DEVELOPMENT OF A NOVEL TOLL-LIKE RECEPTOR-BASED TWO-HYBRID ASSAY FOR DETECTING PROTEIN-PROTEIN INTERACTIONS AND ITS APPLICATION IN THE STUDY OF CD14 DIMERIZATION AND FcγRIIA ACTIVATION

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2007

ACKNOWLEDGEMENTS

I would especially like to thank my supervisor Associate Professor Lu Jinhua, for accepting me as a PhD student, for always reminding me that science is a passion and especially for inspiring me to become an independent thinker. Without his advice and inspiration this thesis would not have been written.

I also thank Dr. Chua Kaw Yan (Department of Paediatrics) and her lab members for the generous loan of the flow cytometry machine.

I would like to express my sincere gratitude to all my colleagues, past and present members of this laboratory who have helped and supported me during these years. Appreciation also to the people from DNA lab, NUMI, especially to Ng Chai Lim, Karen Poh, Chong Hui Da and Koh Jia Yan.

Special thanks to Goh Wee Kang Jason and Lee Kiew Chin for being great and inspiring friends all these years, for sharing so many happy moments, for being patient with me at difficult times, for providing support and encouragement when I needed them most.

I am grateful to the National University of Singapore for awarding me a research scholarship and for giving me the opportunity to work here.

Last, but not least, my deepest love and appreciation to my husband and my family members for their love, care and support. Without your love I could never be what I am today. The thesis is dedicated to them with love.

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SUMMARY

Protein-protein interactions that form functional complexes, play an important role in many biological and physiological processes. In order to identify, characterize and quantify such interactions in mammalian cells, there has been a need for techniques that allows protein-protein interactions to be monitored in live cells specifically in the cellular compartments where they naturally interact. We describe here a method that allows us to detect protein-protein interactions on the cell surface of live mammalian cells. This method is based on the mechanism of TLR2 activation through extracellular (EC) domain-mediated heterodimerization with TLR1. In this assay, the EC domains of TLR2 and TLR1 are replaced by the EC domains of test receptors to express hybrids with the transmembrane/cytoplasmic (TM/Cyt) domains of TLR1 and TLR2, i.e. tmTIR1 and tmTIR2. The hypothesis is that dimerization of test proteins causes TIR1/TIR2 dimerization which is detected using NF-κB luciferase reporter plasmids. To evaluate whether TIR1/TIR2 dimerization can be used to detect receptor-receptor interactions, we expressed IL-4 and the EC domains of IL4Rα and γC as chimeras with tmTIR1 and tmTIR2. At low doses of expression plasmids, co-expression of IL-4Rα-TIR1 and γC-TIR2 did not significantly activate NF-κB. However, it was efficiently induced by IL-4. Co-expression of IL4-TIR1 with IL4Rα-TIR2, but not γC-TIR2, led to NF-κB activation which is consistent with previous report that IL-4 binding to IL4Rα and its lack of direct binding to γC. Co-expression of IL4-TIR1/TIR2, IL4Rα-TIR1/TIR2, or γC-TIR1/TIR2 constitutively activates NF-κB suggesting that IL4, IL4Rα and γC naturally form constitutive homodimers.

Next, this TIR1/TIR2-based two-hybrid assay was used to investigate CD14-CD14 interactions. It showed that the CD14 form homodimers. CD14 was also predicted based on its crystal structure involving β13 and the 'loop' between β12 and β13. Mutation of amino acids L290 or L307 in this region markedly reduced CD14-CD14 interactions. Functionally, these two residues are also required for CD14-mediated LPS signalling of NF-κB activation involving TLR4.

Since IL-4 induced IL4Rα and γC dimerization effectively causes TIR1/TIR2, we used this to investigate whether FcγR dimerizaiton is sufficient to cause NF-κB activation. The TM/Cyt domains of TLR1 in the IL4Rα-TIR1 and γC-TIR1 chimeras were replaced by the TM/Cyt domain of FcγRIIA to generate IL4Rα-FcγRIIA and γC-FcγRIIA chimeras. IL-4 induced dimerization of these chimeras did not induce NF-κB activation suggesting that higher degrees of FcγR oligomerization are probably required to cause signaling. To address this, different forms of IgG i.e. plate-immobilized-IgG (imIgG), heat-aggregate IgG (HA-IgG), beads-coated IgG (IgG-beads) were used to induce FcγRs signaling on human macrophages. The result showed that imIgG is a more potent stimulus of cytokine production compared to IgG-beads and HA-IgG. In addition, the roles of different FcγR in cytokine induction by imIgG and IgG-beads were examined using blocking antibody specific for FcγRI, FcγRII and FcγRIII.

LIST OF FIGURES

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PUBLICATIONS

Paper published

1. L Wang [ab], H Zhang[b], F Zhong[b], and J Lu[ab]* (2004).

A Toll-like receptor-based two-hybrid assay for detecting protein-protein interactions on live eukaryotic cells. *J. Immunol. Methods* 292, 175-186.

Manuscripts in Preparation

1. L Wang and J Lu

Detection of CD14 dimerization and its role in CD14 signal transduction using the TIR1/TIR2-based two-hybrid assay.

2. X Wu*, L Wang*, T Boon King* and J Lu*

Toll-like receptor activation elicits IL-1β formation inside dendritic cells but its secretion requires Fcγ receptor co-stimulation.

Conference Abstracts

1. L Wang and J Lu

Detection of CD14 dimerization and its role in CD14 signaling using a TIR-based two-hybrid assay. *The 16th European Congress of Immunology, September 6-9 Paris, France*, 2006

ABBREVIATIONS

Nucleotide containing adenine, cytidine, guanine and thymine are abbreviated as A, C, G and T. The single-letter and three-letter codes are used for amino acids. Three-letter names are used for restriction enzymes which reflect the microorganisms from which they are derived. Other abbreviations are defined where they first appear in the text and some of the frequently used ones are listed below.

AP	alkaline phosphatase
BCRs	B-cell receptors
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EC	extracellular
E. coli	Escherichia coli
EDTA	ethylene diamine tetra acetic acid
EtBR	ethidium bromide
FCS	fetal calf serum

FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
hr	hour
IFN	interferon
IL	interleukin
IL-1R	interleukin-1 receptor
Ig	immunoglobulin
kDa	kilodalton
LPS	lipopolysaccharide
LRR(s)	leucine rich repeat(s)
MHCII	major histocompatibility class II
MAPK	mitogen activated protein (MAP) kinase
min	minute
mRNA	messenger RNA
MOPS	3-[N-morpholino] propanesulphonic acid
MyD88	myeloid differentiation factor
NF-κB	nuclear factor *kappa* B
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RPE	R-phycoerythrin

RPMI	RPMI-1640 culture medium developed by Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
TBS	Tris-buffered saline
TCRs	T-cell receptors
TEMED	N, N, N', N'-tetra methylthylene diamine
TLR(s)	toll-like receptor(s)
TIR	toll/interleukin-1 receptor
TM/Cyt	transmembrane/cytoplasmic
TNF	tumour necrosis factor
Tris	tri-hydroxymethyl-aminomethane
µl	microliter(s)
µg	microgram(s)

Chapter 1 Introduction (Literature review)

1.1 Protein-protein interactions form the basis of diverse biological processes

Protein-protein interactions are key to the regulation of diverse biological processes, representing dominant forms of molecular communications inside and between cells. These interactions can be homotypic (interactions between identical proteins) or heterotypic (interactions between different proteins), stable and constitutive or transient and inducible, forming dynamic associations in response to specific stimuli. Irrespective of the nature of the interactions, the temporal and spatial combinations of these interactions can generate considerable functional diversity by triggering distinct signaling cascades and leading to regulated cellular activation. How proteins interact with each other to accomplish the diverse biological and physiological activities remains a formidable task to dissect. High through-put methods are particularly useful in this respect.

1.1.1 Overview and historical aspects for detecting protein-protein interactions

A number of methods have been developed to detect protein-protein interactions that are, to various extents, amenable for high through-put detection (Zhu et al., 2003). In the next sections, the strengths and weaknesses of these methods will be discussed.

(i) One method to understand the functions of a protein is to identify proteins it interacts with. The yeast two-hybrid assay (Y2H), developed by Stanley Field's group (Fields and Song, 1989; Fields and Sternglanz, 1994) has been widely applied. This assay employed the modular nature of the yeast *Saccharomyces cerevisi,* GAL4

protein. GAL4 is a transcriptional activator required for the expression of enzymes involved in galactose utilization. GAL4 contains a DNA binding domain (BD) (Keegan et al., 1986) and a transcription activation domain (AD) (Brent and Ptashne, 1985) which are separately folded and functions independently of each other. In the Y2H system (Fields and Sternglanz, 1994), the BD and AD of GAL4 was expressed as separate proteins; neither alone exhibits the transcriptional activity of GAL4. To test the interaction between protein X and Y, BD is expressed in fusion with protein X, whereas AD is fused to protein Y, yielding two hybrid molecules. These two hybrids are expressed in yeasts which are also transfected to express one or more reporter genes under the GAL4 promoter. The upstream of these reporter genes contain activation sequence (UAS) of GAL4. If the X and Y proteins interact with each other, they can regenerate a functional GAL4 by bringing AD into close proximity with BD which is detected by the expression of the reporter genes (Fig.1.1). This method has been widely used to investigate interactions between proteins, particularly intracellular soluble proteins.

The Y2H assay is highly sensitive in the detection of protein-protein interactions in transfected yeasts. It allows the identification of binding partners for a known protein by expressing the protein in fusion with BD domain and then screening proteins in fusion with AD. It also allows the identification of specific binding sites on proteins in combination with mutagenesis (Uetz and Hughes, 2000; Legrain and Selig, 2000).

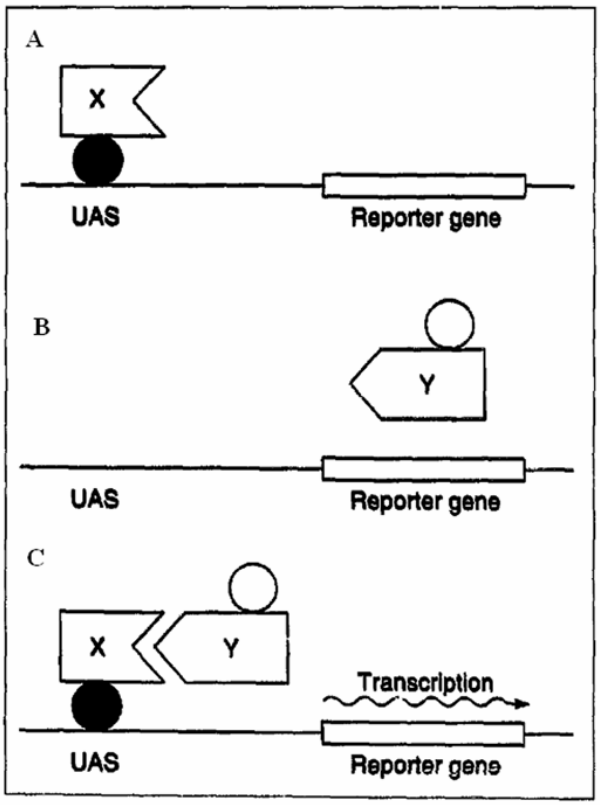


Figure 1.1 Schematic illustration of the Y2H system. (A) A hybrid protein is generated that contains a BD (filled circle) and protein X. This hybrid can bind to DNA but will not activate transcription because protein X does not have an activation domain (B) Another hybrid protein is generated that contains an AD (open circle) and protein Y. This hybrid protein will not activate transcription because it does not bind to the upstream activation sequence (UAS) of the reporter gene. (C) Both hybrid proteins are expressed in the same transformant yeast. If X and Y bind to each other, this brings BD and AD together to activate the transcription reporter gene. Adopted from Fields et al., (1994).

While the use of this method yielded a large body of data in protein-protein interactions, it also has obvious limitations. Firstly, this method generally cannot detect interactions involving three or more proteins and those critically depending on post-translational modifications e.g. phosphorylation. Secondly, it is not suitable for the detection of lateral interactions between membrane-anchored proteins. This is

because of the requirement for nuclear localization of the hybrid transcription factor to activate a reporter gene. Finally, in practice, the high-sensitivity of the assay is accompanied with reduced fidelity and the inferred interactions are often physiologically irrelevant. Therefore, although modified Y2H methods have been successfully applied by many laboratories, other methods are required to complement this assay

(ii) Independently, a method has been developed that allows membrane protein interactions to be detected and it potentially allows protein-protein interactions to be monitored in real time in the cellular compartment where these interactions naturally take place. This method is based on two β-galactosidase (β-gal) mutants which individually lack activity. However, its enzymatic activity is restored after dimerization of the two mutants. Intracistronic β-gal complementation is a phenomenon whereby its mutants α and ω, which harbor inactivating mutations in different regions of the molecule, are capable of reconstituting an active enzyme by sharing their intact domains (Langley and Zabin, 1976; Marinkovic and Marinkovic, 1977). In this method, two distinct but weakly complementing deletion mutants of β-gal, α and ω, are each expressed in fusion with a test protein. If the two test proteins interact with each other the β-gal activity is reconstituted (Fig. 1.2) (Rossi et al., 1997).

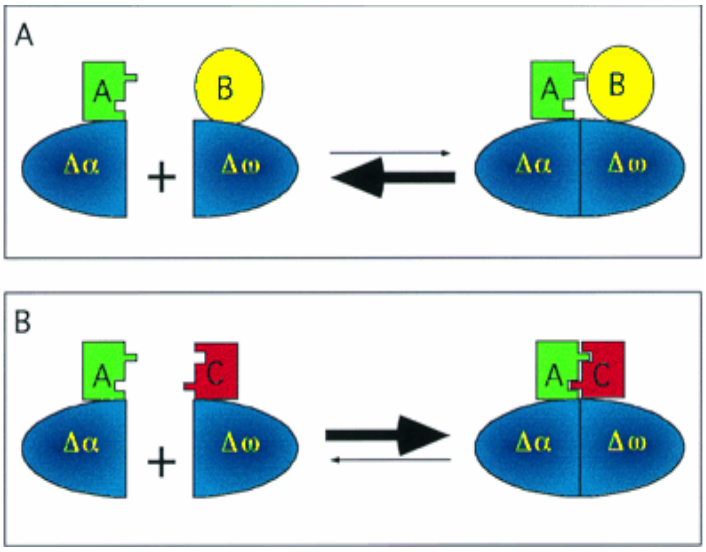


Figure 1.2 Schematic illustration of β-gal-based method for detecting protein-protein interactions. (A) When the Δα and Δω β-gal mutants are fused to test proteins that do not dimerize, their association is not favored and β-gal activity not detected. (B) When the Δα and Δω β-gal mutants are fused to proteins that dimerizes, the formation of active β-gal is favored where it reconstitutes the β-gal activity. Adopted from Rossi et al., (1997).

The strengths of this method are: (a) it allows protein-protein interactions to be investigated in live mammalian cells in the compartment in which they naturally take place, such as on the membrane or in the cytoplasm; (b) the enzymatic activity of β-gal amplifies signals, allowing protein-protein interactions to be detected without over-expression; (c) it provides quantitative and kinetic readout of protein-protein interactions (Rossi et al., 2000). The major limitation of this method is the large size of the β-gal mutants. They are approximately 80 kDa and require the employment of retro-viral vectors. When plasmid vectors are used, limited capacity is left for the cloning of test proteins. The detection of protein-protein interaction by intracistronic complementation is also hindered by steric constraints that may prevent the formation of an active enzyme.

(iii) The method of fluorescence resonance energy transfer (FRET) allows detection of protein-protein interactions and protein conformational changes in live mammalian cells. This method is based on non-radioactive energy transfer from an excited fluorescent donor molecule to an acceptor molecule through the dipole-dipole coupling mechanism (Selvin, 2000). In this method, one test protein is labeled with a flruorochrome such as yellow fluorescent protein (YFP) which act as an energy acceptor and the other test protein is labeled with a different fluorochrome such as cyan fluorescent protein (CFP), acting as an energy donor (Uster and Pagano, 1986; Truong and Ikura, 2001) (Fig 1.3). The emission spectrum of the donor fluorochrome significantly overlaps with the absorption spectrum of an acceptor. If the two test proteins interact with each other, the fluorochrome tags will be brought close to each other. Provided that dipoles of the donor and acceptor fluorochromes are in favourable mutual orientation, energy that directly activates the fluorochrome on the donor will indirectly activate the fluorochrome associated with the acceptor through energy transfer. This results in sensitized fluorescence emission from the acceptor, indicating that the test proteins are <10 nm apart or they bind to each other. The distance over which FRET occurs is 1 to 10 nm (Stryer, 1978; Wu and Brand, 1994).

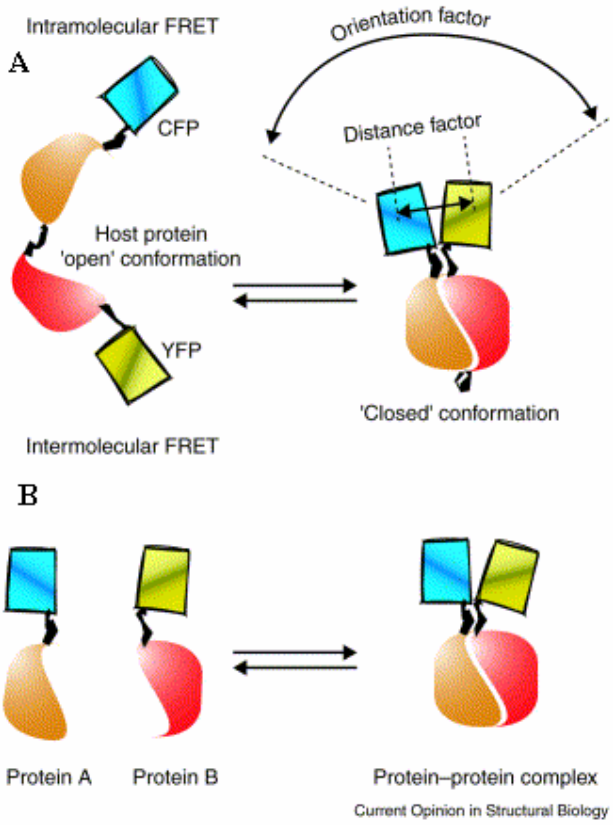


Figure 1.3 The principles of FRET. (A) Intramolecular FRET can occur when both the donor and acceptor chromophores are on the same host molecule, which undergoes a transition, for example, between 'open' and 'closed' conformations. In each square box corresponding to CFP or YFP (shown in cyan or yellow, respectively), a diagonal line represents the chromophore. The amount of FRET transferred strongly depends on the relative orientation and distance between the donor and acceptor chromophores. (B) Intermolecular FRET can occur between one molecule (protein A) fused to the donor (CFP) and another molecule (protein B) fused to the acceptor (YFP). When the two proteins bind to each other, FRET occurs. When they dissociate, FRET diminishes. Adopted from Truong et al., (2001).

FRET can be performed to detect conformational changes within a protein as well as interactions between proteins. FRET microscopic imaging has the unique advantage to verify close molecular interactions between co-localized donor and acceptor labeled fusion proteins beyond the resolution of traditional fluorescent microscopy (Chen et al., 2003). However, the limitation of this method is the low expression of fluorescent labeled proteins which often results in insufficient amounts of donor and acceptor fluorochrome in close vicinity, reducing the efficiency of energy transfer.

The low expression is because FRET uses transfected cell cultures and the expression is influenced by a number of factors including transfection efficiency of the given cell type, the quality and quantity of DNA taken up by the cells, the cytotoxicity of the transfection reagent and the condition of the cells. These limitations have restricted the widespread use of this technique.

As discussed above, each method has its own strengths and shortcomings which emphasize the need for additional methods to facilitate the investigation of protein-protein interactions. In this study a novel method has been developed based on the principles of Toll-like receptor activation.

1.2 Introduction to Toll-like receptors (TLRs)

1.2.1 Discovery of TLRS

The discovery of Toll-like receptors (TLR) began with the identification of the Toll protein in Drosophila which was essential for establishing dorsoventral polarity during embryogenesis (Anderson et al., 1985; Hashimoto et al., 1988). It has a homologue in Drosophila, i.e.18-wheeler (18W), which is also required for embryogenesis (Eldon et al., 1994). Aside from their role in embryogenesis, Toll, 18W and other homologues (Toll-3 to Toll-8) are also involved in Drosophila immunity (Tauszig et al., 2000). These proteins are essential for Drosophila immune responses against fungal and bacterial infections through the induction of anti-microbial peptides (Lemaitre et al., 1996; Williams et al., 1997). An essential step in the activation of Toll involves the activation of a proteolytic cascade by microbial structures that cleave pro-Spätzle into Spätzle and Spätzle activates Toll. There are

common features found in the different Toll proteins. These are type I receptors and the extracellular regions are characterized by leucine-rich repeats (LRR) which are linked to the transmembrane domain through a cysteine-rich region. The cytoplasmic domains, known as Toll/IL-1R homology (TIR) domains, share striking homology with that of the type I IL-1 receptor (IL-1R) (Gay and Keith, 1991). Moreover, signaling through Toll follows the signaling pathways induced by IL-1R. One of the signaling pathways elicited through IL-1R is the NF-κB/I-κB pathway. In Drosophila, Toll mediates the activation of intracellular proteins Dorsal and Cactus, homologues of mammalian NF-κB and I-κB respectively (Hultmark, 1994). However, IL-1R differs from Toll in the extracellular domain as they have immunoglobulin-like rather than leucine -rich. The first mammalian homologue of Toll, i.e. hToll (human Toll), was discovered based on its homology to Toll and IL-1R over the TIR domain (Medzhitov et al., 1997). Poltorak et al (Poltorak et al., 1998) showed that TLR4 was responsible for host recognition of LPS leading to septic shock.

To date, at least 11 mammalian genes have been identified encoding mammalian TLRs (TLR1-11) (Rock et al., 1998; Rock et al., 1998; Chaudhary et al., 1998; Takeuchi et al., 1999b; Sebastiani et al., 2000; Chuang and Ulevitch, 2000; Chuang and Ulevitch, 2001; Zhang et al., 2004). TLRs are pattern recognition receptors (PRRs) that enable host cells to recognize and differentiate between different pathogens to initiate appropriate signaling cascades and host cell activation. Antigen presenting cells (APCs) express many TLRs and TLR activation on APCs bridge innate and adaptive immunity by increasing the expression of various co-stimulatory molecules and effecter cytokines (Zhang and Ghosh, 2001).

All TLRs are type I transmembrane proteins with an extracellular domain consisting of LRRs that recognize conserved structures on pathogens and a cytoplasmic TIR domain. Based on sequence similarity, human TLRs can be divided into five subfamilies (Fig. 1.4): the TLR3, TLR4, TLR5, TLR2 and TLR9 subfamilies (Takeda et al., 2003; Gangloff et al., 2003). The TLR2 subfamily is composed of TLR1, TLR2, TLR6 and TLR10. TLR1 and TLR6 are highly similar in sequence (69.3% identity) with over 90% identities in their TIR domains (Takeuchi et al., 1999b). The TLR9 subfamily is composed of TLR7, TLR8 and TLR9. The other subfamilies consist of single members so far. With respect to amino acid sequences, among all known Drosophila Tolls (dTolls), only dToll9 exhibits significant similarity with human TLRs. The rest of dTolls are more related to each other than to human TLRs (Gangloff et al., 2003). Cell signaling downstream of Toll, TLRs and IL-1R is very similar owing to the presence of the TIR domain in these receptors.

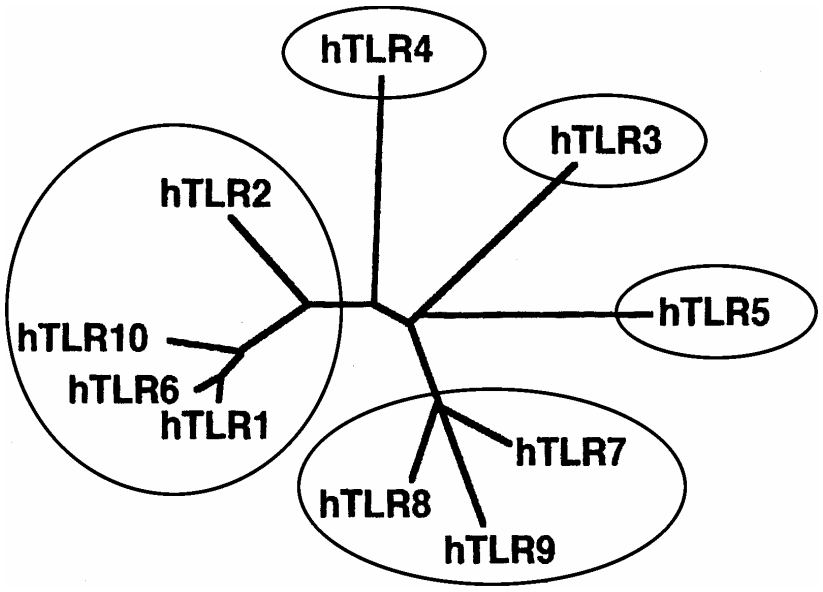


Figure 1.4 Phylogenetic tree of human TLRs. The phylogenetic tree was derived from an alignment of the amino acid sequences for the human TLR members using the neighbor-joining method. Adopted from Takeda et al., (2003).

1.2.2 Toll-like receptor 1 (TLR1) and Toll-like receptor 2 (TLR2)

1.2.2.1 Genes and structure

Human TLR1 and TLR2 genes have been mapped on chromosome 4p14 and 4q32 respectively (Rock et al., 1998). Human TLR1, TLR6 and TLR10 have similar genomic structure, consisting of a single exon and are located in tandem on chromosome 4. They may represent the products of evolutionary duplicates. TLR2 is the next most homologous TLR to TLR1. TLR2 and TLR1 sequences share 32% identity and 53% similarity (Takeuchi et al., 1999b). Both human and mouse TLR2 genes consist of three exons, of which the first and second exons are non-coding. The entire TLR2 open reading frame is located on exon three. Alternatively spliced forms also exist for TLR2 (Haehnel et al., 2002). 5'-flanking regions of both human and mouse TLR2 genes have been cloned (Matsuguchi et al., 2000; Musikacharoen et al., 2001; Haehnel et al., 2002). Sequence homology has not been detected between the human and mouse TLR2 genes over the promoter region. In the 5' untranscribed region of the mouse TLR2 gene, two NF-κB binding sites were identified which play a role in regulating TLR2 gene expression. TLR1 and TLR2 consist of 18-20 LRRs in the extracellular domains (Kirschning and Schumann, 2002).

The LRR motif was first described in α2-glycoprotein as a 24-residue repeated sequence with characteristically spaced hydrophobic residues (Takahashi et al., 1985). Each LRR is a conserved 11 residue segment with the consensus sequence LXXLXLXXNXL (X=any amino acid; N=can be replaced by C, S or T; L=can be replaced by hydrophobic amino acids) followed by a variable region. LRR-containing proteins can be classified into subfamilies based on sequence similarity, length and

structure of the variable region (Kajava, 1998; Kobe and Kajava, 2001). These include typical (including TLRs and other LRR-contian portiens), ribonuclease-inhibitor-like (RI) (e.g. GTPase-activating protein rna1p), SDS22-like (e.g. spliceosomal protein U2A'), cysteine-containing (e.g. SKp2), bacterial (e.g. YopM) and plant-specific subfamilies. The primary sequences of LRR in various proteins are shown in Figure 1.5 (Bell et al., 2003). The consensus sequence for majority of the LRRs in TLRs is a 24-residue motif that resembles the LRR of CD42b, exhibiting less curvature than RI. Two other LRR subtypes - SDS22 and bacterial - are also present in some TLRs. Large insertions that occur at positions 10 or 15 of the LRR are also present in some of the TLRs. Therefore, different TLRs can be distinguished by the presence of LRRs that deviate markedly from typical TLR-LRRs. The variations are conserved in TLRs of the same subfamilies e.g. TLR7, TLR8 and TLR9 have the same LRR variations.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
TLR	*x*	*L*	*x*	*x*	*L*	*x*	*L*	*x*	*x*	*N*	*x*	*Φ*	*x*	*x*	*Φ*	*x*	*x*	*x*	*x*	*F*	*x*	*x*	*L*	*x*				
RI	x	L	x	x	L	x	L	x	x	N	x	L	x	x	x	x	x	x	x	L	x	x	x	L	x	x	x	x
CD42b	x	L	x	x	L	x	L	x	x	N	x	L	x	x	L	P	x	x	x	x	x	x	x	x				
SDS22	x	L	x	x	L	x	L	x	x	N	x	L	x	x	L	x	x	L	x	x	L	x						
Bacterial	x	L	x	x	L	x	L	x	x	N	x	L	x	x	L	P	x	L	P	x								

Figure 1.5 Primary structures of LRRs. LRR consensus sequences. Shaded in green are the first ten residues that form the concave, β-face of the LRR solenoid (shown in Fig. 1.6). This portion is common to all LRR subtypes. Shaded in pink are the portions that form the outer, convex side of the solenoid. This portion is variable between subtypes. Shown in the alignment are LRR consensus sequences for Toll-like receptors (TLRs), ribonuclease inhibitor (RI), CD42b, SDS22 (a yeast protein having 11 LRRs each with 22 residues) and proline-rich subtypes. X refers to any amino acid, L and F are frequently replaced by other hydrophobic residues, and Φ is any hydrophobic residue. The consensus N residue at position 10 is often replaced by C, S or T. Adopted from Bell et al., (2003).

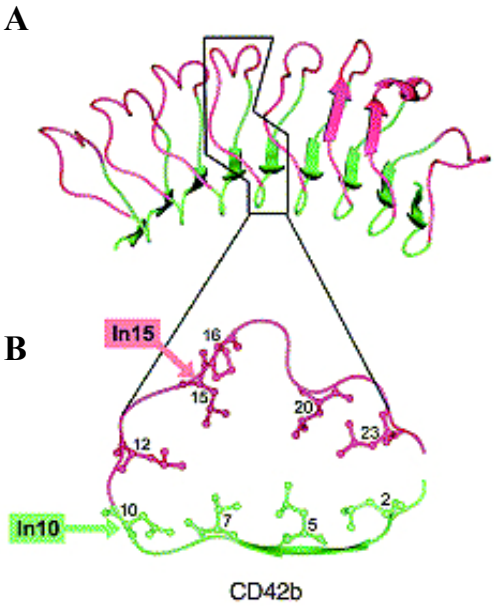


Figure 1.6 Tertiary (A) and secondary (B) structure of the LRR proteins (CD42b). (A) Solenoid structure of CD42b is formed by tandem repeats of individual LRRs. The β-strands together form the concave surface of the horseshoe/solenoid structure and the α-helices form the convex surface, lining the outer circumference of the solenoid structure. (B) Residues shaded green form a strand within each LRR, whereas those shaded pink form a helix in CD42b. Insertions (In10, In15) occur in some of the LRRs of TLRs. Adopted from Bell et al., (2003).

