The development of an assay for evaluating the expression of human interleukin-10 promoter region gene linked to inflammatory bowel disease and its application in turmeric assessment

A thesis presented in partial fulfilment of the

requirements for the degree of

Master of Science

in

Human Nutrition

at Massey University, Manawatū,

New Zealand.

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2013

Abstract

Inflammatory bowel disease (IBD) appears in two forms, Crohn's disease (CD) and ulcerative colitis (UC), which are debilitating diseases with less than satisfactory treatments. Despite years of study, the aetiology of this chronic inflammation remains unclear. Evidence from epidemiological and clinical studies supports that it is a complex interaction among environmental, genetic and immune-regulatory factors. Therefore, gene-nutrition based approaches are suggested to be an appropriate candidate in the future prevention and treatment of IBD.

Different geographic and racial prevalence of IBD are observed in many epidemiological studies, with highest rates found in developed countries and in Caucasian populations. However, the prevalence has increased dramatically in traditional low-incidence areas during the last two decades, and the racial gap is also closing, indicating that both environmental factors such as diet and genetic predispositions contribute to the IBD susceptibility.

The imbalance between pro- and anti-inflammatory cytokines is known to be the key contributor of IBD pathogenesis. Interleukin-10 (IL-10), an anti-inflammatory cytokine, is expressed in many different cells of the adaptive and innate immune system including T regulatory cells, activated macrophages, B regulatory lymphocytes and many other cell types. It plays important part in the regulation of immune response, as was demonstrated in spontaneous colitis in IL-10 deficient mice models, thereforeIL-10 is crucial in the IBD pathogenesis.

Three single nucleotide polymorphisms (SNPs) in the promoter region of IL-10 gene, -1082 G/A, -819 C/T and -592 C/A, have been identified to related to IL-10 production and IBD susceptibility, with -1082 G/A as the most relevant SNP.In this research study, Ideveloped a

cell-based luciferase reporter assay in which the reporter expression is investigated under the control of promoter containing the variants of interest.

Turmeric has a long historical use in Asian medicine for treatment of various diseases. It was shown to exert strong anti-inflammatory effectthrough multiple molecular targets and mechanisms of action. In the second part of my research study, I tested turmeric samples for its ability to alter IL-10 production in the risk polymorphic variant, using the developed assay. The results suggest that curcumin, the bioactive component of turmeric, has the ability to increase IL-10 transcription in the low-producer (ACC)haplotype.

The *in vitro* model of IL-10 promoter assay established in this studyis a novel and valuable tool in assessing IL-10 production at transcriptional level.Furthermore, it provides the possibility of high-throughput screening of food to overcome the functional change of SNPs that are important in human IBD.

<u>Acknowledgements</u>

Table of content

<u>Abbreviations</u>

CD	=	Crohn's Disease
CD14	=	Cluster of Differentiation 14
CDAI	=	Crohn's Disease Activity Index
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethylsulfoxide
ER	=	Endoplasmic Reticulum
FBS	=	Foetal Bovine Serum
GWAS	=	Genome-wide Association Studies
HEK293	=	Human Embryonic Kidney cell line 293
JAK1	=	Janus Kinases 1
LPS	=	Lipopolysaccharide
IBD	=	Inflammatory Bowel Disease
IEC	=	Intestinal Epithelial Cell
IFN-γ	=	Interferon-γ
MD-2	=	Myeloid Differentiation factor 2
MAPK	=	Mitogen Activated Protein Kinase
MetLuc	=	*Metridia* Luciferase
NFκB	=	Nuclear Factor kappa B
NOD	=	Nucleotide-binding Oligomerisation Domain
PRR	=	Pattern Recognition Receptors
ROS	=	Reactive Oxygen Species
SEAP	=	Secreted Alkaline Phosphatase
SNP	=	Single Nucleotide Polymorphism

STAT	=	Signal Transducer and Activator of Transcription
Th2	=	Type 2 T-helper
TLR	=	Toll-like Receptor
Tr1	=	Type 1 T-regulatory
Tyk	=	Tyrosine Kinases
TNF-α	=	Tumour Necrosis Factor-α
TGF	=	Transforming Growth Factor
UC	=	Ulcerative Colitis
WST-1	=	Water Soluble Tetrazolium-1

List of Tables

x

List of Figures

List of Appendices

1 Introduction

1.1 Background:

Ulcerative colitis (UC) and Crohn's disease (CD) are two primary forms of inflammatory bowel diseases (IBD) − that are characterised by chronic, uncontrolled inflammation of intestinal mucosa. The two disorders are usually distinguished by their location and severity. IBD diagnosis is often based on presence of architectural distortion and acute inflammatory cells. However, diagnostic gold standard remains elusive(Andres & Friedman, 1999).

IBD is a chronic inflammatory and frequently relapsing disease that leads to impairment of the intestinal tissue. The symptoms of IBD are extremely unpleasant and may affect all aspects of a patient's life. Diarrhoea, abdominal pain, rectal bleeding, fever, weight loss are the most commonly seen symptoms of IBD(Lomer, 2011). In severe cases or if left untreated, dehydration, malnutrition, anaemia or even death may occur(Steidler, 2001). With proper management, however, many patients are able to live normal, productive lives.

The state of chronic intestine inflammation in IBD is the result of an inability to properly control inflammatory response to environmental challenge. The precise aetiology of IBD remains unclear. The susceptibility of IBD appears to be multi-factorial, in which genetic predisposition, immune-regulatoryfactors and environmental triggers all contribute (Abreu, 2002; Bouma & Strober, 2003;Xavier & Podolsky, 2007).

Epidemiological evidence has linked IBD incidence rates with age, ethnicity, geographic locations and other demographic features. Both CD and UC have the highest prevalence in developed regions such as northern Europe, the United Kingdom, and North America. However, there is a significant rise in IBD incidence from other parts of the world in the past

two decades, including Asia, Africa, Latin America, etc.(Lakatos, 2006; Loftus Jr, 2004; Ouyang *et al*., 2005).An increase in IBD rates have also been reported by immigrants to developed countries, suggesting an important role for environmental and lifestyle factors, among which smoking and western diet are believed to have the strongest association with IBD risk. Sanitation and childhood exposure to infection have also been implicated(Hanauer, 2006; Lomer, 2011). The role of nutrition played in IBD appears to be important during disease onset. It can help modify the risk, and also use as a potential treatment in disease progression or in symptom management.

A genetic predisposition for IBD has been demonstrated in epidemiological and family studies. However, it is not inherited as in a single gene model but rather on a complex multi-genetic basis. Recent advances in genetic studies have identified 99 IBD susceptibility loci: 71 related with Crohn's disease, 47 related with ulcerative colitis and 28 with both CD and UC(Anderson *et al*., 2011; Franke *et al*., 2010). Approximately 51 of these IBD genes overlap with 23 different diseases, most of which being immune-mediated diseases, suggesting key immune pathways involved in IBD(Lees, Barrett, Parkes, & Satsangi, 2011; Zhernakova, van Diemen, & Wijmenga, 2009).

The fundamental nature of IBD pathogenesis is believed to be characterised by abnormal mucosal immune responses in the gut, facilitatedpartly by changes in intestinal microflora composition(Khor, Gardet, & Xavier, 2011). Therefore, an imbalanced pro- and anti-inflammatory profile is considered to be key contributor to IBD pathogenesis.

Interleukin-10 (IL-10) is a central component of immune system. As an important immune-suppressive cytokine, it acts by dampening the immune response through repressing the effect of pro-inflammatory cytokines(Sabat *et al*., 2010). Abnormal IL-10 activity has been

associated with IBD, and its genetic defects are of great interest in relation to IBD pathology (Russell & Satsangi, 2004).

Three common single nucleotide polymorphisms (SNPs) have been associated with IL-10 production, namely rs1800872 (-592 C/A), rs1800871 (-819 C/T), and rs1800896 (-1082 G/A). These SNPs occur in distinct risk haplotypes as shown in Table 1. The three variants have been related with different IL-10 productions(Tagore *et al*., 1999; Turner *et al*., 1997). It is reported that the allele -1082G (haplotype GCC) is associated with higher IL-10 synthesis while -1082A (haplotype ACC and ATA) is linked with lower production of IL-10(Castro-Santos, Suarez, López-Rivas, Mozo, & Gutierrez, 2006). However, the evidence remains inconclusive. In addition, some studies have shown that contribution of -1082 allelic variants is more important than that of alleles at the -819 and -592 position to the regulation of IL-10 mRNA levels (Suárez, Castro, Alonso, Mozo, & Gutiérrez, 2003).

Table 1. IL-10 haplotypes of the three promoter SNPs of interest in IBD pathogenesis

IL-10 haplotype	Associated SNPs	SNP variant	Hypothesised IL-10 production
GCC	**rs1800896** rs1800871 rs1800872	**-1082 G** -819 C -592 C	High producer
ACC	**rs1800896** rs1800871 rs1800872	**-1082 A** -819 C -592 C	Low producer
ATA	**rs1800896** rs1800871 rs1800872	**-1082 A** -819 T -592 A	Low producer

1.2 Significance of the study

According to a recent systematic review, IBD is considered a global disease with increasing incidence and prevalence in different regions(Molodecky *et al*., 2012). Consequently, an

estimated growing public health burden around the world is anticipated with considerable amount of health care resources input. In addition, IBD tends to affect patients in their most productive years of life, leading to long-term cost to both individual and society.

Both CD and UC are recognised as complex multi-factorial polygenic diseases. The increasing understanding of gene-environmental/gene-diet interactions has given rise to the new field of nutrigenomics and nutrigenetics, which are used to demonstrate how gene polymorphisms affect individual's response to specific food, thus dietary requirement, and vice versa(Lee & Buchman, 2009). The development of the *in vitro* model in our present study serves as a desirable tool in assessing the ability of food componentsto changegene expression. It fills in the gap between SNP identification and selecting foods in animal model investigation. Ultimately, it facilitates an individualised nutrition and treatment-- the future for promoting optimal health and dealing with heterogenic disorders such as IBD.

1.3   Aim and objectives:

1.3.1   Aim:

It is thought that altered production of cytokines underlies IBD susceptibility (Fiocchi, 1998). Polymorphisms at the IL-10 gene promoter control IL-10 cytokine levels, and thus affect IBD susceptibility,therefore my hypotheses are:first, polymorphisms at the IL-10 gene promoter region lead to changes in IL-10 transcription, and second, food components have an effect onthe altered IL-10 promoter activity.The aim of the present study is to develop an *in vitro* model and use it to investigate if food components have theability toalter the abnormal activity of IL-10 promoter region polymorphisms, therefore to alleviate IBD in genetic susceptible individual.

1.3.2 Objectives:

My hypotheses were investigated by the following objectives:

- Establishinga cell-based IL-10 promoter assayto evaluatethe IL-10 promoter activity in different haplotypes.

- Assessment of turmeric samples to alter the activity of the rs1800896 (-1082 G/A) risk variantof the IL-10 promoter (ACC haplotype)

1.4 Overview of the study:

My research study can be dividedinto two parts. The first part is the development of an IL-10 promoter assay, in which the promoter activity of IL-10 in different haplotypes can be assessed.The second part is applying the IL-10 promoter assay in food component tests. More specifically, the assay was used to evaluate the turmeric samples for its ability to change IL-10 transcription in ACC haplotype.Mammalian cell culture technique and *in vitro* bioactivity assays were performed throughout thisresearch study.

The assay development was carried out in six steps. The first step was to culture the 293-hTLR4a-MD2-CD14 cells (referred to as 293TLR4 cells), which havealready been stably transfected with the human toll-like receptor 4a (TLR4a), myeloid differentiation factor 2 (MD2) and cluster of differentiation 14 (CD14) genes. These cells were cultured and maintained throughout the entire study. The second step was transfecting the cells separately with two control vector genes (pMetLuc2-control vector and pSEAP-control vector) in order to optimisethe amount of transfection reagents and DNA to use. The third step was co-transfecting the cells with both pMetLuc2-control and pSEAP-control vectors and then tuning the co-transfection condition to achieve satisfactory signal. After that, pMetLuc2-IL-10-variant (pMetLuc2-GCC/pMetLuc2-ACC/pMetLuc2-ATA) and pSEAP-control vectors were

co-transfected in cells according to previous determined experimental conditions. The IL-10 activity was then induced by lipopolysaccharide (LPS). The final step wasto establish a positive control with a known inhibiting effect on IL-10 activity. With the establishment of a positive control, the food samples of interests were ready to be tested using this model.

Lastly, the second partof my research study was the turmeric samples tests, in which oneturmeric extract andtwelve fractions with a series of dilutions were added into cells prior to the IL-10 promoter activity was assessed. By comparing the IL-10 promoter activity with untreated samples, the effect of the food sampleson IL-10 transcription was concluded.

2 Literature Review

2.1 What is inflammatory bowel disease?

Inflammatory bowel disease consists of Crohn's disease and ulcerative colitis, both of which arecharacterised by chronic inflammation and impairment of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors (Danese, Sans, & Fiocchi, 2004; Podolsky, 2002).

Despite their shared clinical and pathological characteristics-- abdominal pain, diarrhoea, bowel obstruction, blood loss, etc. -- these two diseases have distinctive features(Lomer, 2011). Ulcerative colitis is a condition restricted to large intestine whereas Crohn's disease predominantly, but not exclusively, affects the small intestine. Additionally, the histology of the two diseases differs in that Crohn's disease affects all layers of the intestine, whereas ulcerative colitis only produces lesions within the mucosal layer. There is no diagnostic gold standard identified for the two disorders. In 10% of the cases, diagnosis of UC and CD can also be complicated by so-called "indeterminate colitis", in which patients have indeterminate features between UC and CD (Podolsky, 2002; Price, 1978).

IBD is a chronic condition without medical cure, which means lifetime care is often needed. With proper treatment, however, many patients are able to live normal, productive lives. Depending on the severity of the IBD described in clinical practice, from mild, moderate to severe, treatment may involve oral anti-inflammatory medicines, steroids and antibiotics to control the symptoms (Baumgart & Sandborn, 2007). Immunosuppressive agents are also used for maintenance. In severe cases, surgical removal of the affected parts may berequired. However, due to the undesirable side effects and the failure to respond to conventional

treatment, new therapeutic strategies are urgently needed to induce remission and prevent recurrence.

Despite years of investigation, the exact aetiology of IBD has not yet been identified. Epidemiological studies have revealed the multi-factorial nature of the disease. Great effort has also been made to unravelling the complex pathophysiology of IBD. Although it has not been fully elucidated, there is accumulating evidence suggesting the interplay between several different mechanisms, including genetic predisposition that leads to derangement of immune regulatory response, defects in the intestinal mucosal barrier function, and a susceptibility to environmental factors including both specific antigens and commensal bacteria. In this chapter, I reviewed the current information related to the aforementioned aetiological theories, focusing especially on the immune-related mechanisms and gene-environment interactions.

2.2   Inflammatory bowel disease epidemiology

IBD is a global disease with highest incidence found in developed countries. These includeCanada, United States, United Kingdom, and other parts of north Europe, where the rates have now reached a plateau (Bernstein *et al*., 2006; Loftus *et al*., 2007; Rubin, Hungin, Kelly, & Ling, 2000). Recent studies have also found high IBD rates in Southern Australia and New Zealand(Gearry *et al*., 2006; Wilson *et al*., 2010). However, IBD prevalence in traditional low-incidence areas such as south or central Europe, Asia, Africa and Latin America has increased dramatically during the past two decades and continues to rise(Jacobsen *et al*., 2006; Munkholm, Langholz, Nielsen, Kreiner, & Binder, 1992; Thia, Loftus, Sandborn, & Yang, 2008). The geographic distinction has become less prominent, as shown in Figure 2.1 and 2.2(Hanauer, 2006; Loftus Jr, 2004; Molodecky, *et al*., 2012).
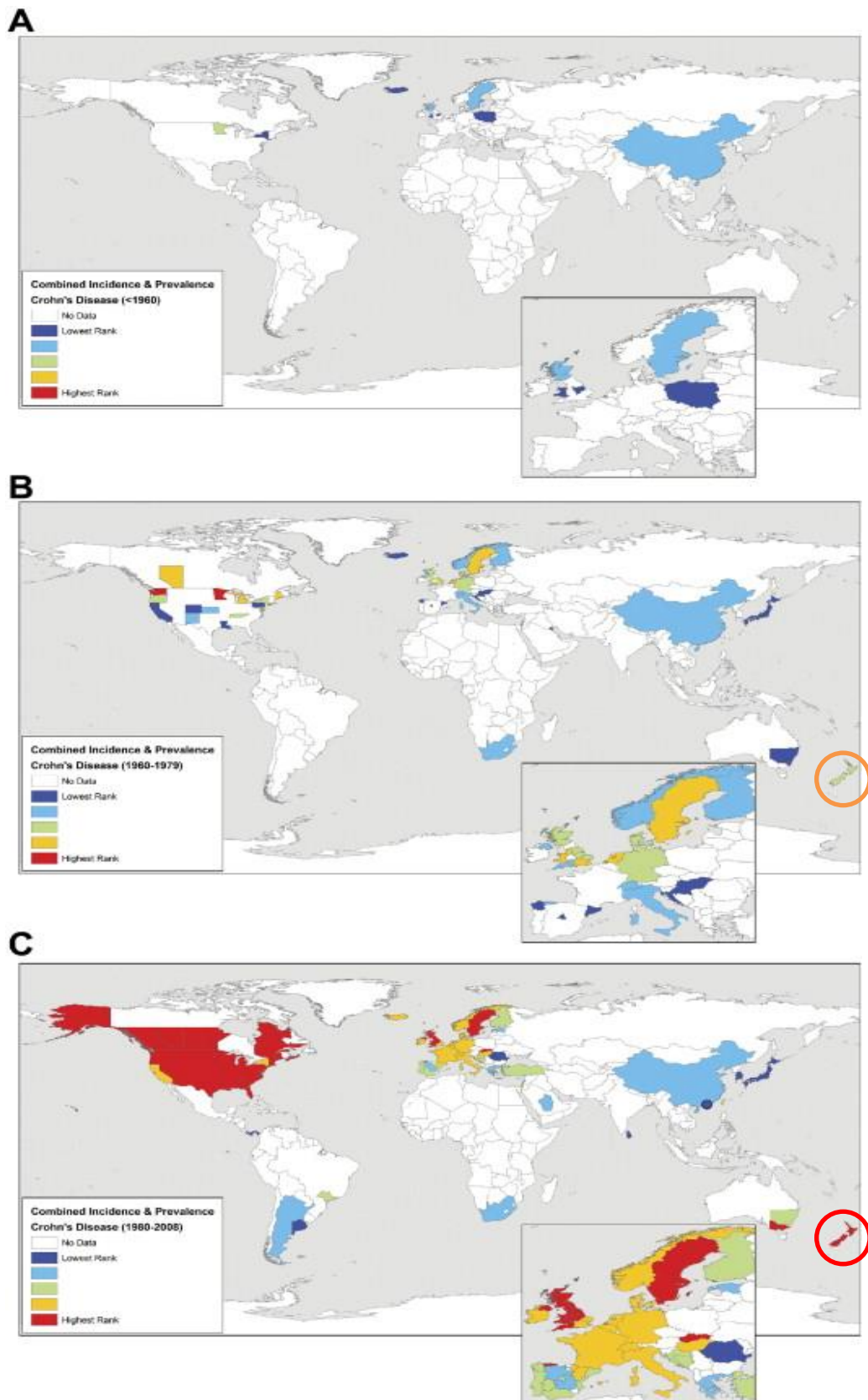
Figure2.1 CD prevalence worldwide. **(A)** data reported before 1960s, **(B)** data reported from 1960 to 1979, and **(C)** data reported after 1980. CD incidence in New Zealand increased from medium risk to high risk. (Adapted from Molodecky, *et al*., 2012)
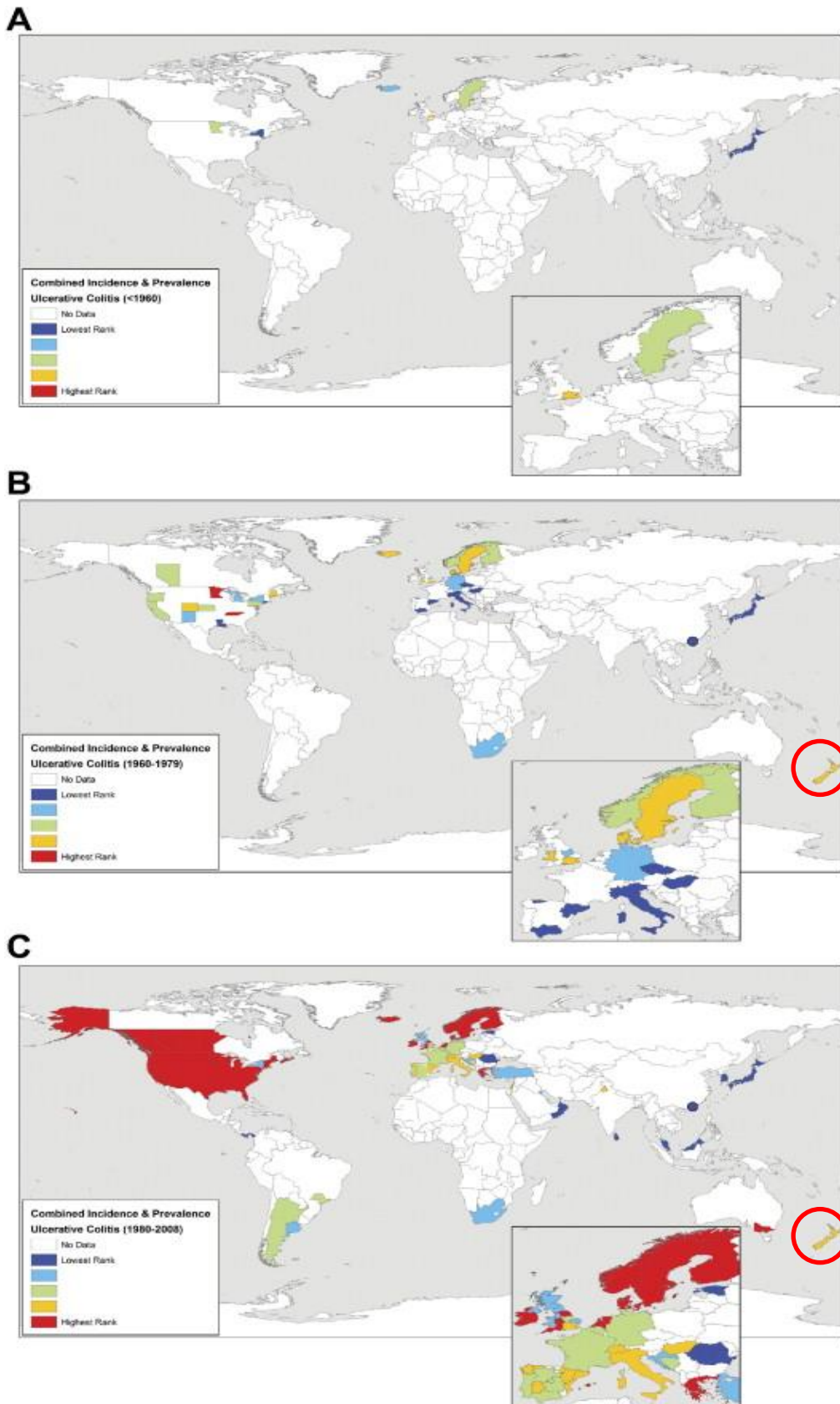
Figure2.2 UC prevalence worldwide. **(A)** data reported before 1960s, **(B)** data reported from 1960 to 1979, and **(C)** data reported after 1980. New Zealand has high risk of UC from 1960 till now.(Adapted from Molodecky, *et al*., 2012)

Previous epidemiological studies in New Zealand suggested a lower prevalence of IBD, compared with other developed countries (Eason, Lee, & Tasman-Jones, 1982; Gaya, Russell, Nimmo, & Satsangi, 2006; Wigley & Maclaurin, 1962). However, a more recent Canterbury population-based cohort study found that IBD in this region is as common as in other Western regions (Gearry, *et al*., 2006). Maori and Pacific Islanders have the least incidence of IBD, which raise the issue of genetic predispositions in the onset and development of IBD (Wigley & Maclaurin, 1962).

Although the data have provided us with strong evidence in relation to different geographic prevalence of IBD, they could also result from various other factors, such as existing differences in IBD diagnosis, access to and quality of health care between developed and developing regions. Since most epidemiological studies have examined only hospital admission rate, it is very likely that IBD incidence in rural communities may be under-reported due to lack of proper health care and poor diagnostic performance. This is especially the case in rural areas of developing regions, for example China and African countries. Study quality such as sample size or methodology is also a contributor to the heterogeneity between studies. In addition, majority of the IBD epidemiological data in the current systematic reviews are from developed countries. There is still a lack of studies on incidence and prevalence of IBD in developing countries. Future effort is required to provide further information on developing region of the world.

IBD is most commonly diagnosed in late adolescence and early adulthood, although it may occur at any age. Both CD and UC follows a bimodal age distribution, in that the peak onset age is between 15 to 30 years followed by a smaller onset peak occurs between age 50 to 70 years old (Andres & Friedman, 1999). However, the second peak has not been confirmed by any further studies (Loftus, 2004). Paediatric IBD accounts for 7% to 20% of all IBD cases,

11

with a higher incidence ratefor CD than UC (Cosnes, Gower–Rousseau, Seksik, & Cortot, 2011).

Gender-related differences in disease onset have also been noted from epidemiological evidence. In some studies, Crohn's disease was found to be more common in women than men, especially in their late adolescence and early adulthood, whereas in some newly diagnosed paediatric IBD cases, most patients were male (Bernstein, *et al*., 2006; Sawczenko *et al*., 2001). However, thegender difference was not reported worldwide and the research evidence is not conclusive (Abdul-Baki *et al*., 2006; Bernstein, *et al*., 2006; Loftus Jr & Sandborn, 2002). Perhaps the difference of gender distribution can provide some clues to the disease aetiology.

Overall, higher rates of IBD occur in people of Caucasian and Ashkenazi Jewish origin than in other racial and ethnic groups. However, this gap is narrowing, with an increased disease onset in Hispanic and Asian population. The low reported incidence in the past could partly due to limited hospital care among these non-white ethnic groups. Increased incidence has also been reported in African-Americans and the second generation of Asians migrants in developed countries(Hanauer, 2006). In some migrant populations such as African-American children, the incidence is even higher than in white population (Ogunbi, Ransom, Sullivan, Schoen, & Gold, 1998). The IBD distribution among ethnic and racial backgrounds still remains dynamic. More data are required from population-based studies evaluating ethnicity from developing countries to address the important gap in the literature.

In conclusion, the current epidemiological evidence indicates that both genetic and environmental factors contribute to IBD risk and that environmental factors play more important roles in the aetiology of IBD than were previously realised.

