acids. (Table 1)

Table 1: Human Toll-like receptors and their ligands

Receptor	Ligand	References
TLR1	Triacyl lipopeptides	Takeuchi et al.,2002
TLR2	Lipoprotein/lipopeptides	Aliprantis et al., 1999
	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Porphyromonas gingivalis lipopolysaccharide	Hirschfeld et al., 2001
		Asai et al., 2001
	Porphyromonas gingivalis fimbriae	Means et al., 1999
	Mycobacterial lipoarabinomannan	Underhill et al., 1999
	Zymosan	
TLR3	Double-stranded RNA	Alexopoulou et al., 2001
	Polyinosine-polycytidylic acid	Alexopoulou et al., 2001
TLR4	*Escherichia coli* Lipopolysaccharide	Tapping et al., 2000
	Porphyromonas gingivalis LPS	Darveau et al., 2004
TLR5	Flagellin	Gewirtz et al., 2004
TLR6	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Diacyl lipopeptides	Takeuchi et al.,2002
	Zymosan	Ozinsky et al., 2000
TLR7	Imidazoquinoline	Hemmi et al., 2002
TLR8	Single-stranded RNA	Heil et al., 2004
	Imidazoquinoline	Jurk et al., 2002
TLR9	Bacterial DNA	Bauer et al., 2001
	CpG Oligonucleotides	Hemmi et al.,2000
TLR10	N.D.	

N.D. = not determined

Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct the development of antigen-specific acquired immunity. Current information suggests that TLR signaling pathways are divergent, although Myeloid differentiation primary-response protein 88 (MyD88), a key adaptor molecule, is used by most TLRs and this signaling pathway is called MyD88-dependent. Stimulation of TLR3 and some TLR4 utilize different adaptor molecules and the signaling pathway is called MyD88-independent pathways (Takeda and Akira, 2005).

TLRs are predominantly expressed on cells of the innate immune system, including neutrophils, monocytes/macrophages, and dendritic cells. These cells express different TLRs, allowing them to induce a wide variety of immune responses to specific pathogens. Recent observations demonstrate mRNA expression of TLR in the non-immune innate cells such as epithelium, fibroblasts and endothelium, all of which were previously considered as inert cells (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003; Kusumoto et al., 2004). These findings imply the possible function in pathogen recognition via TLR signaling performed by the non-immune cells in the innate defensive system.

2.2 Periodontitis and cigarette smoking

Periodontitis is a chronic bacterial infection of tooth supporting structures. It causes destruction of periodontal connective tissue and bone and, in severe cases, tooth loss. Key oral plaque bacteria including

Porphyromonas gingivalis, *Actinobacillus actinomycetemcomitans*, and *Tanerella forsythia* are recognized as etiologic agents in periodontitis. The disease initiation and progression results from the host response to plaque bacteria. Immunohistochemistry studies reveal dense cellular infiltration including numerous T and B cells in periodontitis lesions. In addition, high levels of inflammatory mediators such as TNF-α, IL-1β, prostaglandin E_2, IFN-γ, and IL-8 can be detected in inflamed gingival tissues and gingival crevicular fluid (Seymour, 1991; Page et al., 1997). Like other infectious diseases, periodontitis is multi-factorial in nature. Genetics and environmental factors such as smoking and stress can influence disease initiation and severity.

Cigarette smoking is the well-known risk factor for periodontal disease that has been investigated extensively in both cross-sectional and longitudinal studies (Sopori, 2002; Tonetti, 1998; Ah et al., 1994; Bergstrom et al., 1989; Haber et al.,1992; Haber et al., 1993; Haber et al., 1994; Preber et al., 1992). Strong epidemiologic evidences indicate a positive association between smoking and periodontal disease. A meta-analysis of six studies concluded that smokers are almost three times as likely to have severe periodontitis compared to non-smoker (Papapanou, 1996). The most recent and largest epidemiological study on smoking and periodontal disease is based on data from the National Health and Nutrition Examination Survey (NHANES) III, the data showed that after adjusting for age, race or ethnicity, income and educational level, current smokers were four times as likely to have periodontitis as compared to non-smokers (Tomar et al., 2000). In 12 months longitudinal study, smokers were shown to be at significantly greater risk for further attachment loss when compared to non-smoker, the odds ratio was 5.4 (Machtei et al., 1997). When the group of light smokers were compared to that