In CD42b, LRR represents its structural units. Each unit consists of a β-strand (formed by invariant residues 1-10) and an α-helix (formed by residues in the variable region) (Fig. 1.6A). In this unit, all the β-strands and helices are in parallel to a common axis, resulting in a non-globular, horseshoe-shaped molecule with the curved parallel β-sheet lining the inner circumference of the horseshoe and the helices flanking its outer circumference (Fig. 1.6B). TLRs seem to share features with CD42b based on their LRR similarity, but TLRs contain three times more LRRs than CD42b. Therefore, the LRR regions of TLRs are predicted to form a larger horseshoe structure with an extended concave β-sheet formed by 19-25 parallel β-strands. The LRRs of TLRs frequently contain insertions at positions 10 and 15. The insertion at position 10 occurs in a loop that connects the β-face with the convex surface (Fig. 1.6B). It is expected to lie in proximity to the β-sheet. Insertions at position 15 might also contact the β-face but are more likely to be located near the convex surface, where they might

interact with a large ligand that spills over the β-face or with an accessory molecule, such as MD2 for TLR4 (Bell et al., 2003). These variations in the LRR consensus of TLRs may provide individual TLRs with their ligand specificities. The properties of the TIR domain are discussed in section 1.2.3.1.

1.2.2.2 Gene expression

Studies on human TLR expression in tissues indicated that most tissues express at least one TLR (Zarember and Godowski, 2002). TLR2 has been found in lymphoid tissues, such as spleen, lymph node, thymus and bone marrow (Kirschning et al., 1998; Yang et al., 1998). It is also found in the lung, heart, muscle and brain (Rock et al., 1998). With respect to cell types, TLRs are expressed by adipocytes (Lin et al., 2000), fibroblasts (Mori et al., 2003), epithelial cells (Cario et al., 2000), keratinocytes (Pivarcsi et al., 2003), smooth muscle cells (Watari et al., 2000), and type II alveolar cells (Droemann et al., 2003). TLR1 is ubiquitously expressed and is apparently more abundant than other TLRs (Rock et al., 1998). Human TLR1 and TLR2 have been detected on the cell surface of monocytes, monocyte-derived immature dendritic cell and neutrophils (Visintin et al., 2001; Hayashi et al., 2003).

The expression of TLR1 and TLR2 is modulated by various microbial products and inflammatory mediators (Miettinen et al., 2001; Mita et al., 2001; Liu et al., 2001; Talreja et al., 2004). Depending on the cell type and stimulus, TLR1 and TLR2 expression is differentially regulated (Muzio et al., 2000).

1.2.2.3 Ligands and functions

TLR2 recognizes many different microbial components, including peptidoglycan and lipoteichoic acid from Gram positive bacteria such as *Staphylococcus aureus* (Lien et al., 1999; Yoshimura et al., 1999; Opitz et al., 2001; Schroder et al., 2003),

lipoproteins from Gram-negative bacteria, mycoplasma and spirochetes (Hirschfeld et al., 1999; Brightbill et al., 1999; Aliprantis et al., 1999), lipoarabinomannan from mycobacteria (Underhill et al., 1999b; Means et al., 1999), zymosan from yeast (Underhill et al., 1999a), parasitic protozoa (Campos et al., 2001), a phenol soluble modulin from staphylococcus epidermis (Hajjar et al., 2001), and porins from the outer membrane of *Neisseria* (Massari et al., 2002). The recognition of peptidoglycan and lipoproteins by TLR2 has been shown using $TLR2^{-/-}$ mice (Takeuchi et al., 2000a; Takeuchi et al., 2000b; Wooten et al., 2002). Direct binding of peptidoglycan to soluble TLR2 has also been demonstrated (Iwaki et al., 2002).

The ability of TLR2 to recognize many different ligands might be due to its ability to form heterodimers with other TLRs. The extracellular domains of the two TLRs can contribute to a combined ligand recognition site. TLR2 alone may be sufficient to recognize certain ligands e.g. peptidoglycan (Iwaki et al., 2002). TLR2 recognition of certain ligands require its pairing with other TLRs was first derived from the observation that dominant negative forms of TLR2 or TLR6 could inhibit monocyte TNF-α production elicited by zymosan (Ozinsky et al., 2000). TLR6 was also found enriched in macrophage phagosomes and physically associated with TLR2 through their extracellular domains. TLR1 association with TLR2 was shown by their co-localizaiton on the membrane upon cross-linking (Sandor et al., 2003), TLR1 and TLR2 are both required for lipoarabionomannan or bacterial lipopeptide to stimulate cytokine secretion from mononuclear cells. These two TLRs are also required to recognize soluble factors released from *Neisseria meningitides* (Wyllie et al., 2000). Analysis of TLR1-deficient mice has demonstrated the importance of TLR1 in the recognition of triacylated lipopeptides (Pam3CSK4) (Takeuchi et al., 2002).

Macrophages from this TLR1-deficient mouse showed impaired production of inflammatory cytokines in response to triacyl lipopeptides and lipoproteins from mycobacteria. Involvement of TLR1 in recognition of the outer surface lipoprotein of *B.burgdoferi* was also reported (Alexopoulou et al., 2002). Thus, TLR2 functionally associates with TLR1 and TLR6 which increases the variety of microbial components that this TLR recognizes.

Recognition of microbial products by TLRs activates innate and adaptive immunity. TLR4, TLR1 and TLR2 stimulation induces dendritic cell maturation (Tsuji et al., 2000; Hertz et al., 2001; Michelsen et al., 2001). TLR2 may also mediate inflammation and tissue repair in responses to endogenous stimuli such as necrotic cells (Li et al., 2001). Recently, TLR2 has been shown to internalize antigens into endosomes which are processed by conventional MHC II pathway for stimulation of antigen-specific $CD4^+$ T cells and it could thus be an efficient vaccination target (Schjetne et al., 2003).

Activation of TLR2 also has harmful effects. TLR2 has been suggested to be a 'death receptor' as it mediates bacterial lipoprotein-induced apoptosis (Aliprantis et al., 1999; Aliprantis et al., 2000). TLR2 assists *Mycobacterium tuberculosis* survived in host cells for chronic infection (Noss et al., 2001). *Mycobacterium tuberculosis* bacilli or its lysate inhibit macrophage expression of MHC II molecules and antigen presentation and thus decrease recognition by T-cells despite the innate immune responses in early infection. This inhibition is mediated by TLR2.

1.2.3 TLR signaling

1.2.3.1 TIR domain

The principal function of the TIR domain is to recruit intracellular signaling molecules through homotypic protein-protein interactions (Kopp and Medzhitov, 1999). The crystal structure for the TIR domains of human TLR1 and TLR2 have been determined and they contain a central five-stranded parallel β-sheet (βA-βE) surrounded by five helices (αA-αE) on both sides (Fig 1.7A and B) (Xu et al., 2000). The core TIR-domain starts from the conserved (F/Y) DA amino-acid motif and ends about eight residues carboxy-terminal to the conserved FW motif (Fig. 1.7C). Most conserved residues in TIR are located in the hydrophobic core with large insertions and deletions occuring in some loop regions in specific TIR domains. Therefore, the sizes of TIR domains from different TLRs can vary considerably between 135 and 160 residues.

A large conserved surface patch is present on TIRs, containing the BB loop, with contributions from the αA helix, βB strand and aromatic side chain of the (F/Y) DA motif. The BB loop, connecting the second β-strand and second helix, extends away from the rest of the TIR domain forming a protrusion on the surface. It contains 3 highly conserved residues: Arg (BB3), Asp (BB4), Gly (BB8) in the box 2 motif RDX$\Phi_1\Phi_2$G (where X = any amino acid, Φ_1 = hydrophobic amino acid). Φ_2 is the conserved proline, i.e. Pro (BB7), present in all TIR domains except that of TLR3 in which the proline residue is replaced by an alanine residue. When this proline is mutated to histidine in TLR2 (Pro681His), it abolishes TLR2 response to yeast and Gram-positive bacteria (Underhill et al., 1999a). This conserved proline residue does

not have a major structural role, as no significant structural difference was observed between the mutant (Pro681His) and wild type TIR of TLR2 (Fig. 1.7C). In TLR2, this conserved proline residue interacts with MyD88 (Xu et al., 2000). The crystal structures of TIR reveal structural differences between TLR1 and TLR2 despite of a 50% sequence homology (e.g. in helices αB, αD) (Fig. 1.7B)

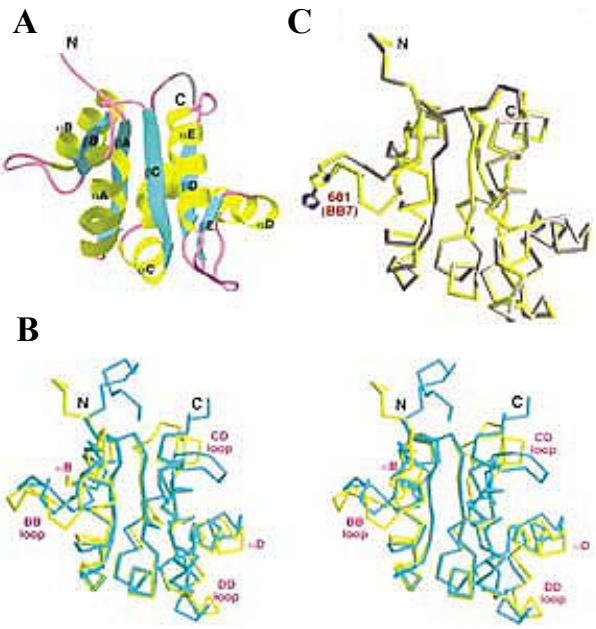


Figure 1.7 Crystal structures of the TIR domains for TLR1, TLR2 and TLR2 mutant. The β-strands or α-helices are given alphabetical denotations. For example, βA and αA are the first β-strand and the first α-helix, respectively. The loops are named by the alphabetical letters of the secondary structures they connect. For example, the BB loop connects the βB strand and αB helix. A residue in a β-strand, α-helix or loop is numbered according to its position in the structure e.g. BB3 is the third amino acid in the BB loop.
(A) Ribbon representation of the TIR domain of human TLR2, with a central five-stranded parallel β-sheet (βA-βE) surrounded by five helices (αA-αE) on both sides.
(B) Superposition of the TIR domains of human TLR1 (cyan) and TLR2 (yellow). Regions with differences between the two structures are labelled. These regions are the BB, CD and DD loops and the αB and αD helices.
(C) Superposition of the TIR domains of human TLR2 (yellow) and the Pro681His mutant (grey). The side chains of residue 681 are shown. Adopted from Xu et al., (2000).

Three interactive interfaces in the TIR domains for TLR signaling has been proposed by (Xu et al., 2000):

i) The first interface (R face) mediates oligomerization between TIR domains, which might be facilitated by ligand-induced association of the extracellular domains of TLRs.

ii) The second interface (A face, likely to be equivalent to the R face in the receptors) mediates interactions between cytosolic adaptor molecules that also contain TIR domains e.g. MyD88 and Mal. These adaptor molecules have been predicted to heterodimerize through hydrophobic residues at the end of the BB loop in the TIR domain and polar residues in the αD helix (Dunne and O'Neill, 2003).

iii) The third interface (S face) mediates association between TIR domains of TLRs and adaptors. The formation of such TIR-TIR interactions recruits adaptors and activate receptor signaling. The S face might be highly conserved among TIR domains, as one common adaptor molecule MyD88 can interact with the TIR domain of most TLRs. An S face has been identified in TLR2 to contain the conserved proline residue, but this does not seem to be conserved in TLR4. TLR4 interaction with MyD88 does not involve this conserved proline residue. TLR4 is predicted to interact with MyD88 via the CD loop and interact with Mal via the αC helix (Dunne and O'Neill, 2003).

TIRs play a pivotal role in TIR signal transduction since mutations in this domain can completely abolish TLR signaling of cell activation (Poltorak et al., 1998; Qureshi et al., 1999). The signaling cascades elicited by TLRs are initiated through TIR interaction with different adaptor molecules.

1.2.3.2 MyD88-dependent TLR signaling pathway

Most members of the IL-1R and TLR family transduce signals by recruiting MyD88. Evidence of MyD88 as a universal adaptor has been provided in studies with MyD88$^{-/-}$ mice. These mice do not produce IL-1, TNF-α, IL-6 and IL-12 in response to immuno-stimulatory microbial structures such as LPS (Kawai et al., 1999), PGN (Takeuchi et al., 2000c), lipoproteins (Takeuchi et al., 2000b), CpG DNA (Hacker et al., 2000), flagellin (Hayashi et al., 2001) and imidazoquinolines (Hemmi et al., 2002). Thus, MyD88 is essential to common signaling events of TLRs leading to inflammatory cytokine production. The MyD88-dependent pathway is illustrated in Figure 1.8.

Upon activation, TLRs or the IL-1R family of receptors interact with MyD88. MyD88 contains TIR domain in its C-terminal portion and a death domain (DD) in its N-terminal portion. Activation of TLR or IL-1R recruits MyD88 to the receptor. The TIR domain of MyD88 binds to the TIR domains of activated TLRs, whereas the death domain interacts with the death domain of IL-1R associated kinase I (IRAK1) and (IRAK4) (Medzhitov et al., 1998; Burns et al., 1998; Muzio et al., 1997; Wesche et al., 1997). Thus, the kinase domains on IRAK1 and IRAK4 are brought in close association. In the MyD88/IRAK1/IRAK4 complex, activated IRAK4 phosphorylates IRAK1 to activate the kinase activity of IRAK1, leading to IRAK4 phosphorylation. IRAK-4 is a central molecule in IL-1R/TLR signaling, as IRAK4$^{-/-}$ mice have almost completely lost response to IL-1, LPS or other bacterial components (Suzuki et al., 2002). The phosphorylated IRAK1 associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) (Cao et al., 1996). The formation of the IRAK4/IRAK1/TRAF6 complex causes conformational change, leading to their

disengagement from the receptor complex. The IRAK4/IRAK1/TRAF6 complex interacts at the membrane with another preformed complex consisting of TGF-β-activated kinase (TAK1) and its two adaptor proteins, TAK1-binding protein (TAB) 1 and TAB2. TAB2, an adaptor linking TAK1 to TRAF6, is associated with cell membrane under unstimulated conditions. TAK1 is a MAPKKK (i.e. mitogen-activated protein kinase kinase kinase), is activated by TAB1, and is essential for IL-1 and TNF-α induced NF-κB activation (Jiang et al., 2002). Interaction of the IRAK4/IRAK1/TRAF6 and TAK1/TAB1/TAB2 complexes induces phosphorylation of TAB2 and TAK1, resulting in the translocation of both complexes into the cytosol. TAK1 is then activated in the cytoplasm through ubiquitination, leading to the activation of IκB kinase (IKK), IKKα and IKKβ (Wang et al., 2001). Inactive IκBα sequesters NF-κB in the cytoplasm. Activation of IKK leads to the phosphorylation and degradation of IκB, resulting in the release and translocation of NF-κB from cytoplasm into the nucleus (Mercurio et al., 1997; Zandi et al., 1997). NF-κB is a transcription factor that induces the production of inflammatory cytokines such as TNF-α and IL-6 and expression of co-stimulatory molecules on APCs (Medzhitov et al., 1997).

TAK1 also activates the p38, JNK and p42/p44 MAPK signaling pathways. p38 in turn activates the transcription factor activation protein 1 (AP-1) (Johnson and Lapadat, 2002).

A second TIR domain-containing molecule TIRAP or Mal is also required exclusively for MyD88-dependent signaling of TLR2 and TLR4 (Horng et al., 2001; Fitzgerald et

al., 2001). In Mal/TIRAP$^{-/-}$ mice there was delayed NF-κB activation and no induction of TNF-α, IL-6 or IL-12p40 (Yamamoto et al., 2002).

1.2.3.3 MyD88-independent signaling pathway

MyD88-independent signaling was initially suggested by responses of MyD88$^{-/-}$ mice to LPS and poly (I:C) stimulation (Kawai et al., 1999; Kaisho et al., 2001; Alexopoulou et al., 2001). The MyD88$^{-/-}$ macrophages exhibited NF-κB and MAPK activation in response to LPS but the response was delayed. LPS stimulation of MyD88$^{-/-}$ macrophages also leads to interferon regulatory factor-3 (IRF-3) activation and induced production of IFN-β and IP-10 (Kawai et al., 1999). Therefore, macrophage response to LPS can be mediated through both MyD88-dependent and independent pathways. Response to poly (I:C) through TLR3 is not impaired in MyD88$^{-/-}$ macrophages (Akira, 2003). TLR3 signaling induces the production of a large amount of type I IFNs and activates many IFN-inducible genes although it also induces a small amount of inflammatory cytokines. This suggests that TLR3 utilizes MyD88-independent signaling pathway.

Through MyD88-independent signaling, TLR3 and TLR4 can mediate the activation of IRF-3 and this induces IFN-β expression. IFN-β in turn activates signal transducer and activator of transcription 1 (STAT1) leading to the production of several IFN-induced genes which are protective against viral infection (Doyle et al., 2002; Toshchakov et al., 2002). However, not all TLRs utilize this alternative signaling pathway. Apart from TLR3 and TLR4, stimulation of TLR7 and TLR9 with respective ligands also causes IRF-3 activation in a MyD88-dependent manner

(Hoshino et al., 2002). Therefore, there seems to be a common MyD88-dependent pathways and distinct MyD88-independent pathways utilized by different TLRs.

The upstream molecular mechanism of MyD88-independent signaling involves two adaptor proteins; i.e TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). Studies from $TRIF^{-/-}$ mice showed no activation of IRF-3 and impaired expression of IFN-inducible genes in response to TLR3 and TLR4 ligands (Yamamoto et al., 2003a; Hoebe et al., 2003). In contrast, $TRAM^{-/-}$ mice showed defects in cytokine production only when they were challenged with TLR4 ligand. IFN-β production was abolished in these mice when the TLR4-mediated MyD88-independent signaling pathway was stimulated but not the TLR3 signaling pathway (Fitzgerald et al., 2003b; Yamamoto et al., 2003b). Thus, TRIF functions downstream of TLR3 and TLR4 whereas TRAM functions downstream of TLR4 only.

TLR3-mediated NF-κB and MAPK activation involves an IRAK-independent pathway where the TLR3-TRAF6-TAK1-TAB2-PKR (dsRNA protein kinase) cascade is employed. It has been demonstrated that Tank-binding kinase 1 (TBK1) and IKKε/IKK, (kinases that are distantly related to IKKα and IKKβ) are required for activation of IRF3 elicited by TRIF and TLR3 (Fitzgerald et al., 2003a). Embryonic fibroblast cells lacking both TBK1 and IKKε/IKK showed impaired activation of IRF-3 and expression of IFN-β and IFN inducible genes in response to TLR3 and TLR4 ligands (McWhirter et al., 2004; Hemmi et al., 2004). Thus, TBK1 and IKKε/IKK are critical regulators of IRF-3 activation in the MyD88-independent pathway.

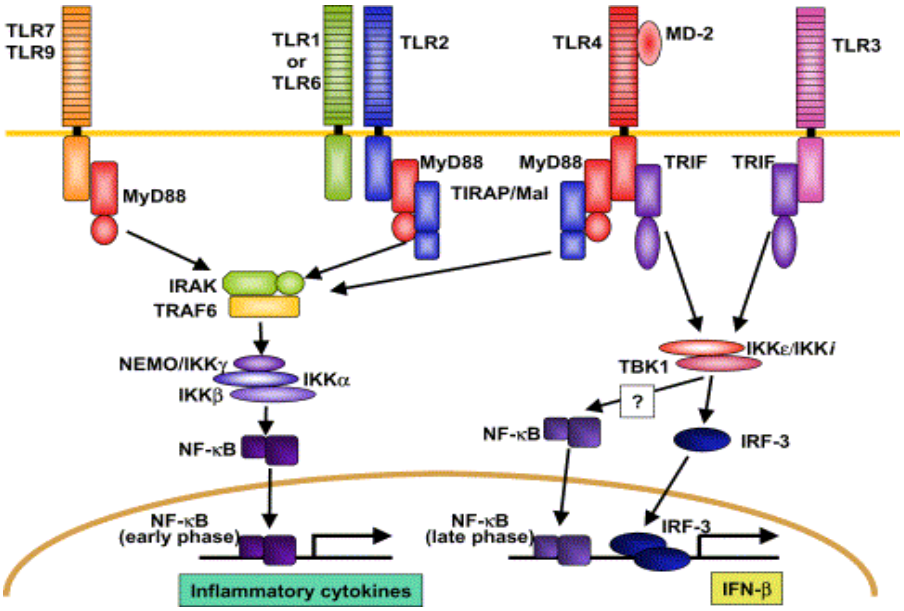


Figure 1.8 TIR domain-containing adaptors and TLR signaling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via all the TLRs. TIRAP/Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In the TLR4 and TLR3-mediated signaling pathways, a MyD88-independent pathway exists that leads to activation of IRF-3 via TBK1 and IKKε/IKKi. The TIR domain-containing adaptor TRIF mediates this MyD88-independent pathway. Adopted from Takeda and Akira et al., (2004).

1.2.4 Mechanism of TLR1 and TLR2 activation

Molecular studies of the IL-1R family suggest that ligand-induced heterodimerization might be the mechanism for signal activation in TIR domain containing receptors. The extracellular domain of IL-1RI binds IL-1α and IL-1β (Vigers et al., 1997) which then recruits the IL-1R accessory protein IL-1RAcP (Greenfeder et al., 1995; Casadio et al., 2001) to form a heterodimer. Optimal IL-1RI response requires the synergistic actions of IL-1RAcP and IL-1RI to recruit MyD88 (Wesche et al., 1997). Lack of IL-1RAcP results in non-responsiveness to IL-1 (Korherr et al., 1997; Cullinan et al., 1998).

Signaling induced by TIR heterodimers has been observed in some TLRs. Dominant negative TLR6$^{-/-}$ and TLR2$^{-/-}$ independently and completely inhibited TNF-α production induced by Gram-positive bacteria. This suggests that the two receptors co-operate to elicit inflammatory cytokine production. In fact, TLR6 was also found enriched in macrophage phagosomes and physically associated with TLR2 through interactions between the two extracellular domains (Ozinsky et al., 2000). TLR2 requires TLR1 to recognize soluble factors released from *Neisseria meningitides* (Wyllie et al., 2000). Confocal microscopy and cross-linking studies have shown TLR2 association with TLR1 on the cell surface and the requirement for both TLR1 and TLR2 to recognize lipoarabinomannan and lipopeptide (Sandor et al., 2003). TLR2 was also shown to physically associate with TLR1 upon co-transfection and the association between TLR2 and TLR1 is ligand-independent (Tapping and Tobias, 2003).

The requirement of dimerization with another TLR for TLR2 signaling is further demonstrated by expression of the CD4 extracellular domain (EC) in fusion with the transmembrane and cytoplasmic domains of TLRs (CD4-TLRs). The CD4 extracellular domain naturally form homodimers and CD4-TLR chimeras therefore form constitutive TLR dimers (Wu et al., 1997). CD4-TLR2 homodimers could not activate TNF-α production in macrophages. In contrast, CD4-TLR4 homodimers constitutively induce TNF-α production (Medzhitov et al., 1997). When the extracellular domains of TLR1, TLR2, TLR4 or TLR6 were similarly expressed as CD4-TLR fusion proteins, only CD4-TLR4 homodimer induced TNF-α production in macrophages. In contrast, homodimers of CD4-TLR1, CD4-TLR2 and CD4-TLR6

failed to induce TNF-α production (Ozinsky et al., 2000). These data indicate that the signaling domains of TLR1, TLR2 and TLR6 are not functionally equivalent to that of TLR4. The finding that CD4-TLR2 homodimers are not active suggests that TLR2 needs a signaling partner to activate TNF-α production. When CD4-TLR2 was co-expressed with CD4-TLR6 or CD4-TLR1, TNF-α production was induced. Co-expression of CD4-TLR1 and CD4-TLR6 did not induce cytokine production. Thus, TLR2 can pair with either TLR6 or TLR1 to induce cell signaling. An independent study using integrin αV and β5 to express TIR fusion proteins subunit showed the same result (Zhang et al., 2002). When αV-TLR2 was co-expressed with β5-TLR6 or β5-TLR1 in 293T transfected cells, NF-κB was activated. These data may imply that TLRs form pairs in response to different pathogens and may induce different signaling pathways.

In this project, we examine whether it is feasible to detect protein interactions by expression of test proteins as the EC domains of TLR1 and TLR2. The well-characterized interactions between IL-4 and its receptors were used to evaluate this method.

1.3 Interleukin-4 (IL-4)

1.3.1 IL-4 and its functions

Human IL-4 is a 129 residue cytokine initially characterized as a proliferation and differentiation factor for B cells (Howard et al., 1982; Vitetta et al., 1985). It is now known as a pleiotropic cytokine that can stimulate a wide variety of cell types

including T cells, macrophages, fibroblasts and endothelial cells (Paul, 1991), mast cells (Plaut et al., 1989), and basophils (Seder et al., 1991).

By analysis of the DNA of human and mouse somatic cell hybrids and in situ chromosome hybridization, its gene has been mapped to 5q23-3, where the IL-3 and GM-CSF genes are also found (Le Beau et al., 1989). Functionally, these cytokines are designated as the 'IL-4 family of lymphokines'. IL-4 is one of the short chain, four-helix-bundle cytokines (Rozwarski et al., 1994).

IL-4 plays a central role in regulating the differentiation of antigen-stimulated naïve T-cells. It causes such cells to develop into Th2-like cells capable of producing IL-4 and a series of other cytokines including IL-5, IL-10 and IL-13 (Seder et al., 1992; Hsieh et al., 1992). In particular, IL-4 has a major physiological role in controlling the specificity of immunoglobulin class switching. For example, stimulation of human B cells with anti-CD40 or anti-CD58 antibody, or with Epstein-Barr virus, induces the production of IgE and IgG4 (Thyphronitis et al., 1989; Gauchat et al., 1990; Gascan et al., 1991; Diaz-Sanchez et al., 1994). In IL-$4^{-/-}$ (Kuhn et al., 1991) and IL-$4R^{-/-}$ (Noben-Trauth et al., 1997) mice as well as in mice that lack a principal signaling substrate of the IL-4 receptor, Stat-6 (Shimoda et al., 1996; Kaplan et al., 1996), IgE production is diminished by 100 fold or more. The productions of IgE antibodies induced by IL-4 that are associated with atopic diseases, associated with allergens suggest a pre-eminent role for IL-4 in the regulation of such conditions. IL-4 also exerts a wide variety of effects on hematopoietic and non-hematopoietic cells. It enhances the expression of CD23 (Defrance et al., 1987) and MHC class II molecules (Noelle et al., 1984) in B cells and upregulates surface expression of the receptor

complex for IL-4 (Ohara and Paul, 1988). On vascular endothelial cells, IL-4 together with TNF-α induces the expression of vascular cell adhesion molecule-1 (VCAM-1) (Thornhill et al., 1991) and downregulates the expression of E-selectin (Bennett et al., 1997) thereby changing the adhesive characteristics of endothelial cells. The altered adhesive characteristics of endothelial cells facilitate tissue infiltration by allergic inflammatory cells, such as eosinophils.

1.3.2 IL-4 and its receptor complex

IL-4 mediates its biological responses by binding to the IL-4 receptor complex (Nelms et al., 1999). The IL-4 receptor complex consists of two subunits. One subunit is the IL-4R α-chain (IL-4Rα), a high-affinity receptor present in hematopoietic and non hematopoietic cells including human fibroblasts, epithelial cells, hapatocytes, endothelial cells and muscle (Mosley et al., 1989; Idzerda et al., 1990; Galizzi et al., 1990). IL-4Rα is a member of the type I cytokine receptor super family which is characterized by the presence of four conserved cysteine residues and a WSXWS motif (W, tryptophane; S, serine; X, non conserved amino acid) in their extracellular domain (Bazan, 1990). The WSXWS motif is required for maintaining the receptor in a conformation favourable to cytokine binding (Livnah et al., 1996). The cytoplasmic domain of IL-4Rα contains a short amino acid sequence termed box 1 which is conserved among members of this cytokine receptor-family (Fukunaga et al., 1991; Murakami et al., 1991). In addition to this conserved domain, IL-4Rα bears a region termed insulin-IL-4 receptor (I4R) motif in amino acids 437-557 in the cytoplasmic domain. This region is essential for cellular proliferation (Harada et al., 1992; Seldin and Leder, 1994; Keegan et al., 1994; Nelms et al., 1999).

The other IL-4R subunit is the common γ-chain (γC), which also belongs to the type I cytokine receptor super-family. It was initially identified as the third component of the IL-2 receptor complex (Takeshita et al., 1992). It is now known that γC is a common component for several receptors: IL-2 (Takeshita et al., 1992), IL-4 (Russell et al., 1993), IL-7 (Noguchi et al., 1993a) and IL-15 (Giri et al., 1994). Mutation of the human γC gene can cause X-linked severe combined immunodeficiency (XSCID) syndrome, which is characterized by the absence of T cells (Noguchi et al., 1993b). Mice deficient for the γC gene exhibit more severe forms of the syndrome than humans, displaying the absence of B cells, natural killer cells and T cells (Cao et al., 1995; Sugamura et al., 1996). These phenotypes reflected the loss of cytokine functions mediated through γC and an essential role for γC is the development of a normal immune system.

1.3.3 Mechanism of IL-4R activation

IL-4 triggers the formation of a heterodimer consisting of IL-4Rα and γC (Duschl and Sebald, 1996). This elicits an ordered sequential mechanism. Soluble IL-4 primarily binds with IL4Rα. Subsequently, this intermediate IL-4/IL-4Rα complex recruits the γC to form a IL-4Rα/ γC heterodimer (Russell et al., 1993; Kondo et al., 1993).

The crystal structure of the IL-4/IL-4Rα complex has been characterized (Hage et al., 1999; Mueller et al., 2002). It revealed that the IL-4 binding site on IL4Rα is a mosaic-like structure consisting of three discrete clusters of trans-interacting residues. Two of them exhibit a conspicuous amphipathic structure with an outer surface of hydrophobic side chain moieties and an inner core of polar groups. These two clusters have been termed the ‘avocado cluster’, since an avocado fruit also has a core that is surrounded by a hydrophobic layer (Hage et al., 1999; Mueller et al., 2002). The

amino acid residues involved in IL-4 binding to IL-4Rα have also been identified by mutational and quantitative analysis and the findings support the functional significance of these residues and also confirm the existence of a mosaic binding structure between IL-4/IL-4Rα (Wang et al., 1997).

γC interaction with IL-4 is distinct from that of IL-4 and the IL-4Rα. Cells expressing γC alone cannot bind to IL-4 even at concentrations as high as 100 nM (Sugamura et al., 1995). However, direct interaction between IL-4 and γC has been shown in cells that express both the IL-4Rα and γC (Sugamura et al., 1995). Iodinated IL-4 becomes efficiently cross-linked to γC if IL-4Rα is also present. The binding affinity between γC and IL-4 has been determined by plasmon resonance technology (Letzelter et al., 1998).

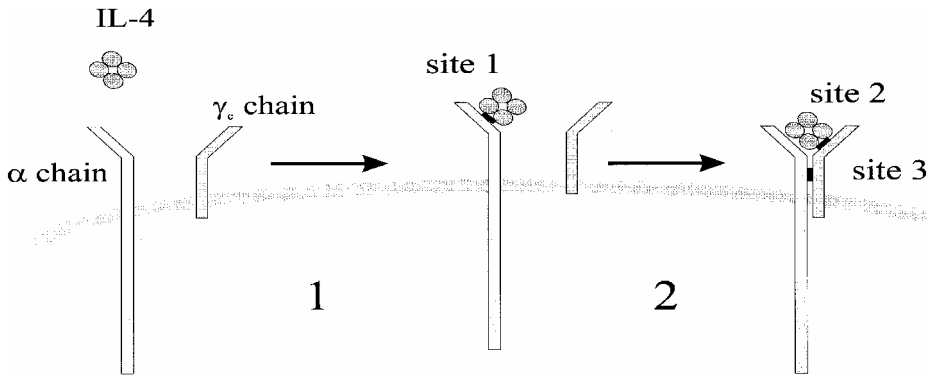


Figure 1.9 Model of the two-step mechanism for IL-4R activation. (1) Soluble IL-4 binds via site 1 to the IL4Rα-chain first. (2) Dimerization on the membrane is driven by interaction between IL-4 site 2 and γC as well as by a weak direct interaction between the receptor chains site 3. Adopted from Letzelter et al., (1998).