2.3 Nature and nurture: modifying inflammatory bowel disease risk

Despite years of study, the fundamental cause of IBD remains unknown. There is a complex interaction of environmental, genetic and immune-regulatory factors in the disease onset. Interactions between the mucosal immune system, the host genetic susceptibility and lifestyle factors such as diet and gut microflora have been the recent research focus(Tagore, *et al*., 1999).

The different prevalence of IBD among racial/ethnical groups found in epidemiological studies revealed the role of genetic variations in determining the disease risk. Family and twin studies also provide strong evidence of genetic makeup in the IBD susceptibility(Bonen & Cho, 2003). And it is believed that genetic factors may be more important in CD than in UC, supported by monozygotic twin studies.Variations in environmental factors, such as diet among different populations, could help explain the significant distinct IBD risks across geographic regions and also the increased disease incidence in migrant populations. Smoking is another important lifestyle risk factor which modifies the disease risk; however, its association between UC and CD are interestingly in contrast. Observational studies have indicated some other IBD-related factors, such as appendectomy, oral contraceptives, domestic hygiene, perinatal infection and mode of feeding (Loftus Jr, 2004). Both genetic and environmental factors are critical in IBD onset. Gene-environment interplay thus serves essential role and underlies the complexity of disease phenotype.

2.3.1 The role of genetic predisposition

IBD is a genetically complex disease, and cannot be explained by just one or more penetrant mutations, but a rather wide range of mutations that each affects disease risk in different

levels. It is believed that ulcerative colitis and Crohn's disease share some but not all of the susceptibility loci.

2.3.1.1 Familial and ethnic studies

The incidence rate of IBD in first-degree and second-degree family members can be up to 40%. There is also a higher relative risk among siblings (Binder, 1999; Farmer & Michener, 1986). The strongest evidence supporting inherited factors in disease susceptibility comes from twin studies. Higher rates of concordance of IBD are found in monozygotic twins (36%) compared to dizygotic twins (4%), the latter of which is almost the same as in non-twin siblings (Orholm, Binder, Sørensen, Rasmussen, & Kyvik, 2000; Subhani, Montgomery, Pounder, & Wakefield, 1998; Thompson, Driscoll, Pounder, & Wakefield, 1996). This difference is noted to be more pronounced in CD than in UC (Gaya, *et al*., 2006; Järnerot, Halfvarson, & Tysk, 2007). The level of concordance between monozygotic twins in UC is 6-14%, whereas in CDis much higher—44 to 50%.

Studies of families with high IBD incidence show that approximately 75% of patients are concordant for the two forms of IBD, whereas the other 25% have developed both UC and CD among family members, which indicates multiple and overlapping genetic predispositions in the disease onset (Järnerot, *et al*., 2007; Tysk, Lindberg, Järnerot, & Floderus-Myrhed, 1988).

A significant higher risk of IBD in Jewish population has been found in various studies, leading to the assumption that genetic makeup may play a larger role in some ethnic subgroups (Yang *et al*., 1993).

In familial and ethnical studies, both genetic and environmental factors are associated with the disease risks. For patients who have shared genetics and also shared lifestyle factors, it is

very difficult to discriminate between the two. This could lead to inaccurate results while quantifying their contributions to IBD risks. Selection bias and the short length of follow-up time also raise problems in the existing studies (Russell & Satsangi, 2004).

2.3.1.2 Genetic studies

Recently, great progress has been made in interpreting the genetic architecture of IBD. Large scale, genome-wide association studies (GWAS), carried out by international collaborative research groups, have been particularly successful in identifying the genetic loci that contribute to IBD susceptibility. To date, 99 non-overlapping genetic risk locihave been confirmed: 71 related with Crohn's disease, 47 with ulcerative colitis, and 28 with both CD and UC (see in Figure2.3) (Anderson, *et al.*, 2011; Franke, *et al.*, 2010; Lees, *et al.*, 2011).However, only a fraction of the documented loci are known for their causative functional mutations. Additionally, there is still significant gap in translating the associating genotypes to disease phenotypes.

The identified susceptible genes account for only 23% of heritability in Crohn's disease and 16% in ulcerative colitis reported in GWAS (Anderson, *et al.*, 2011; Franke, *et al.*, 2010). This may have been substantially underestimated due to the limitation of their models in analysing true attributable SNPs risk, which screen for only the most common SNPs so the unusual genetic variants are left unidentified. So far, only the most significant associations have been identified. Increasing samples sizes may help in detecting other genes with moderate effects on diseases; however, it may be less effective due to the limit of statistical tool and the contribution of inter-population heterogeneity. As a result,it is clear that the missing heritability cannot be explained by GWAS alone. Other genetic, epigenetic and non-genetic components contribute to the heritability gap.
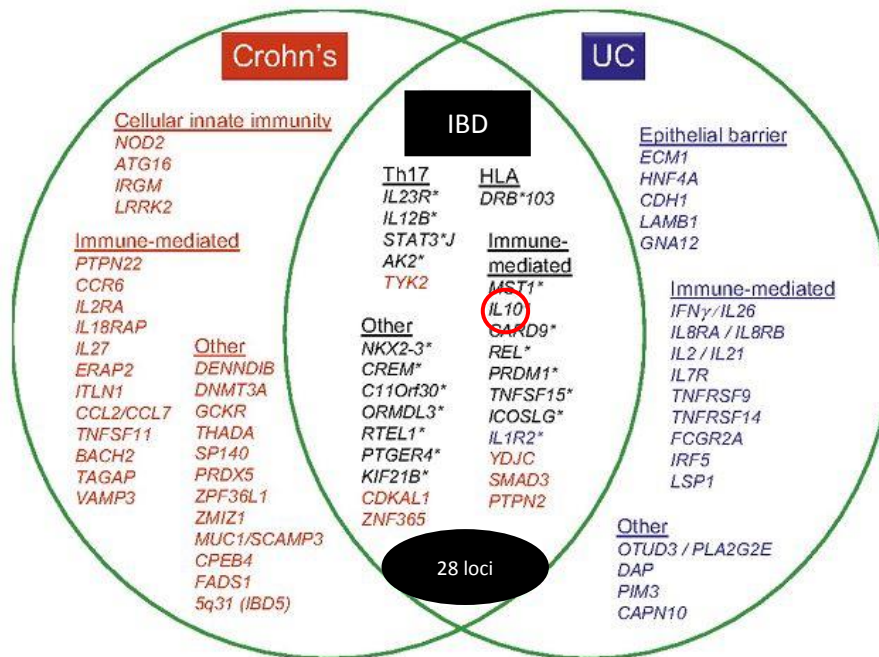
Figure2.3 Inflammatory bowel disease susceptibility loci (Adapted from Lees, *et al*., 2011)

Shared susceptibility genes between IBD and other diseases have been reported in recent reviews. To date, approximately 51 IBD genes overlap with 23 different diseases, including other gastrointestinal conditions, autoimmune diseases, mycobacterial infection and non-immune diseases. Many of the overlap diseases are immune-mediated, but others are not, such as type 2 diabetes mellitus, colorectal cancer (Lees, *et al*., 2011). Furthermore, the overlapping genes are reported to have contrasting effects in different diseases, i.e. serving as risk factor in some diseases but being protective against others. These findings provide new insights into diseases pathogenic mechanisms.

2.3.2   The role of environmental factors

One of the lesser known and most difficult puzzles in IBD pathogenesis is the role played by environmental factors. Many aspects of lifestyle have been postulated to contribute to IBD, including prenatal events, breastfeeding, childhood infection, smoking, oral contraceptives, diet, hygiene, occupation, physical activity, seasonal variability, pollution and miscellaneous components such as appendectomy, tonsillectomy, etc. Among all these, smoking is by far the most well established risk factor. The most complex associations exist between diet and nutrition with IBD aetiology (Mahid, Minor, Soto, Hornung, & Galandiuk, 2006; Reif, Klein, Arber, & Gilat, 1995; Rubin & Hanauer, 2000).

2.3.2.1   Smoking

The relationship between smoking and IBD incidence has been investigated since 1980s (Calkins, 1989; Somerville, Logan, Edmond, & Langman, 1984). There is a striking distinction between CD and UC regarding their associations with smoking behaviour. The unusual protective effect of cigarette smoking is found for ulcerative colitis, which was later confirmed by many studies (Loftus Jr, 2004). Current smokers have a 40% chance of developing UC than non-smokers do. In ex-smokers, however, the risk appears to be higher than current smokers, but still is only 70% as likely as those who have never smoked (Calkins, 1989). The mechanism for this puzzling relation is unknown.

In contrast to UC, smoking is noted to be a risk factor for Crohn's disease. The reported risk to develop CD was as high as twice in smokers as it is in non-smokers. People who have quit smoking have a significantly reduced risk of developing CD than current smokers, yet still higher than non-smokers (Calkins, 1989; Loftus, Schoenfeld, & Sandborn, 2002). However, smoking was not found to be a risk factor for CD in Israeli studies, suggesting that it may

have different impact on modifying disease risk among different ethnicities (Reif, *et al*., 1995).

Smoking may also influence the course of IBD. The positive effect of smoking is noted in many studies. Active smokers were reported to have a 50% lower chance of hospitalisation, compared to non-smokers. UC patients who quit smoking experienced a higher need for corticosteroids and the hospitalisation rate was also increased. It is unclear from randomised controlled trials as to whether the nicotine is the contributor to the protective effect of smoking or not. In the course of CD, cigarette smoking is found positively associated with disease progression. It is reported that active smokers have an increased risk of recurrence after surgical resection together with a higher need for immunosuppressive agents. In addition, other evidence indicates that less medical attention is required after patients have quit smoking(Birrenbach & Böcker, 2004; Loftus Jr, 2004). A number of mechanisms in the pathogenic effect of smoking were postulated, which include modification of intestinal immune system, changes of gut permeability and motility, alteration of cytokine levels and production of free oxygen radicals (Birrenbach & Böcker, 2004).

2.3.2.2   Diet

Dietary and nutritional factors are known to have an important role in the development and treatment of IBD. It has been observed from epidemiological data that IBD risk is increasing in countries where diet is becoming westernised or in immigrants who have adopted a western diet (Hou, Abraham, & El-Serag, 2011). Research evidence available to support this hypothesis, however, is scarce and the underlying mechanisms are still poorly understood.

First of all, it is noteworthy that studies examining the diet-disease relationship are difficult to perform due to the recall bias in diet record, the possibility that people subconsciously change their diet during disease course, etc. Therefore, prospective studies provide stronger evidence

than case-control studies. It is essential to take into account of various confounding factors while determining associations between diet and IBD.

Simple sugar. One of the most consistent results in dietary studies is the link between high sugar intake and IBD onset, especially CD(Riordan, Ruxton, & Hunter, 1998). Numerous case-control studies confirmed this relationship; however their retrospective nature serves as a major limiting factor. Furthermore, this type of studies does not reveal a causal relationship, in that a higher sugar intake may increase the risk of IBD, or it may also be a consequence of IBD patients' altered diet.

Fatty acids. Overall fat intake and saturated fats have been shown to increase the IBD risk by various studies(Shoda, Matsueda, Yamato, & Umeda, 1996; Amre, *et al.*, 2007). Epidemiological data have also linked polyunsaturated (PUFA) to IBD risk. It is suggested that n-3 PUFA, including EPA and DHA, have anti-inflammatory effect, whereas n-6 PUFA have pro-inflammatory effect. Increased n-3 PUFA intake and decreased n-6 PUFA intake have been related to increased onset of both CD and UC(Amre *et al.*, 2007). A high n-6:n-3 ratio was indicated to have contributed to the development of CD (Shoda, *et al.*, 1996). However, from the available data, the sources of n-3 PUFA included in the input measurement were not consistent. Some studies measured only food sources, while others use fish oil supplementation or both. Ratio of products and chain length varies in different sources, which could lead to unreliable results.

Others. Other dietary factors, such as protein, may also play a role in the aetiology of IBD. Dairy products have been shown to be a risk factor for IBD (Shrier, Szilagyi, & Correa, 2008). Studies on high intake of animal proteins and IBD risk have shown conflict results. In some other studies, high fruit and vegetable intake, and dietary fibre consumption were observed to have a protective effect against IBD, but results appear to be inconclusive (Amre,

et al., 2007; Yamamoto, Nakahigashi, & Saniabadi, 2009). Higher consumption of vitamin C and vitamin E -- two well-known antioxidants — were reported to modify disease risk in opposite manner (Sakamoto *et al*., 2005). Overall, the available data regarding micronutrient intake are insufficient to establish its association with IBD risk.

Current observational data have provided little conclusive evidence in relation to diet and cause of IBD. More long-term and more prospective investigations are needed. Furthermore, it is possible that dietary patterns rather than individual foods that are critical in modifying the risk of IBD.

2.3.2.3 Perinatal and childhood factors

The infant feeding mode, domestic hygiene and perinatal infection have been proposed to have a modifying effect on the risk of IBD. Most convincing evidence on the protective effect of breast-feeding derives from epidemiological studies. A recent meta-analysis included four high methodological quality studies, in which the protective effect against IBD was reported. However, the effect appeared to be heterogeneous in UC and CD (Klement, Cohen, Boxman, Joseph, & Reif, 2004; Klement & Reif, 2005). Confounding factors such as the genetic background of the subjects, differences in breast milk composition and variations in diagnostic standards all contribute to the inconsistency of the results.

Childhood exposure to infection may be a protective factor for IBD. The degree of sanitation was inversely associated with future onset of IBD, suggesting an altered gut microflora due to lack of important bacterial exposure in improved hygiene (Krishnan & Korzenik, 2002).

2.3.2.4 Appendectomy and other factors

The inverse relationship betweenappendectomyand UC has been reported in many epidemiological data. The reduction in CD occurrence was observed consistently in

severalcase-control studies,ranging from 13% to 69%(Koutroubakis & Vlachonikolis, 2000). However, the result was not reproduced in other large cohort studies (Koutroubakis, Vlachonikolis, & Kouroumalis, 2002). Additionally, the clinical course of CD also seems to be altered by previous appendectomy, but the evidence available is limited and controversial. It was suggested that the risk of Crohn's disease was increased in relation to appendectomy (Corrao *et al*., 1998). But, statistically significant difference was not consistently found in all of them. Several hypotheses, such as its influence on gut mucosal immune response and existing antigenpresented in appendix were proposed around the mechanism of how appendectomy could modify the IBD risk(Loftus Jr, 2004).

Other factors that have been raised to modify the IBD risk include psychological stress, use of non-steroid anti-inflammatory drugs and infections (both intra- and extra-colonic). However, they have not been demonstrated in a reproducible fashion.

2.4   Intestinal homeostasis and immune-biology: mediating the inflammatory bowel disease process

A dynamic interplay among intestinal epithelial cells (IECs), intestinal microbial agents and local immune cells underpin the fundamental features of intestinal homeostasis. These interactions are responsible in maintaining a healthy intestinal environment and organising normal immune responses to pathogen exposures, as well as in contributing to the pathogenesis of IBD, leading to the loss of intestinal homeostasis.

Several pathways are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, generation of reactive oxygen species (ROS), autophagy, regulation of adaptive immunity, endoplasmic reticulum (ER)

stress and metabolic pathways. Susceptible loci found in genome-wide meta-analysis have paralleled these pathways (Khor, *et al*., 2011; Shkoda *et al*., 2007).

Inflammatory pathways underlying IBD have been investigated by an increasing number of studies. Recentevidence indicates that pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of IBD. Genetic predisposition for developing an inadequate immune response towards luminal factor seems to be one of the underlying causes of the disease. Autoimmune mechanisms against colon epithelial cells may be involved in the cause of tissue damage in UC. The reduced ability of mucosal immune system to eliminate certain microorganism or to tolerate non-pathogenic microorganism are believed to contribute to the inflammatory process of CD (Fiocchi, 1998; Gasche *et al*., 2000).

In this section, I will discuss the structures and functions of intestinal barrier, the immune-regulatory circuits in the gut homeostasis, and how their defects involved in IBD pathogenesis.

2.4.1   The role of intestinal barrier

The intestinal barrier is a complex system which separate intestinal tissue from its content. It also regulates nutrient absorption and the interactions between microflora and mucosal immune system. A monolayer of intestinal epithelial cells is located at the centre of the barrier, with a thick layer of mucus on the luminal side and an underlying set of cells on the basolateral side. The barrierforms a physical as well as a functional boundary to maintain the homeostasis in the intestine (Roda *et al*., 2010). (As shown in Figure 2.4)
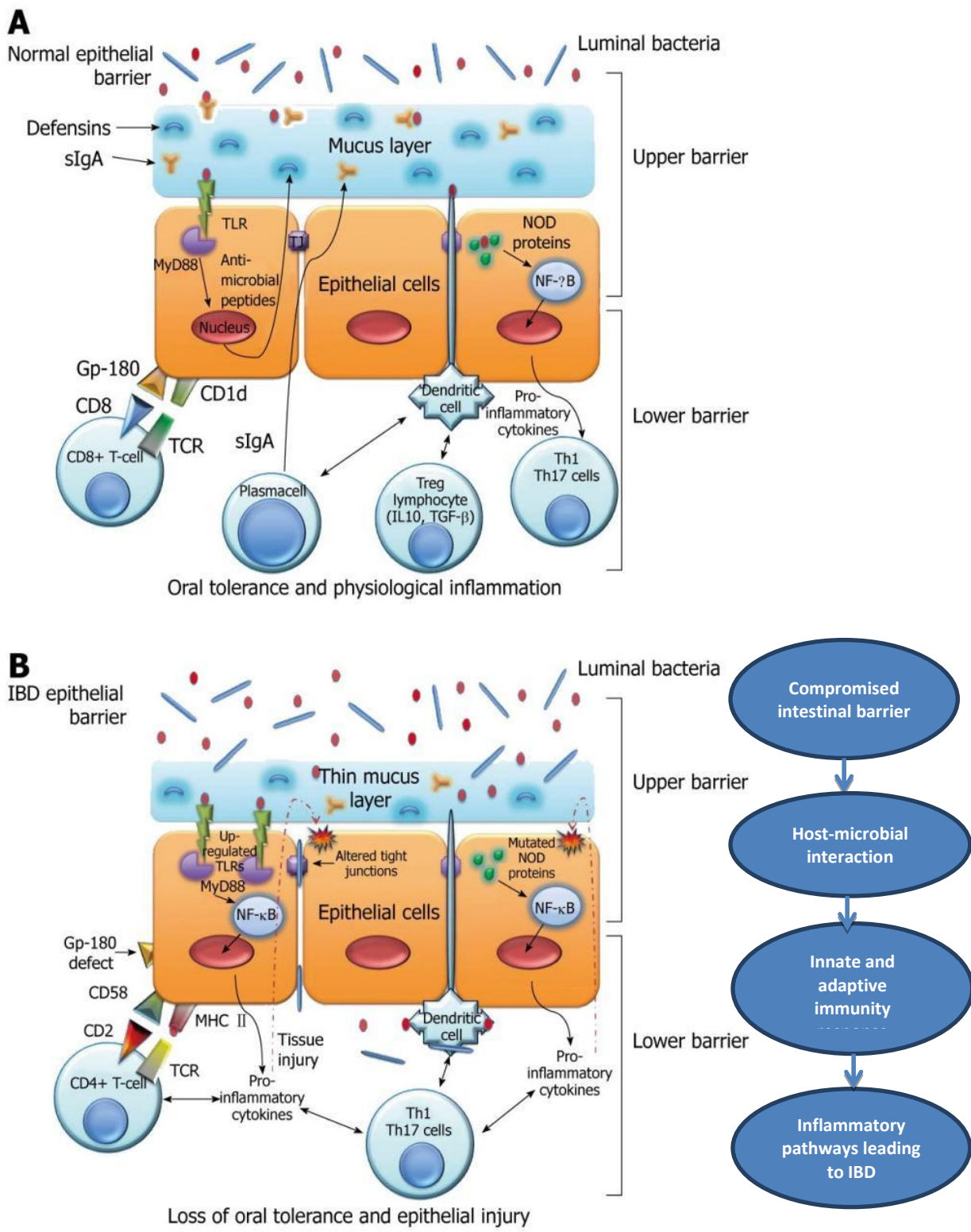
Figure2.4 The epithelial barrier system. (**A**) Normal epithelial barrier; (**B**) IBD epithelial barrier. TLR: Toll-like receptors; MyD88: myeloid differentiation factor 88; TJ: Tight junctions (Adapted from Roda, et al., 2010).

Several types of IECs are presented at the upper part of the barrier. The enterocytes are completely coated with thick mucus secreted by goblet cells that is impenetrable to most of the bacteria. The layer of enterocytes can also be interrupted by entero-endocrine cells which produce hormones used in tissue repair, angiogenesis and differentiation. Paneth cells can secrete antimicrobial peptides, called defensins, to assist in pathogen killing.

At the lower part of the intestinal barrier, the intestinal epithelial cells play important part in the mucosa immune system as non-professional antigen-presenting cells. They process and present antigens to mucosal lymphocytes and promote T-cell response. An altered lymphoid-epithelial interaction is believed responsible in IBD pathogenesis (Roda, *et al*., 2010).

IBD pathogenesis has been related to increased permeability and loss of barrier function of the intestinal epithelial lining, in which the bacterial products and other antigens could cross the mucosal barrier and have direct contact with mucosal immune cells, leading to a continuous stimulation of the mucosal immune system (Hanauer, 2006; Hollander *et al*., 1986; Welcker, Martin, Kolle, Siebeck, & Gross, 2004).

In IBD patients, it was found that the quality (thickness and viscosity) of the mucus layer were reduced, which leads to weakened barrier function (Welcker, *et al*., 2004). Dysfunction of Paneth cells and insufficient defensin production are also noted in IBD patients. However, it is still not clear whether those defects are primary or are secondary effects in response to the inflammatory state (Wehkamp, Schmid, & Stange, 2007; Welcker, *et al*., 2004). Epithelial tight junctions also have an important part in preserving the integrity of intestinal barrier. They are regulated by various cytokines. Studies indicate that overproduction of pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), are responsible in the impairment of tight junction and consequently the loss of proper barrier function (Roda, *et al*., 2010).

2.4.2   Intestinal microbial agents and host immune regulations

2.4.2.1   The functions of gut microflora

Gut microbiota consists of a complex and dynamic society of microorganisms that live in the digestive tract. The exact composition has not been identified, but it is believed that at least 500 species are present in the gut (Xu *et al*., 2003). Their relationship with the host is considered mutualistic as they play important part in host nutrition, physiology and immune system modification (Round & Mazmanian, 2009).

Metabolic functions: Intestinal bacterial can ferment non-digestible dietary carbohydrates into short chain fatty acids, which can be used by the host as energy source or in improving the absorption of some dietary minerals, such as calcium, magnesium and iron. The bacteria also facilitate the absorption of needed vitamins. The overall outcome of the bacteria metabolism in the gut is the supply of energy and nutritive products for the host as well as for bacterial growth (Guarner & Malagelada, 2003).

Trophic functions: The differentiation of epithelial cells is largely influenced by the resident microorganisms. Another important function of the short chain fatty acids produced by gut bacteria is that they can stimulate epithelial cell proliferation and differentiation (Kau, Ahern, Griffin, Goodman, & Gordon, 2011).

Protective functions: Intestinal microbiota also has a protective effect -- limiting potential harmful species from colonising the host gut. This is known as the barrier function of microbiota. High density of intestinal microbes leads to constant communication between host cells and the microbial community (Round & Mazmanian, 2009). The dynamic interactions between gut bacteria and host immune system also play key roles in the

development of oral tolerance, prevention of allergy, modulation of immune response towards pathogens (Cerf-Bensussan & Gaboriau-Routhiau, 2010).

The composition of gut microbial agents varies greatly among individuals, with a unique collection of bacterial species in each person (Fava & Danese, 2011).Various factors can modify the composition of gut microbial communities. They include diet and other environmental exposure to microorganisms, antibiotic therapy, and microbial colonisation in the neonatal period (Kau, *et al*., 2011). The heterogeneity of microbial agents is a major challenge in understanding the interactions between microbiota and its host. A high degree of similarity in microbiota profile is found in identical twins. Siblings also reported to have shared microflora make-up, while spouses who shared the same environment had the least similarity (Zoetendal, Akkermans, Akkermans-van Vliet, de Visser, & de Vos, 2001). These findings highlight the genetic factors in the gut microbiota development and suggest a modifying effect of environmental factors.

2.4.2.2 The balance of tolerance and defence

Human infants form their oral tolerance to the microbiota and food-derived antigens mostly in their first two to three years of life. Microbial exposure and colonisation takes place at this time, and so the mucosal immune system matures (Baumgart & Carding, 2007).

After the establishment of oral tolerance, the host immune system continuously monitors and regulates the intestinal microorganisms, to prevent the overgrowth of resident microbes and to eliminate pathogenic microbes. This is commonly known as the intestinal homeostasis(Abraham & Medzhitov, 2011). In IBD, this symbiotic relation between commensal bacterial and host is interrupted and the tolerance to luminal microflora is lost, triggering inflammatory responses.

By far, it is not completely understood how intestinal immune tolerance is established and maintained, or how it is lost in inflammatory disease state.The underlying mechanisms involve complex interactions between microbiota and its host and intricate immune regulations, which are believed to be subject to genetic predispositions and environmental exposures.

In the healthy gut, the exposure to normal luminal components is tolerated without triggering the inflammatory response. It is hypothesised that commensal bacteria could down-regulates the inflammatory genes and block the activation of nuclear factor kappa B (NFκB) pathway, therefore avoiding destructive immune responses (Xavier & Podolsky, 2007).

In the intestinal tract, luminal antigens are sensed and processed by pattern recognition receptors (PRR). The basal PRR activation is believed to be crucial in maintaining intestinal barrier function and homeostasis. However, the abnormal signalling by PRR may also contribute to IBD pathogenesis.This has been the interest of numerous genetic studies linking PRR genes, such as Nucleotide-binding oligomerisation domain 2(NOD2) and toll-like receptors (TLR), with IBD susceptibility.

The membrane-associated TLR are one of the most important pattern-recognition receptors identified. TLR members expressed by intestinal epithelial cells include: TLR2, which recognised peptidoglycan of Gram+ bacterial cell wall; TLR3, a receptor for viral double-stranded RNA; TLR4, which recognised the major component of Gram- bacteria cell wall -- lipopolysaccharide (LPS); and TLR5, which binds bacterial flagellin. The NFκB pathway is activated as a result of the TLR2 recognition, leading to the triggered innate and adaptive immune responses.

Another PRR --NOD, which contain NOD1 and NOD2 are expressed in antigen-presenting cells. On recognition of peptidoglycan by NOD2, NFκB pathways are activated. Both TLR

and NOD receptors are involved in host-response to microbes in the intestinal tract, either pro-inflammatory (e.g., TNF-α, interleukin-12.) or anti-inflammatory (interleukin-10, transforming growth factor β). (Figure 2.4)

2.4.2.3 IBD: immune dysfunction or abnormal gut microflora?

It is well known that IBD is the result of dysfunctional interaction between intestinal bacteria and the gut mucosal immune system. However, this imbalance can be interpreted in two ways. Either a defective immune system or abnormal gut microflora could contribute to this imbalance, or a combination of both. Several aetiological theories therefore have been proposed, based on the loss of microbial tolerance observed in IBD.