of heavy smokers in relation to periodontal attachment loss, a strong dose-response relationship between smoking and periodontitis could be found (Grossi et al.,1994; 1995; Calsina et al., 2002). Grossi et al. (1994) demonstrated that the odds for more severe attachment loss ranged from 2.05 for light smokers to 4.75 in heavy smokers (Grossi et al., 1994). They also demonstrated the odds for severe bone loss ranging from 3.25 to 7.28 for light and heavy smokers respectively (Grossi et al., 1995).

2.2.1 Nicotine

Cigarette smoke contains thousands of different compounds such as nicotine, tar, carbon monoxide, ammonia, formaldehyde that are directly noxious/poisonous to living organisms and cells (Palmer et al., 2005). Many of the toxic effects of tobacco have been attributed to nicotine, a major component of the particulate phase of tobacco smoke. Nicotine is a tertiary amine composed of a pyridine and a pyrrolidine ring that is distilled from burning tobacco and is carried proximally on tar droplets and probably also in the vapor phase which is inhaled (Pool et al., 1985).

Many of the adverse effects of smoking may result from the ability of cigarette smoke to weaken the immune system by inhibiting both innate and adaptive immunity. It was found that alveolar macrophage, an important innate immune cell in the lung from smokers have a reduce ability to phagocytose and/or kill microbial pathogens (King et al., 1988; Martin, 1977). As for the adaptive immunity, several studies have demonstrated the marked reduction in serum levels of immunoglobulin in long-term smokers (Ferson et al., 1979). T cells from smokers and animals that were exposed to cigarette smoke have a

decreased ability to proliferate in response to T cell mitogen which indicates a deficient cell-mediated immune response (Holt, 1987). On the basis of particle size cigarette smoke is composed of 2 phases; the vapour phase and the particulate phase. Chronic exposure to the vapour phase does not suppress the immune system (Sopori, 1998). The data suggest that one or more component(s) in the particulate phase is immunosuppressive. Nicotine is associated with the particulate phase and represents one of the major constituents of cigarette smoke that has immune suppression activity (Geng et al., 1996).

Many *in vitro* studies showed the deteriorated effects of nicotine on human gingival fibroblasts and HGECs (Hanes et al., 1991;Tipton et al., 1995; Wendell et al., 2001; Giannopoulou, 2001; Wendell et al. 2001). Nicotine could alter human gingival fibroblast function that decrease collagen production while increasing collagenase production (Tipton et al., 1995). In vitro study, nicotine can directly stimulate human gingival fibroblast IL-6 and IL-8 production. Moreover, the combination of high doses of nicotine and either *E. coli* and *P. gingivalis LPS* can synergistically upregulate cytokine production (Wendell et al., 2001). Cultured gingival keratinocytes exposed to nicotine produce higher amounts of the proinflammatory cytokines IL-1 (Johnson et al., 1997). So far, there is no study that investigate the effect of nicotine on HBD-2 production from human gingival epithelium.

2.3 Gingival epithelial cell

Epithelium is an avascular tissue which is composed of cells, tightly-bound to each other, with no intercellular connective tissue. It lines the

surfaces of the body and is mainly located at the border between the external and internal environments. The epithelium is not only considered as a physical barrier, but also able to perform a number of very important protective innate immune responses. Owing to different strategic location in human body, many types of epithelium are found with varying structure and function (Bartold et al., 2000).

In the oral cavity, epithelial cells protect the underlying connective tissues from microorganisms and other harmful agents. They play an important role in innate immune response and homeostasis. Oral epithelial cells are a multiple layer whereas gut epithelial cells are a single layer. However, both of them continually expose to large numbers of both commensal and pathogenic bacteria. In intestinal epithelium of mice, commensal bacteria are recognized by TLRs under normal steady-state conditions, and this interaction plays a crucial role in the maintenance of intestinal epithelial homeostasis and protects against gut injury that associated mortality (Rakoff et al., 2004).