It was proposed by (Letzelter et al., 1998) that the ligand-induced IL-4Rα/γC dimerization proceeds in a two-step mechanism. Soluble IL-4 binds to the extracellular domain of IL-4Rα first which is a three-dimensional reaction and this is followed by lateral or two-dimensional recruitment of γC to the binary IL-4/IL-4Rα complex (Fig. 1.9).

1.4 CD14

CD14 is discussed here because TIR1/TIR2-based two-hybrid-based assay was applied to investigate interactions of this molecule. CD14 is related to TLRs in function and structure.

1.4.1 The CD14 gene and its expression

CD14 was first described as a leukocyte cluster differentiation (CD) antigen mainly expressed on the surface of monocytes, macrophages and, at a lower level, on neutrophils (Ziegler-Heitbrock and Ulevitch, 1993; Antal-Szalmas et al., 1997). CD14 is a 55 KDa glycoprotein that exists as a glycosylphosphatidylinositol (GPI)-linked protein on the surface of these myelomonocytic cells (Haziot et al., 1988; Simmons et al., 1989; Haziot et al., 1993) and soluble CD14 is also found in the serum (Bazil et al., 1986; Bazil et al., 1989).

The CD14 gene was mapped to chromosomes 5q23-31 (Ferrero and Goyert, 1988). In this region of the genome, the genes for other growth factors and their receptors such as IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), the platelet-derived growth factor (PDGF) receptor, and the adrenergic receptor are also found (Goyert et al., 1988). CD14 consists of 356 amino acids which are predominantly LRRs as in the extracellular

domains of TLRs. Its gene contains two exons separated by a 88-bp intron with the first exon coding for the first methionine residue (Ferrero et al., 1990).

1.4.2 Structure

The human CD14 transcripts encodes 375 residues with the first 19 residues forming the signal peptide (Ferrero et al., 1990). The C-terminal end is a 21 residue hydrophobic domain, but it lacks the characteristic basic residues for a typical transmembrane domain. Human CD14 (hCD14) has four potential N-linked glycosylation sites (Asn-X-Thr/Ser) and in addition bears O-linked carbohydrates. All these sites are conserved in mouse CD14 (mCD14). Carbohydrates account for 20% of the CD14 protein (Setoguchi et al., 1989; Stelter et al., 1996). It contains 10 cysteine residues which are important in determining the conformation of CD14 through the formation of specific disulfide bridges. CD14 contains 10 LRRs located between residues 86 and 329 (Ferrero et al., 1990; Setoguchi et al., 1989). These LRRs can mediate protein-protein interactions in other proteins (Hofsteenge et al., 1988; Tan et al., 1990; Krantz and Zipursky, 1990; Kobe and Deisenhofer, 1995). Alignment of the amino acid sequences of hCD14 and mCD14 revealed 66% amino acid identity (Setoguchi et al., 1989).

Crystallographic studies have shown that a monomeric CD14 contains thirteen β strands, and eleven of them, from β3 to β13, overlap with conserved LRRs (Fig. 1.10A). The concave surface of the horseshoe-shaped structure consists of a large β-sheet of 11 parallel and two anti-parallel β strands. The convex surface of CD14 contains both helices and loops in an irregular pattern. CD14 has been suggested to form a dimer based on the crystallographic data (Fig. 1.10B). CD14 dimerization is likely to involve residues in β13 and in the 'loop' between β12 and β13. Parallel β-

sheets from two CD14 monomers interact in an anti-parallel fashion which form a large and continuous β-sheet encompassing the entire CD14 dimer (Kim et al., 2005).

The most striking feature of the CD14 structure is the NH_2-terminal pocket. The pocket is located on the side of the horseshoe near the NH2-terminus and it is entirely hydrophobic (Fig. 1.10B). The main pocket contains a smaller sub-pocket at the bottom. This sub-pocket is formed by hydrophobic residues from β4 to β6, α4, α5, and the connecting loops (Fig. 1.10).

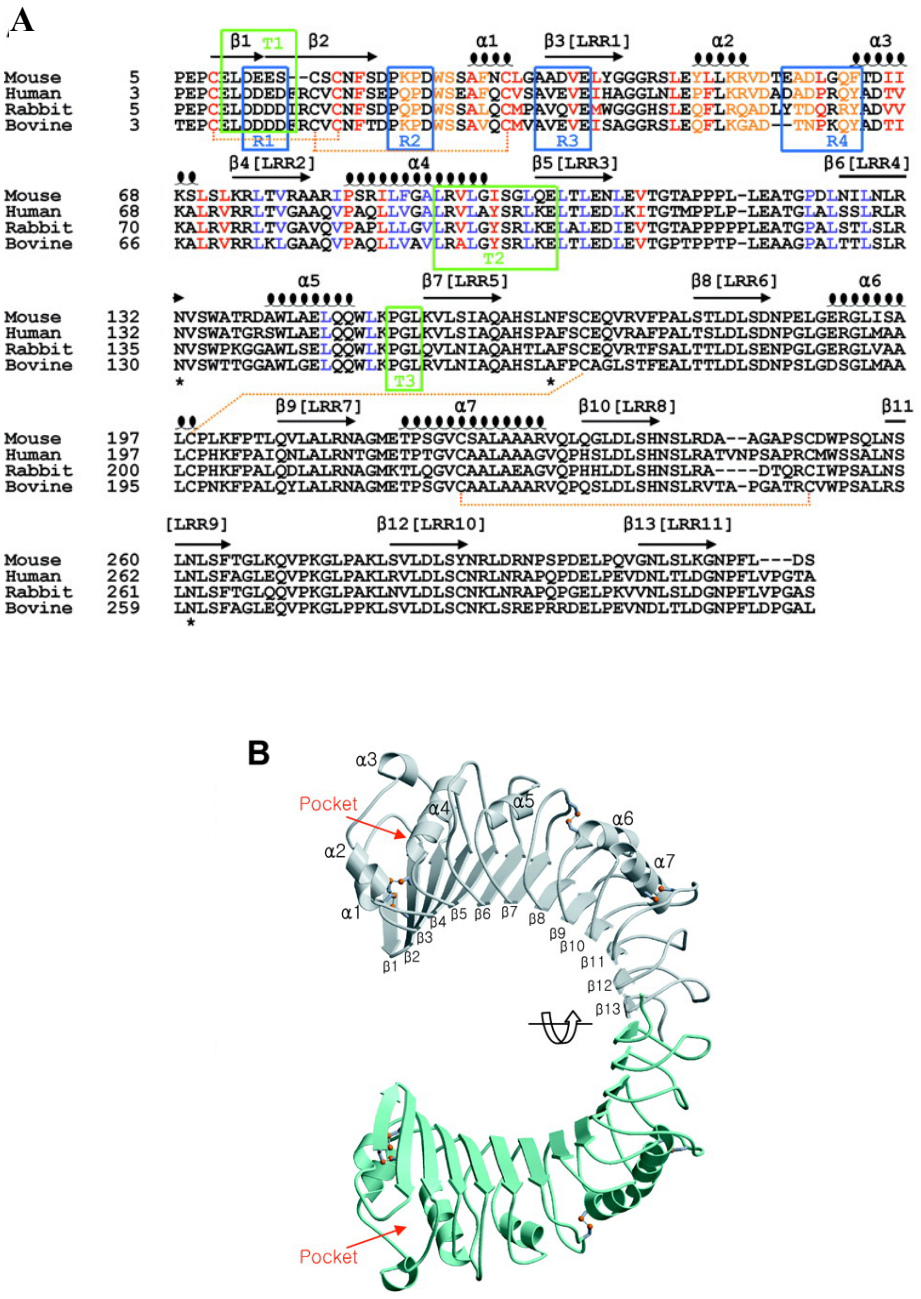


Figure 1.10 Overall structure of mouse CD14. (A) Alignment of mouse, human, rabbit, and bovine CD14. Secondary structures are shown schematically above the sequences. N-Glycosylation sites identified in the crystal structure are marked with an asterisk. Cysteines involved in disulfide bridges are linked by broken yellow lines. Residues on the rim of the NH_2-terminal pocket are written in yellow, residues on the walls and base of the main pocket are written in orange, and residues on the sub-pocket are written in purple. LPS-binding regions identified are labeled R1–R4 and enclosed within blue boxes. Residues important for LPS signaling (36–38) are enclosed within green boxes and labeled T1–T3. (B) Overall structure of the CD14 dimer. Two CD14 monomers in the crystal are colored in gray and cyan. Disulfide bridges are shown in orange. The position of the NH_2-terminal pocket is indicated by an arrow. Adopted from Kim et al., (2005).

1.4.3 CD14 functions

CD14 plays a key role in the recognition of LPS and induces leukocytes to produce inflammatory cytokines and upregulate integrin functions (Wright et al., 1990; Wright et al., 1991). Blocking of cell surface CD14 using anti-CD14 antibody abolishes the cytokine production from myeloid cells in response to LPS. Recognition of LPS by CD14 requires LBP (LPS-binding protein), an acute-phase reactant found in serum (Wright et al., 1990; Mathison et al., 1992; Hailman et al., 1994). LBP also plays a role in LPS internalization as LBP antibodies have been reported to prevent LPS endocytosis (Gegner et al., 1995).

Cross-linking experiments showed that LPS directly associate with membrane-bound CD14 on macrophages and LPS binding to CD14 is essential for macrophage activation (Tobias and Ulevitch, 1993). These findings were supported by data derived from $CD14^{-/-}$ mice. $CD14^{-/-}$ mice were resistant to septic shock induced either by LPS or Gram-negative bacteria (Haziot et al., 1996). The ability of peripheral blood mononuclear cells from $CD14^{-/-}$ mice to respond to LPS was partially restored after addition of soluble CD14 (sCD14) to this assay (Haziot et al., 1996). In contrast, over-expression of hCD14 in transgenic mice rendered these mice hypersensitive to LPS as evidenced by increased susceptibility to endotoxic shock (Ferrero et al., 1993). These results indicate that membrane-bound CD14 plays a central role in LPS-induced cell activation. sCD14 may play the role as either an activator or inhibitor in LPS-mediated cell activation. At high concentrations, sCD14 dampens response to LPS. Recombinant sCD14 can inhibit oxidative burst in isolated human monocytes (Schutt et al., 1992), inhibit TNF-α release from whole blood (Haziot et al., 1994), inhibit LPS priming of neutrophils (Troelstra et al., 1997), and protect mice from

LPS-induced lethality (Haziot et al., 1995). Therefore, membrane-bound and soluble CD14 play important roles in LPS recognition.

1.4.4 LPS binding to CD14

Early work on LPS binding to CD14 was performed using ^{3}H-labeled LPS (Couturier et al., 1991). It was shown that, in the presence of serum, LPS could bind to monocytes in a dose dependent, saturable and displaceable fashion. This specific LPS binding to monocytes was demonstrated to occur through CD14 on the cells. Heumann et al (1992) showed similar results using fluorescent-labeled LPS and the MY4 anti-CD14mAb and by enzymatically removing CD14 from monocyte surface. The LBP-dependent binding of LPS to CD14 was further established using serum depleted of LBP with anti-LBP antibodies (Heumann et al., 1992). Studies using ^{3}H-labeled LPS and human CD14 expressed on transfected Chinese hamster ovary cells or the human monocytic cell line (THP-1) indicated that LPS binding occurred within 10 min and was relatively unaffected by temperature over the range of 4-37°C. Quantitative binding studies were performed at 10°C and 30°C using cells depleted of ATP to prevent LPS internalization. At 10°C, each CD14 bound 20 LPS molecules and it was reduced to 8 LPS molecules at 37°C, which was very different from the expected 1:1 stochiometry. It was suggested that each CD14 might have multiple binding sites for LPS or alternatively CD14 bound to small LPS aggregates (Kirkland et al., 1993). This idea of multiple LPS binding by CD14 was also raised in another study where it was demonstrated that, in addition to its ability to compete with LPS for binding to LBP and CD14, deacylated LPS potently antagonized LPS-induced cell activation at an unknown site distal to the LPS binding site of CD14 (Kitchens and Munford, 1995).

Studies using soluble CD14 mutants have demonstrated that the NH_2-terminal (152 aa) portion of the molecule was sufficient for LPS binding and cell response (Juan et al., 1995c; Viriyakosol and Kirkland, 1995; Viriyakosol and Kirkland, 1996). In another mutational study, specific amino acids in regions 9-13 and 57-64 were found to be critical for LPS binding (Juan et al., 1995a; Stelter et al., 1997). Epitope-mapping studies using LPS blocking antibodies revealed that four different hydrophilic regions over the NH_2-terminal portion of CD14, which were located on one side of the protein surface, were involved in LPS binding. These studies suggest that CD14 employs a charged surface, in a manner similar to the macrophage scavenger receptor, to 'capture' LPS and 'present' them to other components of the innate host defense system (Cunningham et al., 2000). Interestingly, different LPS (from *E.coli* to *P. gingivalis*) may have distinct binding sites on CD14. For example, a point mutation at E47 does not affect the binding of *E.coli* LPS to CD14 but it abrogated *P.gingivalis* LPS binding (Shapiro et al., 1997).

Kim identified the regions on CD14 for LPS binding by crystallographic studies (Kim et al., 2005). Residues from the turn between the β1 and β2 strands constitute region 1. Region 2 is the loop between the β2 strand and the α1 helix (Fig. 1.10 A). Monoclonal antibodies that recognize this area inhibited LPS binding (Viriyakosol and Kirkland, 1995; Dziarski et al., 1998; Cunningham et al., 2000). Region 3 consists of residues from the β3 strand. Region 3 is the most frequent target of LPS blocking antibodies. At least nine monoclonal antibodies that recognize region 2 and 3 reduced LPS binding to soluble CD14 (Viriyakosol and Kirkland, 1995; Dziarski et al., 1998; Cunningham et al., 2000). Region 4 includes residues from the loop connecting α2 and α3 helices. This area is liable to proteolysis in the absence of

bound LPS (McGinley et al., 1995). Collectively, these mutagenesis and epitope mapping data strongly suggest that the NH_2-terminal pocket is the principal LPS-binding site on CD14. In terms of LPS signaling, some studies showed that some CD14 mutants may have minor defects in LPS binding, but they have virtually lost signaling activity (Juan et al., 1995b; Stelter et al., 1999; Muroi et al., 2002). They are alanine mutations of Glu^{7}-Asp^{10}, Asp^{9}-Phe^{13}, or Leu^{91}-Glu^{101} in human CD14 or Pro^{151}-Leu^{153} in mouse CD14. These regions are labeled as T1-T3 in Fig. 1.10A.

1.4.5 CD14 and its receptor complex

CD14 has been recognized for many years as a major receptor mediating LPS effects on monocytes, macrophages and neutrophils (Wright et al., 1990). However, numerous studies suggested that CD14 alone is not able to induce signaling and needs to associate with a transmembrane signal-transducing receptor. Beutler and coworkers mapped the Lps locus in the LPS-resistant C3H/HeJ mice and identified TLR4 as a candidate gene responsible for LPS hypo-responsiveness of these mice (Poltorak et al., 1998). The TLR4 gene was found deleted in the LPS non-responsive C57B1/10ScCr mouse (Qureshi et al., 1999; Hoshino et al., 1999). $TLR4^{-/-}$ mice also exhibited hyporesponsiveness to LPS (Takeuchi et al., 1999a). In contrast, $TLR2^{-/-}$ mice remained responsiveness to LPS. These results consistently show the critical role of TLR4 in host response to LPS. TLR4 alone was not fully functional but required an additional protein, MD2, to confer LPS responsiveness of cells (Shimazu et al., 1999). MD2 lacks a membrane anchoring domain but after secretion, it physically associates with the extracellular portion of TLR4 (Akashi et al., 2000; Yang et al., 2000). These results suggest a CD14, TLR4 and MD2 receptor complex for LPS. Biochemical

studies have shown that LPS binds directly to all three members of this tripartite LPS receptor complex (da Silva et al., 2001; Visintin et al., 2003).

1.4.6 CD14 and its signaling cascade

As TLR4 has been found to be involved in LPS signaling, the LPS-induced intracellular signaling cascade leading to NF-κB activation has been studied in depth which has been described in the TLR signaling section (section 1.2.3). The current hypothesis is that, in the presence of LPS, CD14 binds to TLR4 and MD2. This induces homodimerization of TLR4 which in turn recruits MyD88 or TRIF/TRAM to initiate MyD88-dependent and MyD88-independent signaling.

1.5 Fc gamma Receptors (FcγRs)

These families of receptors are discussed here because TIR1/TIR2-based two-hybrid assay was used to study FcγRIIA dimerization and activation.

1.5.1 Overview of FcγRs

Antibodies represent an important arm of the humoral immune system which enhances cell mediated immunity through immunoglobulin Fc receptors on leukocytes (FcRs). FcRs exist for all antibody isotypes and different FcRs may be expressed on different leukocytes. These include: FcαR (IgA), FcδR (IgD), FcεR (IgE), FcγR (IgG), and FcμR (IgM) (Lefranc, 2001).

Fc receptors for IgG (FcγRs) were first identified through the analysis of cytophylic antibodies and their mechanism of interaction with macrophages when presented as opsonized RBCs (Berken and Benacerraf, 1966). In mammals four different classes of

FcγRs have been identified: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV (Nimmerjahn and Ravetch, 2006).

All the FcγRs belong to the Ig super-family of molecules. The FcγRs share a common ancestral gene and highly homologous Ig-like extracellular (EC) domains. Most of their structural differences are located in the transmembrane and cytoplasmic regions, suggesting that they transduce different signals (Daeron, 1997). In mouse, each class is encoded by a single gene, whereas in human a total of eight genes have been identified: three genes for the high-affinity receptor FcγRI (A, B, C) and five genes for the two low-affinity receptors FcγRII (A, B, C) and FcγRIII (A, B) (Qiu et al., 1990). The structural charecteristics of the different FcγRs are shown in Fig. 1.11.

FcγRI displays a high affinity for the constant region of IgG with restricted isotype specificity, whereas FcγRII and FcγRIII have low affinity for the Fc region of IgG but a broader isotype binding pattern (Ravetch and Kinet, 1991; Hulett and Hogarth, 1994). In addition to the variation in FcγR affinity, each FcγR binds an IgG subclass with distinct specificities (Table 1.1). Very recently mouse FcγRIV has been mapped in the 75 Kb genomic interval between FcγRII and FcγRIII (Mechetina et al., 2002; Davis et al., 2002; Nimmerjahn et al., 2005). It binds to IgG2a and IgG2b antibody with intermediate affinity. The surface expression of FcγRIV is γ-chain dependent and restricted to myeloid lineage cells. There is a putative human FcγRIV gene, but the biological functions of the protein, such as ligand specificity and cellular expression, are as yet unknown (Nimmerjahn et al., 2005). The general characteristics of human

FcγRs are summarized in Table 1.1. In the following section, the three best known classes of FcγRs will be discussed in more details.

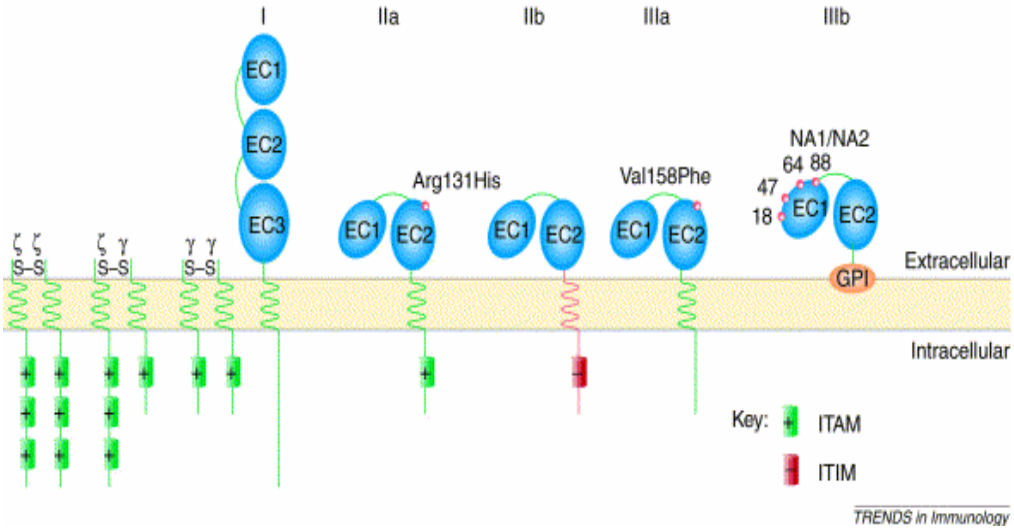


Figure 1.11 Structural diversity and heterogeneity of human FcγRs. All receptors belong to the Ig super-family, with their extracellular regions (ECs) composed of disulfide (S–S)-bonded domains. FcγRI, FcγRIIa and FcγRIIIa are activating receptors, characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain of the receptor (FcγRIIa) or in co-receptors in signaling subunit (γ and/or ζ chains associated with FcγRI and FcγRIIIa). FcγRIIb is an inhibitory receptor, containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. FcγRIIIb is linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. Functional polymorphisms in FcγRIIa, FcγRIIIa and FcγRIIIb are indicated by red circles. NA refers to neutrophil antigen polymorphism. Adopted from Dijstelbloem et al., (2001).

Table 1.1 General characteristics of human FcγRs.

Receptor class (CD)	Genes	Transcripts	MW (kDa)	Isoforms	Ligand specificity for hIgG	Affinity for hIgG (K_a)
FcγRI (CD64)	FcγRIA FcγRIB FcγRIC	FcγRIa FcγRIb1 FcγRIb2 FcγRIc	72		1,3>>4>>>2	10^8–10^9 M^{-1}
FcγRII (CD32)	FcγRIIA FcγRIIB FcγRIIC	FcγRIIa1 FcγRIIa2 FcγRIIb1 FcγRIIb2 FcγRIIb3 FcγRIIc	40 41 37	 FcγRIIa-131H FcγRIIa-131R	1,3>>>2,4 3>1,2>>4 3> 1>>2,4 3≥1>4>>>2	 < 10^7 M^{-1} < 10^7 M^{-1} < 10^7 M^{-1}
FcγRIII (CD16)	FcγRIIIA FcγRIIIB	FcγRIIIa FcγRIIIb	45–72 on monocyte 47–58 on lymphocyte 50–80 45–60 60–75 60–75	 NA1-FcγRIIIb NA2-FcγRIIIb SH-FcγRIIIb	 1,3>>>2,4 3> 1>> 2,4 3 ≥ 1 >> 2,4	3 ×10^7 M^{-1} 10^8 M^{-1} 5 × 10^8 M^{-1}

Table with modification from van de winkel et al., (1993)

1.5.2 Genes, structure and cellular distribution of Human FcγRs.

FcγRI (CD64)

Human FcγRI (CD64) is encoded by three homologous genes, i.e. FcγRIA, FcγRIB and FcγRIC (van de Winkel et al., 1991; Ernst et al., 1992), mapped to chromosome 1 band q21.1 (de Wit et al., 1993). FcγIA is a 72 KDa transmembrane glycoprotein distinct from the other FcγR members by having three extracellular Ig-like domains (Fig. 1.11). Of the three hFcγRI genes (A, B, C), only FcγRIA encodes a high-affinity receptor. FcγRIB has two transcripts, one with a stop codon in extracellular domain 3

(EC3) and the other lacking this domain. Although it is expressed in myeloid cells, this transcript resides in the endoplasmic reticulum and therefore does not contribute to known cellular activation (van Vugt et al., 1999). It has not yet been clarified whether FcγRIC encodes for a functional protein (Porges et al., 1992).

FcγRI binds monomeric IgG with high affinity ($K_A=10^8$-10^9 M^{-1}) as well as aggregated IgG. This unique high affinity is dependent on the additional third Ig domain. The affinity for IgG subclasses is IgG1=IgG3>IgG4>>>IgG2 (Allen and Seed, 1989) (Table 1.1). It has been shown that FcγRI associates with the FcRγ chain, a 20 KDa disulfide-linked homodimeric signaling subunit (Ernst et al., 1993) (Fig.1.11). Although FcRγ is not needed for FcγRI folding and targeting to the cell surface, it is critical for FcγRI signaling and stable expression in vitro and in vivo (van Vugt et al., 1996). The association of FcRγ with FcγRI and FcγRIIIA also increases affinity for IgG (Miller et al., 1996). FcγR association with FcRγ is mediated through the FcRγ transmembrane domain and the cytoplasmic domain of the receptor is not involved.

In humans, FcγRI is constitutively expressed on monocytes, macrophages and granulocytes (van de Winkel and Capel, 1993; Hulett and Hogarth, 1994) and can also be induced on neutrophils and eosinophils by IFN-γ (Guyre et al., 1983; Perussia et al., 1987). Monocyte FcγRI expression can also be up-regualted by IFN-γ or IL-10 (te Velde et al., 1992) and by bacterial infections in vivo (Simms et al., 1989).

FcγRII (CD32)

The three FcγRII genes, i.e. FcγRIIA, FcγRIIB and FcγRIIC, are located on chromosome 1q23-24 (Ravetch and Kinet, 1991; Hulett and Hogarth, 1994). All three FcγRII genes encode 40 KDa glycoproteins, containing two extracellular Ig-like domains (Fig. 1.11). FcγRIIC is likely to result from an unequal crossover event between the first part of FcγRIIB and the terminal part of FcγRIIA (Warmerdam et al., 1993) and has been postulated to be a pseudogene.

FcγRII binds monomeric IgG with low or undetectable affinity ($K_A=10^6\ M^{-1}$) specific for human IgG1 and IgG3 (van de Winkel and Capel, 1993) (Table 1.1). Under physiological conditions, the low affinity of FcγRII for monomeric IgG ensures that this receptor interacts only with aggregated IgG through multivalent interactions. A functional polymorphism has been identified in FcγRIIA (Warmerdam et al., 1990). Sequencing revealed that FcγRIIA either expresses an arginine (R) or a histidine (H) at amino acid position 131 (Fig. 1.11). These polymorphic variants have been shown to interact differently with various ligands, with FcγRIIA-H131 binding to human IgG2 whereas FcγRIIA-R131 showing no binding (Parren et al., 1992).

FcγRII is the only family member known to contain its own signaling motif. FcγRI and FcγRIII are dependent on association with other subunits for signal transduction. Several isoforms of FcγRII exist, which are highly homologous in their extracellular and transmembrane regions but differ in their intracellular domains (Brooks et al., 1989; Warmerdam et al., 1990). FcγRII contains, depending on the isoform, either an immunoreceptor tyrosine-based activation motif (ITAM) or an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIA and FcγRIIC contain an ITAM motif

and are therefore activating receptors, whereas FcγRIIB contains an ITIM motif and is thus an inhibitory receptor (Fig. 1.11) (Van den Herik-Oudijk IE et al., 1995; Van den Herik-Oudijk IE et al., 1994).

hFcγRIIs are the most widely expressed FcγRs and are present on almost all leukocytes, including neutrophils, eosinophils, basophils, platelets, Langerhans cells, B cells and some T-cell subsets (Hulett and Hogarth, 1994; van de Winkel and Anderson, 1991). Monocytes have also been shown to express both FcγIIA and FcγRIIB2 (Pricop et al., 2001). Interestingly, IFN-γ and IL-4 influence the balance of FcγRIIA and FcγRIIB2 on monocytes; IL-4 upregulates FcγRIIB2 while it simultaneously downregulates FcγRIIA, whereas IFN-γ has the opposite effect (Pricop et al., 2001). FcγRIIB1 are expressed mainly on B cells and are the only FcγRs on these cells and its co-ligation with the B cell receptor (BCR) inhibits BCR activation (Van den Herik-Oudijk IE et al., 1994). NK cells have been reported to express FcγRIIC which enhances the cytotoxicity of these cells (Metes et al., 1998; Sulica et al., 2001).

FcγRIII (CD16)

There are two homologous FcγRIII genes, i.e. FcγRIIIA and FcγRIIIB, both being located on chromosome 1q23-24 (van de Winkel and Anderson, 1991; Hulett and Hogarth, 1994). Both gene products show heterogeneous sizes, ranging between 50-80 KDa, as a result of variable N-linked glycosylation. FcγRIIIA and FcγRIIIB have two extracellular Ig-like domains and their extracellular domains differ by only 6 amino acids. In contrast to FcγRIIIA, FcγRIIIB does not contain a transmembrane domain or a cytoplasmic tail but it is anchored to the cell membrane via a

glycosylphosphatidylinositol (GPI) anchor (Fig. 1.11) (Ravetch and Perussia, 1989). Unlike FcγRI, FcγRIIIA is critically dependent on FcRγ and/or FcRζ for trafficking to the cell surface as FcγRIIIA has an endoplasmic reticulum retention signal (Kurosaki et al., 1991).

Like FcγRII, FcγRIII is a low affinity receptor (K_A=5.5 x 10^5 M^{-1}) and it preferentially interacts with immune complexes containing human IgG1 and IgG3 (Galon et al., 1997) (Table 1.1). Genetic polymorphisms affecting IgG subclass specificites have been found in both FcγRIIIA (de Haas et al., 1996; Koene et al., 1997) and FcRIIIB (Huizinga et al., 1989). In FcγRIIA, a polymorphism at amino acid 158, a valine or phenylalanine, has been identified (Fig. 1.11). As a result, FcγRIIIA-V158 has a higher affinity for IgG1 and IgG3 than FcγRIIIA-Phe158 (de Haas et al., 1996; Koene et al., 1997). Allelic variations in FcγRIIIB have been found in four amino acids, referred to as neutrophil antigen 1(NA1) and NA2 (Fig. 1.11) (Huizinga et al., 1990). FcγRIIIB-NA1 internalizes IgG1- or IgG3-opsonized particles more efficiently than FcγRIIIB-NA2 (Huizinga et al., 1989).

Monocytes and natural killer (NK) cells are the major cell population expressing FcγRIIIA (van de Winkel and Anderson, 1991; Hulett and Hogarth, 1994) whereas, FcγRIIIB is solely expressed by neutrophils and eosinophils (Huizinga et al., 1988). FcγRIIIA can be induced by TGF-β on monocytes (Wahl et al., 1992). In contrast, IL-4 strongly inhibits FcγRIIIA expression on these cells (Wong et al., 1991).

1.5.3 Functions of FcγRs

The current understanding of FcγR functions has been greatly enhanced by the generation of mice deficient in the different FcγRs. Analysis of FcγR$^{-/-}$ mice confirmed the importance of FcγRs in mediating IgG effector functions and the development of inflammatory disease (Ravetch and Bolland, 2001; Takai, 2002).