Both CD and UC occur in areas with the highest luminal bacterial content.How the intestinal immune system discriminates between coexisting resident microflora and pathogenic microflora is not fully understood. Subtle changes in bacterial compositions may have profound effect on mucosal function and immune response. In IBD patients, the amount of bacteria attached to the epithelial surface were found to be larger than in healthy individuals. And the bacteria were identified both in epithelial layer and in intracellular locations (Swidsinski *et al*., 2002). It has been suggested that pathological organisms that could establish a low-grade infection in the mucosa are responsible in the inflammatory processesof IBD, although in most cases, no specific pathogen can be identified (Thompson-Chagoyán, Maldonado, & Gil, 2005).

The direct contact of commensal microbiota and theintestinal mucosa is thought to stimulate inflammatory activity. A body of evidence indicates a loss of intestinal epithelial barrier function which allows a close juxtaposition of non-pathogenic organisms to the mucosal immune system offers explanation to the IBD onset. An increased mucosal permeability in intestinal inflammatory conditions has been reported, and this is believed to activate intestinal

immune cells and cause local damage and further disrupt the mucosal barrier function (Farrell & LaMont, 2002).By studying animal models of IBD, in which the inflammation is found to be dependent on the presence of normal microbiota, it was realised that the antigens driving the gut inflammation are derived from normal intestinal microflora (Madsen, Doyle, Jewell, Tavernini, & Fedorak, 1999; Sellon *et al*., 1998; Taurog *et al*., 1994).

After acute inflammatory response to bacterial stimulation, the progression or resolution of the inflammation state largely depends on the delicate balance between pro-inflammatory and immunosuppressive forces. Hyper-reactivity to normal intestinal microflora has been observed in a number of studies with different underlying genetic defects in cytokines crucial in immune regulations, such as IL-2 and IL-10. This leads to abnormal immune responses (Strober, Fuss, & Blumberg, 2002). Mouse models have demonstrated that microbial components could trigger IBD in individuals with immune defects (Watanabe *et al*., 2006).In IBD, chronic inflammation occurs due to the defective regulation of immune response. As a result, the polymorphisms in genes encoding cytokines in the innate immune system are believed to affect the course of inflammatory process and the risk of IBD.

In general, most of the experimental models established are the result of genetic manipulation with deletion (knockout) or over-expression (transgenic) of certain selected genes. These models have significantly increased our understanding towards the cellular and molecular mechanisms of intestinal homeostasis and its break-down in disease. However, IBD in humans are likely to be complex, multi-factorial, and more subtle than those observed in these animal models. Therefore, models occur spontaneously are better at mimicking the complexity of the human disease. Since inflammation arises from multiple defects ina wide variety of immune-modulatory molecules, and that none of the animal models can be used as exact replicates of a human disease, it is important to coordinate multi-centre studies and to

observe the difference between IBD models in order to contribute to the understanding of the pathogenesis of IBD.

2.4.3   The role of IL-10

2.4.3.1   IL-10 in immune regulations

Interleukin-10 has long been known for its potent anti-inflammatory activity. Since it was first identified in 1989(Fiorentino, Bond, & Mosmann, 1989), the number of different cell typesfound to produce and respond to IL-10 has increased rapidly. T cell, including type 2 T-helper (Th2), type 1 T-regulatory (Tr1), Th1 and Th17 cells, and CD8$^+$, monocytes and macrophages, as well as B cells and some subsets of dendritic cells are all known to produce IL-10(Mosser & Zhang, 2008). Non-immune cells such as keratinocytes and epithelial cells are also capable of producing IL-10 (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001; Mosser & Zhang, 2008). The importance of each cellular source of IL-10 has not yet been fully understood and it may vary according to the type, strength, and location of the stimuli.

IL-10 signals through the receptor complex of IL-10 R1 and IL-10 R2, both of which have two subunits with associating Janus kinases 1 (JAK1) and tyrosine kinases (Tyk). Signal transducer and activator of transcription 3 (STAT 3) is then recruited and translocated into nucleus and bind to immune responsive genesfor activation (Sabat, *et al.*, 2010). (As shown in Figure 2.5.A detailed IL-10 signalling pathway is included in the Appendix 3). The IL-10 receptor ligation blocks the NFκB pathway, therefore down-regulates the immune response.
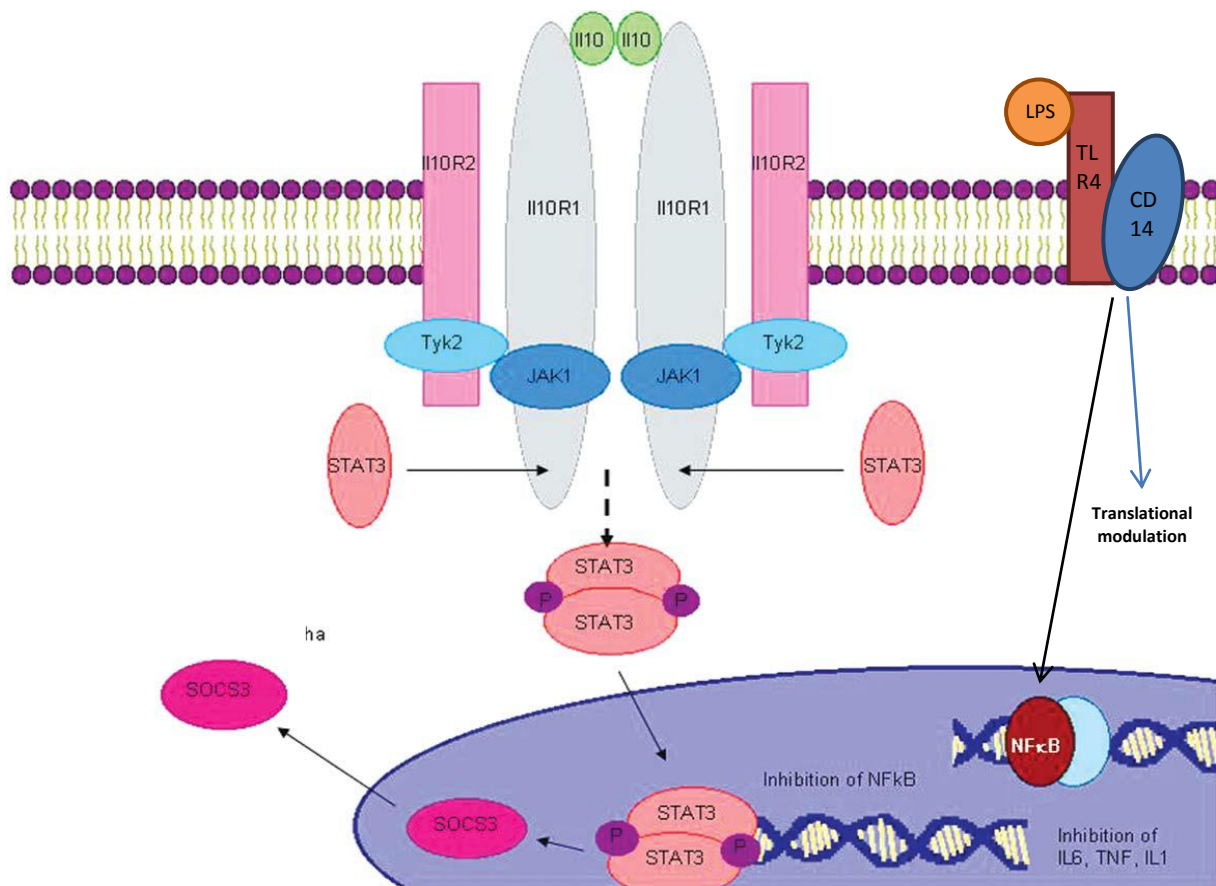
Figure 2.5 Simplified version of the IL-10 signalling (Adapted from Thompson & Lees, 2011)

There are various types of cytokines secreted during the IBD process which attract and activate effector cells. IL-10, as an anti-inflammatory cytokine, down-regulates pro-inflammatory cytokines such as TNF-α through its inhibitory activities on macrophages and monocytes. In addition, IL-10 also could inhibit the production of interferon and has an anti-proliferative effect on human intestinal T cells(Stefan Schreiber, Heinig, Thiele, & Raedler, 1995). Therefore, it is an essential immunoregulator in the intestinal tract. Insufficient production of IL-10 is thus hypothesised to be a key contributor to the imbalance between pro- and anti- inflammatory mechanisms, which is responsible for chronic intestinal

inflammation found in IBD(Stefan Schreiber, *et al*., 1995; Van Deventer, Elson, & Fedorak, 1997).

The first convincing evidence for IL-10's role in intestinal immune regulations and IBD pathogenesis wasdemonstrated with animal models, in which mice lacking IL-10 gene spontaneously developed IBD-like inflammation (Fiocchi, 1998; Kühn, Löhler, Rennick, Rajewsky, & Müller, 1993). However, IL-10 knockout (IL-10$^{-/-}$) mouse models do not develop colitis in germ-free environment, highlighting the role of intestinal bacteria in the stimulation of inflammation(Kühn, *et al*., 1993; Sellon, *et al*., 1998). Appropriate production of IL-10 could thus regulate the immune response to the intestinal microflora, preventing chronic inflammation from developing. Patients with severe CD and UC have been found with decreased IL-10 production (Stefan Schreiber, *et al*., 1995). In *in vitro* and animal models, administration of IL-10 has proven to be effective in ameliorating the inflammation (Ishizuka *et al*., 2001). This has also been confirmed in human observational study (Lowe, Galley, Abdel-Fattah, & Webster, 2003).

2.4.3.2 IL-10 promoter polymorphism

The human IL-10 gene is located on chromosome 1q21-32 and contains five exons and four introns. Several studies have reported correlations between disease susceptibility and IL-10 gene polymorphisms. The strong association between ulcerative colitis and IL-10 gene identified in European GWAS provide exciting evidence on the possibility of multiple causative variants, which, however, remains to be further investigated (Anderson, *et al*., 2011; Louis, Libioulle, Reenaers, Belaiche, & Georges, 2009).

The production of IL-10 varies among individuals, and it is mainly determined by the mRNA synthesis rate (Crawley *et al*., 1999). Changes in the promoter activity are thought to be one of the mechanisms in the alteration of IL-10 production (Turner, *et al*., 1997). This is

regulated by gene polymorphisms at their promoter region(Eskdale, Kube, Tesch, & Gallagher, 1997; Gibson *et al*., 2001), and it is the focus of my study.

European GWAS have confirmed the association between IL-10 gene and IBD susceptibility. The evidence is especially strong in relation to ulcerative colitis(Anderson, *et al*., 2011).Three common SNPs have been relatedwith IL-10 production, namely -1082 G/A, -819 C/T and -592 C/A. Three out of eight possible haplotypes were found in normal population -- GCC, ACC and ATA, which are related to different IL-10 productions (Balding *et al*., 2004; Tagore, *et al*., 1999; Turner, *et al*., 1997). The allele -1082G (haplotype GCC) is associated with higher IL-10 synthesis while -1082A (haplotype ACC and ATA) are associated with lower production of IL-10, reported in several recent studies (Castro-Santos, *et al*., 2006; Koss, Satsangi, Fanning, Welsh, & Jewell, 2000; Ouma *et al*., 2008; Turner, *et al*., 1997). In addition, it has been reported that contribution of -1082 allelic variants is more important than that of alleles at the -819 and -592 position to the regulation of IL-10messenger RNA (mRNA) levels (Castro-Santos, *et al*., 2006; Suárez, Castro, Alonso, Mozo, & Gutiérrez, 2003; Zhu, Lei, Liu, & Wang, 2012).A strong statistical difference was observed between the GCC variant and other low-producer genotypes. Therefore, -1082 G was considered an important genetic factor regulating the constitutive IL-10mRNA levels (Suárez, Castro, Alonso, Mozo, & Gutiérrez, 2003).

It is generally believed that IBD patients are more likely to have the low IL-10 producer genotype and phenotype. However, genotyping of IBD patients and healthy individuals showed conflict results in relating SNPs frequencies with their susceptibility to IBD. Several large, well-characterised cohort studies have related IL-10 polymorphisms with IBD susceptibility in Caucasian populations. The frequencies of -1082 genotypes were statistically different between IBD patients (especially UC patients) and controls. Significantly higher -1082 A allele frequency was demonstrated in IBD patients (Amre *et al*., 2009; Fernandez *et*

al., 2005; Fowler *et al*., 2005; Franke *et al*., 2008; Koss, *et al*., 2000; Parkes, Satsangi, & Jewell, 1998; Tagore, *et al*., 1999). Furthermore, in one study, the association was demonstrated in a gender-specific manner (Amre et al., 2009; Fernandez et al., 2005; Fowler et al., 2005; Franke et al., 2008; Koss, et al., 2000; Parkes, Satsangi, & Jewell, 1998; Tagore, et al., 1999; Tedde et al., 2008).

However, some other studies failed to show the relationship (Andersen *et al*., 2010; Glocker, Kotlarz, Klein, Shah, & Grimbacher, 2011; Klein *et al*., 2000). This may due to the limitations of small number of participants in these studies and the exclusion criteria which exclude the patients with stronger genetic determinants. In addition, the clinical disease pattern in terms of extent and severity was not usually taken into account in the studies, which also contributes to the inconsistency.

A recent New Zealand population genotyping analysis has demonstrated a significant relationship between IL-10 promoter SNPs and serum IL-10 levels, and the SNP at -1082 was associated withCD risk. This association has also been revealed in an earlier study in New Zealand Caucasian population (Hong *et al*., 2008).

2.5 Current treatment of IBD

With the absence of an aetiological therapy, the current treatments of IBD focus on the inflammation process, either to reverse the inflammation state or to maintain the remission state. The choice of medical therapy is usually based on the extent and severity of the disease. Classic anti-inflammatory and immunosuppressant drugs include corticosteroids, mesalamine compounds, azatioprine and its derivatives (Steidler, 2001). However, these drugs exert their effect by non-specifically suppressing inflammation, and long-term use of these drugs may result unpleasant side effects, thus raising safety issues. In addition, many patients will

experience recurrent activity of the disease or do not respond to such treatments (Carter, Lobo, & Travis, 2004). Therefore, novel and improved strategies are required in the IBD treatment.

Recent techniques allow a more localised delivery of therapeutics into inflamed tissues, lowering the risk of unnecessary drug exposure to healthy tissues and organs. They include colonic instillation of conventional chemical therapy or interleukins such as IL-10, intranasal instillation of DNA delivery system, and IL-4 injection and IL-10 gene transfer using recombinant human adenovirus (Barbara, Xing, Hogaboam, Gauldie, & Collins, 2000; Hogaboam *et al*., 1997; Kitani *et al*., 2000; Lang & Peppercorn, 1999).

Novel anti-inflammatory therapies use neutralising monoclonal antibodies or anti-inflammatory cytokines to modulate the immune dysregulation in IBD, such as the systemic administration of recombinant IL-10 or anti-TNF monoclonal antibodies (Lindsay & Hodgson, 2001; S Schreiber *et al*., 1998; Steidler, 2001; Van Deventer, *et al*., 1997). With the understanding of the importance of gut bacteria content in the IBD pathology, antibiotics and probiotic therapeutics are now used in treating IBD. Other than suppressing the host immune action in the conventional methods, manipulating the gut microflora in restoring the tolerancestate has become another promising approach to treat IBD patients. The rationale regarding the therapeutic manipulation of intestinal microflora and the use of probiotics includes both preclinical and clinical models (Rolfe, Fortun, Hawkey, & Bath-Hextall, 2006). A number of successful human trialsshowed improved clinical status after short-term or long-term probiotics treatment, indicating this as new therapeutic opportunities for IBD (Naidoo, Gordon, Fagbemi, Thomas, & Akobeng, 2011).

2.6 The role of personalised nutrition in IBD

As previously mentioned, dietary and nutritional factors are thought to have an important role in the development and treatment of IBD. They can serve as risk factors or exert beneficial effect towards IBD (Hou, *et al*., 2011). A variety of foods are identified from epidemiological studies to raise the IBD risk including total fat, simple sugar, dairy products together with animal protein, fast food that are commonlyseen in westernised diet.On the other hand, vegetables, fruits, fishand dietary fibre intake appear to be protective against IBD (Amre, *et al*., 2007). However, it is impossible to identify the role of nutrients in IBD without understanding its complex interactions with genetic and immunologic factors. A recent study on categorising foods in relation to their effects on CD symptoms concluded that it is almost impossible to identify a specific food group that CD patients should avoid eating. The wide range of foods identified to be detrimentalsuggests the importance of personalised diet in the disease management.

Increased understanding from immunologic and genomic research has led us to the discovery of various genomic loci involved in the immune-pathogenesis of IBD. Some single-nucleotide variations in the DNA sequence havecrucialeffects on health or disease. Nutrigenetic and nutrigenomic approaches help demonstrate how these gene polymorphisms affect individual's responses to specific food and vice versa. Because the influence and/or the requirement of diet are remarkably variable among different people, individualised nutrition based on the understanding of individual genetic predisposition is needed to better prevent or treat IBD and to promote optimal health (Triggs *et al*., 2010).

Our experiment on testing food for its ability to change IL-10production in people with abnormal IL-10 activity aims to contribute to a more individualised nutritional and

therapeutic approach for not only IBD patients but also people in greater risk of developing IBD.

2.7 Turmeric/Curcumin and IBD

Curcumin is a natural yellow compound found in the plant *Curcuma longa* and is the active component of turmeric, which is used as a food spiceor colouring agent. Traditionally, the rhizomes of turmeric plants were gathered annually, after which they are boiled and dried, then ground to a orange-yellow powder and used for various purposes, as shown in Figure 2.6 (Tayyem, Heath, Al-Delaimy, & Rock, 2006). In addition to its use as a food additive, turmeric has a well-documented historical use in Chinese, Hindu, and Ayurvedic medicine over the centuries to treat a wide range of diseases including inflammatory disorders. Increasing evidence suggests that turmeric consumption leads to many protective biological effects, which can be attributed to its main bioactive constituent -- curcumin. For the last few decades, extensive work has been done to establish its biological activities and pharmacological actions (Hatcher, Planalp, Cho, Torti, & Torti, 2008; Zingg & Meydani, 2012).

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). Curcumin (3-4%), which is responsible for the yellow colour, has mainly presented in three forms--curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). Curcumin, together with the two derivatives -- Demethoxy and bis-demethoxy curcumin, are referred to as curcuminoids (Chattopadhyay, Biswas, Bandyopadhyay, & Banerjee, 2004). The chemical structures of natural curcuminoids can be found in Appendix 7.
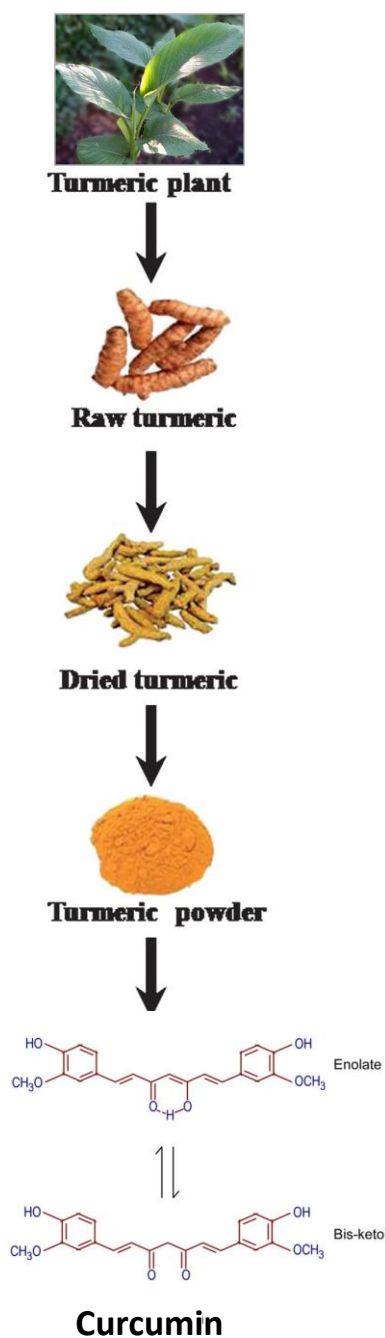
Figure 2.6 Obtaining curcumin from turmeric (Adapted from Baliga et al., 2012)

2.7.1 Characteristics of turmeric/curcumin

Curcumin is chemically unstable, as it is subject to degradation in alkaline pH, exposure to oxygen, ultraviolet and visible light. Its instability was reported even at physiological pH (Wang *et al*., 1997). However, the major degradation products due to curcumin auto-oxidation have not received much attention in metabolism studies by far. Much work is

needed to evaluate the congeners and metabolites of curcumin (see in Appendix 8) for their effect on human health.

Being a natural product, turmeric/curcumin was demonstrated to be safe in animal and human studies at high doses. Humans were shown to be able to tolerate high concentrations (100mg/day of curcumin) without significant side effects (Ammon, 1991; Chainani-Wu, 2003). However, the absorption of turmeric/curcumin after oral administration appears to be very poor, suggesting low intestinal bioavailability (Maheshwari, Singh, Gaddipati, & Srimal, 2006).

2.7.2 Biological activities of turmeric/curcumin

The anti-inflammatory, anti-oxidant, anti-proliferative, and pro-apoptotic properties of curcumin have been discovered in increasing number of experiments with *in vitro* and *in vivo* models (Gupta, Kismali, & Aggarwal, 2013; Modasiya & Patel, 2011). Its anti-inflammatory and anti-cancer effects are believed to be mediated through the cell signalling pathways, such as NFκB, mitogen activated protein kinase (MAPK) and JAK/STAT, altering gene expression, such as genes affecting inflammatory cytokines, growth factors, enzymes and cell cycle proteins, or through direct interaction with multiple molecular targets(Shehzad, Rehman, & Lee, 2012; Shishodia, 2012) (shown in Figure 2.7). However, due to its poor solubility (water-insoluble), poor stability in gastro-intestinal tract and low bioavailability (poor intestinal absorption after oral administration), clinical use of curcumin has given limited results(Anand, Kunnumakkara, Newman, & Aggarwal, 2007).

Enzymes
Arylamine N-acetyltransferase↓, ATPase↓, COX-2↓, Desaturase↓, DNA polymerase↓, FPTase↓, GST↑, GCL↑, HO-1↑, iNOS↓, 5-LOX↓, MMP↓, NQO↓, ODC↓, PLA2↓, SHP-2↑, Telomerase↓, XO↓

Inflammatory Cytokines
CXCL1↓, CXCL2↓, IL-1β↓, IL-2↓, IL-5↓, IL-6↓, IL-8↓, IL-12↓, IL-18↓, MCP-1↓, MIP-1α↓, TNFα↓

Apoptosis Related Proteins
Bak↑, Bax↑, Bim↑, Bcl-2↓, Bcl-XL↓, Caspase-3↑, Caspase-7↑, Caspase-8↑, Caspase-9↑, c-FLIP↓, DR4↑, DR5↑, Fas↑, FADD↑, IAP↓, Noxa↑, Puma↑, Survivin↓

Curcumin

Protein Kinases
AK↓, CDPK↓, EGFR kinase↓, ERK↓, FAK↓, IRAK↓, JAK↓, JNK↓↑, p38MAPK↓↑, mTOR↓, PhK↓, cPK↓, PKA↓, PKB↓, PKC↓, pp60ᶜ⁻ˢʳᶜ↓, Syk↓

Multidrug Resistance
P-glycoprotein↓, MDR1↓, MRP1↓

Transcription Factors
AhR ↑, AP-1↓, ATF3↑, β-catenin↓, C/EBP↓, CHOP↑, CTCF↓, Egr-1↓, EpRE↑, HSF-1↓, HIF-1↓, Notch-1↓, Nrf2↑, NF-κB↓, PPAR-γ↑, STAT-1↓, STAT-3↓, STAT-4↓, STAT-5↓, WT-1↓

Figure 2.7 Molecular targets of curcumin (Adapted from Shishodia, 2012)

In IBD, it has been shown that curcumin has inhibitory effects on major inflammatory mechanisms such as NFκB, a key factor in the up-regulation of inflammatory cytokines that have a high profile in IBD, as well as cyclooxygenases 2, lipoxygenase, TNF-α and IFN-γ. With its anti-inflammatory properties, curcumin has been a candidate for IBD treatment in a range of human and experimental studies, which have achieved encouraging results. In a pilot study, all five proctitis patients improved after the two-month curcumin treatment and four out of five Crohn's disease patients had a lowered CDAI (Crohn's Disease Activity Index) (Holt, Katz, & Kirshoff, 2005). A randomised, double-blind, placebo-controlled trial which aimed to evaluate the efficacy of curcumin found a significantly lower recurrence rate in UC patients treated with curcumin than it in the placebo group (Hanai *et al*., 2006; Hanai & Sugimoto, 2009).

In animal models, it is demonstrated that curcumin has very limited effect on IL-10 deficient mice, suggesting its effects may be IL-10 dependent (Larmonier *et al.*, 2008). Another study on IL-10 deficient mice demonstrated that curcumin had anti-inflammatory effects only when it was emulsified in carboxymethyl cellulose – a viscosity modifier, which indicates a bioavailability issue for curcumin treatment (Ung *et al.*, 2010).In a study investigating the effect of solid lipid micro-particles of curcumin on experimental colitis, a reduced level of disease activity was shown in the rats that received this colon-specific delivery approach, compared with those using pure curcumin (Yadav, Suresh, Devi, & Yadav, 2009).

Based on the existing evidence, it is concluded that turmeric/curcumin can modify the anti-inflammatory responses in the intestine and be used as potential treatment for IBD. However, the molecular mechanisms involved are not fully understood. Therefore, in my second part of my study, I usedthe IL-10 promoter assay developed earlier to further understand the effects and the underlying mechanisms of turmeric onits ability to affect IL-10 (a major anti-inflammatory cytokine) production, specifically IL-10 transcription.

3 Materials and Methods

In this research project, a cell-based luciferase reporter assay with inducible expression of each of the IL-10 promoter SNPs was developed. Then it was used to study how certain food components or extract, such as curcumin could affect the IL-10 promoter transcription.

Assay development and sample testing were the two stages in this experiment. The methods include mammalian cell culture, *in vitro* reporter assay and bioactivity assay.

The entire IL-10 promoter assay was completed inthe 293TLR4 cell line that has been genetically modified to express the risk haplotypes of the IL-10 promoter associated with IBD, using transient transfection. A secreted luciferase reporter system was then used to measure the level of promoter activity.