Epithelial cells are recognized as metabolically active and capable of reacting to microbes by synthesizing a number of cytokines, adhesion molecules, growth factors and enzymes. More importantly, the epithelial cells generate a family of potent antimicrobial peptides for protection against infection that cause microbial death by lysis through disruption of the integrity of bacterial membranes. These peptides are β-defensins, cathelicidin LL-37, and calprotectin, all of which have been identified in the oral epithelium (Han et al., 2000; Dale et al., 2001; Ross and Herzberg, 2001; Kusumoto et al., 2004). With the accumulation of bacterial plaque, the epithelial cells also begin to overexpress adhesion molecules such as intercellular adhesion molecule-1

and cytokines such as IL-1β and IL-8, which are involved in neutrophil recruitment and migration. Thus, the gingival epithelium is an important initiator, regulator and mediator of the host immune response against microbial infection (Bartold et al., 2000).

2.3.1 Toll-like receptors on human gingival epithelium

Little is known about the role of TLR in human gingival epithelium. TLR expression on human oral epithelial cells was investigated in patients' gingival biopsies obtained during periodontal surgery. The cells constitutively express variety of TLRs: TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, TLR 10 (Kusumoto et al., 2004). Although gingival epithelial cells express negligible levels of TLR4, their expression can be enhanced by treatment with IFN-γ (Uehara et al., 2002). TLR2 expression is denser in the spinous epithelial layer than in the basal epithelial layer (Kusumoto et al., 2004). An abundance of TLR2-positive cells was also observed in connective tissue subjacent to pocket epithelium (Mori et al., 2003). Thus, TLR2 may be especially important given its strategic location in the outermost layer where continual direct contact with oral bacterial products occurs.

Using immortalized HGECs, Asai et al. (2001) demonstrated that *P. gingivalis* fimbriae and *Staphylococcus aureus* peptidoglycan induced IL-8 production via TLR2 expressed on epithelial cells. In another study, however, *P. gingivalis* fimbriae (prepared similar to the first study) induced negligible production of IL-8 from HGECs. However, a sonic extract of the bacterium was able to strongly induce IL-8 and monocyte chemoacttractant protein (MCP)-1

production (Kusumoto et al., 2004). The conflicting results on fimbriae-induced IL-8 production from gingival epithelial cells requires further clarification.

2.4 Human β-defensin-2

Defensins are small cationic antimicrobial peptides that have 30-40 amino acid residues with molecular weight of about 3-4 kD. To date, six human β-defensins (HBD-1 to HBD-6) have been identified in humans (Bensch et al.,1995; Harder et al.,1997; 2001; Garcia et al., 2001a; 2001b; Yamaguchi et al., 2002). They share a conserved motif, which is composed of six spaced cysteines. HBD-1 was isolated from human plasma and its expressed in most epithelial cells (Bensch et al., 1995; Zhao et al., 1996). HBD-2 was originally isolated from human skin and widely expressed in epithelia such as lung, trachea, foreskin, skin, gingival and leukocyte (Harder et al., 1997; Bals et al., 1998; Hiratsuka et al.,1998; Mathews et al.,1999; Duits et al., 2002; Fang et al., 2003). HBD-3 was found recently that expressed in both epithelial tissue and non-epithelial tissue (Knowles et al., 1997; Harder et al., 2001; Garcia et al., 2001a). HBD-4 had more limited distribution than HBD-1, HBD-2, HBD-3. (Garcia et al., 2001b; Fahgren et al., 2004). The most recently identified family members, HBD-5 and HBD-6, were localized to the epididymis and airways (Yamaguchi et al., 2002; Kao et al., 2003).