FcγRI (CD64) and FcγRIII (CD16)

FcγRI mediates multiple biological functions upon cross-linking. This includes phagocytosis of opsonized particles (Anderson et al., 1990; Davis et al., 1995), killing of opsonized cellular targets via antibody dependent cell cytotoxicity (ADCC) (Graziano and Fanger, 1987), superoxide production (Pfefferkorn and Fanger, 1989) and cytokine release (Debets et al., 1990; Krutmann et al., 1990). The role of FcγRIII is associated with clearance of immune complexes and ADCC (Fossati-Jimack et al., 2000).

Macrophages from FcRγ$^{-/-}$ mice, lacking FcγRI, FcγRIII and FcεRI, are unable to phagocytose IgG-opsonized particles (Takai et al., 1994). These mice also exhibit defective ADCC by NK cells and diminished mast cell-mediated allergic response. The specific roles for FcγRI (Barnes et al., 2002; Ioan-Facsinay et al., 2002) and FcγRIII (Hazenbos et al., 1996) have been studied with cells from mice deficient for the specific receptors. FcγRI$^{-/-}$ mice showed multiple inflammatory and immune responses such as (i) loss of uptake of immune complexes of the T cell-independent immunoglobulin IgG3 as well as a profound reduction in IgG2a uptake; (ii) reduction in antibody-dependent killing of cells by macrophages; (iii) impaired immune complex-dependent antigen presentation to primed T cells; (iv) decreased immune complex-induced inflammation; and (v) enhancement of antibody responses (Barnes

et al., 2002). FcγRIII$^{-/-}$ mice lack NK cell-mediated ADCC and phagocytosis of IgG-coated particles by macrophages (Hazenbos et al., 1996; Hazenbos et al., 1998). These mice also lack IgG-mediated mast cell degranulation and are resistant to IgG-dependent passive cutaneous anaphylaxis and exhibit an impaired Arthus reaction.

Targeting of FcγRI, FcγRII or FcγRIII by Ag/anti-FcγR conjugates on the human monoctyes in vitro resulted in enhanced Ag presentation, although Ag presentation was most efficient by targeting FcγRI (Gosselin et al., 1992). Moreover, mice with transgenic FcγRI expressed selectively on myeloid cells have enhanced Ab responses when targeted by Ag-mAb conjugates in vivo (Heijnen et al., 1996). In addition, macrophages from FcRγ$^{-/-}$ mice, which are required for FcγRI and FcγRIII signaling, failed to upregulate IL-10 production in response to immune complexes (ICs). However, mice lacking either the FcγRII or the FcγRIII were fully capable of upregulating IL-10 production, implicating FcγRI in this process. IL-10 down-regulates pro-inflammatory responses in these processes, suggesting FcγRI also mediates immunostimulatory functions (Sutterwala et al., 1998).

FcRγ$^{-/-}$ mice, lacking FcγRI and FcγRIII are resistant to the development of experimental immune hemolytic anaemia and experimental immune thrombocytopenia (Takai et al., 1994; Clynes and Ravetch, 1995). FcRγ has also been shown to play a major role in IC-mediated inflammatory reactions since FcRγ$^{-/-}$ mice are impaired in Arthus reaction, i.e protected from fatal glomerulonephritis (Sylvestre and Ravetch, 1994). In addition, FcRγ$^{-/-}$ mice are protected from the development of

experimental autoimmune arthritis induced by collagen, suggesting an additional role for FcγRI and/or FcγRIII in the development of arthritis (Kleinau et al., 2000).

FcRγ$^{-/-}$ mice also showed enhancement of mast cell degranulation and IgG1-mediated anaphylaxis in FcγRIII-dependent manner (Dombrowicz et al., 1997; Miyajima et al., 1997).

FcγRII

FcγRIIA has been shown to have very similar biological functions as FcγRI. The role of FcγRIIA has been demonstrated in vivo by generating human FcγRIIA transgenic mice. In comparison with matched wild-type littermates that are negative for the FcγRIIA transgene, induction of thrombocytopenia by Abs was more severe. In contrast, FcRγ$^{-/-}$ mice that lack functional expression of FcγRI and FcγRIII on splenic macrophages did not demonstrate anti-platelet Ab-mediated thrombocytopenia. This suggests that hFcγRIIA plays a significant role in the immune clearance of platelets and independent of FcγRI, FcγRIII and FcRγ (McKenzie et al., 1999). Study on transgenic mice that expresses human FcγRIIA are susceptible to collagen induced arthritis (Tan et al., 2005). A surprising characteristic of the FcγRIIA transgenic mice was the spontaneous development of multisystem autoimmune disease with features of rheumatoid arthritis, systemic lupus erythematosus, pneuminitis and glomerulonephritis. In addition immune complex induced TNF-α and IL-1β production was observed from FcγRIIA transgenic macrophages.

Signaling initiated by ITAM-containing receptors such as BCR, FcγRII or FcγRs associated FcRγ can be attenuated by ITIM-containing receptors (Daeron et al., 1995a). This can be caused by BCR co-ligation with FcγRIIB which also leads to B

cells apoptosis (Ashman et al., 1996; Pearse et al., 1999). FcγRIIB stimulation also affect B cell proliferation (Phillips and Parker, 1984; Sidman and Unanue, 1979), signaling (Choquet et al., 1993; Muta et al., 1994), and antigen presentation (Minskoff et al., 1998; Wagle et al., 1999). Co-ligation of FcγRIIB with FcεRI on mast cells inhibits degranulation and cytokine production, suggesting that FcγRIIB is able to modulate mast cell activation through FcεRI (Daeron et al., 1995b).

FcγRIIB$^{-/-}$ mice show augmented Ab responses and mast cells from FcγRIIB$^{-/-}$ mice are more sensitive to degranulation, resulting in increased passive cutaneous anaphylaxis reaction (Takai et al., 1996). Moreover, FcγRIIB$^{-/-}$ mice are more susceptible to IC-related autoimmune diseases such as collagen-induced arthritis (Yuasa et al., 1999; Nabbe et al., 2003) and Goodpasture's syndrome (Nakamura et al., 2000). It has also been indicated that FcγRIIB is crucial for maintaining self-tolerance. Depending on the genetic background, FcγRIIB-deficient mice can develop a spontaneous autoimmune disease (Bolland and Ravetch, 2000; Yajima et al., 2003). Increased ADCC against tumors has also been observed in FcγRIIB$^{-/-}$ mice (Clynes et al., 2000). Although FcγRIIB contains an ITIM, this receptor has been shown to have phagocytic capacity in mice in the absence of inflammation. In the absence of the activating receptors FcγRI and FcRγIII, using FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ double-knock out mice, FcγRIIB reduces joint inflammation in arthritis by promoting endocytosis and clearance of ICs from the joint (van Lent et al., 2003).

1.5.4 FcγR-mediated signal transduction

FcγRs are characterized by the presence of, or association with, the ITAM motif (Ravetch and Kinet, 1991; Daeron, 1997). The ITAM motif consists of two pairs of tyrosines and leucines within the consensus sequence D/E-X7-D/E-X2-Y-X-X-L-X7-Y-X-X-L, (X is any amino acid) (Cambier, 1995; Isakov, 1997). It has been shown to be important for the activation of signal transduction. On the other hand, FcγRIIB harbors an ITIM (the consensus sequence is I/V/L/Sx/YxxL/V) motif (Unkeless and Jin, 1997; Long, 1999; Bolland and Ravetch, 1999), which recruits signaling molecules to inhibit ITAM-mediated cellular activation. The signaling cascades elicited by activating FcγRs (FcγRI, FcγRIIA and FcγRIII) is similar to that elicited by BCRs and TCRs (Kurosaki, 1999; Ravetch and Bolland, 2001). This is illustrated in Figure 1.12.

(i) Upon cross-linking of activating FcγRs, tyrosine residues in the ITAM motif is phosphorylated by the action of non-receptor tyrosine kinases of the Src family with subsequent recruitment of Src homology domain-containing signaling molecules that bind to the phosphorylated ITAM, most notably the Syk kinase family molecules. Depending on the particular cell types, FcγR triggers the activation of different tyrosine kinases. For example, FcγRIIIA aggregation activates Lck in NK cells (Salcedo et al., 1993), while FcγRIIA or FcγRIIIA activate Lyn and Hck in monocytic and mast cells (Wang et al., 1994; Ghazizadeh et al., 1994). Likewise, Syk is activated in mast cells and macrophages (Agarwal et al., 1993; Crowley et al., 1997), whereas the related kinase ZAP70 is activated in NK cells (Cone et al., 1993).

(ii) In turn, phosphorylated Syk activate numerous downstream signaling molecules. One is the recruitment and activation of phospahtidylinositol 3-kinase (PI3-K) (Chacko et al., 1996). It has been shown that PI3-K activity is decreased in

macrophages from $Syk^{-/-}$ mice when PI3-K was immunoprecipitated with anti-phosphotyrosine antibodies (Crowley et al., 1997). PI3-K activity is essential for phagocytosis. Phagocytic cells incubated with wortmanin, which binds to the catalytic subunit of most PI3-K and inhibits their function, bind particles but are unable to internalize them. It catalyzes the generation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) from a membrane phospholipid phosphatidylinositol (4,5)-biphosphate (PIP2) (Ninomiya et al., 1994; Araki et al., 1996).

(iii) PIP3 recruits molecules containing pleckstrin homology (PH) domains such as phospholipase-Cγ (PLCγ) and Tec kianses (Ferguson et al., 1995; Salim et al., 1996). Myeloid cells contain several Tec kinases, e.g. Btk, Itk and Emt (Kawakami et al., 1996), that can be activated upon FcγR aggregation. The PLCγ1 and PLCγ2 of PLCγ family are responsible for the break down of membrane-bound PIP3 to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Ting et al., 1992; Divecha and Irvine, 1995).

(iv) IP3 is the principal second messenger to induce cytosolic calcium (Ca^{2+}) rise (Berridge, 1993). IP3 binds its receptors (IP3Rs) on the endoplasmic reticulum (ER) leading to calcium release from this internal Ca^{2+} stores. Calcium (Ca^{2+}) induces some isoforms of protein kinase C (PKC) translocation to the plasma membrane where it is activated by DAG. An increase in cytoplasmic Ca^{2+} concentration is necessary for the granule release from NK cells involved in the delivery of the lethal hit (Kagi et al., 1994; Gumperz and Parham, 1995).

(v) GTP-binding protein such as Ras and Rac also participates in FcγR signaling pathway. The Ras family together with PKC, regulate the mitogen-activated protein kinsae (MAPK) cascades (Galandrini et al., 1996). Ras directly activates Raf-1. Raf-1 inturn activates MEK (MAPK kinase), and MEK activation directly leads to ERK

(extracellular signal-regulated kinase) activation (Macdonald et al., 1993; Marshall, 1994). The JNK pathway is activated by FcγR through Rac1 and Cdc42 GTPases (Minden et al., 1995). Regulation of p38 MAPK is also achieved through PKC (Akiba et al., 1999), Rac and Cdc42 (Zhang et al., 1995; Rose et al., 1997). These signaling cascades ultimately activates transcription factors nuclear factor κB (NF-κB) (Sizemore et al., 1999; Reddy et al., 2000).

However, when the FcγRIIB is crosslinked with activating FcRγs, the ITIM motif on FcγRIIB is phosphorylated by the protein tyrosine kinases lyn. The phosphorylated ITIM recruits phosphatases such as Src homology 2 (SH2) domain-containing inositol polyphosphate phosphatase (SHIP) which inhibit ITAM-triggered calcium mobilization and cellular proliferation (Fig. 1.13) (Daeron et al., 1995a; Ono et al., 1996). These inhibitory activities result from the hydrolysis of PIP3 by SHIP and thus dissociation of PH domain-containing proteins like Btk and PLCγ from the membrane (Ono et al., 1996; Bolland et al., 1998). As a result calcium influx is inhibited which prevents sustained calcium signaling and calcium dependent processes such as degranulation, phagocytosis, ADCC and cytokine release. B cell proliferation can be Arrested through ITIM-dependent activation of the adaptor protein Dok and subsequent inactivation of MAP kinases (Yamanashi et al., 2000; Tamir et al., 2000).Through its phosphatase domain, SHIP can prevent recruitment of the PH domain survival factor Akt by hydrolysis of PIP3 (Aman et al., 1998; Helgason et al., 2000).

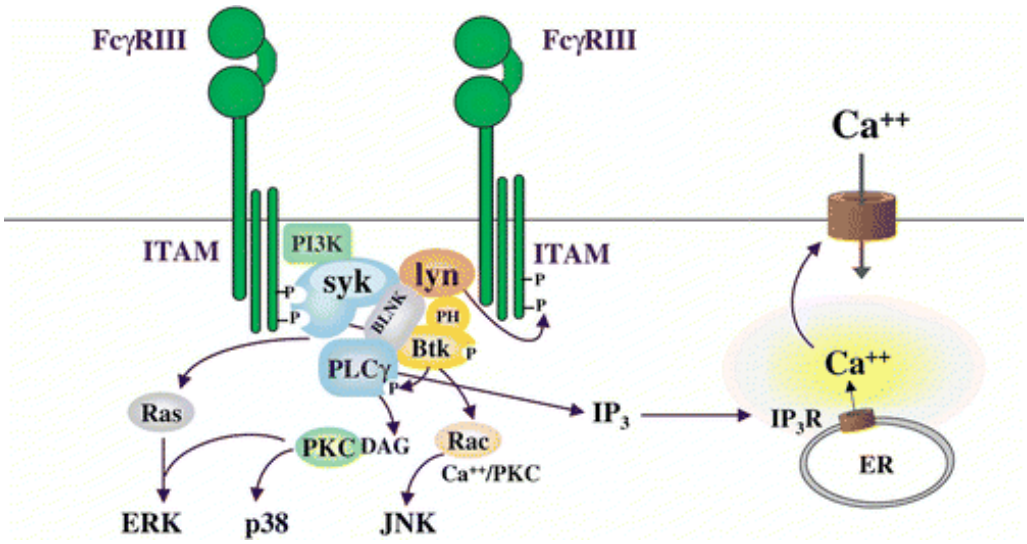


Figure 1.12 FcγRIII signaling
This figure shows an example of the possible signaling pathways initiated upon FcγR ITAM phosphorylation, (Ravetch et al., 2001).

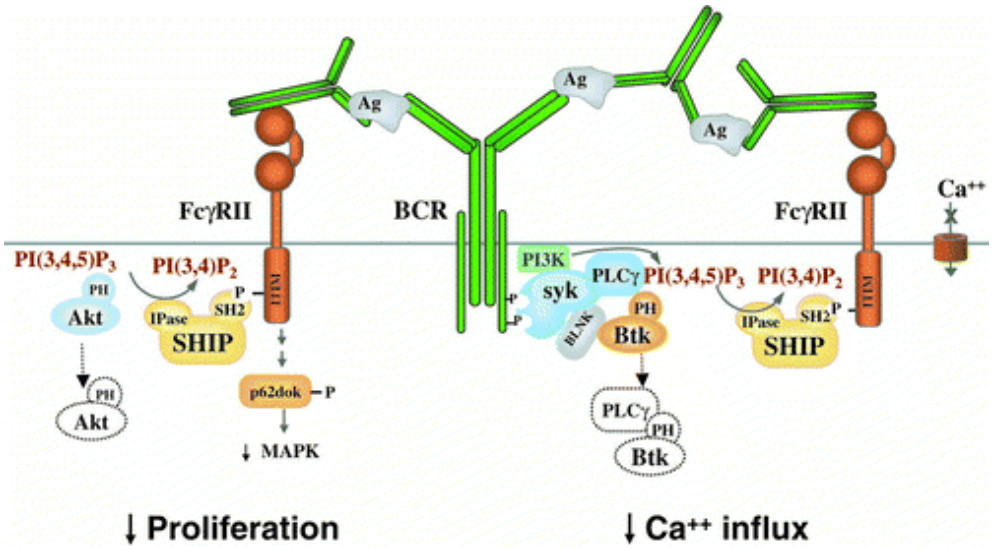


Figure 1.13 Signaling pathways triggered by BCR-FcγRII co-ligation.
SHIP-mediated inhibition of FcγRs mediated signaling. FcγRIIB is shown as an example of this class of inhibitory receptors, (Ravetch et al., 2001)

1.6 Inflammatory cytokines

Cytokines consist of a large group of soluble polypeptide mediators which regulate diverse events, including inflammation, hematopoiesis, development, tissue repair, etc. Micro-organisms induce cytokine secretion by host cells through TLRs (Haddad, 2002). Immune-complexes cause cytokine secretion through stimulation of FcγRs signaling.

The activation of NF-κB is common to these receptors signaling leading to transcriptional activation of various cytokine genes.

These cytokines can be pro-inflammator or anti-inflammatory. Pro-inflammatory cytokines such as IL-1 and TNF-α is produced soon after infection to induce tissue inflammation. Anti-inflammatory cytokines, such as IL-10, are counteractive which restrict the scale of infalmamiton (Haddad, 2002). Some cytokines that will be investigated in this study are briefly described below.

1.6.1 Tumor necrosis factor-α (TNF- α)

TNF-α was first discovered as a serum factor produced in LPS-treated mice. Later, it found to have strong anti-tumor activity against tumors implanted in the mouse skin (Carswell et al., 1975) and molecularly defined as Tumor necrosis factor (Aggarwal et al., 1985). TNF-α is expressed by macrophages, lymphocytes, fibroblasts, Kuppfer cells and many other cells. It is secreted by monocytes in response to immune complex (Debets et al., 1988; Debets et al., 1990). In immune complex-mediated diseases, such as rheumatoid arthritis, high concentrations of TNF-α is secreted by tissue macrophages (Tan et al., 2003; Tan et al., 2005).

It is synthesized as a trimeric type II membrane protein which is cleaved by a membrane-bound enzyme into a soluble, pyramid-like 51 KDa homotrimer. There are two types of TNF-α receptors: TNFα-RI and TNFα-RII. TNFα-RI is responsible for most of the biologic effects of TNF-α (Darnay and Aggarwal, 1999).

TNF-α has many biological effects. It induces the production of other cytokines, e.g. IL-1 (Dinarello et al., 1986) and IL-6 (Zhang et al., 1988), induces T-cells apoptosis (Screaton and Xu, 2000), proliferates of fibroblast, upregualtes adhesion molecules of endothelial cell and differentiate of myeloid cells (Cain et al., 1999; Suryaprasad and Prindiville, 2003; Aggarwal, 2003). TNF-α promotes macrophage oxidative burst and increase the killing of intracellular pathogen (Drapier et al., 1988; Vassalli, 1992). In sepsis, it induces the production of prostaglandin, resulting in fever (Beutler and Cerami, 1987). In liver, it induces the production of acute pahse proteins (Perlmutter et al., 1986). It may cause cardiac dysfunction by disturbing the calcium influx in cardiac myocytes and thereby inhibitng contractility (Bolger and Anker, 2000). It also interfere insulin-signaling mechanism by inhibiting the tyrosine kinase activities of the insuling receptor and serine phosphorylaiton of the insulin receptor substrate (Hotamisligil et al., 1994).

1.6.2 Interleukin-1 (IL-1)

IL-1 is another pro-inflammatory cytokine which possesses many characteristics of TNF-α. There are two isoforms of IL-1, i.e. IL-1α and IL-β, and their biological activities are thought to be similar. The naturally occurring IL-1 receptor antagonist (IL-1ra) is a third member of the IL-1 family. It is structurally similar to IL-1β but has antagonistic activity (Bresnihan et al., 1998). The main cellular source of IL-1 is

activated macrophages by bacterial components and other cytokines. Both IL-1α and IL-1β are synthesized as 33 KDa precursors which are then enzymatically cleaved into the 17 KDa bioactive forms and secreted. IL-1α is also active in its precursor form but remains mostly intracellular (Dinarello, 1996). It is also induced by immune complexes from monocytes and macrophages (Chou et al., 1985; Remvig et al., 1990)

IL-1activates a wide range of cells including endothelial cells, fibroblasts, epithelial cells, dendritic cells and T lymphocytes. IL-1 activates target cells through IL-1 receptor type I (1L-IRI) (Labow et al., 1997). A type II receptor, which lacks a cytosolic domain, appears to act as a decoy receptor, through competitionwith IL-1RI in IL-1 binding (Slack et al., 1993).

The physiological effects of IL-1 are in some aspect, very similar to that of TNF-α and the two cytokines often exhibit additive or synergistic effects to induce inflammatory reactions

1.6.3 Interleukin-6 (IL-6)

IL-6 was first known as a BSF-2 (B cell stimulator factor-2), because it was identified as a T cell factor that induces B cells differentiation (Hirano et al., 1986). It is constitutively produced by some tumor cells. In normal cells, its expression must be induced. TLR ligands are potent in IL-6 induction as for TNF-α and IL-1 (Akira et al., 1993). Immune complex also induces IL-6 production in monocyte (Krutmann et al., 1990). High levels of IL-6 is found in the plasma of patients with acute bacterial infections (Helfgott et al., 1989).

The IL-6 receptor consists of two different subunits, i.e. IL-6R and the signaling subunit gp130. Binding of IL-6R by IL-6 induces homodimerization of gp130, which

initiates the signal transduction cascade. IL-6R is mainly expressed in immunocompetent cells and hepatocytes, whereas gp130 is ubiquitously expressed (Saito et al., 1992). IL-6 has many biological functions including stimulation of Ig secretion by B cells, activation of osteoclasts, maturation of megakaryocytes and growth of T cells and myeloma cells (Kishimoto, 1989; Kishimoto et al., 1992).

1.6.4 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine expressed by activated T-cells, B-cells, monocytes, macrophages, mast cells and keratinocytes. Its physiological function is the limitation and final shut-off of inflammatory reaction in immune defense once invading pathogens have been eliminated. IL-10 expression by T-cells is activated by anti-CD3 antibodies and phorbal esters and is inhibited by IL-4 and IFN-γ (de Waal et al., 1991a; de Waal et al., 1992). IL-10 is also produced by monocytes in response to immune complex and it inhibits the production of pro-inflammatory cytokines TNF-α and IL-1β (Berger et al., 1996). IL-10 also inhibits MHC class II. However, it co-stimulates the growth of several hematopoietic cells, including T-cells, B-cells, mast cells, megakaryocytes and erythroid (Fiorentino et al., 1991; de Waal et al., 1991b). It also inhibits the production of reactive oxygen species (ROS) and nitric oxide (NO) by macrophages (Bogdan et al., 1991).

1.6.5 Granulocyte macrophage-colony stimulating factor (GM-CSF)

GM-CSF is a 144 residue glycoprotein secreted by activated monocytes and macrophages upon LPS stimulation (Hamilton, 1994). It also secreted by endothelial cells, fibroblast cells and chondrocytes upon proinflammatory cytokine stimulation

such as IL-1 and TNF-α (Bagby, Jr. et al., 1986; Leizer et al., 1990; Campbell et al., 1991). Solid-phase IgG also induce the secretion of GM-CSF from monocytes (Herrmann et al., 1992). In inflammatory and non-inflammatory arthritis the affected joint contained detectable amount of GM-CSF(Feldmann et al., 1996).

The functions of GM-CSF are mediated by binding to high-affinity receptors comprising a GM-CSF-specific α-chain and a signal transducing β-subunit, which is shared by IL-3 and IL-5 receptors (Kitamura et al., 1991; Tavernier et al., 1991). It amplifies the inflammatory response by increasing the production of IL-1 and TNF-α by macrophages. Moreover, this cytokine has a role as a growth factor by maintaining the survival and by stimulating the proliferation and the differentiation of granulocyte, macrophage and eosinophil (Lopez et al., 1992).

1.6.6 Interleukin 8 (IL-8)

IL-8 was discovered as neutrophil chemotactic factor (Yoshimura et al., 1987). It is a chemokine secreted by activated macrophages, monocytes, neutrophils, natural killer cells and other cell types in response to TNF-α, IL-1 (Matsushima et al., 1988) or directly in response to bacterial products (Baggiolini et al., 1997; Oppenheim et al., 1991). It also secreted by monocytes via FcγR in response to immune complexes such as Immobilize IgG (Marsh et al., 1995). In immune complex-mediated diseases, such as rheumatoid arthritis, high concentrations of both IL-8 and neutrophils are present in monocyte and macrophage rich compartments (Seitz et al., 1991; Verburgh et al., 1993),.

IL-8 mediates its functions by binding to two distinct receptors, IL-8RI and IL-8RII with a similar high affinity (Holmes et al., 1991; Murphy and Tiffany, 1991). IL-8 is

responsible for the various functions of neutrophils, including degranulation and respiratory burst (Mukaida, 2000). It induces chemotaxis of eosinophils together with IL-3 or GM-CSF (Warringa et al., 1991). It also induces basophils to exhibit chemotaxis (Geiser et al., 1993) and to adhere to endothelial cells (Bacon et al., 1994). In the presence of shear stress, monocytes adherence to endothelium cells is markedly increased in responses to IL-8, also suggest a potential role of IL-8 in macrophage migration (Gerszten et al., 1999).

1.7 Aims of the Study

The overall aims of this study are as follows:

(1) Develop a novel method for the detection of ligand-induced receptor-receptor interactions and interactions between secreted protein ligands and their receptors in live 293T cells.

In earlier studies, it was shown that heterodimerization between the cytoplasmic domains of TLR1 and TLR2 i.e. TIR1 and TIR2, caused by the CD4 extracellular domains led to TLR signaling for NF-κB activation (Ozinsky et al., 2000). In another word, TIR1 and TIR2 can report interactions between proteins that are fused with them. In a recent study, TIR1 and TIR2 were also found to detect interactions between the extracellular domains of integrin αV and β5 (Zhang et al., 2002). Based on these results, we decided to evaluate the TIR1/2 pair for general application in the detection of interaction between proteins on the cell membrane. The well-known interactions between IL-4 and the extracellular domains of its receptors IL-4Rα and γC in conjunction with TIR1 and TIR2 are chosen for investigation. Results obtained using a well established pair like the above chosen system will help to validate the efficacy of the TIR1/TIR2 assay. IL-4 like many other growth factors and cytokines transmits

a signal into the cell by promoting the dimerization of receptor chains (i.e. IL4Rα and γC) in the plasma membrane (Stahl and Yancopoulos, 1993; Heldin, 1995). The nature of the interactions between the cytokines and their receptors is therefore of fundamental importance to the understanding of the immune response. It is important to have a system where we can detect IL-4-induced receptor-receptor interactions and interactions between secreted protein ligands and their receptors. This type of studies will provide new insights into more general protein-protein interactions.

(2) Evaluate CD14-CD14 interactions and significance of this interaction in TLR4-mediated NF-κB activation.

CD14 plays an essential role in host response to LPS. The molecular mechanism of LPS binding and transferring to CD14 has been intensively investigated through mutagenesis and antibody-based binding site mapping. In this study, CD14 is fused with TIR1 and TIR2 respectively the CD14-TIR1 and CD14-TIR2 hybrid molecules are expressed on 293T cells. NF-κB activation was examined. Furthermore, amino acid residues implicated in CD14-CD14 interactions based on the crystallography study (Kim et al., 2005) are mutated to assess their roles in LPS signaling.

(3) Investigate whether dimerization of FcγRIIA is sufficient to elicit cell signaling using this TIR1/TIR2-based two-hybrid system.

FcγR elicit cell signaling through cytoplasmic ITAM motif which is found in FcγRIIA but not FcγRIIB which contain ITIM motif instead of ITAM. FcγRIIA is an activating receptor whereas FcγRIIB is an inhibitory receptor that serves to recruit phosphatases to the receptor to reduce cellular activation (Van den Herik-Oudijk IE et al., 1995) . Although FcγRIIA contains ITAM motif in its cytoplasmic tail, FcγRI and FcγRIIIA

rely on FcRγ chain for signal transduction. In addition, these three classes of FcγR can elicit different cellular responses depending on the cell types. FcγR cross-linking in myelomonocytic cells causes the production of cytokines which are important in tissue inflammation, such as IL-6, TNF-α, IL-1 and IL-8. Cytokine induction by specific FcγRs is not fully characterized. In this study, the transmembrane and cytoplasmic domain of FcγRIIA is fused with the extracellular domains of IL-4Rα and γC respectively and expressed in 293T cells. The cells are stimulated with IL-4 and NF-κB activation is measured.

Chapter 2 MATERIALS AND METHODS

The preparation of media and solutions, unless otherwise described in this chapter is given in the Appendix.

2.1 Molecular biology

2.1.1 Materials

2.1.1.1 Bacterial strains

Escherichia coli (*E. coli*) DH5α (Hanahan, 1983) was used for plasmid propagation. Working stocks of DH5α were prepared as colonies on LB agar plates and kept at 4^{o}C. The bacteria were restreaked once a month on fresh LB agar plates. The bacteria were kept at -80^{o}C for long term storage. In this case, the bacteria were stored in the respective culture medium containing 20% glycerol.

2.1.1.2 Plasmid vectors and primers

Plasmid vectors used in this study are listed in Table 2.1.1. The vector based primers used for sequencing purposes, are included in the Table. All plasmids were propagated in *E. coli* DH5α. Either ampicillin (100 μg/ml LB broth) or kanamycin (30 μg/ml LB broth) was used for selection purposes.

Table 2.1.1 Plasmid vectors and primers

Vectors (Sources)	Description	Antibiotic selection in *E.coli*	Primers flanking the multiple cloning sites for sequencing (5'…..3')
pcDNA3.1/Myc-His (Invitrogen)	A 5.5 kb vector for constitutive protein expression with C-terminal myc/His-tags under the CMV promoter	Ampicillin	T7: taatacgactcactataggg BGH reverse: tagaaggcacagtcgaggc
P5xNF-κB-Luc (Stratagene)	A 5.7 kb reporter vector that expresses the firefly luciferase under 5 copies of the NF-κB enhancer	Ampicillin	None
pRL-CMV (Promega)	A 4 kb control reporter that expresses the *Renilla* luciferase constitutively with a CMV promoter/ enhancer	Ampicillin	None

2.1.1.3 DNA primer synthesis

Primers for PCR were designed using the PrimerSelect software (DNAStar) to avoid primer dimer and hairpin loop formation. Primers for PCR and site-directed mutagenesis were designed as described in section 2.4 and 2.5. Primers used for PCR and site-directed mutagenesis were synthesized by Research Biolabs (Singapore) or AlphaDNA (Montreal, Canada).

2.1.2 Methods

2.1.2.1 Isolation of total RNA from cell culture

Total RNA was isolated using the TRIZOL Reagent (Gibco BRL), according to the manufacturer's instructions. Briefly, 2 x 10^6 cells were lysed in 1ml of TRIZOL Reagent and incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added to the lysate with vigorous mixing for 15 sec. After 2 min of phase separation, it was centrifuged at 4°C for 15 min at 12,000 rpm. The aqueous phase was transferred to a fresh tube. 0.5 ml of isopropyl alcohol was added for RNA precipitation and incubated for 10 min at

room temperature. RNA was pelleted by centrifugation for 10 min at 12,000 rpm, washed with 1ml 75% (v/v) ethanol and air-dried. The purified RNA was dissolved in 20 µl of DEPC-treated water and stored at -80°C. DEPC-treated water was obtained from Sigma.

2.1.2.2 Quantitation of RNA

2 µl of RNA samples were diluted in 0.5 ml of DEPC-treated water (i.e. 250 x dilution factor) and optical density was measured at 260 nm (OD260) using a DU® 640B spectrophotometer (Beckman). RNA concentration, in µg/ml, was estimated according to the following formula:

$1OD_{260}$ = 40 µg/ml. Absorption at 280 nm (which measures protein contamination) was also taken, and the ratio of OD_{260}/OD_{280} was used to determine RNA purity. Samples with an OD_{260}/OD_{280} ratio greater than 1.7 were used.