3.1   Cell culture of 293-hTLR4A-MD2-CD14 cells

Human embryonic kidney cells (HEK293) stably transfected with the human TLR4a,MD2 and CD14 genes (293-hTLR4A-MD-CD14, referred to as 293TLR4later) wereused in the entire study.The 293TLR4 cells were co-transfected with hTLR4a, MD2 and CD14 genes and purchased from InvivoGen, USA. Normal HEK293 cell (ATCC, USA, CRL-1573) morphology is shown in Figure 3.1.

The 293TLR4 cell line is designed to study the stimulation of human TLR4 (hTLR4) by LPS -- an important structural component of the membrane of Gram-negative bacteria, whichinitiates the release of critical pro-inflammatory cytokines and enzymes such as IL-10 that are necessary in enabling the immune response.

The cell culture procedures in this experiment were completed in physical contaminant level 2 (PC2) laboratories. The ClassII biosafety cabinet and aseptic techniques were used

throughout the experiment process to ensure a sterile working environment. The working surface, plastic ware or other consumables and cell reagent containers were sanitised with disinfectant (70% ethanol) before use in the biosafety cabinet.
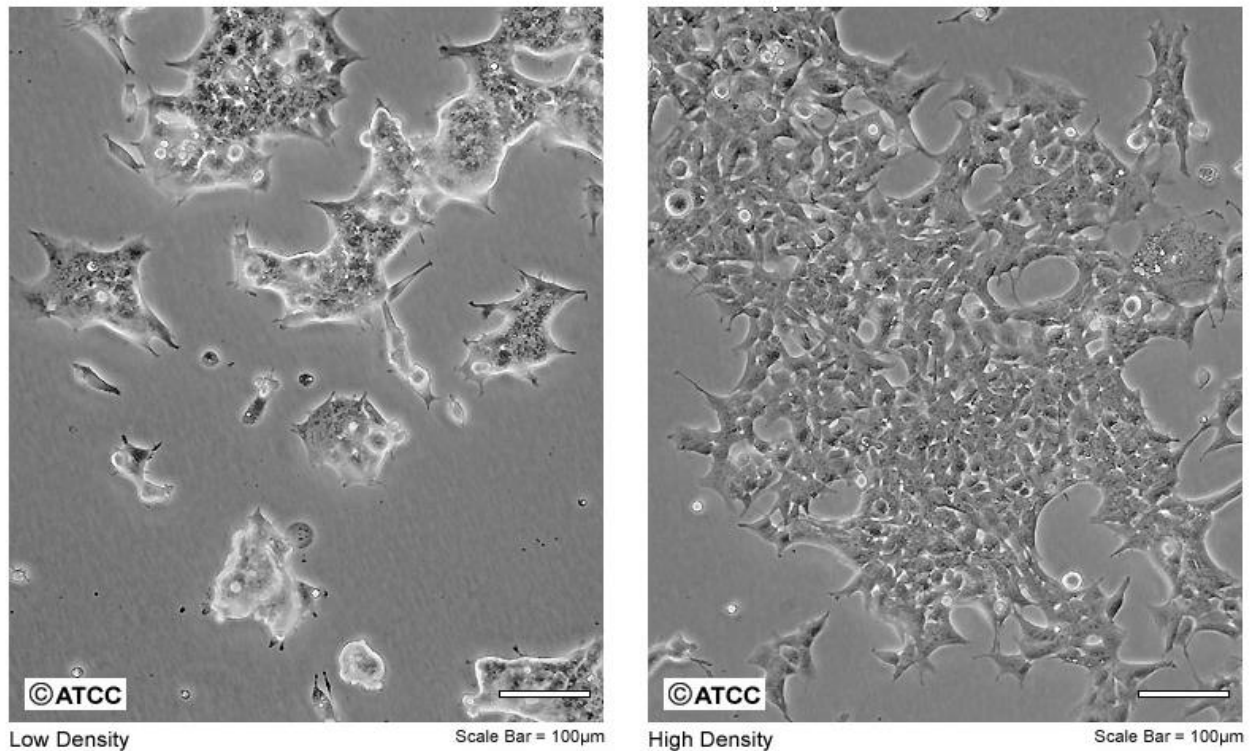
Figure 3.1 HEK293 cell morphology at high and low density

3.1.1 Recovery of cells from cryostorage

The 293TLR4 cell line has a specific media formulation (growth medium) for optimal growth. This formulation was used when the cell line was first purchased from supplier or when recovered from cryostorage. Generally, cells can be stored indefinitely in liquid nitrogen (i.e. cryostorage). In this assay, sufficient cell stocks were made before commencing the experiment. Only cells or cell stocks with a passage number of twenty or less were used, as

recommended by Invitrogen. At any point of the study when cells were needed, they could be recovered from cryostorage.

Cryotubes containing 293TLR4 cells were removed from liquid nitrogen and left to thaw at room temperatureuntil completely thawed. The contents of the cryotube then were transferred to a 50 ml centrifuge tube (Corning Life Sciences, USA). The cryo-preservative was diluted 20-fold with pre-warmed cell culture growth medium. After gentle mixing, the tube was spun at 1200rpm for 5 minutes. The supernatant was discarded, and the cell pellet was re-suspended in fresh growth medium, seeded intoa tissue culture flask (25, 75, or 180 cm^2, Corning Life Sciences, USA) containing appropriate amount of medium, and incubated at 37°C and 5% CO_2.

Growth medium contains advanced Dulbecco's modified Eagle medium (aDMEM, Life Technologies, USA) with 2% of foetal bovine serum (FBS, Life Technologies, USA), 1% of Penicillin/streptomycin solution (PenStrep, Life Technologies, USA) and 100 mg/ml of Normocin (InvivoGen, USA), or Dulbecco's modifiedEaglesmedium (DMEM, Life Technologies, USA) with 10% of FBS, 1% of PenStrep, and 100 mg/ml of Normocin. These media are compositionally identical and only differ in the amount of FBS used.

3.1.2 Cell maintenance and subculture

Each time after cells are seeded into new flasks, they will first experience a latent period of no growth, called the lag period, which lasts from a few hours up to forty-eight hours and allows them to recover and attach. Then, they enter the log phase, also known as exponential growth, during which cell population doubles over certain amount of time, and this time varies among different cell lines. As the cells become packed and the nutrients are used up, they will eventually enter the plateau phase, where the growth rate drops to close to zero. At this stage, some cells exit cell cycle but retain viability. It is preferable that cells are sub-

culturedduring log phase, in which the cells are most healthy. This growth cycle is repeated every time the cells are sub-cultured; therefore the passage number or P number of cells is increased by one.

The level of coverage of the cell layer in the tissue culture dish or flask is referred to as the confluency. Generally, cells are not allowed to become fully confluent to avoid reaching the plateau phase. At 80 to 90% confluent, 293TLR4were seeded and sub-cultured accordingly. 293TLR4 are adherent cells but no dissociation agentwas used while removing them from flask surface. Gentle medium extrusion onto the cells was sufficient to detach them. After centrifugation of cell suspension, they were reseeded into new flasks. There was usually a medium change every forty-eight hours afterthe cells were freshly reseeded. Depending on the cell density, cells weresub-cultured every five to eight days.

293TLR4 cells are maintained in growth medium for at least three sub-cultures before the selective antibiotics were introduced. This is to ensure cells are fully recovered and in healthful condition. The use of culture medium, growth medium supplemented with selective antibiotics (10μg/ml of Blasticidin and 50μg/ml of HygroGold, InvivoGen, USA), after three sub-cultures is required in order to maintain the plasmid integrity for hTLR4a, MD2 and CD14 gene. Genetic instability is a biological phenomenon that occurs in stably transfected cells in normal cell culture conditions. Therefore, cells were not allowed to passage more than 20 times to remain efficiency during the entire experiment, and adequate frozen stocks from early passages were also prepared at the beginning stage of the experiment.

3.1.3 Cryostorage of cells

After the cell line could be grown sufficiently at the beginning of the project, cryopreservation of cells were carried out to secure sufficient amount of cell stocks without aging and other problems, e.g. contamination or incubator failure. The cell density at freezing

is usually 1×10^6 to 1×10^7 cells/ml, with the presence of 10% preservative -- dimethylsulfoxide (DMSO, Sigma-Aldrich, USA). 293TLR4 cell stock were made at 5×10^6 cells/ml and stored at -80^oC overnight before transferred into liquid nitrogen (at approximately -175^oC). When recovering cells from cryostorage, 2 to 5 fold of the normal seeding density was used, and the growth medium was changed at the following day instead of after forty-eight hours to minimise cell recovery time.

3.1.4 Cell counting

Quantification of the cell population is required when assessing medium suitability, determining population doubling times and monitoring cell growth, or simply in seeding a defined number of cells for various assays. Cell numbers are usually determined with a haemocytometer, an instrument for estimating the number of cells in a given volume under a microscope. The improved Neubauer haemocytometer (Camlab, UK) used in this experiment consistsof a thick glass slide with two counting chambers, each 0.1mm deep and can be divided into nine large squares delineated by triple while lines. The centre 1mm^2 area is further divided into 25 squares, each of which is further marked into 16 squares (as shown in Figure 3.2).This is to help orient the counting and avoid counting an individual cell more than once. The area of entire reticulated part is 9 mm^2.

Cell viability can also be determined using trypan blue, a vital stain. Only non-viable cells absorb the dye and appear blue and asymmetrical under the microscope, whereas healthy viable cells are refractory to the dye and rounded. It is assumed that the cell suspension placed into the chamber represents a truly random sample. Therefore, to accurately count cells, it is important to well disperse the cell suspension until without any visible cell clump.
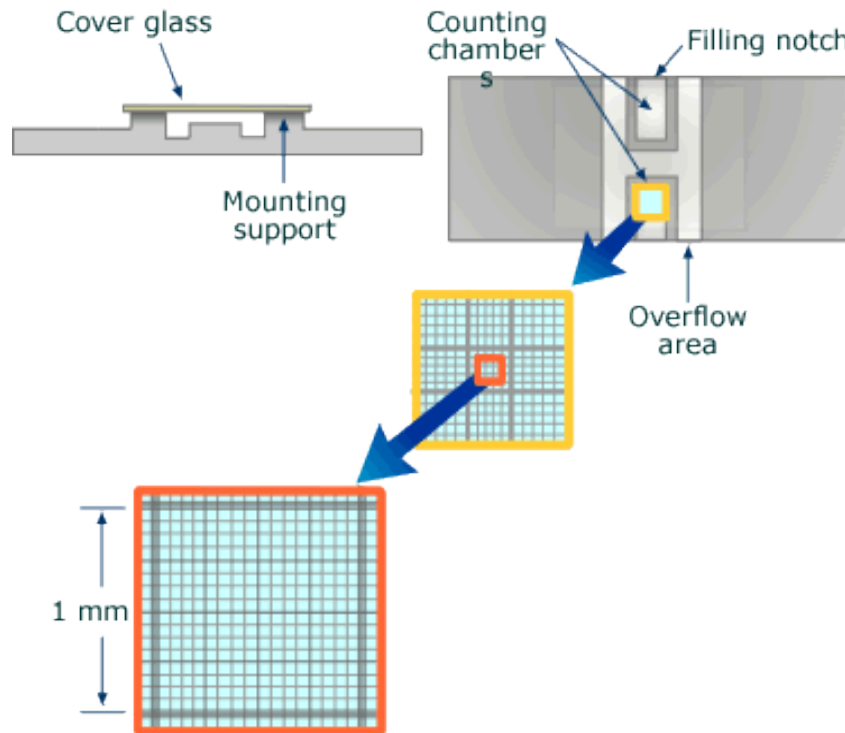
Figure 3.2 A Standard haemocytometer with Neubauer ruling.

When counting 293TLR4cells, thecell suspension to be counted was well dispersed. Then50μl of cell suspension was mixed with 50μl of trypan blue to fill the haemocytometer. The number of cells in the $1mm^2$ centre square (5x5) was counted in both counting chamber.(The difference between them should be less than 10%).Dead cells were counted separately if assessing viability.

The haemocytometer is designed so that the number of cells in the central square is equivalent to the number of cells x 10^4/ml. Therefore, to obtain the final cell number:

- Total cell count/ml (x 10^4 cells/ml) = (Total number of cells / Number of squares) x dilution factor -- 2 (1:2 dilution of cells and cell stain)

- Total cell count = total cell count/ml x volume of cell suspension (ml)

To obtain the cell viability:

- Cell death (%) = (dead cell count) / (total cell count) x 100

- Cell viability (%) = 100 - cell death (%)

3.2 Establishment of IL-10 promoter assay

The IL-10 promoter assay involved using the 293TLR4 cell line that had been genetically modified to express the three risk haplotypes IL-10 promoters related with IBD. This was achieved by transiently transfecting the cells with genes of interest and measuring the amount of luciferase reporter expressed, using a *Metridia* secreted luciferase reporter system. The transfection process, which refers to theintroduction offoreign nucleic acid into cells, was completed by addingcationic lipids reagents to facilitate DNA delivery into cells.

3.2.1 The*Metridia*luciferase reporter system in the assay

The secreted luciferase reporter system uses secreted *Metridia* luciferase (MetLuc) as a reporter molecule to monitor the activity of promoters and enhancers.*Metridia* luciferase,found in *Metridialonga* -- a marine copepod, catalyse a simple bioluminescent reaction of which the product is a blue bioluminescent protein (Markova, Golz, Frank, Kalthof, & Vysotski, 2004). The *Metridia longa* secreted luciferase gene has been cloned and codon-optimised to enhance expression when transfected into a human cell line.

The reporter vector used in this assay, pMetLuc2-Reporter, allows promoter elements to be inserted upstream of the secreted *Metridia* luciferase gene. Activation of the promoter of interest drives the expression of secreted *Metridia* luciferase, which is detected in the supernatant of the transfected cells. (Flowchart of Ready-To-Glow™ Secreted luciferase reporter assay procedure used in this experiment is shown in Figure 3.3).IL-10 promoter gene variants were cloned into the pMetLuc-Reporter vector (by AgResearch Ltd and provided for use in this study) to produce pMetLuc2-variants vectors (pMetLuc2-GCC/ pMetLuc2-ACC/ pMetLuc2-ATA), as shown in Table 2 at section 3.2.4. Activation of IL-10 promoter then

leads to the expression of secreted *Metridia* luciferase, which is then detected in the supernatant of the transfected 293TLR4 cells.

1. Clone response element/
   promoter of interest into
   pMetLuc-Reporter vector

2. Transfect host cell line

Promoter activation leads to
expression of secreted luciferase
protein in the medium

Removal of supernatant

3. At desired time points,
   assay luciferase activity
   • Transfer media
     sample to 96-well plate
   • Add substrate
     reaction buffer
   • Assay luciferase activity
     in a luminometer

Add luciferase
substrate

Metridia
secreted
luciferase
MCS
pMetLuc-
Reporter
Vector

Secreted
luciferase

Product

LIGHT

Detection by
luminometer

Figure3.3 Flowchart of the Ready-To-Glow™ secreted luciferase reporter assay procedure

- *Negative control:*

A negative control is necessary to measure the background signal associated with the cell culture media. To determine the background level, each assay plate included 50μL samples of 293TLR4 cells culture medium only. The values obtained were then subtracted from experimental results.

- *Positive control*:

A positive control is necessary to confirm transfection and expression of exogenous DNA, and to verify the cells are secreting *Metridia* luciferase into the culture media. The pMetLuc2-control vector is designed to function as a positive control. Cells transfected with this vector will constitutively express and secrete *Metridia* luciferase, with high activity. To confirm the expression and secretion of luciferase in transfected cells, each assay plate has included 50 μL samples of culture medium from cells transfected with the pMetLuc2-control vector. This vector was also used in optimising the transfection conditions.

- *Normalisation of transfection efficiency*:

When monitoring the effect of promoter sequences on gene expression, it is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection. This was done by co-transfecting a second plasmid as a marker gene, which constitutively expresses another reporter that can be clearly differentiated from secreted *Metridia* luciferase. The level of this second reporter − Secreted Alkaline Phosphatase (SEAP) was then used to normalise the levels of luciferase due to transient transfection among different groups and transfections.

MetLuc and SEAP can be distinguished from each other by adding reporter-specific substrates. Since the two reporters have similar secretion kinetics, the amount of either reporter in the cell culture supernatant will accurately reflect its promoter activity. Therefore, SEAP can be used as an indicator of transfection efficiency to normalise the value of MetLuc which varies in different IL-10 variants. When 293TLR4 cells are co-transfected with both reporters, the IL-10 promoter activity can be determined by measuring the luminescence in samples of culture media after adding the relevant substrate.

There are mainly three parts in the establishment of IL-10 promoter assay. First of all, 293TLR4 cells were transfected with pMetLuc2-control vector and pSEAP2-control vector separately. This step was to seek an optimal amount of plasmid DNA and transfection reagents to use in order to achieve thehighest luminescence signal. After the appropriate amount was determined, 293TLR4 cells were co-transfected with both pMetLuc2-control and pSEAP2-control vectors to optimise the co-transfection conditions. Lastly, cells were co-transfected with both pMetLuc2-variant and pSEAP2-control vector to monitor their IL-10 promoter activity.

3.2.2   pMetLuc2-control and pSEAP2-control vector transfection optimisation

The aim of the initial step in the IL-10 assay development was to successfully transfect two control vectors in 293TLR4 cells at separate occasions, and to determine an optimal DNA and lipid to DNA ratio to use in the following co-transfection.

After 293TLR4 cell were sub-cultured in growth medium for three times from cryostorage,they were transferred into culture medium with additional antibiotics and maintained in this medium until before transfection. During transfection, Opti-MEM® I reduced serum medium (Life Technologies, USA) was used to dilute DNA and Lipofectamine™ LTX reagents (Life Technologies, USA) before adding into the cells. This is because medium with antibiotics at this stage may result in cell death.Lipofectamine™ LTX reagent is a cationic lipid formulation for the transfection of DNA into eukaryotic cells. Cationic lipid-mediated transfection (or lipofection) works by facilitating DNA delivery into cells with cationic lipids, which are specially designed and positively charged. They can interact with negatively charged nucleic acid and form DNA-cationic lipid complex (or DNA-liposome complex). This then fuses with cell membrane and transfer the genetic material into the cell for gene expression.

The optimal lipid to DNA (i.e. Lipofectamine to DNA) ratios for both the pMetLuc2-control and pSEAP2-control vectors was determined by testing different concentrations of DNA and transfection reagents. Luciferase secretion and SEAP activity were then measured to determine the optimal transfection conditions for each vector.

To obtain the highest transfection performance, a range of DNA and Lipofectamine™ LTX amounts with the PLUS™ reagent (a transfection enhancing agent) were tested in a 24-well format. Depending on the sensitivity of the cell line, the test DNA concentrations are usually 250ng for sensitive cell line, 500ng, 750ng for most cell lines and 1,000ng for robust cell line. As 293TLR4 is recognised as one of the easiest cell line to transfect, only the first three concentrations were testedin the cells. Selected lipid to DNA ratio to test according to themanufacturer recommendations are 1, 2, 2.5, 3, 4, and 5.

- *Step one: cell seeding*

A healthy sub-confluent culture of 293TLR4 cells were harvested, counted, and 1×10^5 cells were seeded into twenty-four wells of two tissue culture-grade 24-well microtitre plates (Corning Life Sciences, USA). Growth medium was added to ensure the final volume was 500 μl. The cells were cultured at 37^oC, 5% CO_2 in a humidified atmosphere for approximately twenty-four hours to allow for attachment.Cells were ready to be transfected when 50-80% confluencywas achieved.

- *Step two: preparation of transfection reagents*

Stock DNA solutions(pMetLuc2-control or pSEAP2-control) with the concentration of 200ng/μl were prepared at the beginning of the experiment and kept in -20°C.They were used to dilute working stocks of 5, 10,and 15ng/μL of DNA in Opti-MEM medium. Each concentration was then mixed with PLUS reagent (2, 4, and 6μLseparately, according to

manufacturer recommendation), followed by 10 minutes incubation at room temperature. Lipofectamine LTX™ was prepared in a 1 in 10 dilution. Certain amount of the diluted lipofectamine was then mixed with 50µL of DNA+PLUS to achieve lipid:DNA ratios of 1, 2, 2.5, 3, 4, and 5 in each column. The mixture of lipid and DNA+PLUS is called drop-wise, and it was incubated at room temperature for 30 minutes to allow lipid-DNA complexing.

- *Step three: transfection*

Before adding the drop-wise into the cells, the cell culture media in each well was removed and replaced with fresh media. This was to eliminate any background signal caused by the accumulation of secreted *Metridia* luciferase in the supernatant before the start of the transfection. After incubation for 30 minutes at room temperature, the DNA/PLUS/Lipofectamine mixture (drop-wise) was then added to the cells in each well containing 500µL of medium, with final DNA concentrations of 250, 500,and 750ng in each row. An example of the 24-well plate layout is shown in Figure3.4. The cells were then incubated at 37°C, 5% CO_2 in a humidified atmosphere. After 24 and 48 hours, a 100 µL sample of medium from each well was collected and stored at -20°C until required.

293TLR4 cells were sampled in a multi-well plate format. In each plate, cells were seeded from one tissue culture flask, i.e. from the same cell population. Transfection of these two vectors were performed both in duplicates from different cell populations in order to obtain reliable results. Other experimental conditions and procedures were maintained the same during each transfection.
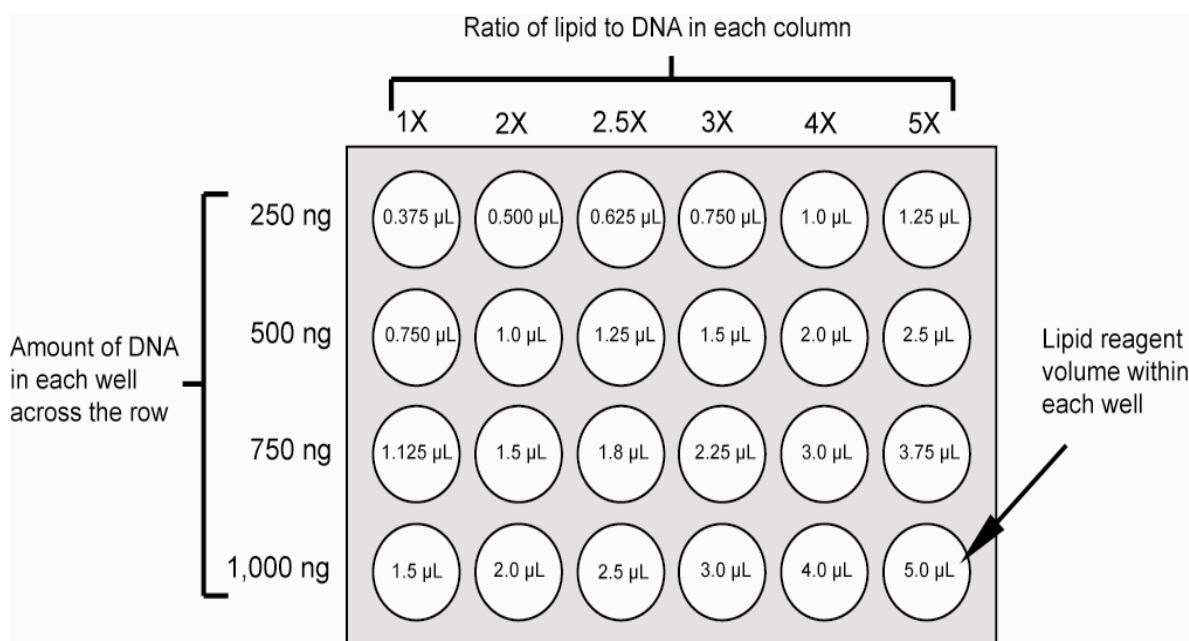
Figure 3.4 Example of a 24-well optimisation plate

- ***Step four: Secreted Luciferase assay***

The secreted *Metridia* luciferase reporter in cell culture medium after pMetLuc2-control vector transfection was quantified using the Ready-To-Glow™ dual secreted reporter kit (Clontech, USA).

The luciferase reagents from the Ready-To-Glow™ dual secreted reporter kit were reconstituted according to the manufacturer's instructions. A 10x substrate stock solution was prepared and kept at -20°C. A 1x substrate/reaction buffer was made in each assay and used within one hour. Duplicate aliquots of stored pMetLuc2-control vector media sample (50 µl) were transferred intoa 96-well white-walled tissue culture grade microtitre plate (Corning Life Sciences, USA) and 5 µl of substrate/reaction solution was added into each well. The 96-well opaque white plates used were the recommended plates for bioluminescence assays and detection. A multichannel pipette was also used in order to reduce the time between substrate addition and signal detection. The luminescence of the samples was measured immediately for 10 minutes using a FlexStation 3 spectrophotometer (Molecular Devices, USA).

- *Step five: Secreted Alkaline Phosphatase activity assay*

The SEAP reporter in cell culture medium after twenty-four and forty-eight hours of pSEAP2-control vector transfection was quantified using Ready-To-Glow™ dual secreted reporter kit (Clontech, USA).

The SEAP reagents from the Ready-To-Glow™ kit were reconstituted according to the manufacturer's instructions. Duplicate aliquots of stored pSEAP2-control vector media (25 µl) were transferred into a 96-well white-walled tissue culture grade microtitre plate and 75 µl of 1x dilution buffer added into each well. The samples were incubated at $65^{\circ}C$ for 30 minutes prior to chilling on ice and recovered to room temperature.100 µl of the solution was added to each sample, followed by incubationat room temperature for 30 minutes. The luminescence of the samples was measured for 10 minutes using FlexStation 3 spectrophotometer (Molecular Devices, USA).

3.2.3   pMetLuc2-control and pSEAP2-control vector co-transfection optimisation

The aim of the second step in the IL-10 assay development was to successfully co-transfecting two control vectors in 293TLR4 cells at the same time, and to optimise the experimental conditions in order to be able to efficiently co-transfect the pMetLuc2-IL-10 promoter vector and pSEAP2-control vector for the food screening assay.

Based on the data from separate transfections, an optimal amount of plasmid DNA and lipid to DNA ration was determined. They were used in the co-transfection of pMetLuc2-control and pSEAP2-control vectors.

- *Step one: cell seeding*

The cell seeding procedures were the same as in previous part (3.2.2). 1×10^5 of healthy sub-confluent 293TLR4 cells were seeded into a tissue culture-grade 24-well microtitre plates. Growth medium was added to ensure the final volume was 500 µl. Cells were cultured at 37°C, 5% CO_2 in a humidified atmosphere for 24 hours oruntil50-80% confluency.

- *Step two: preparation of transfection reagents*

200ng/µl of stock DNA solutions (pMetLuc2-control and pSEAP2-control) were diluted to working stocks of 10ng/µL in Opti-MEM medium. It was then mixed with PLUS reagent, followed by 10 minutes incubation at room temperature. Lipofectamine LTX™ was prepared in a 1 in 10 dilution. Diluted lipofectamine was then mixed with 50µL of DNA+PLUS to achieve chosen lipid:DNA ratio of 2.5.