HBD-1 and HBD-2, are both active against gram-negative bacteria bacteria and have more limited activity against gram-positive bacteria (Harder et al., 1997; Scott et al., 2000). HBD-3 is active against gram-positive bacteria (Sahasrabudhe et al., 2000; Harder et al., 2001) and gram-negative bacteria (Joly et al.,2004). In vitro study, antimicrobial activities of HBD-2 and HBD-3

were assessed against a panel of oral microorganisms that showed HBD-2 and HBD-3 were antimicrobial against aerobic and anaerobic, gram-positive and gram-negative oral bacteria and *Candida* species. MICs ranged from 6.5 to >250 μg/ml for HBD-2 and from 4.5 to >250 μg/ml for HBD-3 (Joly et al., 2004). The mechanism of action in killing microorganism appears to be HBD positively charge that bind to negatively charge-bacteria membrane targets include lipopolysacchalide (LPS) in Gram-negative bacteria and polysaccharide (teicholic acids) in Gram-positive bacteria. Then the bacterial membranes are permeabilized through the formation of multimeric pores. These are initiated through electrostatic interactions so the outer membrane was transient opening that permitting perturbation of membrane resulting bacteria death (Hancock ,1997).

The role of HBD in periodontal health has been well recognized (Krisanaprakornkit et al., 1998 ; 2000 ; 2002). Detection by RT-PCR of HBD-1, HBD-2, and HBD-3 expression in gingival tissues or gingival keratinocytes have been reported (Krisanaprakornkit et al., 1998; Mathew et al., 1999; Dunsche et al., 2001;2002; Bissell et al.,2004; Premratanachai et al., 2004; Joly et al., 2005). They were all expressed in healthy tissues. In contrast, the decreased levels of mRNA expression for all HBD were found in diseased tissues including gingivitis, marginal periodontitis, apical periodontitis and candidiasis. (Dunsche et al., 2002), thus indicating their protective role in periodontal tissues.

Expression of β-defensins could be induced. The effects of proinflammatory cytokines such as IL-1β, TNF-α and IFN-γ and various bacteria components such as LPS on induction of HBD-2 and HBD-3

expression were demonstrated *in vitro* epithelial cell cultures (Mathews et al.,1999; Krisanaprakornkit et al., 2000; Dale et al., 2001; Garcia et al., 2001a; Harder et al., 2001; Jia et al., 2001). But HBD-1 expression were not induced after cytokine or LPS stimulation. However, recent real time PCR study revealed that the magnitude of HBD induction depended on the basal levels of expression and these levels varied between individual gingival tissue cultures (Bissell et al.,2004; Joly et al., 2005). Basal levels of HBD-1 expression was shown to be more profound than for either HBD-2 or HBD-3 basal levels, thus reflecting the inducibility profiles of HBD-2 and HBD-3 (Joly et al., 2005).

Defensin synthesis could be mediated via TLR signalling pathway. In lung epithelium, HBD-2 expression could be induced after stimulation with TLR ligands: 2, 3, 4, and 9 (Birchler et al., 2001; Duits et al., 2003; Jia et al., 2004; Platz et al., 2004), whereas in intestinal epithelium, the HBD-2 expression could be induced after stimulation with TLR ligands: 2, 4, 5 and 6 (Vora et al., 2004; Ogushi et al., 2001;2004). Previous reports using gingival epithelium showed that crude preparations of oral plaque bacterial components (*Fusobacterium nucleatum* and *Porphyromonas gingivalis*) could stimulate the cells to induce HBD-2 expression. More recently, the study showed that HBD-2 expression on HGECs was induced by TLR2 and TLR4 (Sugawara et al., 2006). In this study, we will explore a panel of TLR ligands, highly purified bacterial/viral components, for the ability to induce HBD-2 production from human gingival epithelium. Also the effect of nicotine on epithelium HBD-2 expression in response to *P. gingivalis* LPS, the key component of periodontal pathogen. In this study, we investigated the role of HGECs as an important non-immune innate cells in the periodontium by their expression of TLRs, and their responses to different TLR ligands and cytokine TNF-α. In addition, we

studied the effect of nicotine on anti-microbial HBD-2 production in stimulated HGECs.