2.1.2.3 Reverse transcription

Reverse transcription was performed using the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA), according to the manufacturer's instructions. Briefly, RNA (0.2-1.0 µg) was mixed with 1 µl random hexamers and 1 µl oligo $(dT)_{18}$ primers. The samples were heat-denatured for 2 min at 70°C and rapidly chilled on ice to prevent RNA re-annealing. Reverse-transcription was carried out for 1hr at 42°C in a master mix reaction of 20 µl (Table 2.1.2). The reaction was terminated by heating at 94°C for 5 min and the synthesized cDNA was either used immediately or stored at -20°C for a short period of time.

Table 2.1.2 Composition of a reverse transcription reaction (20 µl)

Reagent	Volume (µl)	Final concentration/ amount
RNA+ DEPC-H_2o	12.5	0.2-1.0 µg total RNA
Random hexamer primer	1	20 pmol
Oligo $(dT)_{18}$ primer	1	20 pmol
5× reaction buffer	4	50 mM Tris-HCl (pH 8.3) 75 mM KCl 3 mM $MgCl_2$
dNTP mix (10 mM each)	1	0.5 mM each
RNase inhibitor	0.5	1 unit/µl
MMLV reverse trancriptase	1	≥200 units/µg RNA

MMLV: moloney murine leukaemia virus

2.1.2.4 Polymerase chain reaction (PCR)

PCR was used to exponentially amplify target DNA sequences. Two types of DNA polymerase were used in PCRs. Taq DNA polymerase (Promega) was used in PCR reactions to identify and verify cloned DNA sequences in plasmid vectors. The Pfu DNA polymerase (which has 3' → 5' proofreading activity) (Promega) was used to generate DNA fragments for cloning into plasmid vectors. 2 µl of cDNA from each reverse transcription reaction or less than 500 ng of plasmid DNA was used as template in each 50 µl PCR reaction. The rest of a PCR reaction contains 10 x reaction buffer, 0.2 mM of each dNTP, 0.5 µM of each primer, 1-2 U of Taq or Pfu DNA polymerase. For Taq DNA polymerase, $MgCl_2$ was added to a final concentration of 1.5 mM. When Pfu DNA polymerase was used, $MgSO_4$ was added to 2 mM. Thermal cycling was typically carried out as follows:

(i) 1 cycle at 94^{o}C for 1 min for initial DNA denaturation; (ii) 35 cycles each at 94^{o}C for 45 sec (DNA denaturation), 50-65^o C* for 45 sec (primer annealing), 72^{o}C for a defined time** (extension) and (iii) a final cycle at 72^{o}C for 10 min (extension).

* Primer melting temperature is calculated based on $4^{o}C$ for every C or G base and $2^{o}C$ for every A or T base and PCR annealing temperature is used at 2-$4^{o}C$ below melting temperature.

** For Taq polymerase, extension was carried out at 1 min/kb of expected DNA products, whilst for Pfu polymerase, it is 2 min/kb of expected DNA products.

2.1.2.5 Ethanol precipitation of DNA

To precipitate DNA, 1/10 volume of 3M sodium acetate (pH 5.4) is added to DNA solutions followed by 2.5 volumes of ice cold 100% ethanol. The solution was mixed by brief vortexing and then left at -80ºC for 30 min. The mixture was centrifuged for 20 min at 13,000 rpm in a microcentrifuge. The pellet was washed with 70% (v/v) ethanol at room temperature, then air-dried and dissolved in an appropriate volume of deionized water or the TE buffer (Appendix).

2.1.2.6 DNA agarose gel electrophoresis

DNA separation was carried out by agarose gel electrophoresis (Sambrook et al., 1989). Agarose was added to Tris-acetate-EDTA (TAE) buffer (Appendix) at 1% (w/v) and boiled in the microwave for 1 min to melt the agarose. The melted agarose is cooled down to approximately 60°C and then ethidium bromide (10,000 μg/ml, Bio-Rad, USA) were added to a final concentration of 0.5 μg/ml. The agarose was then poured in a gel-casting set (Bio-Rad), avoiding bubbles. DNA samples were mixed with 1/5 volumes of 5x sample loading buffer (Appendix), e.g. an 8 μl of DNA sample is mixed with 2 μl of DNA loading dye and loaded to the gels. As a control, 1Kb Plus DNA Size Standard (Gibco BRL) is loaded in parallel. Electrophoresis was carried out at 100V in TAE buffer and stopped when the dye front reached 3/4 of the

gel-length. Ethidium bromide chelates DNA to produce fluorescence signal upon UV illumination. Results were recorded by photography using a Polaroid MP-4 Land camera and Polaroid type 667 films (Polaroid Corporation, UK).

2.1.2.7 Isolation and purification of DNA from agarose gels

DNA separated on agarose gels was isolated using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA). Agarose gel containing the desired DNA band was excised and placed into an Eppendorf tube. 10 µl of capture buffer was added per 10 mg of the gel slice and the contents were vortexed vigorously and incubated at 60^{o}C for 5 to 15 min. After the agarose slice was completely melted, the sample was transferred to a GFX column and incubated for 5 min at room temperature. The column was then centrifuged at 13,000 x g for 30 sec and the flow-through was discarded. 500 µl of wash buffer was added to the column which was then centrifuged at 13,000 x g for 30 sec. This was followed by the addition of 50 µl of autoclaved deionized water to the GFX column and incubation at room temperature for 5 min. The column was then centrifuged at 13,000 x g for 1 min to recover the purified DNA in the flow-through.

2.1.2.8 Rapid isolation of plasmid DNA

The Wizard$^{(R)}$ Plus SV Minipreps DNA Purification System (Promega Co, Madison WI) provides a simple and reliable method for rapid and small scale isolation of plasmid DNA. Briefly, a single colony of transformed *E.coli* DH5α was inoculated in 10 ml LB containing 100 µg/ml ampicillin. After overnight culture, bacteria was pelleted by centrifugation and then resuspended in 250 µl of resuspension solution. Cells were then lysed by adding 250 µl of lysis solution. After incubation for 5 min

with 10 µl of an alkaline protease solution, the cell lysates were neutralized with 350 µl of neutralization buffer to precipitate cell debris. The lysates were centrifuged for 10 min and then transferred to Spin Column. The column was washed twice with wash solution and column-bound DNA was eluted with deionized water and collected into a fresh tube by centrifugation. The purified plasmid was used directly for automated DNA sequencing or restriction enzyme digestion.

2.1.2.9 Plasmid purification for transfection

QIAGEN plasmid Maxi kit (QIAGEN GmbH, Germany) was used for the purification of grade plasmids required for transfection according to manufacturer's instructions. For Maxi-scale plasmid preparation, a single plasmid-bearing bacteria colony was picked from a freshly streaked ampicillin selective agar (LA) plate and inoculated into a starter culture of 2-5 ml LA selective medium. The culture was grown for 8 hr at 37^{o}C with shaking (200 rpm). This starter culture was diluted 1/500 into 250 ml of LA selective medium and cultured overnight (12-16 hr) at 37^{o}C with shaking (200 rpm). The bacterial cells were pelleted by centrifugation for 15 min at 6,000 x g and at 4^{o}C and re-suspended in 10 ml of Buffer P1 (resuspension buffer). 10 ml of buffer P2 (lysis buffer with NaOH and SDS) was then added, mixed and incubated for 5 min at room temperature. This was followed by addition of 10 ml of chilled buffer P3 (neutralization buffer with NaOAc). After mixing, the lysate was incubated on ice for 20 min and centrifuged for 30 min at ≥ 20,000x g and at 4^{o}C. The supernatant was passed through the QBT buffer-equilibrated QIAGEN-tip 500 column (containing proprietary DNA binding resin). The column was washed two times and the bound DNA was eluted with 15 ml of Buffer QF. To precipitate the eluted DNA, 10.5 ml isopropanol was added and mixed, followed by centrifugation for 30 min at ≥ 15,000

x g and at 4°C. The DNA was pelleted with 5 ml of 70% ethanol at room temperature and centrifuged at ≥ 15,000 x g for 10 min. The DNA pellet was air-dried for 5-10 min, dissolved in autoclaved deionized water and stored at -20°C.

2.1.2.10 Quantitation of DNA

DNA was quantitated using the Hoefer DyNA Quant 200 fluorometer (Pharmacia Biotech), which measures fluorescence emitted by the DNA-chelating dye bisbenzimide Hoechet No. 33258 trihydrochloride (Pharmacia Biotech). 2 ml of assay buffer, containing 0.1 µg/ml of dye and 1 x TNE (0.2 M NaCl, 10 mM Tris-HCl and 1 mM EDTA), (pH 7.4) was used as blank. 2 µl of calf thymus DNA standard (100 µg/ml) (Sigma-Aldrich) was added for calibration. 2 µl of diluted DNA (i.e 50-100 x dilution of prepared plasmid) sample was used for quantitation, and DNA concentration was obtained in µg/ul.

2.1.2.11 Restriction endonuclease digestion

The presence of restriction sites in a DNA sequence (e.g. plasmid) was analyzed using the Webcutter 2.0 software (http://www.firstmarket.com/cutter/cut2.html). Restriction digestion of DNA was carried out using restriction enzymes from Promega except for *SfuI* (Roche Molecular Biochemicals, Germany) and *PmeI* (New England Biolabs, USA). Digestion was carried out in a total volume of 20-50 µl reaction containing the appropriate restriction enzyme buffers (as supplied for each enzyme), DNA (2-5 µg of PCR products or plasmid vector), specific restriction enzyme and 10 µg/µl of acetylated BSA. The digestion was carried out at 37°C for 2 hr. For double digestions, DNA was either simultaneously digested with two enzymes using the multicore buffer or sequentially digested using optimal buffers for each enzyme. A useful website for

identifying suppliers of restriction enzymes and restriction sites is http://rebase.neb.com/rebase/

The digested DNA fragments were separated by electrophoresis and purified from the gel slices as described in section 2.1.2.7.

2.1.2.12 DNA ligation

T4 DNA ligase and the ligase buffer (containing 1 mM ATP) (New England Biolabs, USA) were used in 20 µl ligation reactions. The molar ratios between inserts and vectors ranged from 3:1 to 5:1 and ligation was carried out for 2 hr or overnight at 22°C for cohesive-end ligations.

2.1.2.13 Preparation of competent cells

DH5α was streaked onto LB plate and cultured overnight at 37ºC. A single colony was inoculated into 10 ml LB and cultured overnight at 37ºC with shaking at 200 rpm. 2 ml of the overnight culture was transferred to 200 ml of fresh LB (1:50 or 1:100) in a 500 ml flask. When the OD_{600} of the culture has reached 0.5-0.6, the culture was centrifuged at 3000 rpm for 10 min and at 4ºC to pellet the cells. Each pellet was then re-suspended in 10 ml of pre-chilled filtered 0.1M $CaCl_2$ (Stock 1M). The cells were centrifuged at 2500 rpm and 4ºC. The pellet was resuspended in 10 ml of cold $CaCl_2$ and incubated on ice for 30 min. After centrifugation, the pellet was re-suspended in 2 ml of ice cold $CaCl_2$ solution with 14% glycerol (v/v). 200 µl aliquots of this competent cells preparation were dispensed in 1.5 ml chilled eppendorf tubes. Aliquots of the competent cells were rapidly frozen in liquid nitrogen and stored at -80ºC.

2.1.2.14 Transformation of competent cells

Competent cells were thawed on ice and either 50-100 ng of DNA or 10 μl (or more) of the 20 μl ligation reaction was mixed gently with 100 μl of competent cells and incubated for 30 min on ice. Upon heat-shock for 2 min at 42^{o}C, 300 μl of pre-warmed LB was added and the cells were further cultured for 1 hr at 37^{o}C. 50 μl or 200 μl fractions of the transformed cells were plated on LB plates with the appropriate selective antibiotic and cultured overnight at 37^{o}C.

2.1.2.15 Identification of positive clones by PCR

Between ten to twenty transformant colonies were each inoculated in 1 ml LB containing the appropriate antibiotics. After 6 hr of culturing, PCR was carried out for 30 cycles using 1 μl of each culture as templates and primers specific to the insert DNA in 20 μl reactions. 10 μl of each reaction mixture was examined on an agarose gel to identify positive clones. Plasmid DNA was purified (described in section 2.1.2.8) from the culture containing the expected PCR products obtained.

2.1.2.16 Identification of positive clones by restriction enzyme digestion

An alternative method for identifying positive clone, involves restriction digestion. Five transformant colonies were each cultured in 10 ml LB containing the appropriate antibiotics. Plasmid DNA was isolated from the cultures as described in section 2.1.2.8. The purified plasmids were digested with appropriate restriction enzymes and examined on agarose gels.

2.1.2.17 Site-directed mutagenesis

QuikChange™ XL Site-directed Mutagenesis Kit (Stratagene) was used to introduce point mutations into a cloned DNA fragment. This was used to create restriction enzyme cutting sites or to change a codon for amino acid mutation. Primers used are designed according to the following considerations:

i) Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the DNA fragment in which mutations are to be introduced.

ii) Primers should ideally be between 25-45 bases in length, and the melting temperature (Tm) of the primers should be $\geq 78^{o}$ C.

Tm = 81.5 + 0.41(% GC) - 675/N - % mismatch (where N is the primer length in bases and % GC and % mismatch are whole numbers)

iii) The desired mutation should be in the middle of the primer with about 10-15 bases of correct sequence on both sides.

iv) The primer should optimally have a minimum GC content of 40% and should terminate in one or more C or G bases.

It was recommended by the manufacturer to use FPLC or PAGE purified primers, PCR-grade primers (desalted) from Research Bio Labs worked just as well in our case. A 50 µl reaction mixture contains 10 ng of plasmid DNA, 125 ng of each primer and the supplied dNTP mix, QuikSolution, 10 x reaction buffers and Pfu Turbo DNA polymerase (2.5 U/µl). PCR cycling parameters include a first cycle at 95^{o} C for 1 min, followed by 18 cycles each at 95^{o}C for 50 sec, 60^{o}C for 50 sec and 68^{o}C for an appropriate length of time (2 min/kb of plasmid length). The PCR is extended with a final cycle at 68^{o}C for 7 min. The reaction products was then treated with 1 µl of DpnI restriction enzyme (10 U/µl) at 37^{o}C for 1 hr to digest the parental (nonmutated)

double-stranded DNA. 2 µl of DpnI-treated DNA was then used to transform the XL10-Gold ultracompetent cells as provided by the manufacturer.

2.1.2.18 Sequencing

The Bigdye Terminator cycle sequencing reagent (Perkin Elmer) was used to carry out fluorescence-based dideoxy sequencing reactions (Sanger et al., 1977). 200-500 ng of plasmid DNA or 20-50 ng of PCR product was used in a 20 µl reaction mix, containing 5 pmol of primer and 8 µl of terminator ready reaction mix. Cycle sequencing was carried out using a total of 25 cycles, each comprising a DNA denaturation step at 96^{o}C for 30 sec, a primer annealing step at 50^{o}C for 15 sec, and a primer extension step at 60^{o}C for 4 min. After the reaction, sequencing products were precipitated with 2 volumes of cold ethanol after addition of sodium acetate (pH 7.4) to a final concentration of 0.3M. The DNA pellets were washed once with 70% ethanol and air-dried. Polyacrylamide gel electrophoresis or capillary electrophoresis was carried out for the sequencing products using the ABI 374 or 310 DNA sequencer (NUMI, NUS). Sequence data obtained were analyzed using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST)

2.2 Cell biology

2.2.1 Materials

2.2.1.1 Stimulant

Table 2.2.1 Molecular and microbial stimuli used in this study

Stimulant	Bacterial Strain	Supplier	Working concentration
Recombinant IL-4		R & D Systems, Inc	100 ng/ml
Lipopolysaccaride (LPS)	*Escheriachia coli* O5:55	Sigma-Aldrich	0.5 μg/ml
Bacteria	*Escherichia coli* DH5α	Invitrogen	50 bacteria : 1 cell
Purified human IgG		Zymed Laboratories	100 μg/ml
Polymyxin B		Fluka	20 μg/ml
Polystyrene Microsphere 2.0 μm		PolySciences, Inc	50 beads : 1 cell

2.2.2 Methods

2.2.2.1 Mammalian cell culture

The 293T human embryonic kidney cells were originally obtained from the American Type Culture collection (ATCC) (Rockville, MD). The cell were maintained at 37°C in the presence of 5% CO_2, in DMEM (Sigma-Aldrich) supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin (complete medium). The medium was replaced with fresh media every 3 days. The cells were subculture at a ratio of 1:5. To subculture cells, the medium was removed from the flask and the cells were gently washed with 1x PBS (Appendix). Trypsin/0.5 mM EDTA (Sigma-Aldrich) was added to dislodge the cells and incubated the washed cells for 2-5 min at 37°C. The complete DMEM medium was added to the cells to quench the trypsin. Cell clusters were dispersed by repeated pipetting and seeded at desired densities.

2.2.2.2 Storage of cells

To make cell stocks, cells were pelleted at 300x g for 5 min and then resuspended to 0.5 –1 x 10^7 cells/ml in appropriate medium. 20% DMSO was freshly made in the medium and then an equal volume was slowly added to the cell suspension with mixing. The cell suspension was left at room temperature for 5 min to allow for equilibration of DMSO across the cell membrane and then transferred to cryogenic vials (Nalge Nunc International A/S. Roskilde, Denmark). The final storage medium is 10% DMSO in the desired culture medium. The vials were frozen at -80^{o}C and then transfered to liquid nitrogen for long term storage. The cells were maintained at -80^{o}C for short term storage.

To revive stored cells from frozen vials, a cryogenic vial was thawed rapidly in a 37^{o}C water bath and then transfered to the cultured medium (5-10 ml). The cells were centrifuged at 300 x g for 5 min and the pellet was resuspended in appropriate medium and tranfered to culture plates or flasks. All reagents used for mammalian cell culture were sterilized by autoclaving or filtration.

2.2.2.3 Liposome-based cell transfection

Approximately 5 x 10^5 cells were seeded into each well in 6-well plates and cultured at 37^{o}C for 16-24 hr in the presence of 5% CO_2. Transfection of these cells was carried out using the liposome-based GenePORTER^{TM} 2 Transfection Reagent (Gene Therapy Systems, La Jolla, CA) following the manufacturer's instructions. Briefly, 3 μg of plasmid DNA was diluted with 75 μl of the DNA diluent buffer (known as new diluent B) and incubated for 5 min at room temperature. The DNA solution was added to dilute GenePORTER 2 reagent and incubated for 5-10 min at room temperature. This is to allow GenePORTER 2/DNA complex formation (DNA-liposome). The

cultured medium in each well was adjusted to 1 ml by removing the extra medium and the DNA-liposome mixture was added directly to cells. The cells were incubated for 24 hr before stimulation or analysis in functional assays.

2.2.2.4 Using calcium phosphate cell transfection

This is employed when cell transfection is carried out in larger scales. 4 x 10^6 cells were seeded in a T75 cell culture flask the day before transfection and incubated at 37^{o}C in the presence of 5% CO_2. In this setting, the cells were transfected with 20 µg of the expression vectors (details in section 2.4 and 2.5). As negative controls, cells were transfected with the empty pcDNA3.1 vector. Briefly, 20 µg DNA was diluted in 1.2 ml sterile water. 1.4 ml of 2 x HBS buffer (Appendix) was added to the diluted DNA and mixed properly. 150 µl of 2.5 M $CaCl_2$ was added to the DNA mixture dropwisely. The DNA mixture was immediately vortexed for 5 sec and then incubated for 45 min at room temperature before adding to the cells. This DNA mixture was added to the cells and then incubated for 6-8 hr at 37^{o}C in 5% CO_2. After 6-8 hr, the cells were washed once with the DMEM media and then cultured for 24-48 hr before analysis by immunoprecipitation or western blotimg.

2.2.2.5 Dual luciferase assay

In this assay, 293T cells were seeded in 24-well plates at the densitiy of 1.5 x 10^5 cells/well. After 24 hr, the cells were transfected with the plasmids at a total of 300 ng/well using the GenePORTER^{TM} 2 transfection reagent. In negative controls, cells were transfected with the empty pcDNA3.1 vector rather than the test plasmids. When test plasmids were used at < 300 ng/well, pcDNA3.1 was used to normalize the total plasminds in each well to 300 ng/well. The p5×NF-κB-luc (Stratagene) and pRL-

CMV (Promega) luciferase reporter plasmids were co-transfected at 200 ng/well in each experiment.

For example, the chimeric IL4-TIR1/TIR2, IL4Rα-TIR1/TIR2, γC-TIR1/TIR2 and CD14-TIR1/TIR2 expression vectors were used at 19 ng/well each. The IL4Rα-FcγRII and γC-FcγRII expression vectors were also used at this dosage. In every case, pcDNA3.1 was used at 262 ng/well.

To express the LPS receptor complex in 293T cells, TLR4 and MD2 were each used at 5 ng/well and the CD14 plasmid was used at 20 ng/well. To express FcγRs in 293T cells, the FcγRIIA, FcγIIIIA and FcRγ expression vectors were each used at 150 ng/well. Cells were stimulated with different stimuli where stimulation was required. Luciferase expression was determined 24 hr after stimulation with the Dual Luciferase Assay kit (Promega).

Briefly, culture media was removed and the cells were washed once with PBS. Cells were lysed in the wells using 1 x PLB (known as passive lysis buffer). Expression of firefly luciferase (which is directed by the NF-κB promoter) was read upon addition of Luciferase Assay Reagent II (LAR II). Following this, the Stop and Glo® Reagent which contains substrate for the Renilla luciferase, was added to measure *Renilla* luciferase expression under the CMV promoter. All experiments were performed in triplicates and in each experiment, the firefly luciferase values were normalized to the *Renilla* luciferase values.

2.2.2.6 Treatment of cells with specific stimuli

293T cells were cultured for 24 hr after tranasfection. Where the cells were transfected with IL4-TIR1/2, IL4Rα-TIR1/2, and γC-TIR1/2 vectors, the cells were

stimulated with IL-4 at 1, 10 and 100 ng/ml. Where the CD14-TIR/2 vectors were used to transfect 293T cells, LPS (*E. coli* O55:B5, Sigma-Aldrich) was used to stimulate the cells at 200 ng/ml. Where 293T cells were co-transfected with TLR4 or MD2 or CD14 vectros, LPS was used to stimulate the cells at 500 ng/ml. Cells that were transfected with FcγR expression vectors, were stimulated with the bacteria DH5α opsonized with human IgG at 50 bacteria:1 cell.

2.2.2.7 Flow cytometry

In 24-well plates 293T cells were transfected with IL4-TIR1/2, IL4Rα-TIR1/2 or γC-TIR1/2 expression vectors (100 ng/well). As a control, cells were transfected with the pcDNA3.1 plasmid. The cells were cultured for 24 hr before analysis by flow cytometry. Cells were harvested by incubation with 0.5 mM EDTA at 37^{o}C for 5 min and pelleted by centrifugation at 300x g for 5 min. Cells were reuspended in culture media at 2 x 10^6/ml and 5 x 10^5 cells were used for antibody staining. Specific primary antibodies (Table 2.3) were diluted in FACSwash (PBS containing 2.0% (v/v) BCS and 0.05% (w/v) sodium azide). 50 µl of the cells were then incubated with each antibody for 45 min on ice. If secondary antibodies were used, the cells were preblocked for 20 min using 20% (v/v) serum of the same species in which the secondary antibody was raised. The cells were then incubated with specific primary antibodies. This was followed by incubation with flourescent antibody for 30 min. The cells were washed twice with FACSwash and fixed in 1% (v/v) formaldehyde in PBS.

Antibodies against IL4, γC, CD14, FcγRIIA and FcγRIIIA were fluorescently conjugated whereas that for, IL4Rα, was not. Direct conjugated primary antibody was also used for staining macrophage cell surface markers (Table 2.3).

Antibody-stained cells were analysed using FACScalibur (Becton Dickinson Immunocytometry Systems) and 10,000 cells were collected for each sample. Data was analyzed using the WINMDI software, version 2.8 (Scripps Institute, La Jolla, CA).

2.2.2.8 Isolation of human peripheral blood monocytes

Peripheral blood leukocytes were obtained from the NUH blood Donation Centre in the form of buffy coat preparations derived from healthy donors. Monocytes were isolated from the buffy coats essentially as previously described with minor modifications (Lu et al., 1996). Briefly, 15 ml buffy coats were diluted with 30 ml PBS in a 50 ml tube. 30 ml of the diluted buffy coat was layered on 12 ml Ficoll-Paque (Amersham Bioscience Corp) and centrifuged for 30 min at 400x g without brake. Peripheral blood mononuclear cells (PBMCs) were collected from the gradient interface and topped up with PBS to 50 ml. PBMCs were pelleted down by centrifugation at 200x g for 15 min. This was repeated once and followed by two washes at 100x g each for 10 min. The washed cells were resuspended in 60 ml RPMI containing 5% (v/v) iron-supplemented bovine calf serum (BCS) (HyClone) and splitted into three T75 cell culture flasks and cultured for 2 hr at 37^{o}C. Non-adherent cells, mainly lymphocytes, were removed and the adherent cells were washed three times each with 10 ml warm RPMI containing 5% BCS. The adherent fraction, mainly monocytes, was harvested by gentle scraping. Cells were washed once with RPMI supplemented with 10% (v/v) BCS and then cultured in the same medium after addition of M-CSF or GM-CSF. Monocytes isolated by this method were ~95% pure as judged by the expression of CD14.

2.2.2.9 Generation of macrophages from monocytes

Monocytes were cultured in 6-well tissue culture plates at a density of 1 x 10^6/ml in complete RPMI containing 10% (v/v) BCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, and 0.0012% (v/v) β-mecaptoethanol. THe cells were cultured for 6 days in the presence of 20 ng/ml GM-CSF (R & D Systems Inc, Minneapolis, MN). Every other day, half volume of the culture medium was removed and replenished with fresh media containing GM-CSF. During the culture, monocytes underwent morphologic changes, which were characterisitc of macrophages, such as an increase in cell size and adherenace.

2.2.2.10 Cell activation

Macrophages were harvested, washed once with PBS and resuspended at 1 x 10^6/ml in RPMI containing 10% (v/v) heat-inactivated BCS, 100 units/ml penicillin and 100 μg/ml streptomycin. 1x10^5 cells were cultured in each 96-well. After 24 hr, macrophages were stimulated with immobilized IgG (ImIgG), heat-aggregated IgG (HA-IgG) or soluble IgG at a concentration of 100 μg/ml. In some experiments, IgG was coated on latex beads (IgG-beads). As controls, BSA was coated on the beads (BSA-beads). These beads were used to stimulate macrophages.

2.2.2.11 Preparation of ImIgG, Heat aggregated-IgG (HA-IgG) and IgG-beads

Immobilize IgG was prepared by incubating tissue culture plates with purified human IgG (100 μg/ml in PBS) at 4°C overnight. As a negative control, the plates were coated with BSA (100 μg/ml). The plates were washed three times using PBS before macrophages were cultured on these IgG-coated plates. In some experiments, polymyxin B was included to eliminate any endotoxin contamination of human IgG.

HA-IgG was prepared by heating IgG (5 mg/ml in PBS) at 65^{o}C for 60 min. Large precipitates, which are not often observed, were removed by centrifugation at 10,000g for 20 min. HA-IgG in the supernatant was determined by spectrophotometry at 280 nm. IgG was coated on latex beads by incubating polystyrene microspheres (2.0 μm in diameter) (2 x 10^{8}/ml) with human IgG (5 mg/ml) over night at 4^{o}C. The mixture was gently rotated. BSA was similarly coated on latex beads. Beads were then pelleted by centrifugation for 10 min at 3000 rpm. The supernatant was removed without disturbing the beads and the pelleted beads were washed two times with ice-cold PBS. The amount of IgG bound to the beads was estimated by measuring IgG concentration in the supernatant at 280 nm.

2.2.2.12 Macrophage stimulation with different forms of IgG

Macrophages were stimulated with IgG or BSA-beads at a ratio of 50 beads:1 cell. The cells were stimulated with soluble and HA-IgG at 100 μg/ml. In some experiments, macrophages were incubated with blocking antibodies (20 μg/ml) against different FcγRs. The incubation was carried out for 1h at 4°C in medium containing heat-inactivated BCS. The cells were then stimulated with the different forms of IgG. With regard to 293T cells transfected to express FcγRs, the cells were stimulated with human IgG-opsonized bacteria. Opsonization of DH5α was achieved by incubating bacteria (2 x 10^{8}/ml) with human IgG (5 mg/ml) for 1 hr at 37°C. The opsonized bacteria were centrifuged for 5 min at 3000 rpm. The bacteria washed three times in PBS and re-suspended at desired concentrations. 293T cells were stimulated in serum free media for 6 hr at 37^{o}C in 5% CO_2 incubator with opsonized bacteria at a ratio of 100:1.

2.3 Protein Chemistry

2.3.1 Materials

2.3.1.1 Antibodies used in this study

All antibodies used in this study have been listed in Table 2.3.1, together with information of their source, isotype and working concentrations.

2.3.2 Methods

2.3.2.1 Protein concentration determination

Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad). It was based on the method by Bradford (Bradford, 1976). Assays were performed according to the manufacturer's protocol. Briefly, eight serial dilutions of a standard BSA solution were prepared. The linear range of this assay is 0.04 mg/ml to approximately 0.4 mg/ml. Diluted BSA (10 μl) and protein samples (10 μl) were added into 96-well microtiter plate. The dye reagent was diluted (1 part Dye Reagent Concentrate with 4 parts distilled water, filtered through 0.45 μm filter to remove particulates) and 200 μl was added to each well. After mixing thoroughly using a multichannel pipette, it was incubated at room temperature for at least 5 min. The plates should not be incubated at room temperature for more than 1 hr. Absorbance was measured at 595 nm. The standard curve was constructed by plotting the known BSA concentrations. Protein concentration in samples was determined by comparison with the BSA standard curve.