- *Step three: transfection*

Both pMetLuc2-control and pSEAP2-control mixtures from step two were added into the 293TLR4 cells containing freshly changed media after incubation for 30 minutes at room temperature.After 24 and 48 hours, a 100µL sample of medium from each well was collected and stored at -20°C until required.

Co-transfections of these two vectors were performed in triplicate from different cell populations in order to obtain reliable results.

- *Step four & Step five: Secreted Luciferase assay& Secreted Alkaline Phosphatase activity assay*

These two assays were performed following the procedures in section 3.2.2, using the Ready-To-Glow™ dual secreted reporter kit(Clontech, USA).

- *Optimisation of co-transfection experimental conditions*

The initial results of control vector co-transfection showedunsatisfactory luminescence detected, suggesting lowered transfection efficiency in current co-transfection conditions compared to transfecting cells separately with either control vector. In order to increase overall detectable luciferase and SEAP reporter, several variations of the method were assessed to improve the reliability and robustness of the assay.

Increasing the cell density to achieve better signal: half a million cells were seeded rather than one hundred thousand in the first step.

Transfecting before plating to achieve better signal: cells were previously seeded for approximately 24 hours before transfection to allow for attachment.In order to transfect cells more efficiently, transfection reagents were added at the same time when cells were seeded into plates.

Assay samples immediately after collection to lower signal reduction: cell culture medium samples were collected after the transfection and kept frozen until the luciferase and SEAP assays. A loss of reporter activity may occur during this process. Therefore, supernatant samples were assessed immediately after collection to optimise the signal detection.

Adjusting the assay format from 24-well plate to 96-well plate: The assay was scaled and optimised from 24-well to 96-well plates in order to be able to efficiently test food compounds at a later stage. Volumes of media, cell number and reagentswere normalised to the surface area per well to ensure proper scaling.

3.2.4   IL-10 promoter assay

In this part, the reporter vector of three IL-10 promoter variants, pMetLuc2-GCC, pMetLuc2-ACC and pMetLuc2-ATA were co-transfected with pSEAP2-control vector. The promoter activity was induced by LPS and assessed by detecting luminescence in cell culture

supernatants. The adjusted assay format and conditions which showed better and sufficient signals was used in the IL-10 promoter assay. Assay duration was also shortened as no overnight incubation after cell seeding was needed.

- *Generation of pMetLuc2 vector with IL10 variants*

The vectors with the IL-10 variants used in this study were prepared by AgResearch Ltd as shown in Table 2. The IL-10 sequences and plasmid map are shown in Appendix 1 and 2.

Table 2. IL-10 haplotypes of the three promoter SNPs of interest in IBD pathogenesis and the pMetLuc2 with IL-10-variants

IL-10 haplotype	Associated SNPs	SNP variant	pMetLuc2-IL-10-variant
GCC	**rs1800896** rs1800871 rs1800872	**-1082 G** -819 C -592 C	pMetLuc2-GCC
ACC	**rs1800896** rs1800871 rs1800872	**-1082 A** -819 C -592 C	pMetLuc2-ACC
ATA	**rs1800896** rs1800871 rs1800872	**-1082 A** -819 T -592 A	pMetLuc2-ATA

- *Step one: cell seeding and cell transfection*

3×10^5 of healthy sub-confluent 293TLR4 cells were seeded into a tissue culture-grade 96-well microtitre plates (Corning Life Sciences, USA). Growth medium was added to ensure the final volume was 100 μl.

At the same time, transfection reagents and test plasmid DNA were prepared. 200ng/μl of stock DNA solutions (three pMetLuc2-variant and pSEAP2-control) was diluted to working stocks of 10ng/μL in Opti-MEM medium. They were then mixed with PLUS reagent, followed by 10 minutes incubation at room temperature. The diluted lipofectamine was then added to DNA+PLUS with a lipid:DNA ratio of 2.5.

Both pMetLuc2-variant and pSEAP2-control DNA/PLUS/Lipofectamine were added into the 293TLR4 cells containing fresh changed media after incubation for 30 minutes at room temperature. Cells were cultured at 37°C, 5% CO_2 in a humidified atmosphere for 24 hours or until reach 50-80% confluency.

- *Step two: LPS induction*

A stock solution (5mg/ml) of LPS was prepared by adding 1ml of UltraPure water to the lyophilised LPS-EB (InvivoGen, USA). Stimulation of TLR4 with LPS can be achieved with concentrations from 10ng to 10µg/ml, according to manufacturer's instructions.After 24 hours of co-transfection, IL-10 transcription was induced using 1 µg/ml of LPS. After 2, 4, 6 and 24 hours, a 100µL sample of medium from each well was collected and used in the following assays.

- *Step three & Step four: Secreted Luciferase assay& Secreted Alkaline Phosphatase activity assay*

These two assays were performed following the procedures in previous part, using the Ready-To-Glow™ dual secreted reporter kit (Clontech, USA).

The IL-10 promoter assay including co-transfection of the two vectors and the luciferase and SEAP assay were performed in triplicate from different cell populations in order to obtain reliable results. An optimal LPS induction time was also determined, as shown in the Appendix 4.

3.3 Establishment of positive control for IL-10 transcription

Glucocorticoids are well-known to inhibit the expression and production of many cytokines in the inflammatory responses. Dexamethasone is a potent synthetic glucocorticoid and IL-10

inhibitor (Fushimi, Okayama, Seki, Shimura, & Shirato, 2009). A lowered IL-10 promoter activity was thus expected by introducing dexamethasone into 293TLR4 cells transfected with pMetLuc2-IL-10 variants. Therefore, it could be used as a positive control for the assay.

- *Dexamethasone cytotoxicity assessment*

Before assessing the effect of dexamethasonefor its ability to inhibit IL-10 activity,it was important to determine a non-toxic concentration to use. This step would ensure the reduced IL-10 promoter activity observed in the next step is caused by inhibiting effect of dexamethasone rather than cell death. The cytotoxicity of different concentrations was determinedby assessing the proliferation of the transfected 293TLR4 cells, using the premixed water-soluble tetrazolium-1 (WST-1) assaykit (Clontech, USA). This assay is based on the enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye which can be detected by absorbance at 450nm.This bio-deduction occurs only with live cells; therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

293TLR4 cells were first seeded in tissue culture grade flat-bottomed 96-well plates in a final volume of 100 µl per well of culture medium. After 24 hours of incubation, different concentrations of dexamethasone (Sigma-Aldrich, USA) -- 0, 10, 20, 40, 60, 80, 100, 200, 300, 400, and 500 µM were added into each well. Using FlexStation 3, the absorbance was measured after addition of 10 µl WST-1solutions. Each concentration was tested in twenty-four replicates. Based on these data a non-toxic concentration of dexamethasone was then tested for its capacity to block IL-10 transcription.

- *Establishment of dexamethasone as positive control for IL-10 transcription*

A non-toxic concentration of dexamethasone (1.0 µM) was used to test IL-10 inhibition. 293TLR4 cells were seeded and transfected according to the format and procedure as previously indicated. After LPS induction, 1.0 µM of dexamethasone was added into each well. Untreated cells with no dexamethasone were also included in the assay plate. The IL-10 promoter activity of the transfected cells after dexamethasone stimulation was then measured using Ready-To-Glow™ dual secreted reporter kit (Clontech, USA), and the relative metabolic activity to untreated cells was calculated. This assay was repeated three times with different cell populations.

With the completion of IL-10 promoter assay development and the establishment of a valid positive control, food compounds were then ready to be tested for their ability to alter the IL-10 promoter activity.

3.4 Testing of turmericsamplesusing the IL-10 promoter assay

- *Preparation of turmeric samples*

The samples of turmeric (1 ethanol extract and 12 reversed-phase fractions) used in this study were prepared by collaborators in NuNZ based at Plant and Food Research Ltd (the methods used by them was acquired and stated in Appendix 5). The curcumin content of each sample was also measured by them using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) and was shown in Appendix 6.Samples were then sent to us for use in the IL-10 assay.

- *Turmeric sample testing*

The turmeric samples (NuNZ Extraction and Fractions 1-12) were reconstituted using DMSO and/or UltraPure water depending on the solubility. Samples were fully mixed and stored in -20°C.A dilution of 1 in 10, 1 in 100, 1 in 1,000, and a 1 in 10,000 of each sample was

prepared and tested in the IL-10 promoter assay. A detailed description of sample preparation is included in Appendix 6.

The seeding procedures for 293TLR4 cells were followed in the same manner as previously stated in section 3.2.4.Cells were co-transfected with both pMetLuc-ACC (-1082 A) and pSEAP2-control vectors. Twenty-four hour later, each dilution of each turmeric sample in duplicate was added into transfected cells. After an hour treatment, LPS was added to induce IL-10 promoter activity. After two-hour incubation, culture media from each well was collected and assessed for reporter activity.The luciferase and SEAP assay was performed as previously indicated. The experimental steps were summarised into the following flowchart shown in Figure 3.5. Each sample testing with four dilutions was completed in four replicates across two separate experiments.
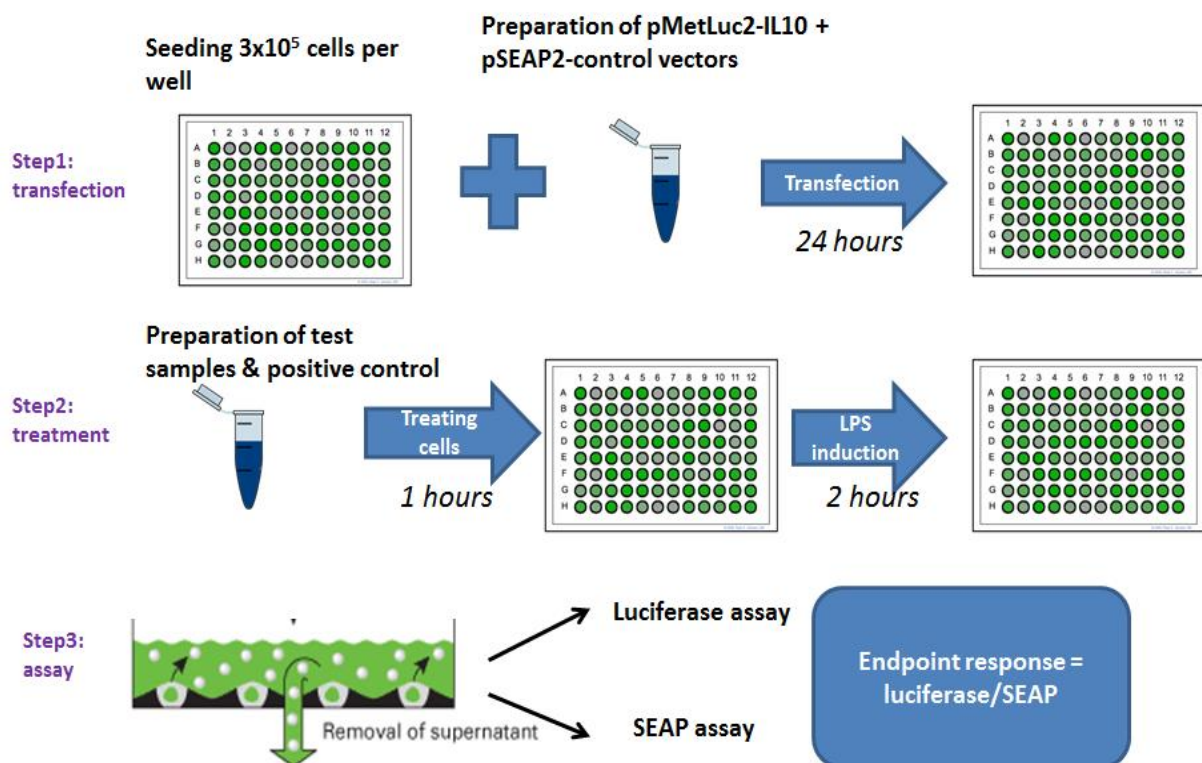
Figure 3.5 Flowchart of IL-10 promoter assay in testing food components

A negative control (culture medium with no cells), a positive control for transfection (cells transfected with pMetLuc2-control and pSEAP2-control vector), a positive control for transcription (dexamethasone stimulated) and untreated cells (no turmeric sample treatment) were all included in each assay plate. An example of the IL-10 promoter assay in 96-well plate is included in Table3.

Table 3. Example of 96-well plate layout for IL-10 promoter assay in testing food samples

	Sample No.	1	2	3	4	5	6	7	8	9	10	11	
A	Negative control												1:10
B	Negative control												
C	LUC CTRL												1:10
D	LUC CTRL												
E	Positive control												1:10
F	Positive control												
G	untreated												1:10
H	untreated												

- *Turmeric sample cytotoxicity assessment*

The WST-1 cell proliferation assay was also included to assess the effect of each turmeric sample on the proliferation of the 293TLR4 cells.The purpose of this step is to confirm the lowered IL-10 promoter activity was caused by reduced IL-10 transcription rather than cytotoxic effect of the turmeric samples.

After the culture media were collected for luciferase and SEAP assay, the remaining of the cell culture content was used in the WST-1 assay by adding the WST-1 solution in the

volume of one tenth of the media left in each well and measuring the absorbance on FlexStation 3 spectrometer (Molecular Devices, USA).

3.5 Data handling

In this study, the endpoint luminescence and absorbance was measured using a FlexStation 3 spectrophotometerand the raw data from each well of 96-well plate was recorded and entered to Sigma Plot (Systat software Inc., USA) for analysis.

First, the replicates of every sample in each assay plate were averaged to conclude a mean value. The background luminescence signal associated with the cell culture media was subtracted from the luminescence mean. Then, the luminescence value was calculated by dividing luciferase value mean withcorresponding SEAP value mean in order to normalise for transfection efficiency.

There is no statistical analysis for the control vector optimisation step in the study, as the aim of which was to determine an optimal amount of DNA and transfection reagents to use in the following food sample tests rather than a statistical comparisons of each separate groups. Therefore, the decided concentration from the control-vector optimisation step was concluded based on the highest luminescence signal detected.

The data of WST-1 assay and turmeric tests were ranked and analysed for statistical significance using a one-way ANOVA with SigmaStat 12.3 software (Systat software Inc., USA). Following ANOVA, significantly different means were identified using Dunnett'sor Dunn's post-hoc test. A P-value of less than 0.05 was considered to show a significant difference.

4 Results

The assay results consist of four parts. First is the separate transfection optimisation of pMetLuc2-control and pSEAP-control vectors. Several combinations of plasmid DNA amount and DNA:lipid ratios were tested. According to the result, the one with the highest luminescence was picked to use in the following assays. The second part is the co-transfection optimisation of pMetLuc2-control and pSEAP-control vectors. This step is to assess if the pre-determined (from the first part) plasmid DNA and lipid:DNA ratio can be efficiently co-transfected in 293TLR4 cells. The next part is to establish positive control for the assay. This step is to confirm the inhibiting effect of dexamethasone on IL-10 transcription. The final part is the result of turmeric samples for its ability to change IL-10 promoter activity in IL-10 gene variant of interest -- ACC.

4.1 pMetLuc2-control and pSEAP-control vector transfection optimisation

The luciferase and SEAP activity of 293TLR4 cells transiently transfected with either one of the two control vectorsover 24 and 48 hours,using different amounts of plasmid DNA and lipid:DNA ratios, are shown in Figures4.1 to 4.4. The relative luminescence units represent the level of reporter activity. They were obtained at 0, 5, and 10 minutes after substrates were added, using a FlexStation 3 spectrophotometer (Molecular Devices, USA).

Figure 4.1 demonstrate the secreted luciferase activity of the culture media from cells transfected with pMetLuc2-control vector for 24 hours. Plasmid DNA concentration of 250ng was shown to have the least luminescence signal detected among the three (top graph of Figure 4.1). The detectable luminescence doubled (approximately 2.0×10^5) using twice the amount of DNA in the transfection (middle graph of Figure 4.1). No increase of

luminescence was found after further increasing the amount of DNA to 750ng (bottom graph of Figure 4.1), suggesting a possible saturation level. Among the six lipid-to-DNA ratios used in this assay, 2.5, 3.0 and 4.0 were shown to have the highest luminescence signals. This was found consistently in the three DNA concentrations.

The results of 48-hour pMetLuc2-control vector transfection shown in Figure 4.2 foundan overall increase in luminescence signal (highest signal detected reached 4.0×10^5) compared to 24-hour transfection, with the exception of using DNA concentration of 750ng. Similar to the 24-hour transfection, the highest signal was also detected in samples using DNA concentration of 500ng. According to the chart, lipid:DNA ratios of 1.0, 2.0 and 2.5 show the highest luciferase activity. There is a significant reduction of luciferase activity with lipid: DNA ratios of 4.0 and 5.0.

Figure 4.3 demonstrates the SEAP activity of the culture media from cells transfected with pSEAP2-control vector for 24 hours. There was almost no SEAP activity detected from using 250ng of DNA (top graph of Figure 4.3). The luminescence signal improved significantly to 2.0×10^5using DNA concentration of 500ng. According to the results, there is a slight decrease of luminescence from using 750ng of DNA, with the exception of lipid: DNA of 1. The highest luminescence signal of pSEAP2-control 500ng was found using lipid to DNA ratio of 2.5, 3.4, and 4.0. This is consistent with the result of pMetLuc2-control vector 24-hour transfection.

The results of 48-hour pSEAP2-control vector transfection shown in Figure 4.4 have illustrated a decrease of SEAP activity in samples using 500 and 750 ng of DNA. The luminescence increased after 48 hours using 250ng of DNA, but highest signal was still found in 500ng of DNA. Lipid to DNA ratio of 2.5 was shown to have the highest SEAP activity.
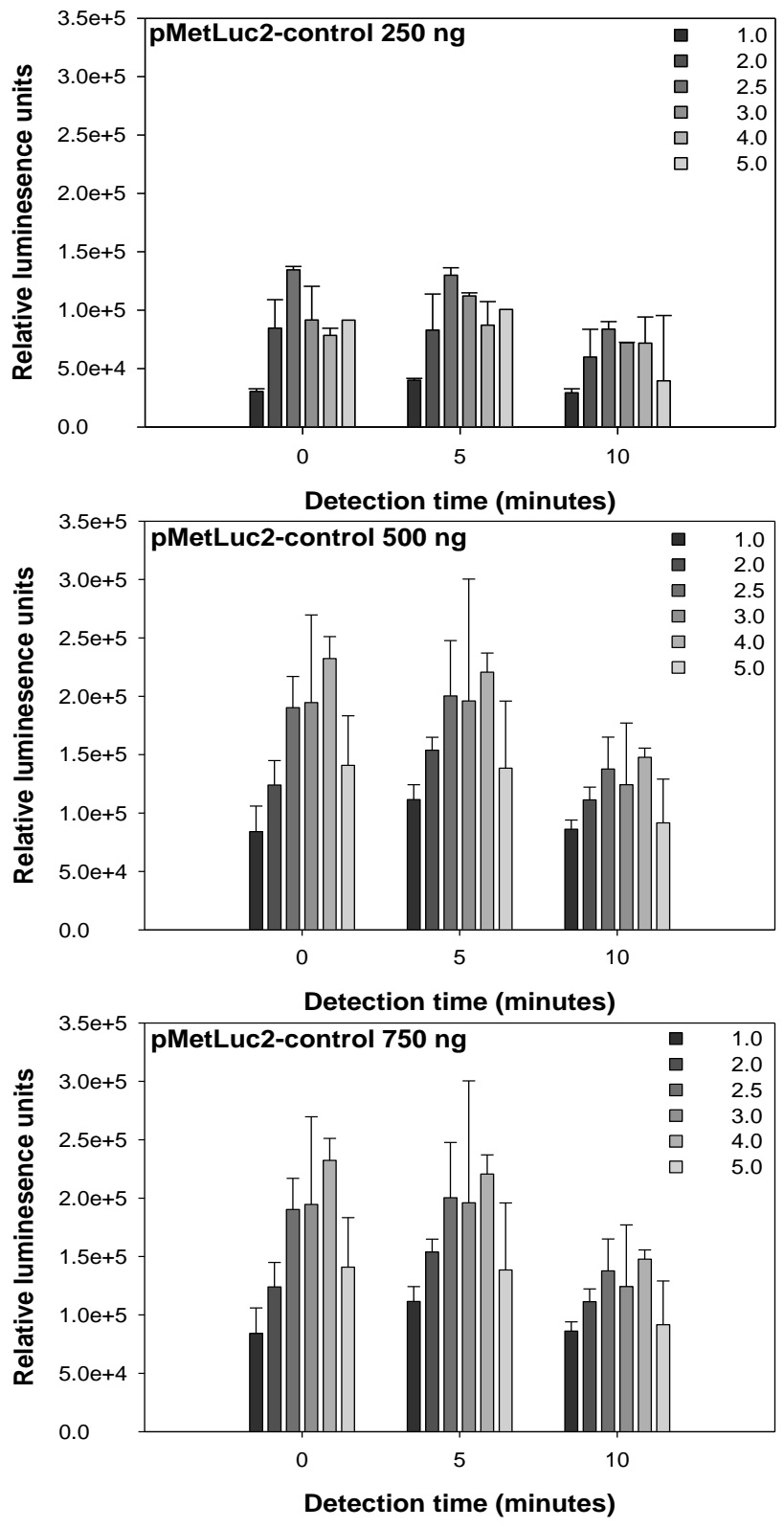
Figure 4.1 Luciferase activity at different amounts of plasmid DNA and lipid:DNA ratios in pMetLuc2-control vector 24hours after transfection. Data are expressed as the mean of three replicates ± standard deviation.
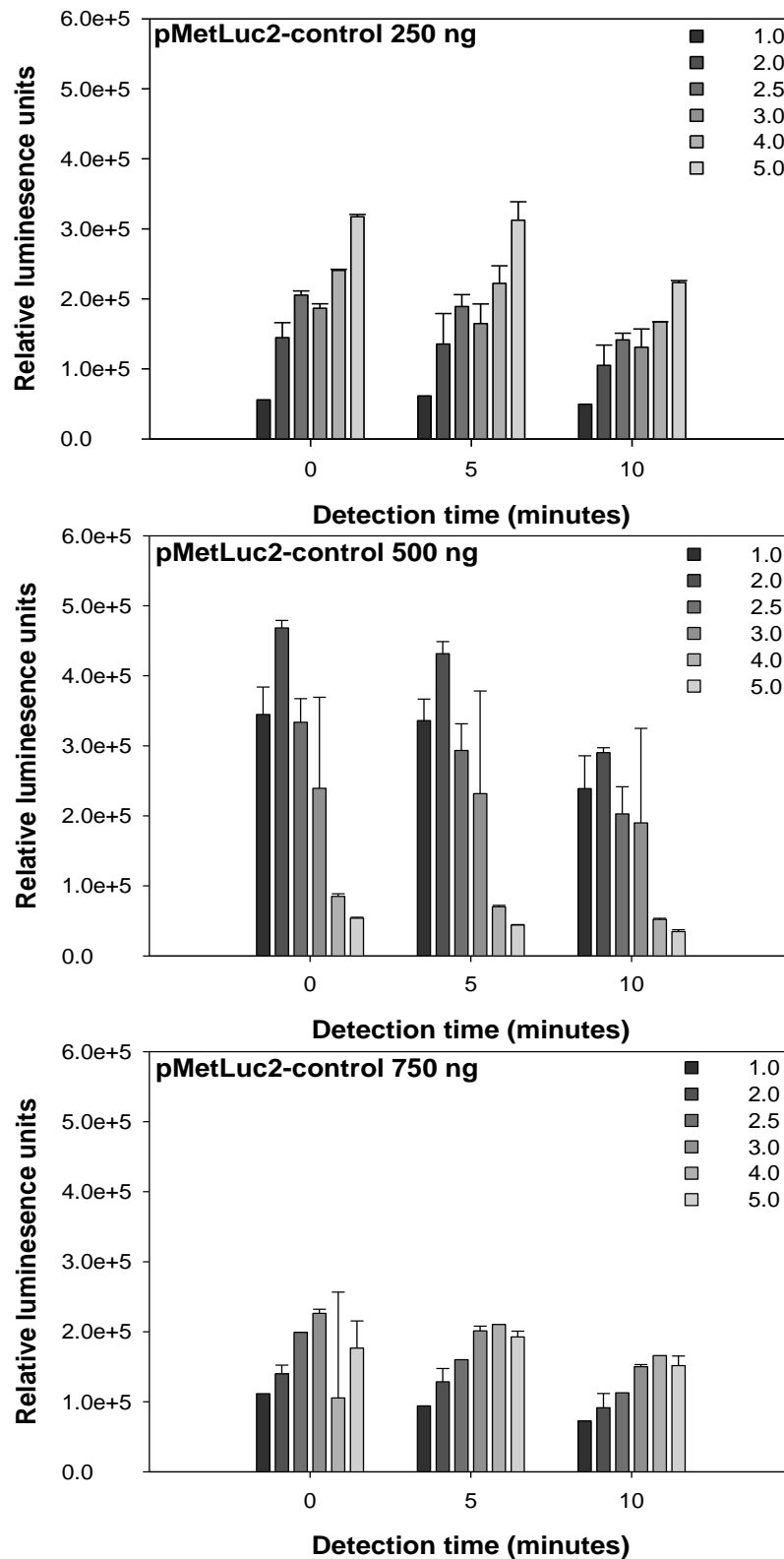
Figure 4.2 Luciferase activity at different amounts of plasmid DNA and lipid:DNA ratios in pMetLuc2-control vector 48hours after transfection. Data are expressed as the mean of three replicates ± standard deviation.
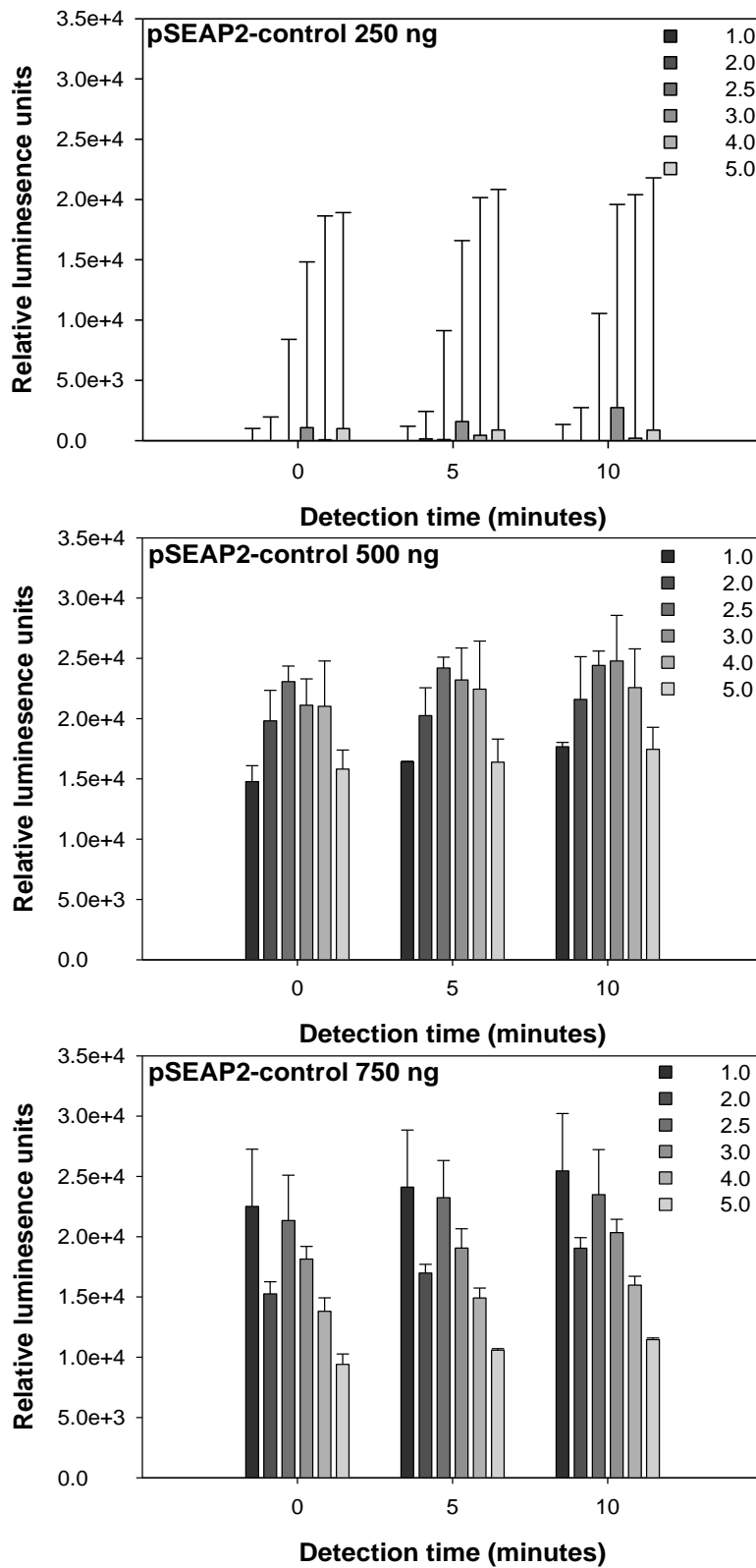
Figure 4.3 SEAP activity at different amounts of plasmid DNA and lipid:DNA ratios in pMetLuc2-control vector 24hours after transfection. Data are expressed as the mean of three replicates ± standard deviation.
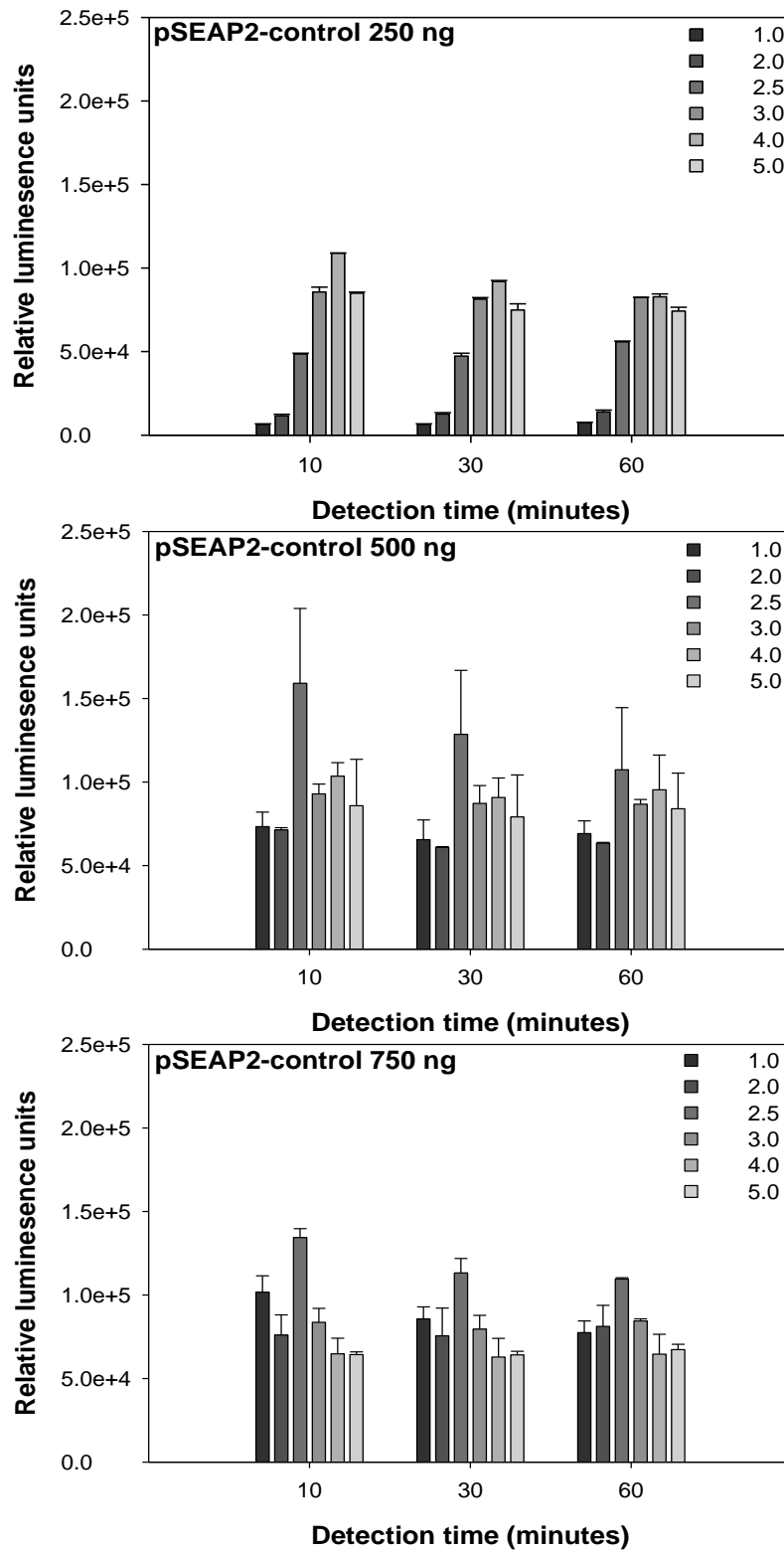
Figure 4.4 SEAP activity at different amounts of plasmid DNA and lipid:DNA ratios in pMetLuc2-control vector 48hours after transfection.Data are expressed as the mean of three replicates ± standard deviation.

In summary, the most abundant luminescence signal was found in samples using 500ng of DNA. Increasing the amount of DNA to 750 ng did not increase the reporter activity. Therefore, using 500ng of DNA, the optimal lipid to DNA ratio for both control vectors is determined to be 2.5. Increasing the transfection time to 48 hours was shown to increase luciferase activity but decrease SEAP activity. And the overall SEAP activity was lower than luciferase activity. According to the previous results from separate vector transfection, the optimal plasmid DNA amount to use in the following co-transfection was 500 ng, and the lipid transfection regents to use was decided based on lipid to DNA ratio of 2.5.

4.2 pMetLuc2-control and pSEAP-control vector co-transfection optimisation

293TLR4 cells were co-transfected with both of the two control vectors, using previously determined DNA and lipid reagent amount (500ng of plasmid DNA and a lipid to DNA ratio of 2.5). The initial results for the luciferase and SEAP activity in the samples from co-transfected cells were less than detectable, indicating poor transfection efficiency for the two control vectors.

A few adjustments were made to optimise the transfection efficiency, including increasing the cell seeding density and changing the timing of transfection. After 24 and 48 hours of co-transfection, the supernatant samples were collected and assessed immediately. The improved luminescence signal is shown in Figure 4.5.

The optimised luciferase and SEAP activity after 24 and 48-hour of co-transfection was shown to have an average of 1.0×10^5 luminescence units from pMetLuc2-control vector transfection and more than 1.0×10^3 luminescence units detected from pSEAP2-control transfection. Overall, the SEAP reporter activity was lower than luciferase reporter activity,

and this is consistent with the results from separate transfections. There is also a slight increase of luminescence after 48 hours, compared to 24 hours.
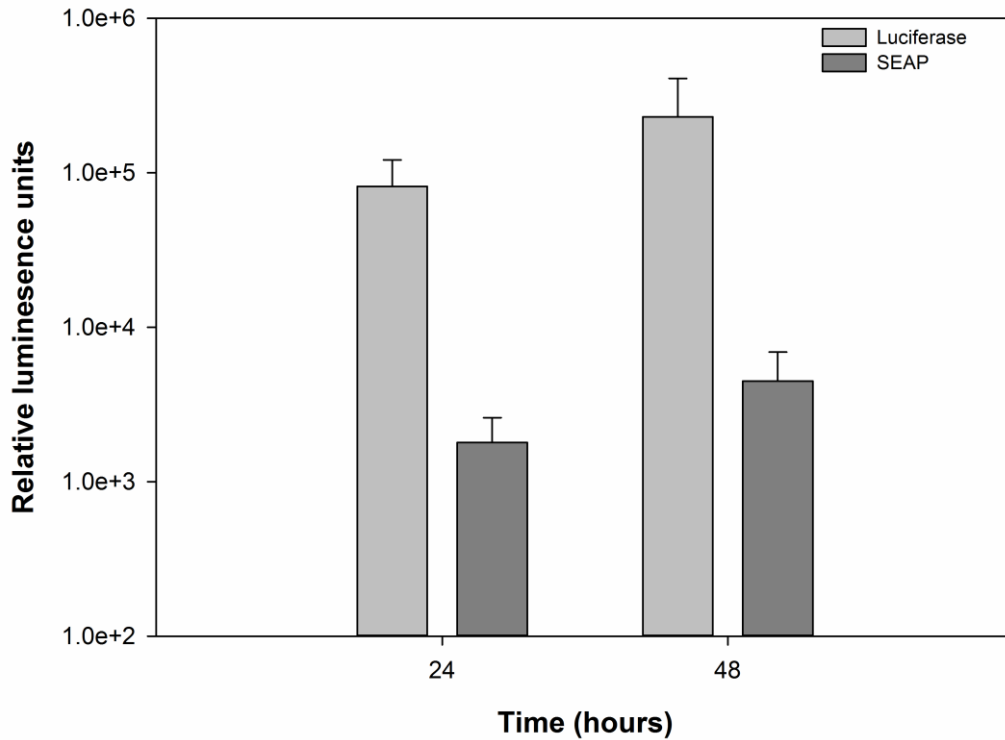
Figure 4.5 Luciferase and SEAP activity in pMetLuc-control and pSEAP-control vectors 24 and 48 hours after co-transfection. Data are expressed as the mean of three replicates ± standard deviation.

4.3 Establishment of positive control for IL-10 transcription

There were two steps in the establishment of positive control. Firstly, dexamethasone at different concentrations (from 10 to 500 µM) was tested for its effects on changing the metabolic activity in 293TLR4 cells (Figure 4.6). Secondly, selected concentration was then used to confirm its ability in inhibiting IL-10 production in transfected cells (Figure 4.7).
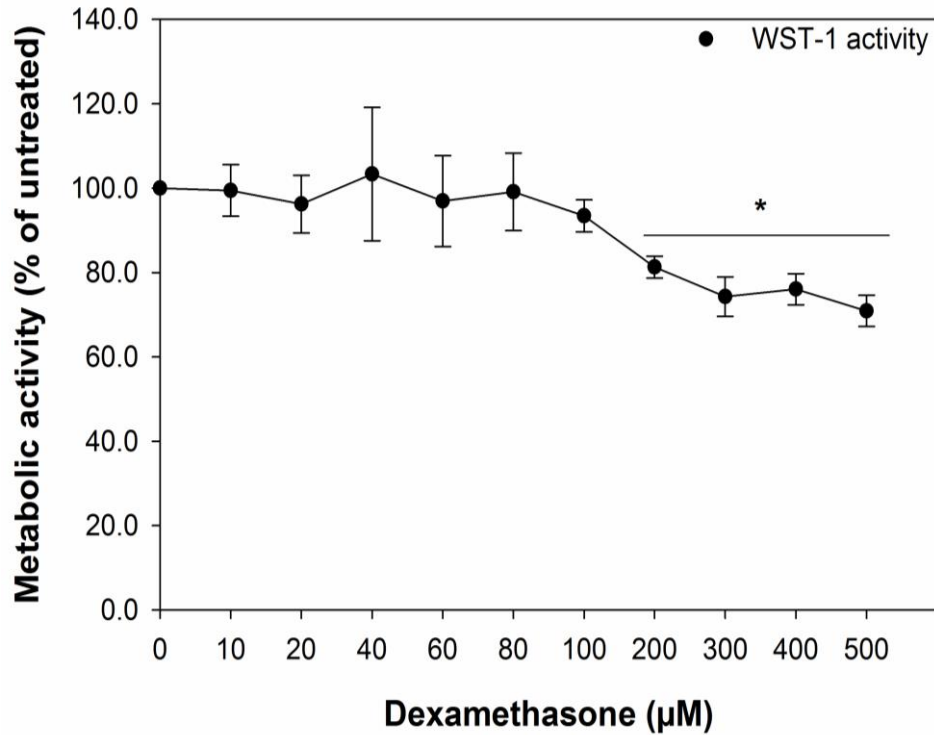
Figure 4.6 The effect of different concentrations of dexamethasone on 293TLR4 cell metabolic activity. The asterisk (*) indicates statistical significance ($P<0.05$) between treated samples (the metabolic activity of which was expressed as % of untreated) and untreated samples.

Based on the data of WST-1 assay, a significant reduction of cell metabolic activity was observed in cells using 200 µM and higher concentrations. The metabolic activity of cells treated with less than 100 µM of dexamethasone was above 90% of untreated cells. The concentration of dexamethasone to use as positive control for IL-10 transcription was 1 µM. Therefore, the cytotoxic effect of the dexamethasone of this concentration was considered negligible.
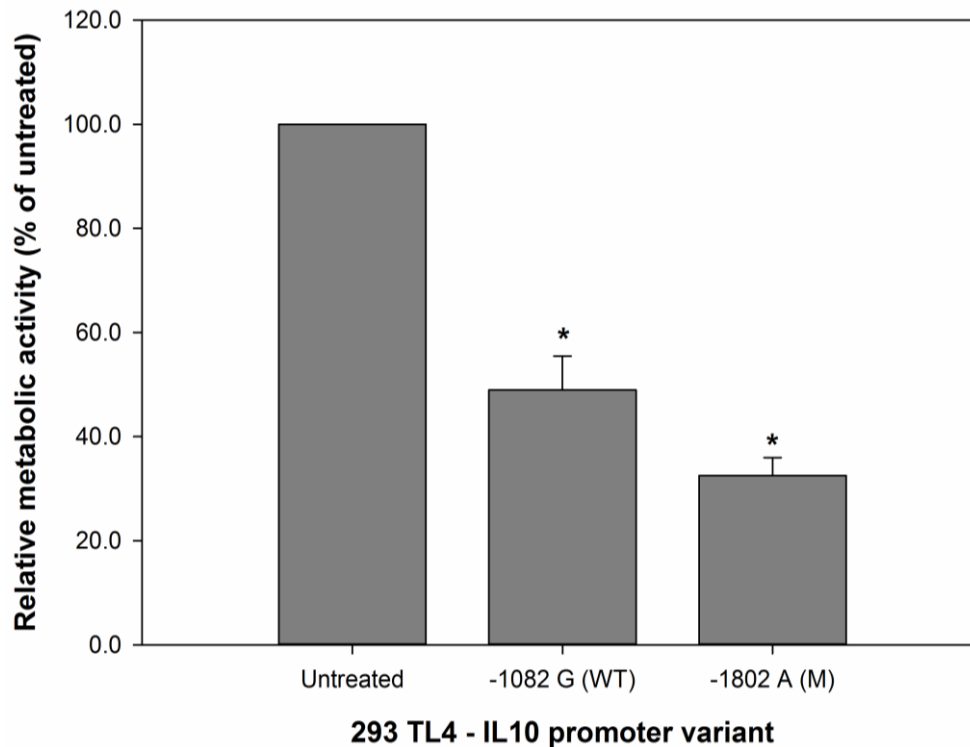
Figure 4.7 The effect of 1 µM dexamethasone on IL-10 promoter activity.Data are expressed as the mean ± standard deviation. The asterisk (*) indicates statistical significance (*P*<0.05) between treated samples (the metabolic activity of which was expressed as % of untreated) and untreated samples.

In this experiment, 293TLR4 cells were transfected with the three IL-10 promoter gene variants (pMetLuc2-IL-10 variant) and then treated with 1 µM of dexamethasone. After LPS induction, the IL-10 promoter activity (luciferase activity in supernatant samples) was measured and shown in Figure 4.7. There is a statistical difference in IL-10 transcription rate between dexamethasone treated cells and controls. Compared with untreated samples, dexamethasone has reduced the IL-10 transcription by more than50%. Therefore, it can use as an appropriate positive control to block IL-10 transcription.

4.4 Turmeric sample tests using the IL-10 promoter assay

With the establishment of the positive control, the IL-10 promoter assay developed in the first part of the study was ready to be used in testing food componentsfor their ability of changing IL-10 production in a genetically modified cell line.

74

In this part, turmeric samples (one extraction and twelve fractions)in a ten-fold serial dilution were preparedandtested using the IL-10 promoter assay. Their ability to alter the IL-10 promoter activity in cells transfected with risk haplotype ACC was shown in Figure 4.8. The curcumin content for each turmeric sample is shown in Figure 4.9.

Compared with untreated cells, the IL-10 promoter activity of the IL-10-ACC variant improved significantly after treatment with turmeric Extract (E) and Fraction 7 (F7). Statistically different significance was found in Extract of 1:10 and 1:100 dilutions, as well as in Fraction 7 of 1:10, 1:100 and 1:1000 dilutions. These samples also have the most abundant curcumin content, suggesting the bioactive role played by curcumin in the assay.

The value from each replicate in these samples however varied greatly. Therefore, the statistical significance should be interpreted with caution. There was also a considerable increase in the IL-10 promoter activity in the first two dilutions of Fraction 8 and in 1:10 dilution of Fraction9.
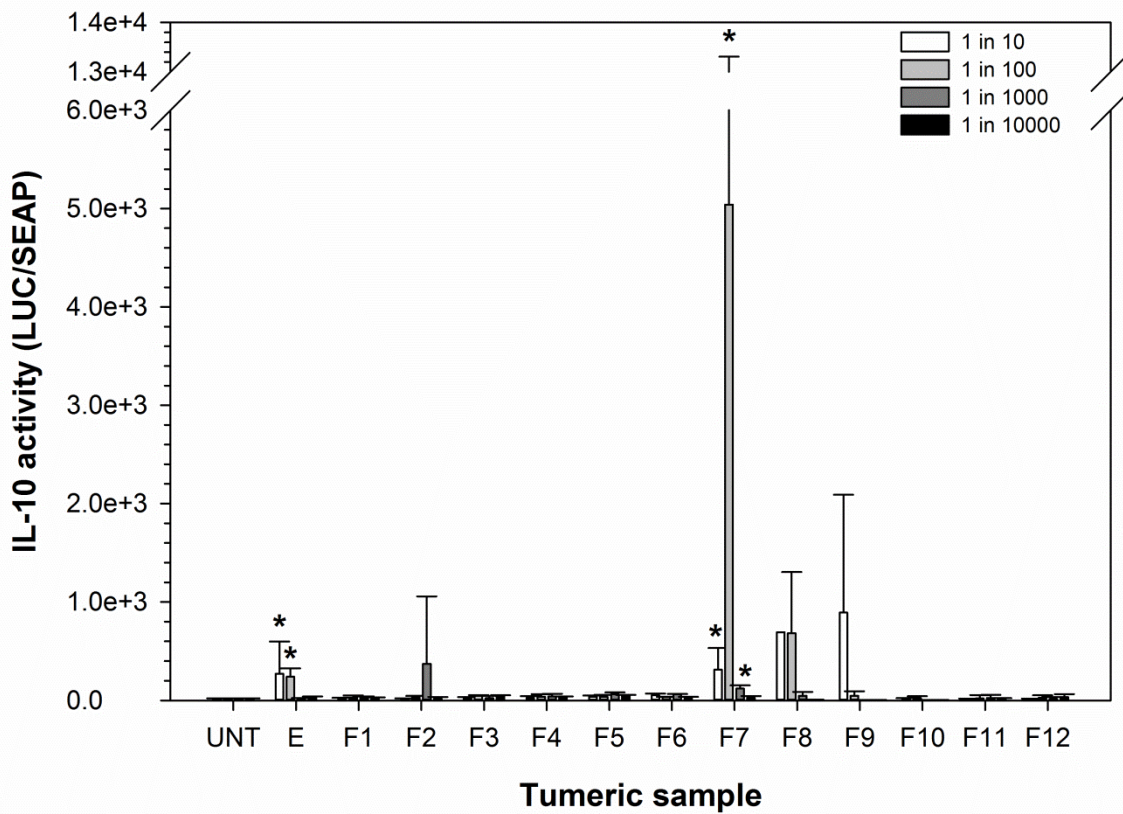
Figure 4.8 Relative IL-10 transcription rate in M-ACC variant after turmeric treatment (13 samples at four concentrations). UNT indicates untreated cells.Data are expressed as the mean of three replicates ± standard deviation.The asterisk(*) indicates statistical significance ($P<0.05$) between treated samples and untreated samples.
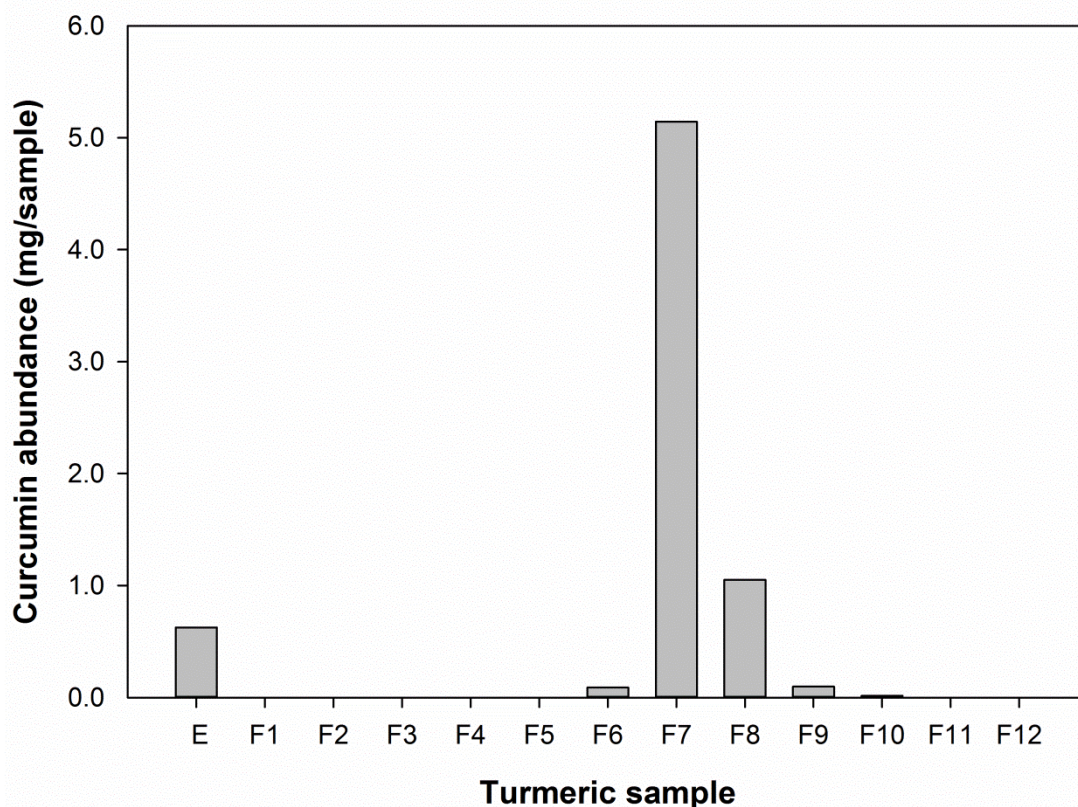
Figure 4.9 The curcumin content of turmeric samples. E indicates Extract. F1 to F12 indicates Fraction 1 to Fraction 12.

Turmeric samples in 1:100 dilutions were also tested for its cytotoxicity, using the WST-1 assay. The absorbance at 450nm in cells treated with turmeric samples of this dilution were shown in Figure 4.10. The white bars indicate the absorbance of treated 293TLR4 cells and dark grey bars indicate the absorbance of the turmeric samples (both after correction for background absorbance at 650 nm). According to these data, turmeric sample itself had strong absorbance at 450 nm, some of which have exceeded the absorbance of the actual cell media. Therefore, it is clear that the colour of the turmeric samples interferes with the metabolic measurement of the cells, suggesting that WST-1 colourimetric assay is inappropriate in assessing the turmeric sample cytotoxicity. A non-colourimetric assay, such as fluorescence-based assay should be considered in the future assessment.
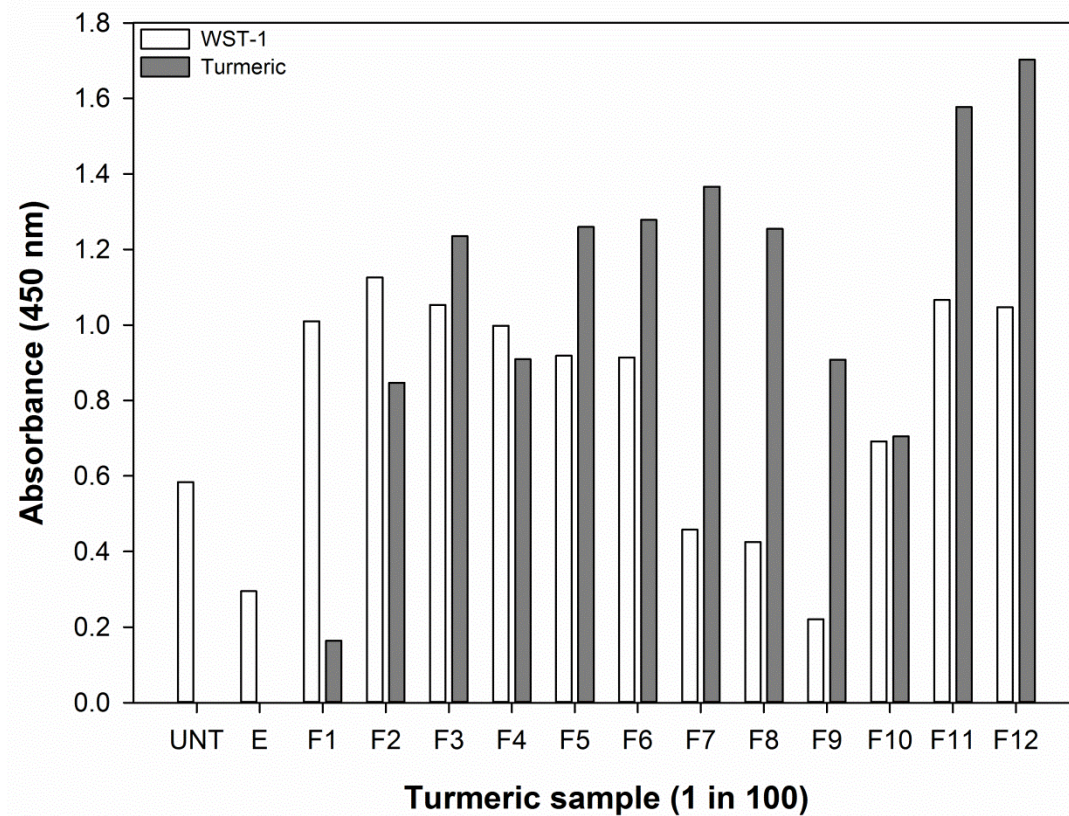
Figure 4.10 The effect of turmeric samples on the metabolic activity of 293TLR4 cells after 24 hours. UNT indicates untreated cells.The white bars indicate the absorbance of treated 293TLR4 cells and dark grey bars indicate the absorbance of the turmeric samples. Strong absorbance of the turmeric sample itself prevents proper cytotoxicity analysis.