Table 2.3.1 Antibodies used in this study

Antibody (anti-)	Clone, isotype Conjugate and stock concentration	Application (working dilution)	Supplier
Human IL-4	MP4-25D2, Rat IgG1 FITC, 100 μg/ml	Flow cytometry (1:100)	BD Pharmingen
Human CD124 (IL-4Rα chain)	X2/45-12, mouse IgG1, Purified	Flow cytometry (1:100)	eBioScience
Human CD132 (IL-2Rγ)	AG184, mouse IgG1, RPE, 200 μg/ml	Flow cytometry (1:10)	BD Pharmingen
Human IL-4	3010.211, mouse IgG1, 500 μg/ml	Western blot (1:500)	R & D Systems, Inc
Human IL-4Rα	25463.111, mouse IgG2, 500 μg/ml	Western blot (1:500)	R & D Systems, Inc
Human IL-2Rγ	8024, mouse IgG1 3500 μg/ml	Western blot (1:500)	R & D Systems, Inc
Mouse IgGs	goat polyclonal, RPE	Flow cytometry (1:25)	DakoCytomation
c-myc tag	9E10, mouse monoclonal IgG1, 200μg/ml	Western blot (1:200)	Roche Molecular Biochemicals
Human CD14	UCHMI, mouse IgG2a, FITC, 0.5 mg/ml	Flow cytometry (1:50)	Ancell
Human CD14	Sheep IgG, 500 μg/ml	Western blot (1:2000)	R & D Systems, Inc
Human CD64	10:1, mouse IgG1, FITC, 200 μg/ml	Flow cytometry (1:50)	Ancell
Human CD32	7.1, mouse IgG1, FITC, 200 μg/ml	Flow cytometry (1:50)	Ancell
Human CD16	3G8, mouse IgG1, FITC, 200 μg/ml	Flow cytometry (1:50)	Ancell
Isotype control	MOPC 31C, mouse IgG1, FITC, 1mg/ml	Flow cytometry (1:100)	Ancell
Human CD16	3G8, mouseIgG1, 1 mg/ml	Blocking(2: 100)	Ancell
Human CD32	7.3, mouse IgG1, 1 mg/ml	Blocking (2: 100)	Ancell
Human CD64	10.1. mouse IgG1, 1 mg/ml	Blocking (2: 100)	Ancell
Isotype control	MOPC 31C, mouse IgG1, 1 mg/ml	Blocking (2: 100)	Ancell
β-actin	AC-15, mouse IgG`	Western blot (1:5000)	Sigma-Aldrich
Mouse IgGs	Goat poly clonal, AP conjugate	Western blot (1:1000)	Bio- rad
Sheep IgGs	Rabbbit polyclonal, AP conjugate	Western blot (1:3000)	Upstate biotechnology

2.3.2.2 Immunoprecipitation Study

293T cells (approximately 1.3 x 10^7 cells in a T75 flask) were transfected with 20 μg of the following expression vectors either singly or in 1:1 pairs: pIL4tm-MH/pIL4tm-TIR2, pIL4Rαtm-MH/ pIL4Rαtm-TIR2, pγCtm-MH/ pγCtm-TIR2 (details of plasmid in section 2.4.3). The cells were harvested after 36 hr and lysed at approximately 2.5 x 10^7 cells/ml in lysis buffer (Appendix) by rotating for 45 min at 4°C. Protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 mM PMSF) were included in the lysis buffer. The lysates were centrifuged for 15 min at 13,000 rpm. The cleared supernatants were either directly analyzed by Western blotting or used for immunoprecipitation. For the latter, the supernatants were pre-cleared for 1 hr at 4°C with protein G-Sepharose (Roche applied science, Germany). The lysates sepharose mixtures were centrifuged for 1 min at 13,000 rpm and the supernatants were removed and incubatated overnight with an anti-myc antibody (1 μg/ml). As controls, the supernatants were incubated with isotype-matched mouse IgG. The cleared lysates were incubated with 50 μl of protein G-Sepharose beads. After 3 hr of rotation at 4°C, the lysate bead mixtures were centrifuged at 13,000 rpm for 2 min. The supernatants were discarded and the beads were washed three times with washing buffer (Appendix) containing protease inhibitor. The bead-bound proteins were eluted in SDS-PAGE sample buffer.

In some experiments, the pre-cleared supernatants were incubated overnight with Ni-NTA-agarose (Sigma) which binds His tags. In this case, the bead-precipitated proteins were eluted with immidazole (250 mM). The eluted proteins were separated on 10% or 12.5% (w/v) SDS-PAGE gels and detected by Western blotting using IL-4, IL-4Rα or γC antibodies.

2.3.2.3 Cell surface biotinylation

293T cells were cultured in 6-well plates and transfected with the following CD14 expression plasmids (3 μg /well). The first group of plasmids expresses CD14-TIR hybrids and mutants based on these hybrids: pCD14-TIR1, pCD14-TIR2, loopdelCD14-TIR1, β13delCD14-TIR1, and pCD14-TIR1 mutants ($CD14^{290A}$, $CD14^{294A}$, $CD14^{298A}$, $CD14^{299A}$, $CD14^{300A}$, $CD14^{302A}$, $CD14^{305A}$, $CD14^{307A}$ and $CD14^{290A, 307A}$). The second group of plasmids include full-length CD14 and CD14 mutants based on full-length CD14 ($CD14^{290A}$, $CD14^{294A}$, $CD14^{298A}$, $CD14^{299A}$, $CD14^{300A}$, $CD14^{302A}$, $CD14^{305A}$, $CD14^{307A}$, $CD14^{290A, 307A}$). Details of these plasmids were in section 2.5. After 36 hr, the cells were washed three times with ice-cold PBS (pH 8.0) and incubated for 30 min at 20°C with membrane-impermeable biotinylation reagent (1 mg/ml) (Link™ sulfo-NHS-LC-biotin; Pierce, Rockford, IL). The biotin reagent was dissolved in PBS (pH 8.0) immediately before use. The reaction was stopped by addition of 20 mM glycine followed by incubation for another 15 min. The cells were lysed in 200 μl lysis buffer (Appendix) and centrifuged to remove insoluble materials. The cell extracts were diluted in 0.5 volume of 1:1 slurry immobilized streptavidin-agarose beads (Pierce) and then incubated with the beads overnight at 4°C under constant shaking. The beads were pelleted by centrifugation at 5,000xg for 1 min and washed three times in the washing buffer containing protease inhibitor (Appendix). Bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer (Appendix) and then subjected to SDS-PAGE and Western blotting.

2.3.2.4 DTSSP-based protein-protein cross-linking on the cell surface

293T cells in 6-well plates were transfected with plasmids for wild type or mutant CD14 (3 μg/well) using the GenePorter 2 reagent. After 36 hr, the cells were washed

three times with PBS and pelleted into 1.5 ml microcentrifuge tubes. The cells were resuspended to 2 x 10^6/ml in PBS and then incubated with membrane-impermeable bifunctional cross-linking reagent, 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP, Pierce) (1mg/ml) in dark for 45 min at 20°C with rocking. The cross-linking reaction was stopped by adding 1M Tris-HCl (pH 7.4) and incubates for 10 min. Next, cells were washed and lysed in the lysis buffer containing the protease inhibitors. THe lysates were subjected to SDS-PAGE in the presence or absence of β-mercaptoethanol and followed by Western blotting.

2.3.2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cells were lysed on ice in the lysis buffer containing protease inhibitors. After removing insoluble materials by centrifugation, protein concentration of the lysates was determined using the BCA reagent (Bio-Rad) (section 2.3.2.1). 50-100 μg protein samples were mixed with 1/5 volume of a 5 x SDS-PAGE sample loading buffer with or without β-mercaptoethanol (Appendix). The samples were boiled for 10 min at 95°C before loading into the SDS-PAGE gels. The gels were run in 1x SDS/Glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3) at 120V until the dye front is approximately 1cm from the bottom (Sambrook et al., 1989).

2.3.2.6 Western blotting

Proteins separated on the SDS-PAGE gels were electro-blotted onto nitrocellulose membranes (Bio-Rad) in the transfer buffer (Appendix) at 100V and 250 mA for 1.5 hr at room temperature using an electroblotting apparatus set (Bio-Rad). The blots were blocked at 4°C overnight in 5% non-fat milk dissolved in the TBS-T buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween-20). The blots were washed three times

(10 min each) and then incuabted with specific primary antibodies for 2 hr at room temperature with constant shaking. The blots were washed three times and then incubated with alkaline phosphatase (AP)-conjugated secondary antibodies for 1hr. After washing the blots were finally developed using the Immun-Star™ Stubstrate Pack (Bio-Rad). The signals were detected using the ECL protein detection system (Amershem).

2.3.2.7 Human cytokine array assay

The Human Cytokine Antibody arrays (RayBiotech, Norcross, GA) provide a highly sensitive approach to the simultaneous detection of multiple cytokine expression in conditioned media. Briefly, membranes are dotted with antibodies in duplicates that are specific for different cytokines. Incubation of the membrane with conditioned media cultures cytokines to the spots. This is detected with secondary antibody for each cytokines. Monocytes derived GM-CSF macrophages (approximately 1 x 10^6) were stimulated with ImIgG, HA-IgG, IgG-beads or soluble IgG. HA-IgG and soluble IgG were used at at 100 μg/ml. IgG-beads or as a control, BSA-beads were used at 50 beads:1 cell. After incubation for 24 hr at 37°C in the presence of 5% CO_2, the conditioned media was harvested and centrifuged for 5 min at 3,000 x g to remove cell debris. Meanwhile, the array membranes (RayBiotech) were blocked with 1x blocking buffer by rotating for 30 min at room temperature. 1 ml of conditioned media was mixed with an equal volume of fresh media and then incubated for 2 hr with array membrane at room temperature by rotating. The membrane was washed with washing buffer I for three times (each for 5 min) and then washed with buffer II (both wash buffers are provided in the kit) for two times. 1 ml of biotin-conjugated primary antibody mixture was added and incubated with membrane at room temperature for 2

hr. The membrane was washed and then incubated with 2 ml of horseradish peroxidase-conjugated streptavidin at room temperature for 1 hr. The membranes were developed using substrate detection buffer A and detection buffer B (provided in the kit) mixed at 1:1 ratio. The membrane was exposed to X-ray films and the films were processed by autoradiography.

2.3.2.8 Enzyme-linked Immunosorbent Assay (ELISA)

Cytokines secreted into the culture medium from IgG-stimulated GM-CSF macrophages or 293 T cells was measured by ELISA using the human DuoSet ELISA Development kits (R&D Systems Inc). These assays were performed according to the manufacture's instructions. Briefly, 96-well BD Falcon™ ELISA plates (BD Biosciences) were added with 1 capture antibodies diluted in PBS (100 μl/well), sealed with parafilm and incubated overnight at 4°C. The plates were washed with wash buffer (0.05% Tween ®20 in PBS, pH 7.2 - 7.4) three times (250 μl/well) and blocked with the Reagent Diluent (1% BSA 5 in PBS, pH 7.2 - 7.4, 0.2 mm filtered) (200 μl/well) for 2 hr at room temperature. The plates were washed three times with wash buffer and samples or purified cytokine standard diluted in Reagent Diluent were added into each well (100 μl/well). Dilution of conditioned media was made in Reagent Diluent so as to achieve the final reading within the linear range of the standard curve. Plates were sealed and incubated overnight at 4°C. The plates were washed three times and biotinylated detection antibodies diluted in Reagent Diluent were added to each well (100 μl/well). After incubation at room temperature for 2 hr, plates were washed three times and incubated with streptavidin-HRP conjugate in Reagent Diluent (100 μl/well) for 1hr at room temperature in the dark. Plates were washed 5 times. Substrate solution was prepared within 15 min prior to use by mixing equal volume of Substrate reagent A

(hydrogen peroxide in a buffered solution) and Substrate B (3,3',5,5' tetramethylbenzidine in an organic solvent) provided in the kit (BD Biosciences). 100 μl substrate solutions were added to each well and the plates were incubated in dark for 30 min. The colormetrical reaction was stopped by adding 50 μl Stopping Solution (1M phosphoric acid) to each well. The optical density was determined immediately using a microplate reader (TECAN) at 450 nm with a wavelength correction at 570 nm. The linearized standard curve was constructed by plotting the cytokine concentrations versus that of the OD reading value (log scale). The concentration of each sample was derived by comparing with the standard curve and then multiplied by the dilution factors.

2.4 Molecular biology techniques

2.4.1 Construction of vector for the expression of TLR chimeras

2.4.1.1 Expression vectors for integrin-TLR chimeras (pβ5-TLR vectors)

Expression vectors were constructed to express the extracellular domains of integrin β5 with the transmembrane and cytoplasmic domain of TLR1 or TLR2. These vectors were constructed by Dr. Zhang Haifeng. Briefly, cDNA encoding the transmembrane and cytoplasmic domains (TM/Cyt) of TLR1 and TLR2 were amplified by PCR using the specified primer pairs:

TLR1 Forward 5'cccttcgaaaacataactctgctgatcgtc3';

Reverse 5'cgggtttaaacgactatttctttgcttgctctg3';

TLR2 Forward 5'cccttcgaaacagcactggtgtctggc 3';

Reverse 5'cgggtttaaacaacctaggactttatcgacg 3';

A *SfuI* site was introduced at the 5' end of each forward primer and a *PmeI* site was introduced at the 5' end of each reverse primer. All PCR products were therefore flanked by SfuI/PmeI sites and cloned in the pβ5/MH vectors which replaced the

Myc/His-coding sequence (Zhang et al., 2002). This led to the pβ5-TIR1/2 expression vectors (Fig. 2.4A).

2.4.1.2 Expression vectors for fusion receptors between IL-4 or the extracellular (EC) domains of IL-4Rα, γC or CD14 and the TM/Cyt domains of TLR1 or TLR2

cDNA encoding IL-4 or the EC domains of IL-4Rα, γC, and CD14 were amplified by RT-PCR using monocyte RNA and the listed primer pairs in Table 2.4. The cDNA fragmants were cloned into the pβ5TLR1 and pβ5TLR2 vectors (Zhang et al., 2002) at the indicated restriction site (Table 2.4)**,** to replace sequences encoding the EC domain of integrin β5 in these vectors. This yields the pIL4-TIR1/2; pIL4Rα-TIR1/2; pγC-TIR1/2; and pCD14-TIR1/2 expression vectors (Fig. 2.4B).

Table 2.4 Primers used in the cloning of IL-4, IL-4Rα, γC and CD14 cDNA

cDNA	Genebank accession number	Sense/antisense primer pairs (5'…..3) nucleotides positions	Restriction sites for cloning
IL-4	XM_004053	cggggtaccaccatgggtctcacctcccaact (bases 66 to 85) tttgttcgaagctcgaacactttgaata (bases 507 to 525)	*KpnI/SfuI*
IL-4Rα	NM_000418	tgtggaattccagcctggtgccttggca (bases 150 to 169) tttgttcgaagtgctgctcgaagggctc (bases 854 to 871)	*EcorI/SfuI*
γC	NM_000206	cttggtaccgaagagcaagcgccatgt (bases 1 to 18) gaagggcccggcttccaatgcaaacag (bases 783 to 801)	*KpnI/ApaI*
CD14	NM_000591	cggggtaccaccatggagcgcgcgtcc (bases 120 to 134) cccttcgaaagggaccaggaagggattc (bases 1103 to 1121)	*KpnI/SfuI*

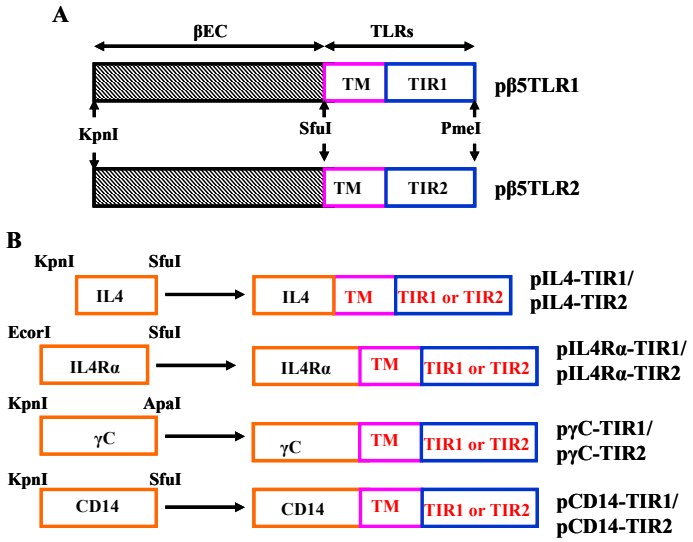


Figure 2.4 Scheme of expression vector construction. A) Sequences encoding the TM/TIR domains of TLR1 and TLR2, in the pβ5TLR1 and pβ5TLR2 vectors (Zhang et al., 2002). The TIR1 and TIR2 sequences are flanked by *SfuI*/*PmeI* sites in frame with 5' sequence encoding the exttracellular domain) of human integrin β5 (β5EC). B) Sequences encoding IL-4 and the EC domain of IL4Rα , γC and CD14 were amplified by RT-PCR using specific primers (Table 2.4). The IL-4 sequence was directly cloned in the pβ5TLR1 and pβ5TLR2 vectors to replace the β5EC sequence. To clone IL4Rα, γC and CD14, EC sequences encoding the TM/Cyt domains of TLR1 and TLR2 were first subcloned into the pcDNA3.1 vector. The PCR-amplified IL4Rα, γC and CD14 sequences cloned into these vectors in frame with the TM/Cyt sequences.

2.4.2 Expression vectors for the expression of fusion receptors between the EC domains of IL-4Rα or γC and the FcγRIIA TM/Cyt domains

To generate the pIL4Rα-FcγRIIA and pγC-FcγRIIA expression vectors, which express the IL4Rα and γC with the Tm/Cyt domain of FcγRIIA, a pair of primers were used to amplify sequence encoding the FcγRIIA TM/Cyt domain (5'tttgttcgaatcaccaatggggatcattg3'/5'cgggtttaacttagttattactgttgacatgg3'). This fragment was flanked by 5'-*SfuI* and 3'-*PmeI* sites and was cloned into the pIL4Rα-TIR1 and pγC-TIR1 vectors in place of the TM-TIR1 sequences. Sequences for the TM/Cyt domain of TIR1 vectors were flanked by 5'-*SfuI* and 3'-*PmeI* sites. This led to the IL4Rα-FcγRIIA and γC-FcγRIIA expression vector (Fig. 2.5).

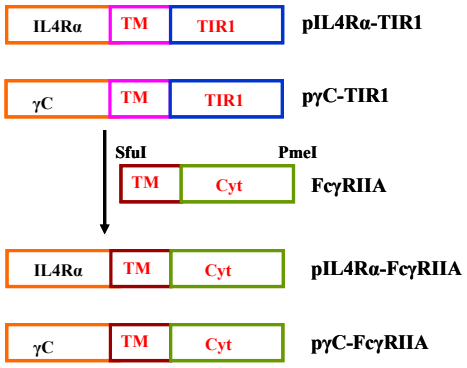


Figure 2.5 Scheme of expression vectors for chimeras between the EC domain of IL-4Rα , γC and Tm/Cyt domain of FcγRIIA. Sequences encoding TM/Cyt domain of FcγRIIA was amplified by RT-PCR using specific primers. This fragment was flanked by 5'-*SfuI* and 3'-*PmeI* and was cloned into the pIL4Rα-TIR1 and pγC-TIR1 vectors in place of TM-TIR1 sequences. This led to the IL4Rα-FcγRIIA and pγC-FcγRIIA expression vectors. All vectors were confirmed by sequencing.

2.4.3 Expression vectors for the expression of fusion receptors between IL-4 or the EC domains of IL4Rα and γC and the transmembrane (TM) domain of TLR2 followed by Myc/His tag

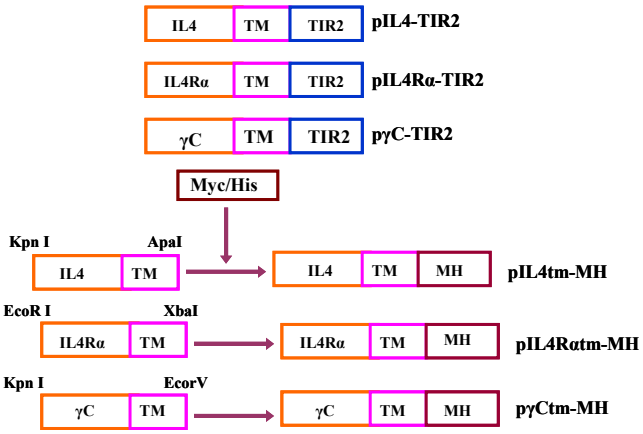


Figure 2.6 Scheme of expression vectors for chimeras between IL4, IL-4Rα , γC and tm-MH. The IL4/TM, IL4Rα/TM and γC/TM sequences were amplified from the pIL4-TIR2, pIL4Rα-TIR2 and pγC-TIR2 vectors by PCR and then subcloned into the pcDNA3.1 vector to generate the pIL4tm-MH, pIL4Rαtm-MH and pγCtm-MH vectors. All vectors were confirmed by sequencing.

The following expression vectors were generated to express IL-4, IL4RαEC and γCEC in fusion with the TM domain of TLR2, followed by Myc/His tags rather than TIR2. Sequences encoding IL-4, IL4RαEC, and γCEC, each followed by the C-terminal TLR2 TM domain, were amplified from the pIL-4-TIR2, pIL4Rα-TIR2 and pγC-TIR2 vectors (Fig. 2.4). This was achieved using the same three sense primers as described in Table 2.4 and a common specific reverse primer (5'gaagggccccgcacaggacccccgtgag 3'; nucleotides 1939 to 1956; accession number U88878) and cloned into the *KpnI/ApaI* site of the pcDNA3.1vector (pIL4tm-MH). Another reverse primer (5'tgctctagagcacaggacccccgtgag 3'), which is identical to the above primer except for a different 3' restriction site, was used to amplify sequences encoding IL4RαEC and γCEC fused with the C-terminal TLR2 TM sequence, from the pIL4Rα-TIR2 and pγC-TIR2 vectors (Fig. 2.4). These vectors were cloned respectively into the *EcorI/XbaI* and *KpnI/EcorV* sites of the pcDNA3.1 Myc/His vector. Thus, this yielded the pIL4Rαtm-MH and pγCtm-MH expression vectors (Fig. 2.6). All DNA inserts were verified by sequencing before use.

2.4.4 Expression of full-length FcγRIIA, FcγRIIIA and FcR γ chain

Full-length cDNA encoding human FcγRIIA, FcγRIIIA and FcRγ were amplified from RNA isolated from human macrophages with the primer pairs listed in Table 2.5. These cDNA fragments were subsequently cloned into the pcDNA3.1 Myc/His vector at the indicated restriction sites to generate the respective expression vectors. All these vectors were constructed with the concensus Kozak sequence CCACC immediately 5' to the ATG codon (Kozak, 1987; Kozak, 1990). In these vectors, the stop codon at the 3'end of the coding sequences was retained and therefore, the 3' Myc/His sequences were not fused to the C-terminal ends of these receptors.

Table 2.5 Primers used to clone FcγR cDNA

FcγR	Genebank Accession	Primer pairs (5'…….3') (forward/reverse)	Restriction sites on pcDNA3.1 ligated into (5'/3')
FcγRIIA	NM_021642	cggggtaccaccatggagacccaaatgtctc gaagggcccttagttattactgttgacatgg	*KpnI/ApaI*
FcγRIIIA	NM_000569	cgcggatccaccatgggtggaggggctgg gaagggccctcatttgtcttgagggtcc	*BamHI/ApaI*
FcR γ	M33195	cgcggatccaccatgattccagcagtggtc gaagggcccctactgtggtggtttctc	*BamHI/ApaI*

2.5 Expression vectors for CD14 mutants

2.5.1 CD14 mutations introduced to the pCD14-TIR1 vector

Two CD14 deletion mutants were generated in the pCD14-TIR1 by removing amino acids 290-300 (the 'loop' between β12 and β13) or 301-315 (β13). This was achieved by introducing new restriction site in the pCD14-TIR1 vector as the backbone (Fig. 2.7) by site-directed mutagenesis (Stratagene, La Jolla, CA). To delete residues 290-300, a pair of primers was used to introduce *EcoRI* restriction cutting sites individually (5'….3'): cagagtgctcgatctcagctgcaacgaattcaacagggcgccgcagcctg (*EcoRI*); gcgccgcagcctgacgagctgcccgaattcgataacctgacactggacggg (*EcoRI*). The mutated pCD14-TIR1 vector was digested with *EcoRI* and simultaneously religated into the pCD14-TIR1. To delete amino acids 301-315, the following primer was used in which *SfuI* cutting site was introduced (5'….3'): cctgacgagctgcccgagttcgaaaacctgacactggacggg. The original pCD14-TIR1 expression vector, have a transmembrane and cytoplasmic domain of TLR1 which was flanked by a 5' *SfuI* and 3' *PmeI* site. The mutated pCD14-TIR1 vectors was digested with *SfuI* and simultaneously ligated into the pCD14-TIR1 vector. This led to generate the ploopdelCD14-TIR1 and pβ13delCD14-TIR1 vector (Fig. 2.7). The Quick change site-directed Mutagenesis kit was also used to introduce alanine substitution in the ploopdelCD14-TIR1 and pβ13delCD14-TIR1 vector. Alanine substitution mutants of CD14 were generated in the same region and Table 2.6 summarizes the names and the

amino acids residues substituted in each mutant. Here the specific amino acid residues which were conserved among mouse, human and rabbit were substituted to alanine. The different sets of primers used for each mutant are listed in the Table 2.7. To generate double mutant (L290 and L307 together) CD14, the same set of primers were used as to generate single mutant of L290A and L307A. This was carried out by introducing one after another mutation in the same mutant CD14 plasmid. The introduction of the appropriate mutation in all vectors was confirmed by DNA sequencing.

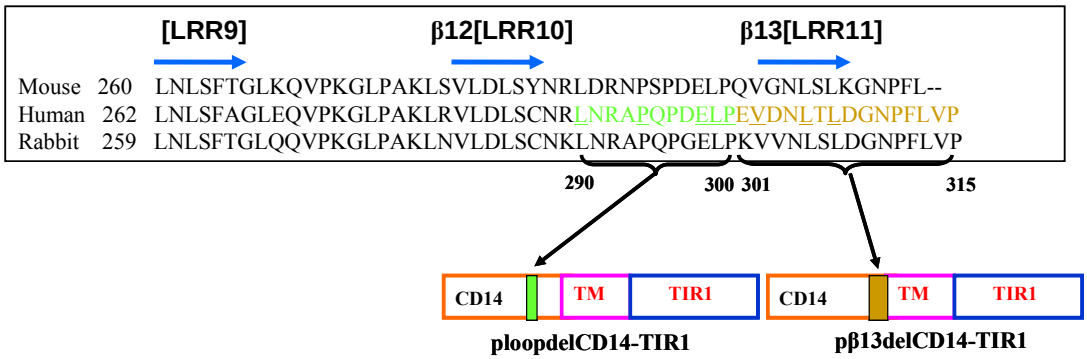


Figure 2.7 Scheme of expression vectors for loopdelCD14-TIR1, β13delCD14-TIR1 and CD14 mutants. Amino acid sequence alignment of mouse , human and rabbit CD14 are shown here. Two deletion mutants were generated in the pCD14-TIR1 by removing amino acids 290-300 (the 'loop' between β12 and β13) or 301-315 (β13). The removed amino acids were highlighted in green and brown. This was achieved by introducing new restriction site (i.e *EcorI* or *SfuI* individually) in the pCD14-TIR1 vector as the backbone by site-derected mutagenesis. The mutated pCD14-TIR1 vectors was digested with the respective restriction enzyme and ligated into the pCD14-TIR1 vector. This led to generate the ploopdelCD14-TIR1 and pβ13CD14-TIR1 vector. A specific amino acid mutant of these regions was also carried out by site-directed mutagenesis. The amino acid codons (underlined) of human CD14 were mutated to that coding for alanine.

Table 2.6 Alanine substitution in CD14 by mutagenesis

Serial No	Name	Position and name of amino acid of CD14 mutants
1	L290A	290Leucine
2	P294A	294Proline
3	E298A	298Glutamine
4	L299A	299Leucine
5	P300A	300Proline
6	V302A	302 Valine
7	L305A	305 Leucine
8	L307A	307 Leucine
9	L290A, L307A	290 and 307 double leucine

Table 2.7 Primers used in site-directed mutagenesis for CD14

CD14 mutants	Forward primer (5'….3') on respective vectors
L290A	gatctcagctgcaacagagcgaacagggcgccgcagcc
P294A	cagactgaacagggcggcgcagcctgacgagctgc
E298A	gcgccgcagcctgacgcgctgcccgaggtggataacc
L299A	gccgcagcctgacgaggcgcccgaggtggataacc
P300A	gccgcagcctgacgagctggccgaggtggataacctgac
V302A	ctgacgagctgcccgaggcggataacctgacactgg
L305A	ctgcccgaggtggataacgcgacactggacgggaatcc
L307A	ctgcccgaggtggataacgcgacagcggacgggaatcccttc
L290A and L307A (double mutant)	The same set of primers was used to generate double mutant as to generate single mutant of L290A and L307A.

2.5.2 Mutations introduced to Wild type (Wt) CD14 vectors

Expression vectors for CD14 mutants (i.e. L290A, P294A, E298A, L299A, P300A, V302A, L305A, L307A and L290AL307A) were obtained by site-directed mutagenesis, using the same set of primers as listed in Table 2.7.

2.6 Brief description of other expression vectors

The expression vectors for the TLR4, MD2 and CD14 were obtained as described by (Zhang et al., 2002). Briefly, cDNA was amplified by RT-PCR from THP-1 with the primer pairs in Table 2.8. The fragments obtained were digested with the respective restriction enzymes and cloned into pcDNA3.1 vector.

Table 2.8 Primers for the cloning of TLR4, CD14 and MD2 cDNA

Expression vectors	**Primers (5'….3') Forward/ Reverse**	**Restriction sites on pcDNA3.1**
pTLR4	gcttggtaccgggccactgctgctcac/gaagggccctcagatagatgttgcttcctg	*KpnI/ApaI*
pCD14	cggggtaccaccatggagcgcgcgtcc/gagtctagattaggcaaagccccgggc	*KpnI/XbaI*
pMD2	cggggtaccaccatgttaccatttctgttttttc/gaagggccctctaatttgaattaggttggtgta	*KpnI/ApaI*

Chapter 3 Development of a TLR-based two-hybrid assay for the detection of protein-protein interactions

3.1 Hypothesis: TLR2 signaling mechanism can be utilized to detect protein-protein interactions

The experiment was designed based on the mechanism by which TLR2-mediated NF-κB signaling is activated. TLR2 transduces signals when it dimerizes with TLR1 (Ozinsky et al., 2000) which causes the dimerization of cytoplasmic TIR domains of TLR1 and TLR2 (i.e. TIR1 and TIR2). When TIR1 and TIR2 was expressed as CD4-TIR1 and CD4-TIR2 fusion proteins, TIR1/TIR2 dimerization induced via the extracellular (EC) domain of CD4 caused NF-κB activation (Fig. 3.1A) (Ozinsky et al., 2000). TIR1 and TIR2 dimerization induced through the EC domains of integrin αvβ5 also leads to NF-κB activation (Zhang et al., 2002). These observations led to the hypothesis that if other receptor EC domains are fused to TIR1 and TIR2, it should allow the detection of interactions between these EC domains. We selected the well-characterized IL-4 receptor to test this hypothesis. As mentioned in chapter 1 (section 3), the IL-4 receptor consists of a high-affinity IL-4 subunit, known as the IL4Rα chain, and a low-affinity subunit, known as the common γ chain (γC) (Nelms et al., 1999). In this experiment, expression constructs were designed in a way that the EC domains of IL4Rα and γC were individually each fused to TIR1 and TIR2 (Fig. 3.1B). The two fusion proteins were co-expressed in 293T cells. NF-κB and the control *Renilla* luciferase reporter plasmids were also co-expressed. The NF-κB luciferase reporter expresses firefly luciferase under the NF-κB promoter whereas *Renilla* luciferase reporter expresses the luciferase under a constitutive CMV promoter. In each experiment, NF-κB activation is registered by the former and the latter serves as an

internal control. IL-4 is expected to induce interactions between the two fusion proteins and therefore cause the association of TIR1 and TIR2 and NF-κB activation (Fig. 3.1B). This assay is expected to detect IL-4 induced interactions between IL-4Rα and γC on live 293T cells.