5 Discussion and conclusion

5.1 Introduction

In the present study, I have developed a cell-based assay to assess the IL-10 promoter activity in cells transiently transfected with the three most common IL-10 promoter gene haplotypes, using a secreted reporter system. I have also optimised the experimental conditions for this assay to be a reliable and efficient tool in food sample testing. And then, I used this assay to test a food candidate for its capacity on affecting IL-10 promoter activity in ACC variant, and observed an increase in IL-10 promoter activity, indicating a beneficial effect of this food component on individuals with abnormal IL-10 production.

5.2 The establishment of IL-10 promoter assay

5.2.1 The use of 293-hTLR4A-MD2-CD14 cell line

In this study, I used human embryonic kidney cells that were stably transfected with TLR4a, MD2 and CD14 genes (293-hTLR4A-MD2-CD14 cells) to establish a model to investigate IL-10 promoter activity by transiently transfecting this cell line with IL-10 promoter gene variants.

The stimulation of TLR and other PRR by LPS, an important structural component of the membrane of Gram-negative bacteria, initiates the release of critical pro-inflammatory cytokines and enzymes such as IL-10 that are necessary in enabling immune response.A series of interactions occur during the LPS stimulation in mammalian cells, including LPS binding protein, CD14, MD-2 and TLR4, which are served as sensors or modulators in the process (Fukata & Abreu, 2008). It is known that TLR4 is a receptor for LPS, and it requires MD-2 to functionally interact with LPS. CD14 also participates in LPS signalling, leading to

NFκB translocation. After transfecting the 293TLR4 cells with plasmid DNA of interest (IL-10 promoter gene variants), the stimulation with LPS leads to the activation of IL-10 transcription, therefore the increase of the promoter activity. In IBD, chronic inflammation occurs due to the defective regulation of immune response. As a result, the polymorphisms in genes encoding cytokines in the innate immune system are believed to affect the course of inflammatory process and the risk of IBD.

In general, the human embryonic kidney293 cell line is easy to grow and sensitive to transfection and has been widely used in various cell biology experiments. Its high transfectability has enabled our investigation towards the expression of protein given limited amount of time and resources. This cell line is not a particularly good model of normal cells, but in this study, it only serves as a tool of delivering reporter protein from genes of interest. The focus of the study is therefore on analysing the expressed reporter protein molecule rather than the behaviour of the cell itself. As a result, 293TLR4 was the best cell line to use for this assay.

5.2.2 The use of *Metridia* luciferase reporter system in the assay

A naturally secreted luciferase found in marine copepod *Metridia longa* has been successfully cloned and adapted for expression in human cells. It catalyses a bioluminescent reaction of which the product is a blue bioluminescent signal that can be detected with high sensitivity in a basic luminometer.In this experiment, the IL-10 promoter gene variants were inserted into the secreted *Metridia* luciferase gene to generate the reporter vector -- pMetLuc-Reporter. This was then usedto transfect 293TLR4 cell line. Activation of the promoter drives the expression of secreted *Metridia* luciferase, which is detected in the supernatant.

The Secreted *Metridia* Luciferase reporter system used in this study has a major advantage in that the reporter molecule is secreted into the culture media, with no cell lysis. It provides a

means to evaluate biological process in real time without sacrificing the cells, which allows flexible endpoint assays and multiple replicates of plates with fast and easy sample preparation. Promoter activation leads to expression of secreted luciferase reporter protein in the medium. Furthermore, the reporter is very stable in the media supernatant, which makes it possible to monitor promoters over extended periods of time.There was also a media changingstep before samples collection to eliminate the chance of any secreted reporter accumulation in the media before the actual experiment.

5.2.3   The optimisation of experimental conditions

5.2.3.1   Selection of DNA and transfection reagents amount:

In the assay development step, the concentration of plasmid DNA and the amount of lipid transfection reagents used (as in lipid to DNA ratio) were established by testing a range of dilutions and selecting the ones that gave highest luminescence signal overall.

After 24 and 48 hours of transfection, supernatant samples were collected and stored for luciferase and SEAP assay. The results of luciferase activity after pMetLuc2-control vector transfection areillustrated in Figures 4.1 and 4.2. And the SEAP activities after pSEAP2-control vector transfection are shown in Figure 4.3 and 4.4.

The data has demonstrated that 250ng DNA with the least luminescence signal, indicating it was an insufficient amount to use in transfecting $1x10^5$ cells. After increasing the DNA concentration to 500ng, much stronger signal was detected. When further increasing the concentration to 750ng, however, the luminescence did not increase in proportion withthe increase of DNA amount, suggesting that it has reached the saturation level. Therefore, 500ng of plasmid DNA was the best amount to use in all three. The lipid to DNA ratio used in the assay was determined based on overall luminescence signal. Although in some cases, more

lipid reagents have showed increased signal, this was not found consistently in all of the concentrations, therefore, 2.5 of lipid to DNA ratio with overall highest luminescence was selected.The luminescence signal was measured at 0, 5 and 10 minutes after assay substrate was added. Based on the data, no significant signal increase or reduction was observed over 10 minutes, therefore, any of the three time points can be used in the assay.

5.2.3.2   Optimisation of co-transfection condition

Using previously determined DNA and transfection reagents amount, 293TLR4 cells were co-transfected with both of the control vectors. After 24 and 48 hours, the reporter activity was measured and showed in Figure 4.5. According to the data, luminescence signal for the two reporters havedecreased, compared to previous separate transfection.A possible explanation for this is that in co-transfection condition, there was twice the amount of DNA-lipid complexadded to the cells at a time whereas in separate transfection only one plasmid DNA-lipid complex was added. This lead to the two plasmid DNA competing for entering cell membrane, which in turn cause low transfection efficiency.

In order to address this problem, cell seeding density was increased in order to make more cells available for DNA-liposome complex. And, the DNA-lipid mixture was added into the cell suspension solution before they were plated. This step was to increase the contact and interaction between DNA-lipid complex and cell membrane. Furthermore, the supernatant samples collected after transfection were assayed immediately, avoiding any signal loss during sample preservation.

Results show theseapproaches have improved the transfection efficiency with satisfactory luminescence detected; therefore it is used in the following IL-10 promoter assay. At the same time, the assay format was scaled upfrom 24-well plates to 96-well plates in order to efficiently test food samples at the next stage.

5.2.3.3 Factors affecting transfection

There are many factors that can affect the transfection process and thus contribute to the inconsistency between each replicate. These include cell health, cell culture conditions (such as confluency, growth phase and number of passages), DNA quality etc. Too few cells could cause poor cell growth and cell contact, while too many cells result in contact inhibition, making cells resistant to uptake DNA. Cell behaviour and characteristic changeover time after repeated passages, therefore, their respond to same transfection conditions may also change. All of the above could affect the transfection efficiency. Although best effort was made to perform the transfection when cells were in optimal health conditions, using high quality plasmid DNA free from protein, RNA or other chemicals, it is still impossible to eliminate the factors above in their role of changing transfection efficiency.

5.2.3.4 Establishment of positive control for transcription

In order to investigate how food component affect IL-10 production, a positive control that will reduce IL-10 activity is needed to be able to establishthe inhibitory effect on IL-10 promoter activity.Dexamethasone, as a potent synthetic glucocorticoid, is expected to lower IL-10 production after adding to cells transfected with pMetLuc2-IL-10 variants.

Before using dexamethasone as positive control for the assay, non-toxic concentrations werefirst determined in order to eliminate the possibility of its cytotoxic effect. According to the WST-1 assay, 293TLR4 cells can tolerate dexamethasone up to the concentration of 200 μM without significant cell metabolic activity changes. Therefore, the toxic effect of 1 μM dexamethasone used in the IL-10 promoter assay is considered negligible.

According to the result, IL-10 promoter activity has reduced by more than 50% after 1 μM of dexamethasone was added, suggesting its inhibiting effect on IL-10 production. This concentration was then used as appropriate positive control for the rest of the assay.

5.3   The test of turmericsamples

The IL-10 promoter assay developed in the first part of the experiment was used to test the ability of turmeric samples to change IL-10 production in cell line transfected with IL-10 ACC variant after LPS stimulation.

IL-10 GCC haplotype has been associated with high cytokine synthesis *in vitro*, while the other two haplotypes - ACC and ATA is related with reduced IL-10 production, indicating that A at position -1082 is related with lower production. This has been confirmed in the first part of our promoter assay. Additionally, it was reported that the contribution of -1082 allelic variants is more important than that of alleles at the -819 and -592 position to the regulation of IL-10 mRNA levels (Suarez, *et al*., 2003). Most recent evidence comes from GWAS studies and a meta-analysis relating -1082 A/G polymorphism with IBD susceptibility. Therefore, IL-10-ACC variant was picked as the first variant to test in the turmeric sample assay since it has the same alleles at -819 and -592 positions with the dominant variant— GCC. The change of promoter activity due to turmeric sample treatment would indicate whether this food sample has a beneficial effect on people carrying high risk IL-10 promoter gene variant.

Extraction and 12 fractions of turmeric samples were prepared based on their polarity. From Fraction 1 to 12, the water solubility decreases, so does their polarity. This process was designed so that if all of an active molecule eluted in one fraction, it would be presented to the assays at ten times the concentration present in the extract. Each turmeric sample was

then diluted in four different concentrations: 1 in 10, 1 in 100, 1 in 1,000, and 1 in 10,000, all of which were included in the sample testing.

Curcumin was identified to be the most bioactive constituent of turmeric (Baliga *et al*, 2012). Curcumin is well-known for its anti-oxidant, anti-cancer, anti-inflammatory and various other beneficial effects. And in this study, the bioactivity of curcumin was further analysed focusing on its ability to change IL-10 promoter activity, and consequently IL-10 production. According to Figure 4.9, F 7, 8 and Extract of the turmeric samples have the most abundant curcumin content. As the results show, the most significant effect on increasing the IL-10 promoter activity in ACC variant was observed also in these three fractions, suggesting that curcumin is the likely candidate for the bioactive component of turmeric and responsible for changing IL-10 transcription. However, the standard deviations of the four replicates were very high in F7, further replication is needed to achieve conclusive results. Due to the limit amount of turmeric samples provided in this study, no further data could be obtained. The possible explanation for such large variation across different replicates could be from the differencesin transfection efficiency during each experiment due to the transient transfection method used. Additionally, curcumin is not the only component in F7 and 8, sub-fractioning the F7 and F8 can provide us with more information on the responsible component of the anti-inflammatory effect. In order to confirm the effect, purified curcumin canalso be used in the future assays.

The most significant increase on IL-10 promoter activity is observed in the 1:100 dilution of Fraction 7, rather than in the more concentrated 1:10 dilutions, suggesting that high concentrations of turmeric samples have an inhibiting effect on IL-10 promoter activity. Another study investigating curcumin on Th-1 mediated colitis in IL-10$^{-/-}$ mice had the similar observation in that higher concentrations of dietary curcumin did not provide with highest efficacy but may be detrimental*in vivo*(Larmonier, *et al*., 2008).However, there

wasnot enough evidence in the current assay to explain if this inhibiting effect was caused by sample toxicity, or change of cell metabolism, or change of gene expression. A possible explanation is that the curcumin bioactivity may have been inhibited by other components in turmeric sample. When diluting, their inhibiting effect becomes less effective.

In Fraction 7 and 8 and Extract of the turmeric, as the samples become more diluted, their ability of changing IL-10 promoter activity reduced, suggesting a dose response between 1:10 to 1:1000 dilutions in fraction 7 and 8 of curcumin samples. This could be further investigated in testing a range of smaller serial dilutions to determine the half effective concentration (EC_{50}).

The data from cytotoxicity assessmentindicate that although turmeric extract and fractions appear to alter metabolic activity, turmeric itself absorbs strongly at 450 nm and therefore any interpretation of metabolic activity is obscured by the colour of the turmeric sample.As a result, the appeared absorbance change in the cells after treatment cannot be used as true indicator of metabolic activity change.

However, according to the promoter assay, the IL-10 promoter activity has increased after treating with turmeric samples at 1:100 dilution, therefore, it is highly unlikely that the samples were toxic to cells at this concentration. However, the cytotoxic effect was not known at a higher concentration. This can be included in the future investigation using other assay method that measures luminescence rather than absorbance.

5.4 Limitations of the study and future thoughts

5.4.1 Transient transfection to stable transfection

In this study, we have employed transient transfection of plasmid DNA vector for the reason of rapid result protein expression. In 293TLR4 cell line, the reporter molecule can be detected

24 hours after transfection. Compared to stable transfection, in which the introduced DNA is integrated into the genome and passed to their progeny, each transient transfection of DNA requires much less time input. Therefore, it was preferable to use in the initial step of establishing the assay and in studying short-term expression of genes on a small scale.

However, the transient transfectionis limited in that the transfection efficiency varies in each individual well in the same experiment and also in each individual experiment (different cell population). Therefore, the results need to be interpreted after the correction of transfection efficiency and it could be a contributing factor to the inconsistency among experiments. Additionally, the cells only express the transiently transfected DNA for a limited period of time, usually several days. Therefore, repeated transfection is needed throughout the experiment, resulting in a longer and more expensive experimental process.

In the future, in order to further study the effect of more food samples in relation to IL-10 promoter region gene polymorphism on a much larger scale, stably transfected cell lineshouldbe used to efficiently produce more consistentdata. Plus, control vector to normalise the transfection efficiency will no longer be needed in stably transfected cells.

5.4.2   IL-10 promoter gene variants to test

Three SNPs in IL-10 promoter region were described in a number of literatures with conflicting results on how they modify the IL-10 production and IBD risk. It is generally believed that individuals who are genetically predisposed to produce low levels of IL-10 are at greater risk of developing IBD. Most convincing evidence found in GWAS and a recent meta-analysis has related -1082 A/G polymorphism with IBD susceptibility. And, evidence suggested that reduced level of IL-10 observed in ACC and ATA genotypes leads to the increased risk of IBD. Therefore, the focus of my study was on how food samples change the IL-10 production in the rs1800896 (-1082 G/A) risk variant (ACC) of the IL-10 promoter.

The results of IL-10 promoter curcumin assay in relation to IL-10 variant ACC have confirmed that the -1082 A/G polymorphism in IL-10 promoter region are associated with abnormal IL-10 production. An improved IL-10 production was seen in this genotype after curcumin treatment. However, we were unable to include the dominant type--GCC variant in the curcumin assay, thus a lack of information on how curcumin sample would affect IBDlow risk variant.

Ideally, food samples would restore the IL-10 production in mutant cell lines without altering the wild-type response. This is the desirable outcome as the active food components might be beneficial to the risk haplotypes (ACC and ATA); no potentially adverse effect would appear on people with normal IL-10 production.In the future, the other two IL-10 promoter variants should also be tested to understand how curcumin sample would modify their disease susceptibility.

5.4.3   Future application of IL-10 promoter assay

The IL-10 promoter assay established in this experiment is a validated tool to assess food of interest in discovering food-disease relationship in people with specific genotype. In order to test the food more efficiently, a few adjustments can be made to produce more reliable data and to increase the throughput of the assay.

Firstly, the cell line can be stably transfected with the IL-10 promoter gene, so that no repeated transfection is required, thus no inconsistency due to different transfection efficiency each time. This can be achieved by applying antibiotic selection to the cells. Secondly, the 96-well format can be further scaled up to 384-well plate to fit more test compounds in one plate. Thirdly, if the 384-well format is validated, the use of liquid handling robotics (an automated pipetting system that can be manipulated using programming software) will considerablysave   manual   labour   and   offer   precision   in   sample   preparation.

Thesemodifications above will improve the IL-10 promoter assay into a realistic and reliable high-throughput screening approach.

In addition, the cytotoxicity of the food compound in this study was evaluated using WST-1 assay, which is based on the absorbance change before and after the treatment. This can easily be affected be the colour of the food sample itself, therefore is not the ideal measurement. In the future, it is recommended to use other assay test that measures luminescence rather than absorbance.

5.4.4 Theoretical considerations

IL-10 is a potent anti-inflammatory cytokine with central role in the pathogenesis of inflammatory bowel disease. Food samples that can increase IL-10 transcription in low-production variant are expected to be protective to IBD development. However, whether or not these food samples will be beneficial to IBD is unclear at present due to the lack of understanding on complex immunological regulations and genetic regulation involved.

In this experiment, the developed assay was aiming to test how food affects the IL-10 promoter assay in transcriptional level. To further understand the polymorphism in its role in regulating IL-10 expression, mRNA and protein level of the IL-10 expression should also be tested.Epigenetic regulation of microRNA is also proposed to play important part in the modulation of gene expression but still poorly understood at present.

Due to the complexity of the cytokine network, there still does not appear to have a clear-cut correlation between IBD occurrence and genotypes that are associated with lower anti-inflammatory cytokine or higher pro-inflammatory cytokine production. It is the imbalanced level of pro- and anti- inflammatory cytokines that are thought to be the most important contributing factor in the IBD development. It is possible that food samples which promote or

inhibit a specific dysregulated immune pathway could either be beneficial or exacerbate disease susceptibility. Therefore, it is also important to evaluate the polymorphism within the regulatory sites of other inflammatory and anti-inflammatory cytokines in understanding the multi-genetic aspects of IBD.

Curcumin is chemically unstable to alkaline pH, and has extremely poor bioavailability in gastrointestinal tract. However, many studies have demonstrated its anti-cancer and anti-inflammatory effects in stomach, intestine, colon, skin, breast and other peripheral tissues, which traditionally has extremely low concentration of curcumin after oral administration.Due to the inadequate knowledge on the curcumin degradation and metabolism, it is reasonable to assume that the beneficial effect observed is a result of metabolites or degradation products of curcumin. Unfortunately, very few studies are there to confirm. Before curcumin can be accepted as a pharmaceutical or nutraceutical, much more evidence is needed to establish its pharmacokinetics, toxicity, as well as *in vivo* activity.

5.5 Conclusions

IBD is a debilitating disease characterised by chronic inflammation of digestive tract, with unknown aetiology. A combination of genetic predisposition and environmental triggers are thought to contribute to the immune dysregulation observed during IBD onset and development. IL-10 has critical functions in maintaining the homeostatic state of immune system by repressing pro-inflammatory responses to attenuate the excessive immune response due to inflammation. Its association with IBD was first recognised by spontaneous colitis developed in IL-10$^{-/-}$mice models. The importance of IL-10 in IBD was further demonstrated by its identification as a UC susceptible gene in genome-wide association studies.

In the present study, I have developed an *in vitro* model for evaluating the level of IL-10 transcription. It is a cell-based assay to assess the IL-10 promoter activity in cells transiently transfected with IL-10 promoter gene haplotypes. Using this method, I have observed an increase in IL-10 promoter activity in the IL-10 ACC variantafter introducing turmeric samples, indicating a beneficial effect of turmeric/curcumin on individuals with abnormal IL-10 production.Transfection conditions, including amount of lipid reagent, timing of transfection, and duration of transfection were all optimised in the assay development.

Curcumin, the primary active component of turmeric, has long been recognised for its beneficial effects on various diseases including inflammation. It is postulated that curcumin exerts anti-inflammatory effects through multiple molecular targets and pathways, which involves direct interaction with the target or through the modification of transcription factors, enzyme activity or gene expression.However, there is still a lack of understanding about the underlying mechanism of curcumin at cellular and genetic level. Therefore, the second part of my study (turmeric tests with IL-10 promoter assay)aimed to address this issue. In this current study, the anti-inflammatory property of turmeric was evaluated at gene transcription level, and the results have indicated its ability of up-regulating anti-inflammatory cytokines by promoting the gene activity in promoter region. However, more data is required to show conclusive evidence. It is believed that the results from the tests have contributed to a more detailed knowledge regarding the anti-inflammatory property of turmeric through its effect on cytokines via transcriptional regulations.

In summary, the assay developed in this study provides the possibility of a realistic high-throughput approach to investigate the effects of a wide range of compounds present in food on specific genotype involved in nutrient-disease relationship. With the increasing understanding of gene-environmental interactions in the pathogenesis of IBD, it is believed that personalised nutrition possess exciting potential in IBD prevention and treatment.

Thisassay allows us toassess the ability of certain food for its ability to overcome the functional change of SNPs that are important in human IBD, and to help facilitate the ultimate goal of promoting optimal health through personalised nutrition.

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Appendices

Appendix 1

IL-10 promoter sequence (-1149 to -31bp from the start of the IL-10 gene)

The SNPs of interest are highlighted in red bold uppercase typeface.

GCC (WT)

rs1800896 -1082G (WT)

rs1800871 -819C (WT)

rs1800872 -592C (WT)

attaccttctacacacacacacacacacacacacacacacacacacacacacaaatccaagacaacactactaaggcttctttggga**G**ggggaagtagggataggtaagaggaaagtaagggacctcctatccagcctccatggaatcctgacttcttttccttgttatttcaacttcttccaccccatcttttaaactttagactccagccacagaagcttacaactaaaagaaactctaaggccaatttaatccaaggtttcattctatgtgctggagatggtgtacagtagggtgaggaaaccaaattctcagttggcactggtgtacccttgtacaggtgatgtaa**C**atctctgtgcctcagtttgctcactataaaatagagacggtaggggtcatggtgagcactacctgactagcatataagaagctttcagcaagtgcagactactcttacccacttcccccaagcacagttggggtgggggacagctgaagaggtggaaacatgtgcctgagaatcctaatgaaatcggggtaaaggagcctggaacacatcctgtgaccccgcctgt**C**ctgtaggaagccagtctctggaaagtaaaatggaagggctgcttgggaactttgaggatatttagcccaccccctcatttttacttggggaaactaaggcccagagacctaaggtgactgcctaagttagcaaggagaagtcttgggtattcatcccaggttgggggggacccaattatttctcaatcccattgtattctggaatgggcaatttgtccacgtcactgtgacctaggaacacgcgaatgagaacccacagctgagggcctctgcgcacagaacagctgttctccccaggaaatcaactttttttaattgagaagctaaaaaattattctaagagaggtagcccatcctaaaaatagctgtaatgcagaagttcatgttcaaccaatcattttgcttacgatgcaaaaattgaaaactaagtttattagagaggttagagaaggaggagctctaaggagaaaaaatcctgtgccgggaaaccttgattgtggcttttaatgaatgaagaggcctccctgagcttacaatataaaaggggacagagaggtgaaggtctacacatcaggggcttgctcttgcaaaaccaaaccacaagacagacttgcaaaagaaggcatgcacagctcagcactgctctgttgcctggtcctcctgactggggtgaggccagcccaggccagggcacccagtctgagaacagctgcacccacttccaggcaacctgcctaacatgcttcgagatctccgagatgccttcagcagagtgaagactttctttgtgagtatgattccttcctgtcctttctctcttcctgggactgcctgaactagacattctcctggaactataagaa

ACC (M1)

rs1800896 -1082A (M)

rs1800871 -819C (WT)

rs1800872 -592C (WT)

attaccttctacacacacacacacacacacacacacacacacacacacacacaaatccaagacaacactactaaggcttctttggga**A**
ggggaagtagggataggtaagaggaaagtaagggacctcctatccagcctccatggaatcctgacttcttttccttgttatttcaacttctt
ccaccccatcttttaaactttagactccagccacagaagcttacaactaaaagaaactctaaggccaatttaatccaaggtttcattctatgt
gctggagatggtgtacagtagggtgaggaaaccaaattctcagttggcactggtgtacccttgtacaggtgatgtaa**C**atctctgtgcct
cagtttgctcactataaaatagagacggtaggggtcatggtgagcactacctgactagcatataagaagctttcagcaagtgcagacta
ctcttacccacttcccccaagcacagttggggtgggggacagctgaagaggtggaaacatgtgcctgagaatcctaatgaaatcggg
gtaaaggagcctggaacacatcctgtgaccccgcctgt**C**ctgtaggaagccagtctctggaaagtaaaatggaagggctgcttggga
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ctataagaa

ATA (M2)

rs1800896 -1082A (M)

rs1800871 -819T (M)

rs1800872 -592A (M)

attaccttctacacacacacacacacacacacacacacacacacacacacacaaatccaagacaacactactaaggcttctttggga**A**
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ccaccccatcttttaaactttagactccagccacagaagcttacaactaaaagaaactctaaggccaatttaatccaaggtttcattctatgt
gctggagatggtgtacagtagggtgaggaaaccaaattctcagttggcactggtgtacccttgtacaggtgatgtaa**T**atctctgtgcct
cagtttgctcactataaaatagagacggtaggggtcatggtgagcactacctgactagcatataagaagctttcagcaagtgcagacta
ctcttacccacttcccccaagcacagttggggtgggggacagctgaagaggtggaaacatgtgcctgagaatcctaatgaaatcggg
gtaaaggagcctggaacacatcctgtgaccccgcctgt**A**ctgtaggaagccagtctctggaaagtaaaatggaagggctgcttggga
actttgaggatatttagcccacccccctcattttacttggggaaactaaggcccagagacctaaggtgactgcctaagttagcaaggaga
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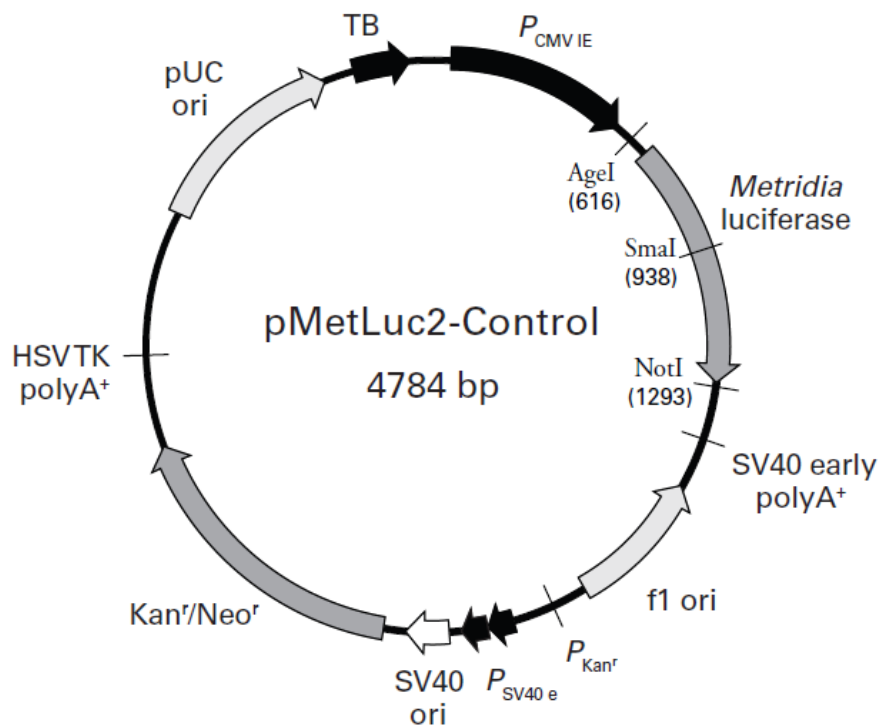ctataagaa

Appendix 2

pMetLuc2-control vector and pMetLuc2-reporter vector information

- **pMetLuc2-control vector map**

Description:

The pMetLuc2-Control vector encodes a sequence-optimized, secreted luciferase from the marine copepod *Metridia longa*. The 24 kDa *Metridia* luciferase (MetLuc) contains a 17 amino acid, N-terminal signal peptide that allows the protein to be efficiently secreted into the cell culture medium, making it easy to detect without cell lysis.