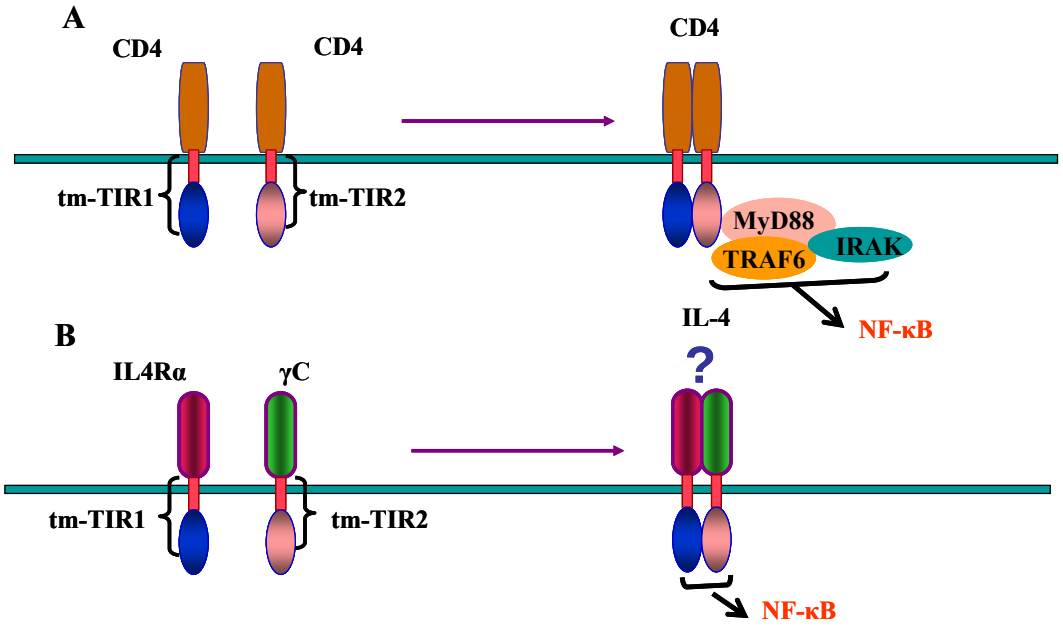


Figure 3.1 Principles underlying the TIR1/TIR2-based two-hybrid assay. This diagram illustrates the underlying mechanism based on which this novel two-hybrid assay was designed. (A) The extracellular (EC) domains of CD4 form dimers. When tmTIR1 and tmTIR2 are fused to the EC domains of CD4, CD4-CD4 interaction leads to tmTIR1 and tmTIR2 dimerization. TIR1/TIR2 dimerization causes NF-κB activation. This has been previously reported (Ozinsky et al., 2000). (B) In this study, the EC domains of IL-4Rα and γC are fused with tmTIR1 and tmTIR2. IL-4Rα and γC association is induced with IL-4. It was expected that IL-4 induces the dimerization of the IL4Rα-TIR1 and γC-TIR2 hybrids, leading to NF-κB activation. This is the hypothesis to be tested in this chapter.

3.2 Expression of TIR1 and TIR2 fusion proteins

Based on the experimental design, vectors were constructed for four fusion proteins, i.e. IL4Rα-TIR1, IL4Rα-TIR2, γC-TIR1 and γC-TIR2 (Fig. 2.7). IL-4 was also expressed as TIR1 and TIR2 fusion proteins, i.e. IL4-TIR1 and IL4-TIR2 (Fig. 2.4). This was to test another hypothesis that interactions between secreted protein ligands and their receptors may also be detected using this assay by expression of the secreted protein as an EC domain in fusion with TIR1 or TIR2. Overall, six expression vectors were generated, pIL4Rα-TIR1, pIL4Rα-TIR2, pγC-TIR1, pγC-TIR2, pIL4-TIR1, and pIL4-TIR2. The expression of these TIR1 and TIR2 chimeras on the cell surface was examined by flow cytometry. These vectors were expressed in 293T cells and the plasmids were used at 100 ng/well. After 24 hr, the cells were stained with specific antibodies for IL4Rα, γC or IL-4. As shown in Fig. 3.2A, all six fusion proteins were detected on the cell surface. When these vectors were over-expressed individually at 150 ng/well, none was able to activate NF-κB (Fig. 3.2B). Addition of IL-4 at 100 ng/ml did not induce NF-κB activation when these hybrids were expressed individually. These results show that individually none of these fusion proteins induces significant NF-κB activation irrespective of IL-4 induction.

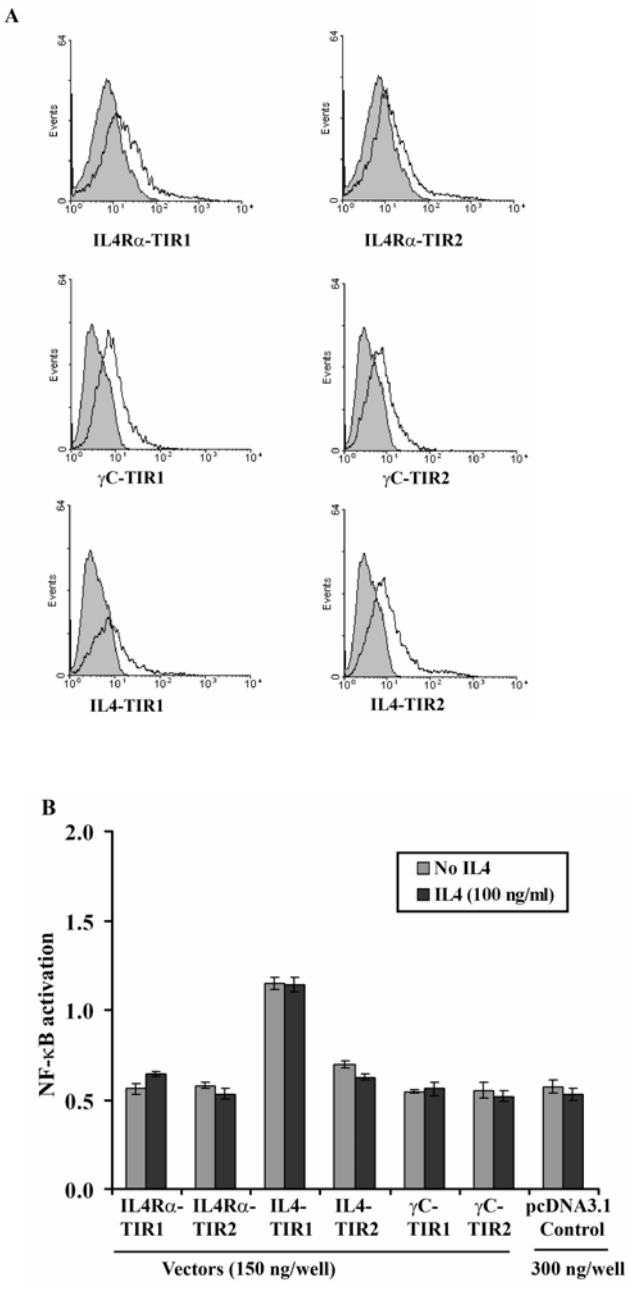


Figure 3.2 Expression of TIR1 and TIR2 fusion proteins. (A) 293T cells were singly transfected in 24-well plates with pIL4Rα-TIR1/TIR2, pγC-TIR1/TIR2, and pIL4-TIR1/TIR2 at 100 ng/well. After 24 hr, the cells were stained with IL4, IL4Rα and γC antibodies and detected by flow cytometry. The filled profiles are signals derived from 293T cells transfected with the pcDNA3.1 vector and the open profiles represent specific signals detected on 293T cells transfected with the respective TIR1 and TIR2 fusion protein vectors. (B) 293T cells were singly transfected with pIL4-TIR1/TIR2, pIL4Rα-TIR1/TIR2, and pγC-TIR1/TIR2 at 150 ng/well. Total expression vector in each experiment was normalized to 300 ng/well with pcDNA3.1. The p5xNF-κB-luc and pRL-CMV luciferase reporter plasmids were co-transfected in each experiment at 100 ng/well each. After 24 hr, the cells were stimulated with or without IL-4 (100 ng/ml) and NF-κB activation was determined by measuring the two luciferases. The firefly (NF-κB-directed) luciferase activity was normalized to the Renilla (CMV-directed) luciferase in each experiment. All experiments were performed in triplicate and the results were expressed as means ± SD.

3.3 Detection of ligand-induced receptor-receptor interactions using the TIR1/TIR2 based assay

Receptor dimerization or oligomerization is a common mechanism in ligand-induced activation of cell signaling. For example, IL-4 activates cell signaling by inducing the dimerization of its two subunits, i.e. IL4Rα and γC. IL-4 binds to IL4Rα with high affinity and then γC is recruited to interact with IL-4 and IL4Rα (Russell et al., 1993; Kondo et al., 1993). In this study, the known interactions between IL-4 and its receptor were used to evaluate the feasibility of this TIR1/TIR2-based assay for detecting protein-protein interactions. Before examining IL-4-induced IL4Rα and γC interaction using this two-hybrid assay, the dosages of the pIL4Rα-TIR1 and pγC-TIR2 plasmids were titrated to identify optimal dosages that give low background NF-κB activation in the absence of IL-4 induction. This was carried out in 24-well plates. 293T cells were co-tansfected with a series of dilutions of the two plasmids at 1:1 ratio from 9-300 ng/well. Total plasmid in every experiment was normalized to 300 ng/well using the pcDNA3.1 vector. As a negative control, only pcDNA3.1 was used. Interactions between IL4Rα-TIR1 and γC-TIR2 were judged by the activation of NF-κB using the luciferase reporter plasmids. Co-expression of IL4Rα-TIR1 and γC-TIR2 resulted in constitutive NF-κB activation in the absence of IL-4 stimulations when high doses of plasmids (75 ng/well or more) were used (Fig. 3.3). Constitutive NF-κB activation was not obvious when plasmids were used at 38 ng/well or less (Fig. 3.3). This suggests that, at these plasmid dosages, IL4Rα-TIR1 and γC-TIR2 are probably not excessively over-expressed. The transfected cells were also examined for dose-dependent response to IL-4 at 1, 10 or 100 ng/ml. IL-4 induced NF-κB activation was observed under all three conditions. This is especially obvious when the vectors were used at low dosages (i.e. 38, 19 and 9 ng/well) (Fig. 3.3). IL-4 induced NF-κB activation was not seen at the

plasmid dosage of 5 ng/well. This result suggests that co-expression of these two plasmids at this dosage may not be sufficient for IL-4 induced NF-κB activation.

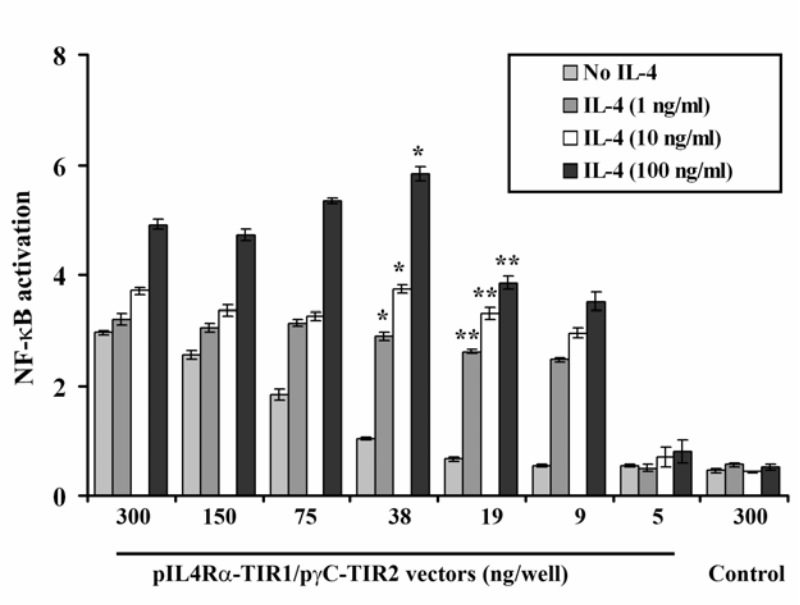


Figure 3.3 Detection of IL-4 induced IL-4Rα and γC interaction (NF-κB activation) using the TIR1/2-based two-hybrid assay. 293T cells were co-transfected with the pIL4Rα-TIR1 and pγC-TIR2 vectors in 24-well plates. The two plasmids were used at 1:1 ratio and the combined dosage was as indicated. Total expression vector in every experiment was normalized to 300 ng/well with pcDNA3.1. As controls, the cells were transfected with 300 ng/well of the pcDNA3.1 plasmid. The p5xNF-κB-luc and pRL-CMV luciferase reporter plasmids were also co-transfected in all experiments at 100 ng/well each. After 24 hr, transfected 293T cells we stimulated for 12 hr with increasing concentrations of IL-4 (1, 10 and 100 ng/ml) as indicated in the inserted legend box. After 12 hr, the activity of NF-κB-directed firefly luciferase and CMV-directed Renilla luciferase expression were determined using the Dual luciferase assay (Promega). The detected NF-κB activation was normalized to the Renilla luciferase activity and expressed as a percentage of the latter. All experiments were performed in triplicate and the results were expressed as means ± SD. *$p \leq 0.010$ and **$p \leq 0.023$ were observed between the effects of different concentrations of IL-4 at 38 ng/well and 19 ng/well.

At the plasmid dosages of 19 ng/well or less, the expression of IL4Rα-TIR1 and γC-TIR2 on the cell surface decreased when checked by flow cytometry (data not shown). Although IL-4 was able to induce NF-κB activation at these plasmid dosages, the levels of NF-κB activation were close to background levels (as induced by the control plasmid). At 38 ng/well co-expression of IL4Rα-TIR1 and γC-TIR2 vectors was well

differentiated and at the same time not over-expressed. At this plasmid dosage of 38 ng/well and IL-4 concentration of 100 ng/ml NF-κB activation was most pronounced as compared to lower plasmid dosages. These optimized condition were then used for subsequent experiments. As a control, NF-κB activation was not induced by IL-4 in 293T cells transfected with the pcDNA3.1 vector.

3.4 IL-4 interacts with IL-4Rα but not γC

The results described above showed the feasibility of the TIR1/2-based assay in detecting lateral receptor-receptor interactions on cell membrane. We then examined if this assay also allows the detection of ligand-receptor interactions, by expression of IL-4 as the EC domain in a IL4-TIR1 hybrid receptor. When IL4-TIR1 was co-expressed with the IL4Rα-TIR2 hybrid receptor, it led to NF-κB activation at the plasmid dosage of 38 ng/well (Fig. 3.4A). As shown in Fig. 3.3, under these conditions, co-expression of IL4Rα-TIR1 and γC-TIR2 caused insignificant NF-κB activation unless the cells were stimulated with IL-4. This suggests that IL-4, expressed as the EC domain of the IL4-TIR1 hybrid receptor, interacts with the EC domain of IL-4Rα in the IL4Rα -TIR2 hybrid receptor. Reducing the plasmid to 19 ng/well did not significantly reduce NF-κB activation and increasing each expression vector to 150 ng/well did not further enhance NF-κB activation (Fig. 3.4A). Addition of soluble IL-4 showed no effect on NF-κB activation mediated by IL4-TIR1 and IL4Rα-TIR2. This shows that NF-κB activation was not simply due to the over-expression of the two-hybrid receptors. These results are consistent with IL-4 having high affinity for IL-4Rα (Nelms et al., 1999).

Protein chemistry and crystallography studies revealed that IL4R is activated through a two-step dimerization mechanism (Hage et al., 1999; Mueller et al., 2002). At physiological concentrations, IL-4 was reported to bind to the IL-4Rα first via its high-affinity binding site 1. Subsequently, its binding site 2 (a low affinity binding site) binds to γC (Letzelter et al., 1998). This appears to be sufficient to cause the association of γC with the preformed IL-4/IL-4Rα complex on cell membrane leading to the activation of cell signaling.

We then examined whether the low affinity between IL-4 and γC could be detected in the absence of IL-4Rα, using this TIR1/TIR2-based assay. IL4-TIR1 was co-expressed with γC-TIR2 in 293T cells. Co-expression of these two-hybrid receptors resulted in NF-κB activation in a dose-dependent manner (Fig. 3.4B). Addition of soluble IL-4 (100 ng/ml) did not inhibit or enhance NF-κB activation. This shows that NF-κB activation observed with IL4-TIR1 and γC-TIR2 was due to over-expression of the two-hybrid receptors which was also observed with IL4Rα-TIR1 and γC-TIR2 co-expression. As another control, co-expression of pIL4Rα-TIR1, pγC-TIR1 or pIL4-TIR1 with the irrelevant pβ5-TIR2 hybrid receptor (19 ng/well for each vector) caused little NF-κB activation which is in contrast with potent NF-κB activation caused by pαV-TIR1 and pβ5-TIR2 co-expression (Fig. 3.4C). These results suggest that, despite the background NF-κB activation caused by over-expression, this TIR1/TIR2-based assay can detect specific IL-4 interaction with IL-4Rα when the assay is performed in a controlled manner.

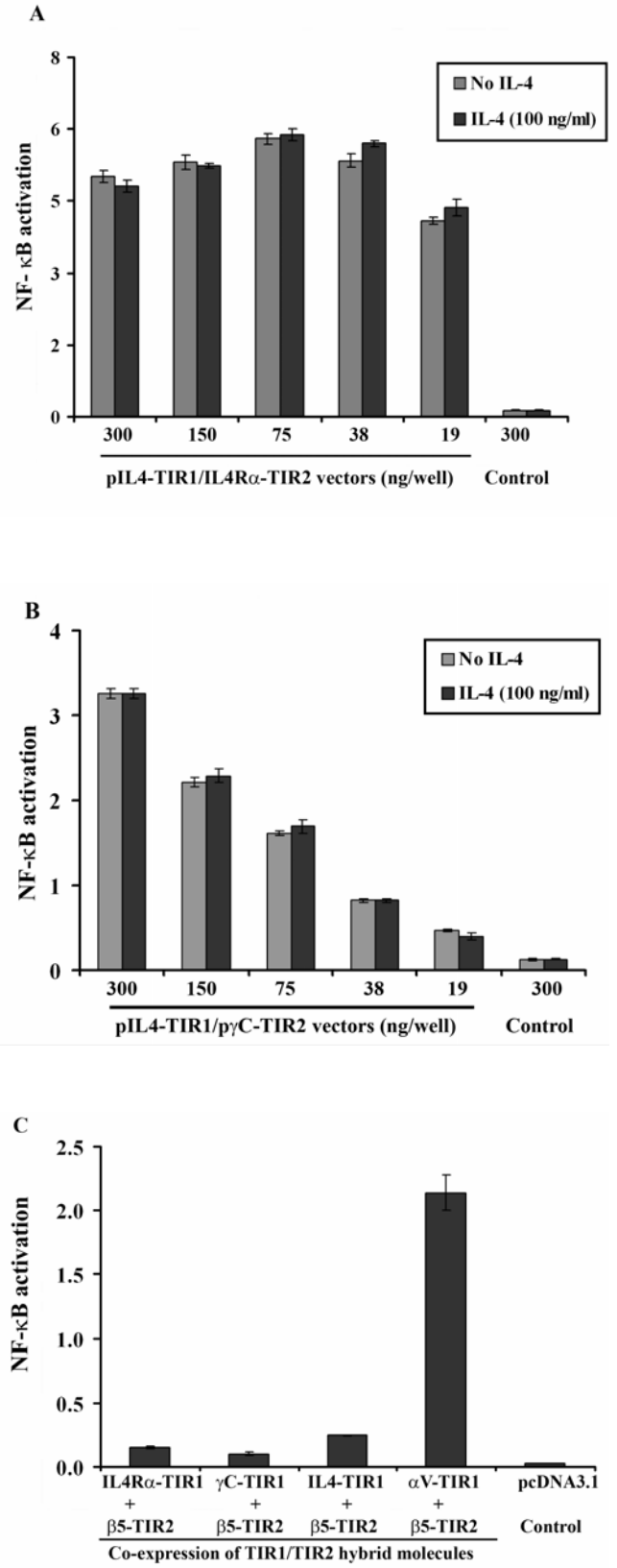


Figure 3.4 IL-4 interacts with IL4Rα but not γC. (A) 293T cells were co-transfected with the pIL4-TIR1/pIL4Rα-TIR2 plasmid pair at a series of dilutions as indicated. (B) The pIL4-TIR1/pγC-TIR2 plasmid pair was co-expressed in 293T cells at a series of dilutions as indicated. As a control, the pcDNA3.1 vector was also transfected at 300 ng/well in both experiments. After 24 hr, transfected cells were either left unstimulated (grey bars) or stimulated with IL-4 for 12 hr at 100 ng/ml (black bars). After stimulation cells were lysed to measure luciferase activities. (C) Co-expression of pIL4Rα-TIR1, pγC-TIR1, pIL4-TIR1 or as a positive control, pαV-TIR1 with pβ5-TIR2. All vectors were each used at 19 ng/well. The luciferase reporter plasmids were similarly co-transfected. NF-κB activation was measured and presented as described in Fig. 3.3.

3.5 Detection of homotypic interactions between IL-4Rα and γC receptors

Some receptors are constitutive homodimers or higher oligomers. As shown in Fig. 3.3 co-expression of IL4Rα-TIR1 and γC-TIR2 at 19 ng/well was unable to induce NF-κB activation unless the cells were stimulated with IL-4. Similarly, co-expression of IL4-TIR1 and γC-TIR2 led to insignificant NF-κB activation (Fig. 3.4B). However, co-expression of either the IL4Rα-TIR1/IL4Rα-TIR2 or γC-TIR1/γC-TIR2 hybrid receptor pair caused NF-κB activation in a constitutive manner (Fig. 3.5A and B). NF-κB activation mediated by both hybrid pairs sustained when each plasmid was reduced to 9 ng/well. Increasing each plasmid to 150 ng/well did not further enhance NF-κB activation (Fig 3.5A and B). It suggests that NF-κB activation through both hybrid pairs reflected specific interactions between the paired hybrid receptors rather than over-expression. Addition of soluble IL-4 at 100 ng/ml had no significant effect on the observed NF-κB activation. These results, together with the lack of NF-κB activation by the IL4Rα-TIR1/γC-TIR2 and IL4-TIR1/γC-TIR2 pairs under the same conditions, suggest homotypic interactions between the IL-4Rα and γC hybrid receptors through the IL-4Rα and γC EC domains. In another word, IL-4Rα and γC are likely to form homo dimers or higher oligomers.

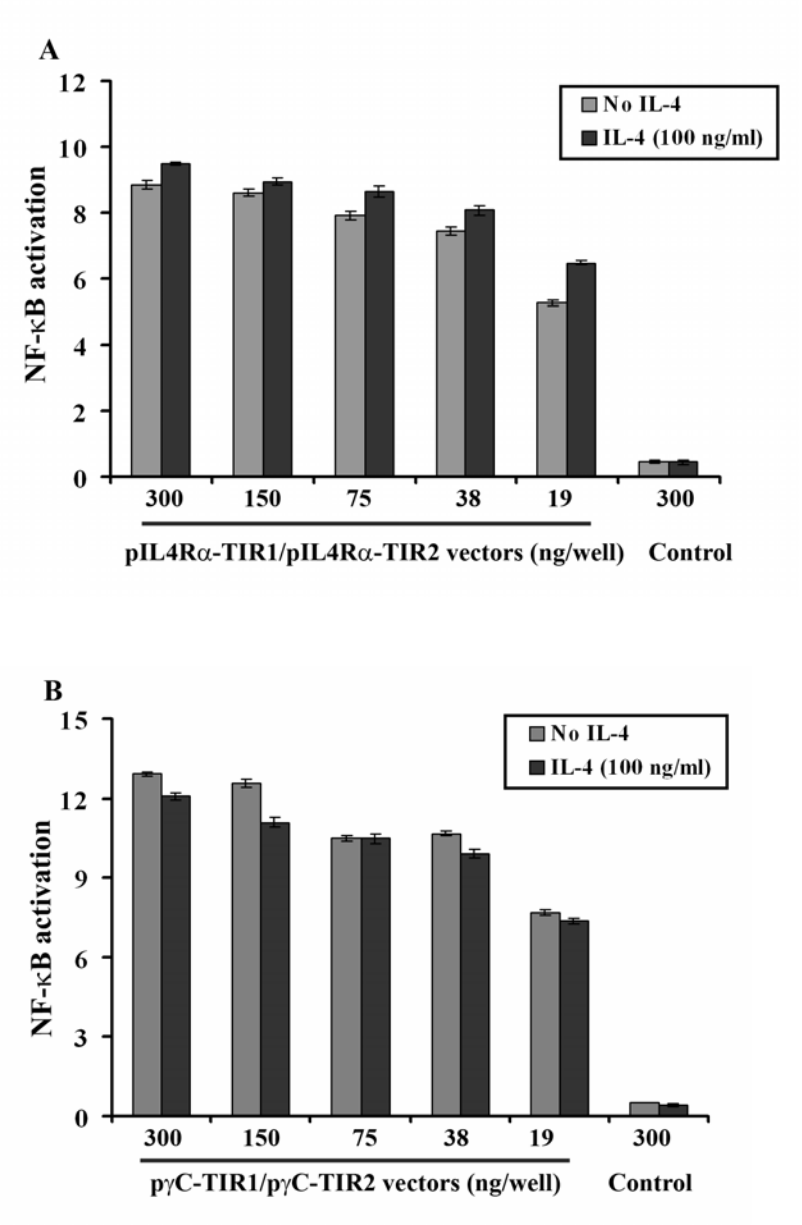


Figure 3.5 Detection of homotypic IL-4Rα and γC interactions. (A) 293T cells were co-transfected with the pIL4Rα-TIR1/pIL4Rα-TIR2 plasmid pair at a series of dilutions as indicated. As a control, 293T cells were also transfected with pcDNA3.1 vector at 300 ng/ml. (B) 293T cells were co-transfected with the pγC-TIR1/pγC-TIR2 plasmid pair at a series of dilutions as indicated. As a control, the cells were tranfected with pcDNA3.1 vector at 300 ng/well. The luciferase reporter plasmids were also co-transfected in these experiments. After 24 hr, the cells were either left unsitmulated (grey bars) or stimulated for 12 hr with IL-4 at 100 ng/ml (black bars). NF-κB activation was determined as described in Fig. 3.3. The data was expressed as means of triplicate experiments ±SD.

Homotypic dimerization has not been reported for IL4Rα or γC. To verify this observation obtained with the TIR1/TIR2-based assay, immunoprecipitation studies were performed. Specifically, the pIL4Rα-TIR2 and pγC-TIR2 expression vectors were modified so that the sequences encoding the TIR2 domain in each hybrid receptor was replaced by that encoding the short Myc/His tags in the pcDNA3.1 vector, i.e. pIL4Rαtm-MH and pγCtm-MH (Fig. 2.5). Here 'tm' indicates the presence of the transmembrane domain which is expected to anchor the hybrid receptor to the cell membrane as for the TIR2 hybrids. The TIR2 domain is approximately 20 KDa in size (Gay and Keith, 1991; Medzhitov et al., 1997; Xu et al., 2000). Therefore these Myc/His-tagged hybrid receptors are expected to be 18 to 20 kDa smaller than IL4Rα-TIR2 and γC-TIR2 respectively. These vectors were co-expressed in 293T cells with the pIL4Rα-TIR2 or pγC-TIR2. The Myc/His-tagged hybrid receptors were precipitated from cell lysates using either a myc antibody or the Ni-NTA-agarose beads. As shown in Fig. 3.6A, when pIL4Rαtm-MH and pIL4Rα-TIR2 were singly expressed in 293T cells, proteins of approximately 50 and 70 kDa (lanes 4 and 5) were detected in the cell lysate by Western blotting using an anti-IL4Rα antibody. These two protein species were not detected in cells transfected with the pcDNA3.1 vector (Fig. 3.6A, lane 6).

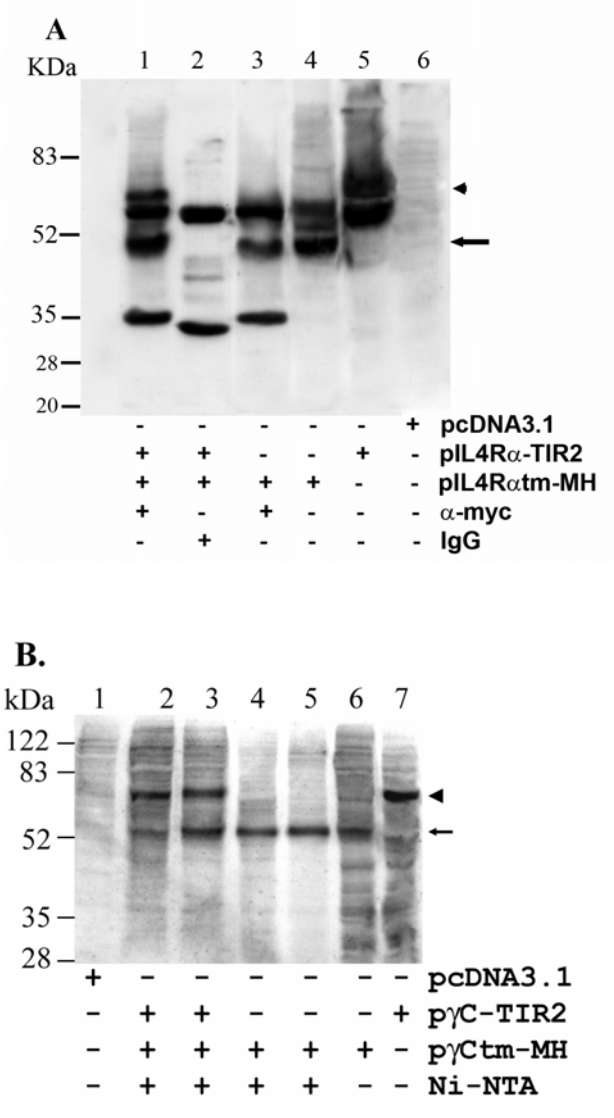
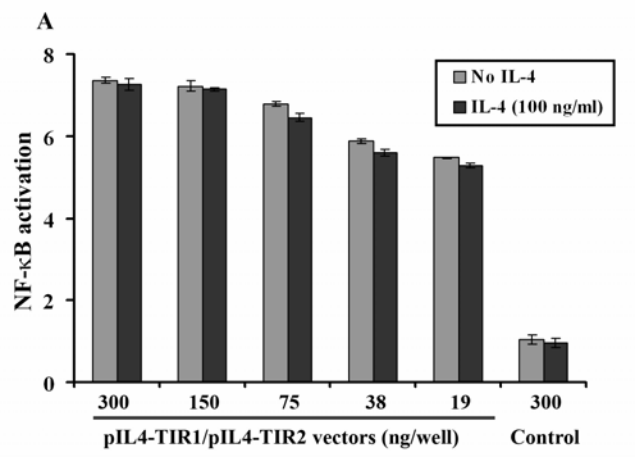


Figure 3.6 Homotypic IL-4Rα and γC interactions detected by immunoprecipitation. (A) 293T cells were singly transfected with pcDNA3.1 (lane 6), pIL4Rα-TIR2 (lane 5), pIL4Rαtm-MH (lane 3 and 4) or co-transfected with the pIL4Rαtm-MH/pIL4Rα-TIR2 plasmid pair (lane 1 and 2). Cells were lysed and the cell lysates were either directly subjected to SDS-PAGE (lanes 4-6) or after immunoprecipitation with an anti-myc antibody (lane 1 and 3) or control isotype mouse IgG (lane 2). The proteins were separated on 10% (w/v) gels under reducing conditions and detected by Western blotting using an anti-IL4Rα antibody. The TIR2 hybrid receptor was indicated with an arrow head and the Myc/His-tagged hybrid molecules in which the TIR1 domain was replaced by the Myc/His tags was indicated with an arrow. (B) 293 T cells were singly transfected with pcDNA3.1 (lane 1), pγC-TIR2 (lane 7), pγCtm-MH (lane 4 and 6) or co-transfected with the pγC-TIR2/pγCtm-MH plasmid pair (lanes 2 and 3). Cells were lysed and the cell lysates were either directly subjected to SDS-PAGE (lanes 1, 6 and 7) or after immunoprecipitation with Ni-NTA-agarose, which precipitates γCtm-MH, followedd by elution with immidazole (lanes 2-5). The first (lane 2 and 4) and second (lane 3 and 5) fractions of the eluted proteins were analyzed. The proteins were separated on 10% (w/v) gels under reducing conditions and detected by Western blotting using an anti-γC antibody expected to detect both γC-TIR2 and γCtm- MH. γC-TIR2 was indicated with an arrow head and γCtm-MH was indicated by an arrow.