Expression of MetLuc is driven by the constitutively active cytomegalovirus immediate early promoter (*P*CMV IE). SV40 polyadenylation signals downstream of the MetLuc gene direct proper processing of the 3' end of the MetLuc mRNA. The vector backbone contains an SV40 origin for replication in mammalian cells expressing the SV40 large T antigen, a
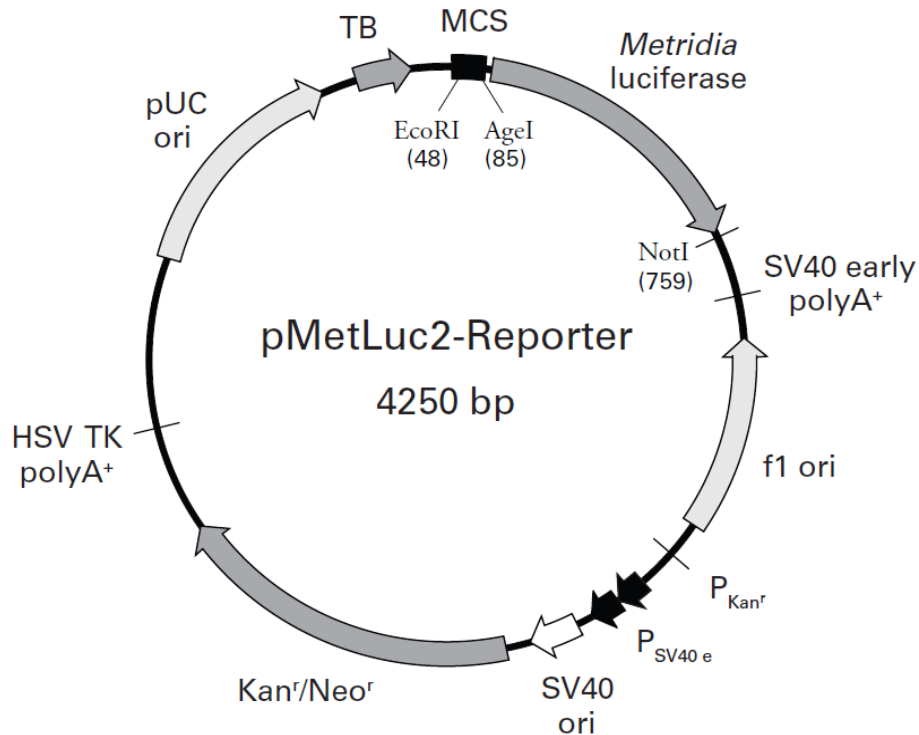
pUCorigin of replication for propagation in *E. coli*, and an f1 origin for single-stranded DNA production. A neomycin-resistance cassette (Neor) allows stably transfected eukaryotic cells to be selected using G418. This cassette consists of the SV40 early promoter, the Tn5 kanamycin/neomycin resistance gene, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV TK) gene. The vector also contains a synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites, that reduces background readthrough transcription. A bacterial promoter (*P*Kanr) upstream of the cassette allows kanamycin resistance in *E. coli.*

Use:

The pMetLuc2-Control vector can be used as a positive control for the constitutive expression and secretion of MetLuc in experiments using the pMetLuc2-Reporter vector. pMetLuc2-control can also be used to monitor the inhibitory effect of drugs, such as Brefeldin A, on protein secretion.

The pMetLuc2-Control Vector can be transfected into mammalian cells using any standard transfection method. Stable transfectants can be selected using G418 when required. The presence of MetLuc in the cell culture medium can be easily detected by adding luciferase substrate to a small aliquot of the medium and analyzing the sample with a luminometer.

- **pMetLuc2-reporter vector map and multiple cloning site (MCS)**

TB MCS *Metridia luciferase*

pUC ori

EcoRI (48) AgeI (85)

NotI (759) SV40 early polyA+

pMetLuc2-Reporter
4250 bp

HSV TK polyA+

f1 ori

P Kan^r

P SV40 e

Kan^r/Neo^r

SV40 ori

SacI
XhoI
PstI   SacII   BamHI
BglII   HindIII   EcoRI   SalI   ApaI   AgeI
27 AGATCTCGAG CTCAAGCTTC GAATTCTGCA GTCGACGGTA CCGCGGGCCC GGGATCCACC GGTC
   TCTAGAGCTC GAGTTCGAAG CTTAAGACGT CAGCTGCCAT GGCGCCCGGG CCCTAGGTGG CCAG

Description:

pMetLuc2-Reporter is a promoter reporter vector that allows the analysis of promoter function in cell-based assays. The vector encodes a sequence-optimized, secreted luciferase from the marine copepod *Metridia longa*. The 24 kDa *Metridia* luciferase (MetLuc) reporter protein contains a 17 amino acid, N-terminal signal peptide that allows efficient secretion of the reporter. When a functional promoter is cloned into the MCS, located upstream of the MetLuc reporter gene, MetLuc is expressed and secreted into the medium surrounding the transfected cells.

SV40 polyadenylation signals downstream of the MetLuc gene direct proper processing of the 3' end of the MetLuc mRNA. The vector backbone contains an SV40 origin for replication in mammalian cells expressing the SV40 large T antigen, a pUC origin of replication for propagation in *E. coli*, and an f1 origin for single-stranded DNA production. A neomycin resistance cassette (Neor) allows stably transfected eukaryotic cells to be selected using G418. This cassette consists of the SV40 early promoter, the Tn5 kanamycin/neomycin resistance gene, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV TK) gene. The vector also contains a synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites, that reduces background readthrough transcription. A bacterial promoter (*P*Kanr) upstream of the cassette allows kanamycin resistance in *E. coli.*

Use:

The pMetLuc2-Reporter vector is used to monitor the activity of promoters cloned into the MCS, located upstream of the *Metridia longa* luciferase coding sequence. Upon induction, functional promoters will drive the expression of secreted luciferase, while nonfunctional promoters will not. The presence of the luciferase can be easily detected by adding luciferase substrate to a small aliquot of the culture medium and analyzing the sample in a luminometer. Promoter function can be quantified by the relative intensity of the bioluminescent signal.

The pMetLuc2-Reporter Vector can be transfected into mammalian cells using any standard transfection method. Stable transfectants can be selected using G418 when required.

Appendix 3

IL-10 pathway
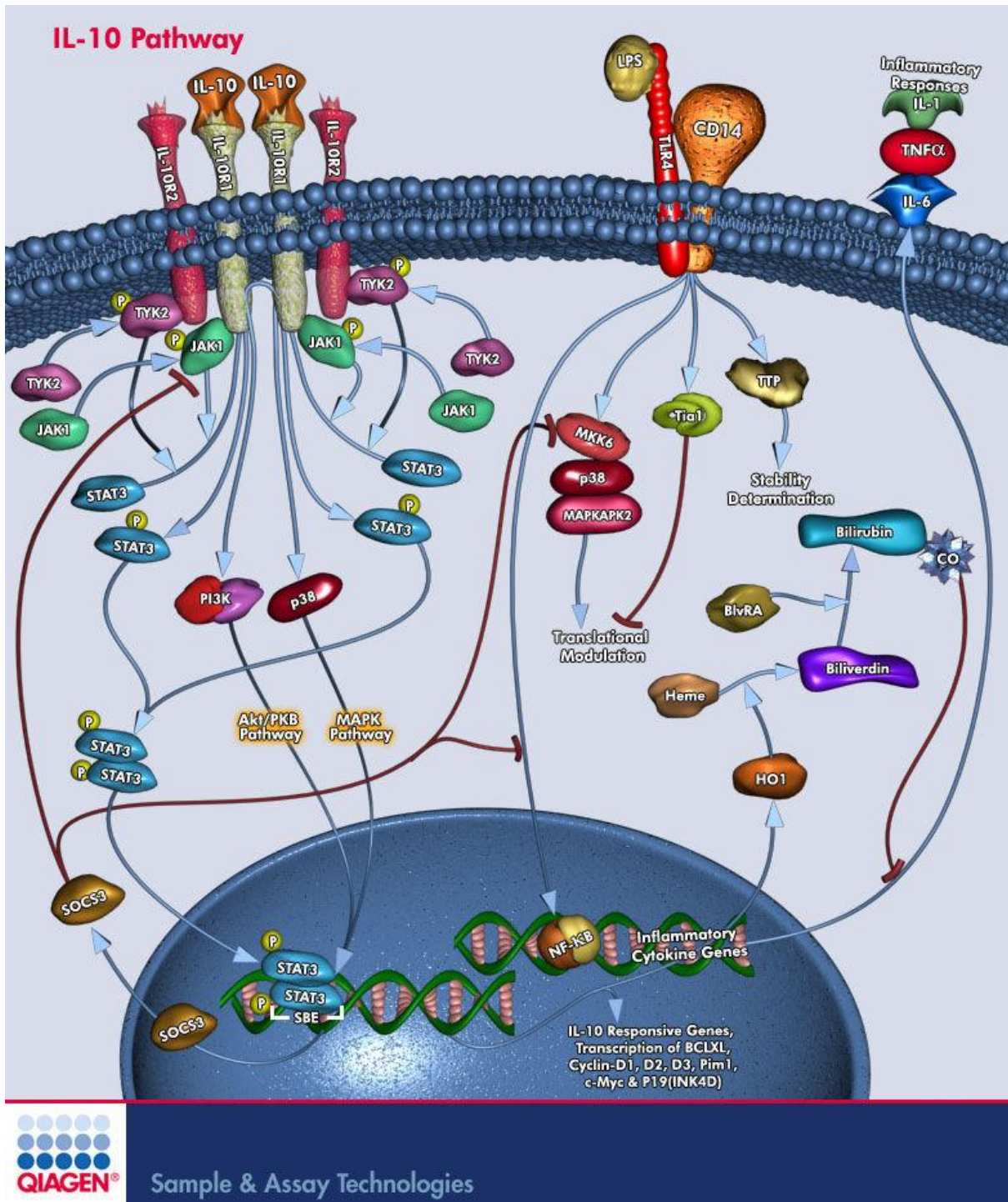
Appendix 4

The effect of 1 μg/ml LPS on IL-10 promoter variant transcription over 24 hours

Time (hours)	IL10 variant	RLU	SEM
2	-1082 A	20967.65	1703.43
	-1082 G	19937.10	632.76
4	-1082 A	21526.75	940.82
	-1082 G	22273.55	1390.01
6	-1082 A	13736.15	923.06
	-1082 G	14027.30	1270.18
24	-1082 A	15618.50	2299.25
	-1082 G	17504.10	98.89

*RLU refers to Relative Luminescence Units, and SEM refers to Standard Error Mean.

According to this table, the RLU value decrease after 2 hours, therefore it was decided that optimal LPS induction time was 2 hours.

Appendix 5

Preparation of ethanol extracts and reversed-phase fractions from food samples

The turmeric samples were extracted and fractionated bycollaborators at Plant and Food Research according to the following steps. Samples were then sent to us for use in the IL-10 assay.

Scope

This method covers preparation of extracts and fractions for screening in the cell based assays. This method will extract molecules of medium polarity and relatively low molecular weight (< 1000Da) which are likely to be bio-available in the assays, i.e. not proteins or polysaccharides. The fractionation aims to concentrate potential active molecules, to give better chance of hits in the assays.

Method summary

Freeze-dried, ground samples are extracted with ethanol.

Reversed-phase fractionation on solid phase extraction (SPE) cartridges to provide 12 fractions based on polarity. Process designed so that if all of an active molecule eluted in one fraction, it would be presented to the assays at ten times the concentration present in the extract.

The extracts and fractions will be dried and distributed to assays, without weighing and adjusting masses. Samples possibly need to be sterilised (gamma-irradiated or ethylene oxide) for all assays except fluorescence polarisation.

Solvents

EtOH = rectified spirits (96% ethanol, 4% water), lab grade re-distilled or HPLC grade

H_2O = distilled water

EtOAc = ethyl acetate, lab grade re-distilled or HPLC grade

Equipment

Schott Duran bottles, 100 ml and 250 ml

Isolute 5 g C18 SPE cartridges

Vacuum manifold for SPE cartridges

Sample preparation and extraction

1. Acquire about 1 kg of most fresh items, and about 400g of dried food choices, except for some expensive items such as herbs and spices which may be purchased in minimal required quantities for extraction.

2. Details of sample, purchase date, source, variety, manufacturer, "use by" date to be recorded at HortResearch with Wendy Smith/Tania Trower, and assigned "FBC" number used to track samples, extracts and fractions. Information to be posted on Wiki.

3. All fresh foods will be freeze-dried. The normally consumed parts (including skins) to be ground as finely as practicable, or finely chopped. Excess dry samples will be stored at $-20\ °C$, sealed under nitrogen.

4. Withdraw representative 10 ± 0.5 g lot of ground/chopped sample for extraction, and place in 250 ml Schott bottle with 100 ml EtOH. Homogenize (UltraTurrax) for 2 min, then close and shake overnight (18 h) at ambient temperature.

5. Filter (coarse, possibly use under vacuum) then put into 100 ml Schott bottle to give bulk extract, labelled with FBC code and 'E", stored at −20 °C.

6. For five sub-samples, filter 1 ml aliquot of bulk extract through 0.45 μm nylon syringe filter (Bonnet, 25 mm diameter) into Eppendorf vial (nylon filter removes some polyphenolics). Dry in Speedvac at ambient temperature to give sub-samples for four different assays, plus reference sample to be kept at extracting lab (at −20°C for further use/reference). Labeled with FBC code and "E". These sub-samples will probably contain 10-50 mg of extract, but will not be weighed before screening assay.

7. Sub-samples (extracts with fractions, see below) couriered to assays at room temp, but stored at −20 °C at other times. Submission of samples to assays recorded on Wiki.

Reversed-phase fractionation

1. Pre-condition a 5 g C18 Isolute SPE cartridge with 10 ml EtOH, then 10 ml 1:1 EtOH:H_2O, then 10 ml H_2O (allow the EtOH to drip through the column by gravity, then a vacuum can be applied for the remaining eluents).

2. Label (FBC code and "F1" to "F12") twelve 15 ml collection tubes (glass or plastic) and place in vacuum manifold.

3. Take 50 ml aliquot of bulk extract (see 6.5) and coat onto 2 g C18 (Aldrich octadecyl-functionalized silica gel) by rotary evaporating at 30 °C. If the coated material is too sticky, add more EtOH and C18 and dry again. Apply coated C18 to the pre-conditioned SPE cartridge.

4. Elute with two times 10 ml each of H_2O, 1:4 EtOH:H_2O, 1:1 EtOH:H_2O, 4:1 EtOH:H_2O, EtOH, and EtOAc. Collect each 10 ml into one tube i.e. two collection tubes per solvent mix.

5. Remove solvent from collection tubes in Speedvac overnight at ambient temperature. Dissolve/resuspend the residue in each sample tube with 5 ml ethanol. Sonicate to mix if required.

6. For five sub-samples, put 1 ml aliquot of fraction into Eppendorf vial. Dry in Speedvac at ambient temperature to give sub-samples for four different assays, plus reference sample to be kept at extracting lab (at $-20°C$ for further use/reference). Label FBC code and "F1" to "F12".

7. Sub-samples (extracts with fractions, see above) couriered to assays at room temp, but stored at $-20\ °C$ at other times.

Appendix 6

Turmeric sample preparation

a. Collect relevant samples (E and F1-12) from storage rack and transfer to cell culture hood. The curcumin content of each sample was shown in the table below.
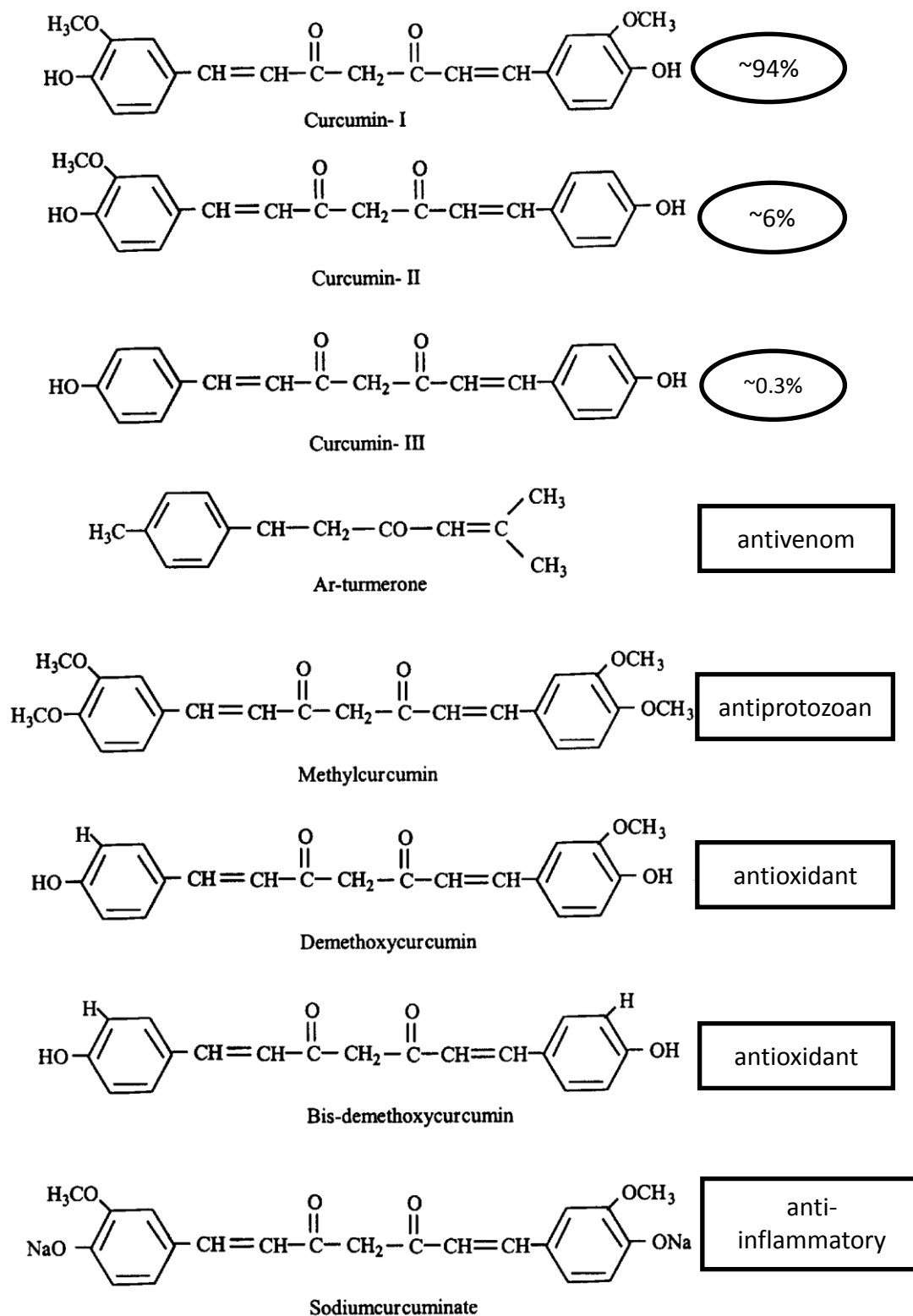
Curcumin	mg/tube
E	0.62
F1	0.00
F2	0.00
F3	0.00
F4	0.00
F5	0.00
F6	0.09
F7	5.14
F8	1.05
F9	0.10
F10	0.01
F11	0.00
F12	0.00

b. Dissolve NuNZ extracts and fractions at either 4x (with 250 μL DMSO) or 1x (with 250 μL DMSO and 750 μL sterile UltraPure water), depending on solubility.

   i. NuNZ crude extracts (E) and Fractions 6-12 should be suspended at 4x in 250 μL DMSO and mixed by vortexing ≥ 20 seconds.

   ii. NuNZ Fractions 1-6 (more hydrophilic fractions) may dissolve better at 1x in 250 μL DMSO and 750 μL sterile UltraPure H_2O. If fraction has a large glassy pellet (sugar), try adding H_2O first before DMSO, vortex mixing ≥ 20 seconds at each step.

   iii. If required, sonicate incompletely dissolved NuNZ samples 5 min at 37°C in bath sonicator.

iv.   Further mix samples by vortexing≥20 seconds.

v.   Record solubility/appearance of each sample.

vi.   Centrifuge any incompletely dissolved samples at 16,000 x g for 1 minute.

vii. If sample forms an oily floating layer, mix thoroughly by vortexing ≥ 20 seconds immediately before preparing dilutions.

c.   Dissolve pure compounds and other non-standard samples as required for assay (typically in DMSO).

d.   Prepare a 4-fold 1:10 dilution for each sample.

Appendix 7

Structure of natural curcuminoids

Curcumin-I ~94%

Curcumin-II ~6%

Curcumin-III ~0.3%

Ar-turmerone — antivenom

Methylcurcumin — antiprotozoan

Demethoxycurcumin — antioxidant

Bis-demethoxycurcumin — antioxidant

Sodiumcurcuminate — anti-inflammatory

Structure of natural curcuminoids (Adapted from Chattopadhyay, *et al.*, 2004)

Appendix 8

Absorption and metabolism of curcumin
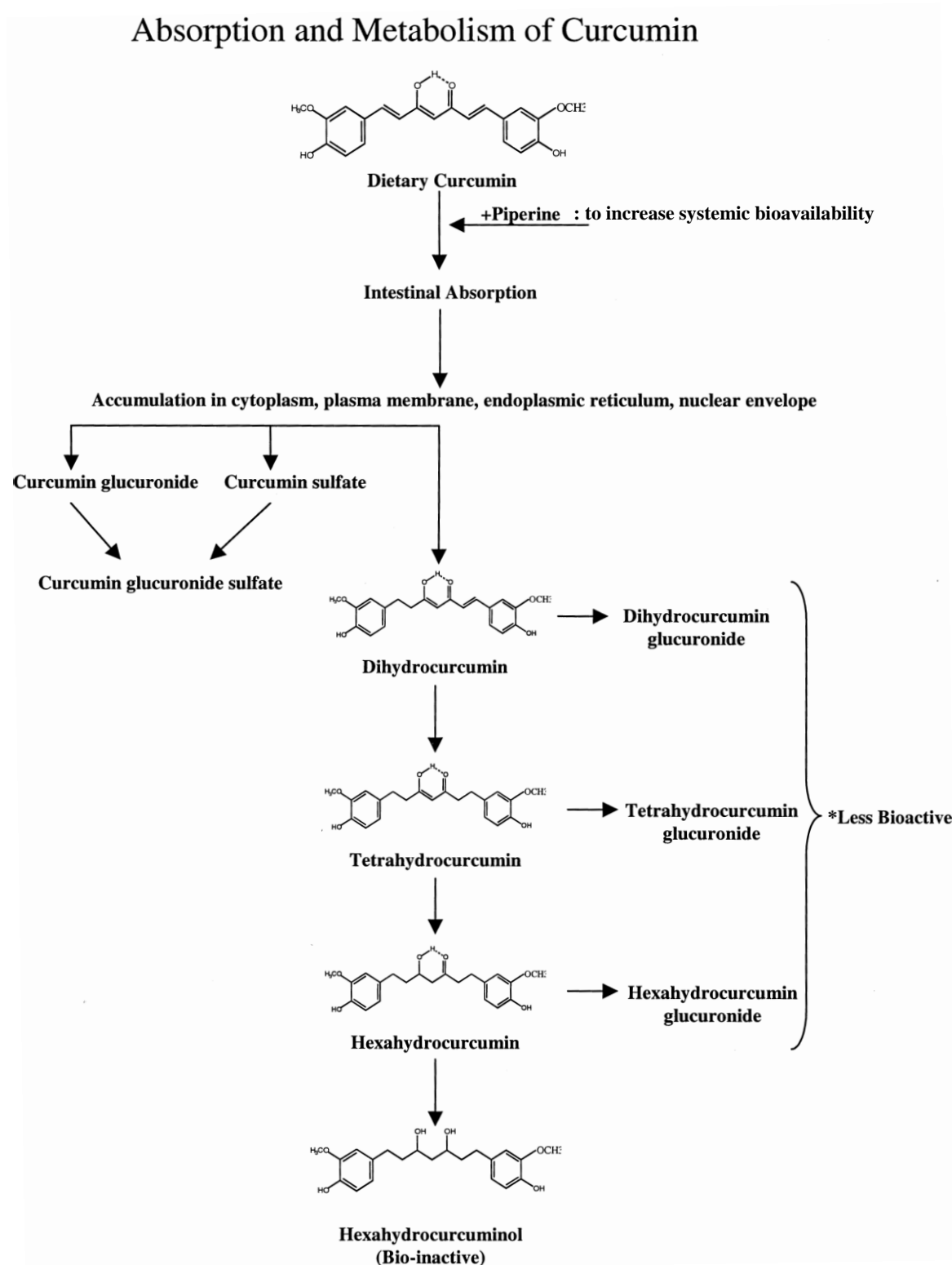
Absorption and metabolism of curcumin (Adapted from Joe, Vijaykumar, & Lokesh, 2004)

Appendix 9

Research Output

McCann MJ, Reilly K, Johnston S, **Men X**, Perry N, and Roy NC. The effect of turmeric (*Curcuma longa*) on the functionality of SLC22A4 and IL-10 variants associated with inflammatory bowel disease. Submitted to Food and Function (June 2013)