When the two-hybrid molecules were co-expressed in 293T cells and IL4Rαtm-MH was immunoprecipitated with an anti-myc antibody, a 50 kDa protein equivalent to IL4Rαtm-MH in size was detected using an anti-IL4Rα antibody (Fig. 3.6A, lane1). A 70 kDa protein, which is equivalent to IL4Rα-TIR2 in size, was also detected with the anti-IL-4Rα antibody in the precipitated proteins (Fig. 3.6A, lane1). Neither protein was precipitated when an isotype-matched mouse IgG was used to mimic IL4Rαtm-MH precipitation (Fig. 3.6A, lane 2). When IL4Rαtm-MH was expressed alone, the 50 KDa protein was precipitated with the anti-myc antibody without the co-precipitation of the 70 kDa protein (Fig. 3.6A, lane 3.). These results show that the EC domains of IL-4Rα interact with each other. Whether the interactions lead to formation of stable homodimers or higher oligomers is unclear. Endogenous wild type IL-4Rα needs investigation to assess the implications of such interactions. In Fig 3.6A (lanes 4 and 5) a protein of approximately 60 KDa was observed. These two lanes contained lysates of 293T cells singly transfected with pIL4Rαtm-MH or pIL4Rα-TIR2. These were not observed in pcDNA3.1-transfected cells. They are likely to represent differentially modified isoforms of IL4Rαtm-MH and IL4Rα-TIR2. Since these proteins co-migrated with the heavy chain of IgG, we could not determine whether they were precipitated in lanes 1 and 3 (Fig. 3.6A)

The γC-TIR1/γC-TIR2 interaction detected using the two-hybrid assay (Fig. 3.6B) was also verified by biochemical studies. In this experiment, the anti-myc antibody was not used to precipitate γCtm-MH as γC-TIR2 was expected to co-migrate with the heavy chain of the IgG antibody on SDS-PAGE. Therefore, it was precipitated using Ni-NTA-agarose which binds to the His tag on γCtm-MH. Bound proteins were eluted using immidazole. When γCtm-MH and γCtm-TIR2 were singly expressed in 293T cells and detected by Western bloting using an anti-γC antibody, the two hybrid receptors were

detected as proteins of approximately 55 and 75 kDa respectively (Fig. 3.6B, lanes 6 and 7). When 293T cells were transfected with pcDNA3.1, neither protein was detected in the cell lysate (Fig. 3.6B, lane 1). When γCtm-MH and γC-TIR2 were co-expressed, Ni-NTA-agarose precipitated the 55 kDa protein, equivalent to γCtm-MH in size. A 75 kDa protein, which is equivalent to γC-TIR2 in size, was co-precipitated (Fig. 3.6B, lanes 2 and 3). When γCtm-MH was expressed singly, it was precipitated as a 55 kDa protein using Ni-NTA-agarose (Fig. 3.6B lanes 4 and 5). In this case, the 75 KDa protein was not detected in the precipitate. These results show that the EC domain of γC is also likely to form homotypic interactions.



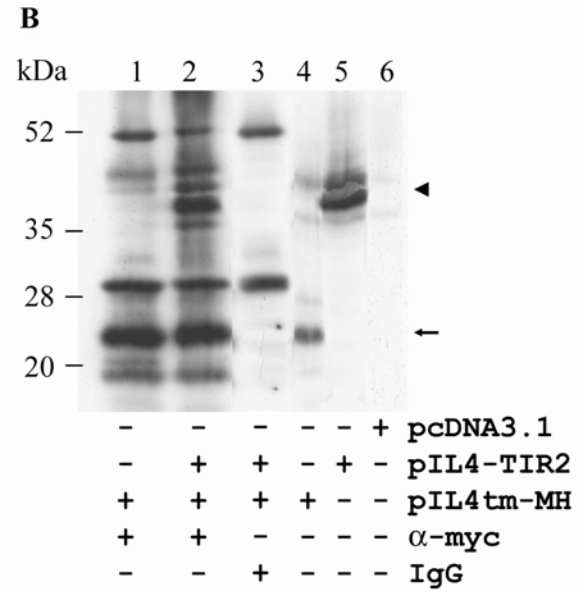


Figure 3.7 Detection of constitutive IL-4/IL-4 interactions. (A) Detection of homotypic interactions between IL-4 using the TIR1/TIR2-based assay. 293T cells were co-transfected with the pIL4-TIR1/pIL4-TIR2 plasmid pair at a series of dilutions as indicated. As a control, pcDNA3.1 (empty) vector was also transfected at 300 ng/well. After 24 hr, the cells were either left unstimulated (grey bars) or stimulated for 12 hr with IL-4 at 100 ng/ml (balck bars). NF-κB activation was measured as described in Fig. 3.4. The data are expressed as means of triplicate experiments. **(B) Detection of homotypic IL-4 interaction by immunoprecipitation.** 293T cells were singly transfected with pcDNA3.1 (lane 6), pIL4-TIR2 (lane 5), pIL4tm-MH (lanes 1 and 4) or co-transfected with the pIL4tm-MH/pIL4-TIR2 plasmid pair (lane 2 and 3). Cells were lysed and the cell lysates were either directly subjected to SDS-PAGE (lanes 4-6) or after immunoprecipitation with the anti-myc antibody (lanes 1 and 2) or isotype mouse IgG (lane 3). The proteins were separated on 10% (w/v) gels under reducing conditions and detected by Western blotting using an anti-IL4 antibody. The TIR2 hybrid receptor was indicated with an arrow head and Myc/His-tagged hybrid molecule was indicated with an arrow.

3.6 Detection of interactions between secreted proteins

As shown in Fig. 3.7A, co-expression of pIL4-TIR1 and pIL4-TIR2 led to potent NF-κB activation when the plasmids were used at 19 ng/well each. Reducing the plasmid dosage each to 9 ng/well did not significantly reduce the observed NF-κB activation and increasing the plasmid dosage to 150 ng/well did not enhance the observed NF-κB activation. This suggests that the observed NF-κB activation reflects specific homotypic interactions between IL-4, when expressed as the EC domains of the hybrid receptors. The homotypic IL-4 interaction detected with the TIR1/TIR2-based assay was also verified by biochemical studies. The pIL4-TIR2 plasmid was modified so that sequence encoding the TIR2 domain was replaced by that encoding the Myc/His-tag (Fig. 2.5). The resultant vector, i.e. pIL4tm-MH, expresses the IL4tm-MH molecule. When IL4tm-MH and IL4-TIR2 were singly expressed in 293T cells, proteins of approximately 22 kDa and 40 kDa were detected in the cell lysates using an anti-IL-4 antibody (Fig. 3.7B, lanes 4 and 5). These two protein species were not detected in 293T cells transfected with pcDNA3.1 (Fig. 3.7B, lane 6). When IL4tm-MH was singly expressed, the anti-Myc antibody only precipitated the 22 kDa protein (Fig. 3.7B, lane 1). When IL4-TIR2 was co-expressed with IL4tm-MH, IL4-TIR2 was co-precipitated with IL4tm-MH using the anti-myc antibody (Fig. 3.7B, lane 2). When a control mouse IgG was used in place of the anti-myc antibody, neither hybrid molecule was precipitated from the co-transfected 293T cells (Fig. 3.7B, lane 3). Taken collectively, these results suggest homotypic interaction of IL-4 at least when it is expressed on the cell surface as the EC domains of the hybrid receptors. It is unclear from three-dimensional crystallography (Walter et al., 1992; Wlodawer et al., 1992) or NMR (Powers et al., 1992; Smith et al., 1992) study whether IL-4 exist as dimer in nature. However, it is possible that IL-4 like other cytokines such as MCSF (Pandit et al., 1992), IL-5 (Devos et al., 1993; Karlen et

al., 1998) and IFN-γ (Greenlund et al., 1993; Walter et al., 1995) may form dimer by presenting its distinct and identical surfaces to attract the second receptor molecule to the initial complex of IL-4/IL-4Rα. This binding may result in the generation of intracellular signals initiating their effector function.

3.7 Conclusion

In this part of the study, it has been shown that the TIR1/TIR2-based two-hybrid assay can detect the known interactions betweenIL-4 and IL-4 receptor subunits (i.e. IL4Rα and γC). Novel homotypic interactions of IL- 4, IL4Rα and γC were also detcted using this TIR1/TIR2-based assay and these interactions were further confirmed by immunoprecipitation studies. These findings have important implications. Firstly, this TIR1/TIR2-based assay proves to be an easy and direct method to investigate the interactions between secreted proteins, i.e. soluble and secreted proteins or membrane-bound proteins. Secondly, many receptors transduce cell signaling through ligand-induced dimerization of different receptor subunits. This assay allows the investigation of three-way protein-protein interactions in live cells i.e. ligand and two recptor subunits. Thirdly, the convenience to express any protein as the EC domain in fusion with TIR1 or TIR2 would allow the identification of novel protein-protein interactions at increased throughput. Finally, this method complements with the yeast two-hybrid method allowing detection of interaction between secreted/membrane proteins whereas the latter detects mainly interactions between cytoplasmic proteins. This method can be used for drug development by screening for inhibitors of protein-protein interactions. The application of this TIR1/TIR2-based two-hybrid assay in the understanding of CD14-CD14 interaction is documented in the next chapter.

Chapter 4 Investigation of CD14 dimerization and its role in CD14 signal transduction

In this chapter the TIR1/TIR2-based two-hybrid assay was used to examine whether CD14 forms dimers. CD14 is a GPI-anchored protein, expressed on the surface of monocytes, macrophages, and polymorphonuclear leukocytes (Simmons et al., 1989). It is responsible for the effects of LPS on these cells (Wright et al., 1990; Hailman et al., 1994). The role of CD14 in LPS signaling has been confirmed using CD14 knock-out mice. CD14-deficient mice are highly resistant to septic shock upon injection of LPS or live bacteria (Haziot et al., 1996). Structurally, crystallography studies have indicated that CD14 may form dimers on the cell surface (Kim et al., 2005), but the functional relevance of CD14 dimerizaiton has not been addressed. Therefore it is of interest to examine CD14-CD14 interactions and how this modulates its function as the LPS receptor. The TIR1/TIR2-based two-hybrid assay will be applied in this investigation.

4.1 Detection of homotypic interaction between CD14 using the TIR1/2-based assay

Soluble mouse CD14 forms a dimer in the crystallographic asymmetric unit as well as in solution (Kim et al., 2005). To verify if CD14 forms dimers on the cell surface, its EC domain were expressed as TIR1 and TIR2 fusion proteins, i.e. CD14-TIR1 and CD14-TIR2 (Fig. 2.4). 293T cells were co-transfected with these two plasmids at 1:1 ratio and different plasmid dosages were used from 19 to 300 ng/well. Total plasmid dosage in all experiments was normalized to 300 ng/well using the pcDNA3.1 vector. As a negative control, 293T cells were transfected with the plasmids pCD14-TIR1, pCD14-TIR2 or pcDNA3.1 individually. Luciferase reporter plasmids were also co-transfected in every experiment. In some experiments the transfected cells were treated

for 12 hr with LPS (500 ng/ml) 24 hr after transfection. Interaction between CD14-TIR1 and CD14-TIR2 was judged by the activation of NF-κB.

Transfection of 293T cells with pCD14-TIR1 or pCD14-TIR2 alone (300 ng/well) did not lead to NF-κB activation (Fig. 4.1A). Similarly, NF-κB activation was not increased in 293T cells transfected with the pcDNA3.1 vector. However, co-transfection of 293T cells with pCD14-TIR1 and pCD14-TIR2 caused NF-κB activation. Decrease of plasmid dosage to 19 ng/well did not reduce NF-κB activation. Increasing each plasmid to 150 ng/well did not further increase NF-κB activation (Fig. 4.1A). This suggests that the NF-κB activation observed after CD14-TIR1 and CD14-TIR2 co-expression reflected CD14-CD14 interactions rather than over-expression of the hybrids. Addition of soluble LPS to the transfected 293T cells had no significant effect on the observed NF-κB activation (Fig. 4.1A). As another set of controls, pIL4Rα-TIR1 or pβ5-TIR1 was used to co-transfect 293T cells with pCD14-TIR2 (each 19 ng/well). Co-expression of IL4Rα-TIR1 or β5-TIR2 with CD14-TIR2 caused little NF-κB activation (Fig. 4.1B). In contrast, co-expression of CD14-TIR1 and CD14-TIR2 led to potent NF-κB activation further excluding the possibility that over-expression of CD14-TIR1 and CD14-TIR2 caused the observed NF-κB activation (Fig. 4.1B). Collectively, these results suggest that CD14 forms dimer thorough its EC domains on the cell surface.

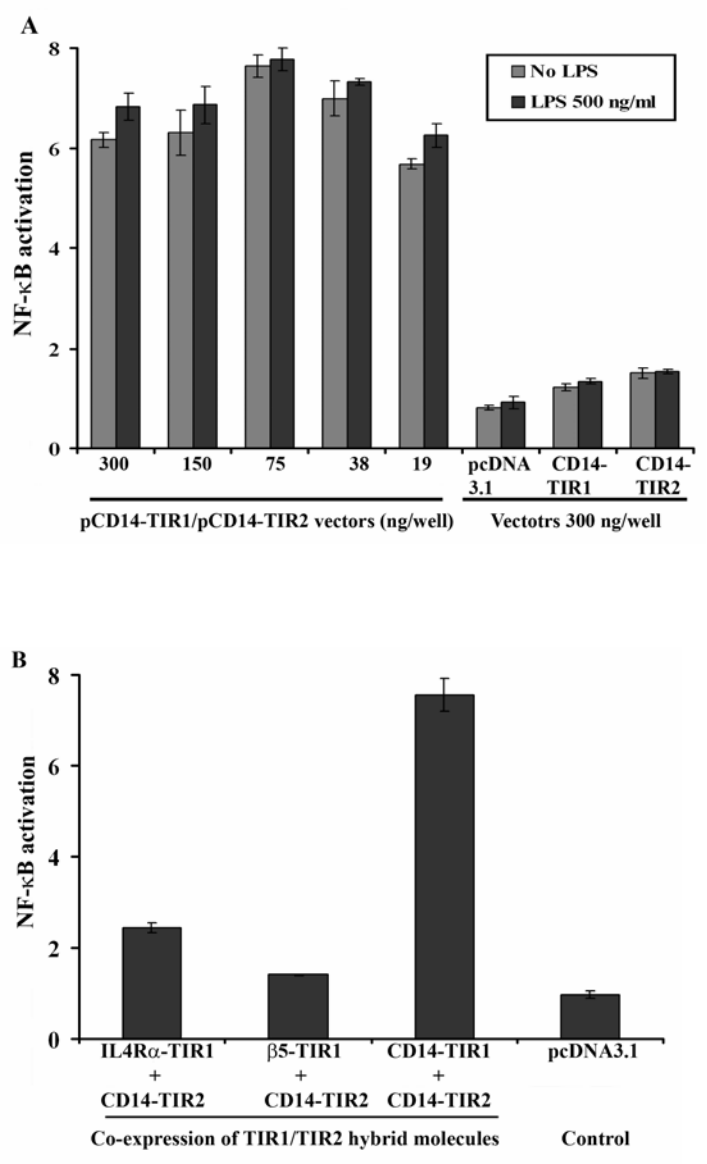


Figure 4.1 Detection of CD14-CD14 homotypic interaction using the TIR1/TIR2-based assay. (A) 293T cells were transfected with pCD14-TIR1 and pCD14-TIR2. The two plasmids were used at a 1:1 ratio and the combined plasmid dosage in each experiment was as indicated. Total expression vector in each experiment was normalized to 300 ng/well with pcDNA3.1. As controls, the cells were singly tranfected with the pCD14-TIR1, pCD14-TIR2 or the pcDNA3.1 plasmid at 300 ng/well. In all experiments, cells were also co-transfected with the p5xNF-κB-luc and pRL-CMV reporter plasmids each at 100 ng/well. After 24 hr, transfected cells were either left unstimulated (grey bar) or stimulated (black bar) with LPS for 12 hr at 500 ng/ml. NF-κB-directed firefly luciferase and CMV-directed *Renilla* luciferase expression were determined using the Dual Luciferase assay (Promega). NF-κB activation was presented after being normalized to the *Renilla* luciferase activity and expressed as the percentage of the latter. (B) pIL4Rα-TIR1, pβ5-TIR1 or pCD14-TIR1 was co-expressed with pCD14-TIR2. Each vectors were used at 19 ng/well. The luciferase reporter plasmids were co-transfected and NF-κB activation was determined using luciferase assay. As a control, 293T cells were also tranfected with pcDNA3.1 (300 ng/well). Results are shown as means ±SD and representative of at least three independent experiments.

4.2 Identification of amino acid residues involved in CD14-CD14 interactions

To investigate the amino acid residues requires for CD14 homo-dimerization, mutagenesis of CD14 was carried out on the conserved residues located on β13 and the 'loop' between β12 and β13. This region has been indicated in CD14-CD14 dimerization in the crystallography study (Kim et al., 2005). Firstly, two CD14 deletion mutants were generated in CD14-TIR1 removing amino acids 290-300 (the 'loop' between β12 and β13) or 301-315 (β13) which were named loopdelCD14-TIR1 and β13delCD14-TIR1 respectively. Details of the deletions are described in section 2.5. 293T cells were co-transfected with CD14-TIR2 and one of these deletion mutants (each 19 ng/well). After 24 hr, luciferase activity of 293T cells was measured. Co-expression of either deletion mutant with CD14-TIR2 showed significantly reduced NF-κB activation compared to 293T cells co-transfected with CD14-TIR1 and CD14-TIR2 (Fig. 4.2A).

Next, specific amino acids in β13 and the 'loop' between β12 and β13 of CD14 were mutated into alanine by site-directed mutagenesis (chapter. 2.5, Table 2.5). Alanine substitution is widely used because it is a small amino acid commonly found in proteins, occurring in all secondary structures without particular steric requirement (Wells, 1991). Such substitutions allows for the elimination of functional properties of the original residues without disturbing local protein conformation. The follwoing alanine substitutions were introduced to the pCD14-TIR1 vector, i.e. L290A, P294A, E298A, L299A, P300A, V302A, L305A, and L307A. These CD14-TIR1 mutants were co-transfected with CD14-TIR2 into 293T cells and each plasmid was used at 19 ng/well. After 24 hr, luciferease activity was measured. As shown in Fig. 4.2B, co-expression of P294ACD14-TIR1 or P300ACD14-TIR1 with CD14-TIR2 caused partially but

significantly reduced NF-κB activation compared to the CD14-TIR1/CD14-TIR2 co-expression. Co-expression of L290ACD14-TIR1 or L307ACD14-TIR1 with CD14-TIR2 caused more significant reduction in NF-κB activation compared to CD14-TIR1/CD14-TIR2 co-expression (Fig. 4.2B). However, co-expression of double mutants CD14-TIR1 (L290A,L307A) with CD14-TIR2 caused reduction of NF-κB activation to almost background level (as shown by the control pcDNA3.1). The rest alanine substitutions on CD14-TIR1 (i.e. E298A, L299A, V302A and L305A) had no effect on its ability to cause NF-κB activation when co-expressed with CD14-TIR2 (Fig. 4.2B). These results suggest that L290 in the 'loop' and L307 in β13 are critical to CD14-CD14 dimerization. P294 and P300 in the same 'loop' may also be involved in CD14-CD14 dimerization.

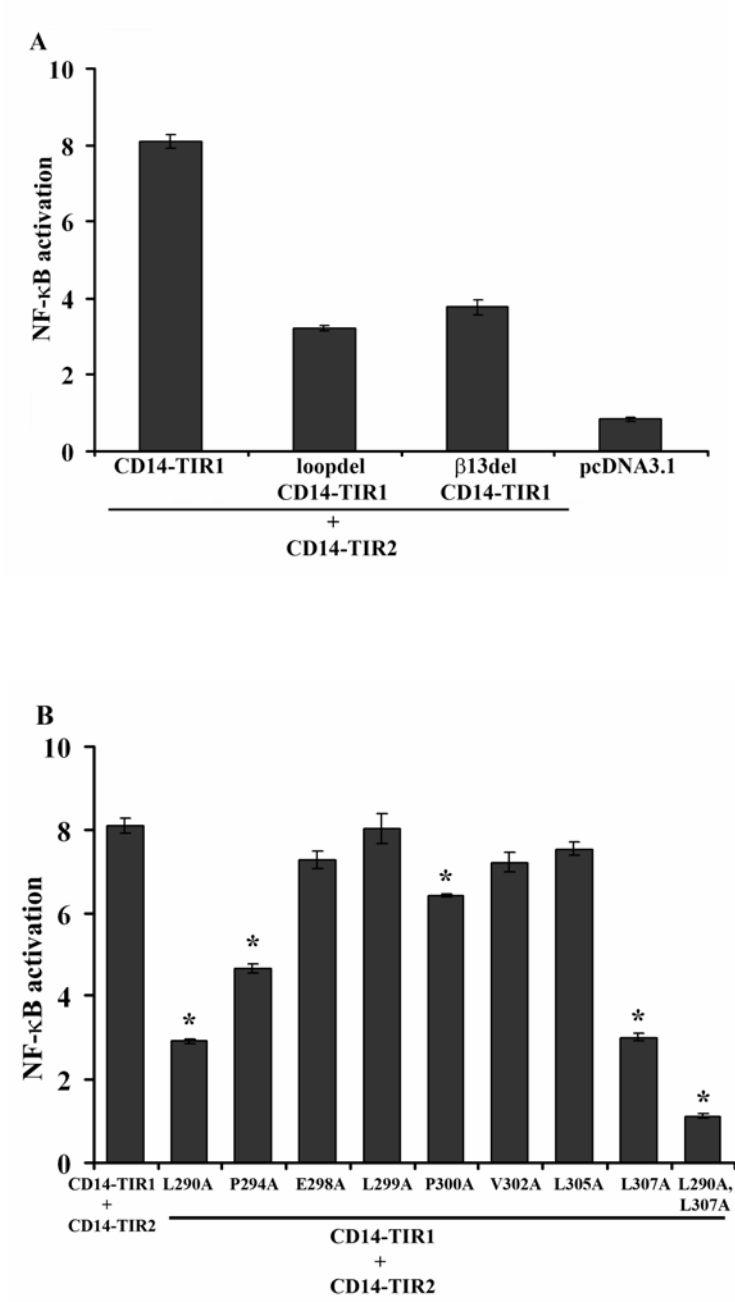


Figure 4.2 Effects of CD14 deletions and point mutations in its loop between β12/β13 and β13 on its dimerization. (A) 293T cells were co-transfected with pCD14-TIR1/pCD14-TIR2 as a positive control. 293T cells were also co-transfected with ploopdelCD14-TIR1 or pβ13delCD14-TIR1 deletion mutant and pCD14-TIR2 (each at 19 ng/well). (B) 293T cells were co-transfected with CD14-TIR2 and either pCD14-TIR1 or one of the residue specific pCD14-TIR1 mutants as indicated. As a negative control, pcDNA3.1 was used to transfect 293T cells at 300 ng/well in both experiments. The luciferase reporter plasmids were also co-transfected. NF-κB activation was determined using the luciferase assay. Results are expressed as mean ±SD of triplicate experiments and each is representative of three similar experiments. * Significant difference ($p \leq 0.002$) was observed between specific mutants CD14-TIR1/CD14-TIR2 and CD14-TIR1/CD14-TIR2.

4.3 Effect of CD14 mutations on LPS-induced NF-κB activation and IL-8 production

While CD14 binds to LPS, it requires the TLR4/MD-2 complex to activate cellular signaling (Shimazu et al., 1999; Akashi et al., 2000; Yang et al., 2000). TLR4-mediated NF-κB activation was investigated in association with WtCD14 and CD14 mutants. Vectors for WtCD14 and mutant CD14 were used at 20 ng/well and vectors for TLR4 and MD2 were each used at 5 ng/well. After 24 hr, the cells were stimulated with LPS (500 ng/ml) for another 12 hr. As shown in Fig. 4.3A, the P294A and P300A CD14 mutants exhibited reduced NF-κB activation in response to LPS compared to WtCD14. The E298A, L299A, V302A, and L305A CD14 mutants showed no difference from WtCD14 (Fig. 4.3A). The L290A and L307A mutants exhibited markedly reduced NF-κB activation compared to WtCD14 (Fig. 4.3A). These two mutations, when introduced in CD14-TIR1, also caused the most reducion in NF-κB activation (Fig. 4.2B). The effects of these CD14 mutations on LPS-induced IL-8 production from 293T cells were also examined. 293T cells co-transfected with the L290A and L307A CD14 mutants also produced the least IL-8 upon LPS stimulation (Fig. 4.3B). However, double mutation of two leucine (L290A and L307A), reduced NF-κB activation and IL-8 production to 70% of WtCD14. Whether the loss of LPS responsiveness by these double mutants is due to their low level of surface expression will be addressed in the following section. Other CD14 mutations showed no difference from WtCD14 in mediating LPS-induced IL-8 production (Fig. 4.3B). The P294A and P300A mutants rendered slightly reduced IL-8 production (Fig. 4.3B). In summary, amino acids L290 and L307, which are required for CD14-CD14 interactions (Fig. 4.2B), are also important for TLR4-mediated NF-κB activation and IL-8 production in response to

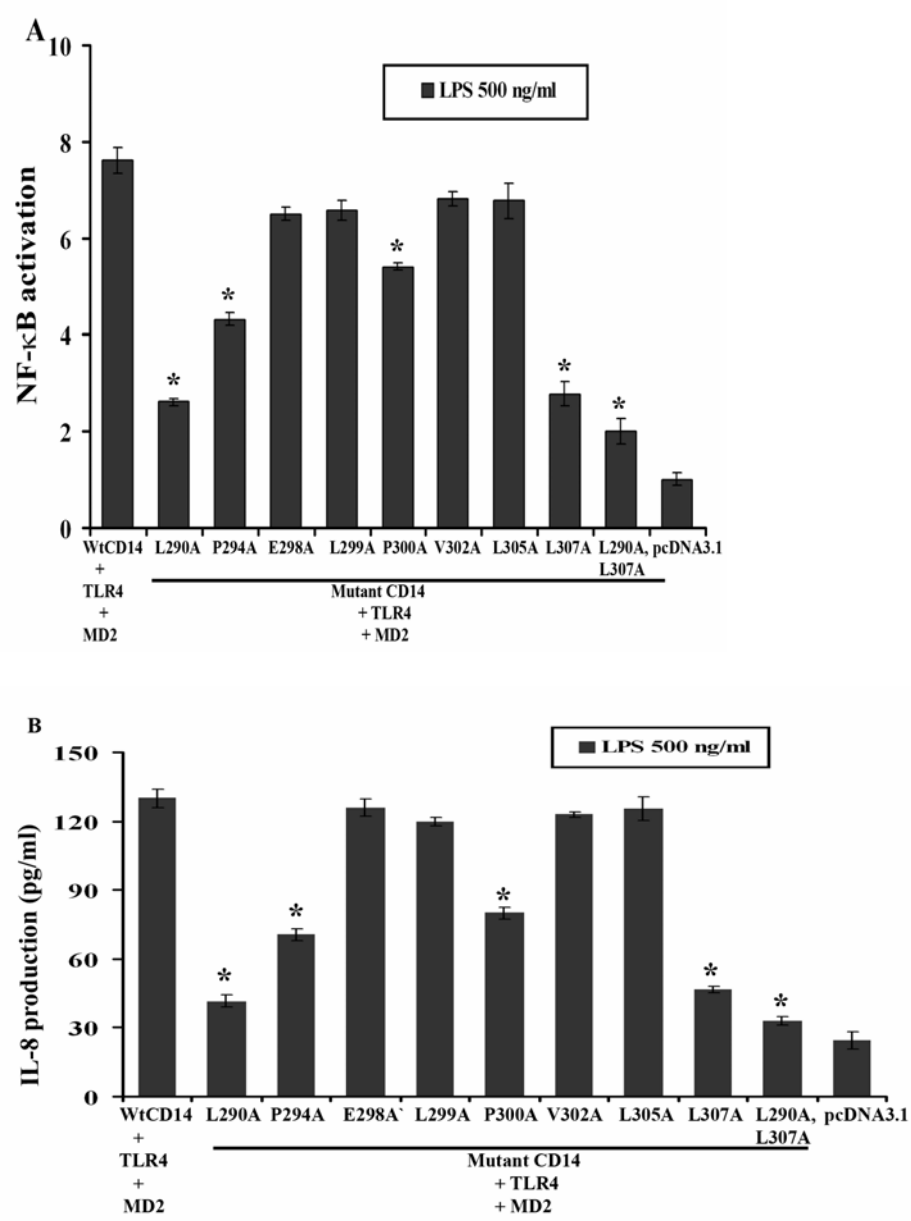


Figure 4.3 Effect of CD14 mutations on its response to LPS. (A) 293T cells were transfected either with wild-type (Wt) CD14 plasmid (40 ng), or the mutant CD14 plasmid indicated (40 ng), together with TLR4 (5 ng) and MD2 (5 ng) plasmids. As a control, the cells were transfected with the pcDAN3.1 plasmid at 300 ng/well. The cells were also co-transfected with the p5xNF-κB-luc and pRL-CMV luciferase reporter plasmid (200 ng/well). After 24 hr, transfected cells were stimulated with LPS at 500 ng/ml for 12 hr and processed for the determination of both NF-κB-directed firefly luciferase and CMV-directed *Renilla* luciferase activities using Dual luciferase assay system (Promega). Firefly luciferase activity, which reflects NF-κB activation, was normalized to the *Renilla* luciferase activity. The final values were expressed as a percentage of the latter. All results are representative of at least three similar experiments and are presented as means ± S.D. (B) Cell free supernatants were assayed for IL-8 production from the same experiments by ELISA. Results are the average of triplicate wells ± S.D. * Significance difference ($p < 0.05$) was observed between specific mutantsCD14 indicated and WtCD14.

LPS. It also shows that CD14 dimerization or oligomerization is physiologically relevant and can potentially modulate host response to LPS.

4.4 Cell surface expression of CD14 and its mutants

Differential expression of the CD14 mutants may also occur which in turn affects NF-κB activation. This was examined by surface biotinylation of transfected 293T cells using a membrane-impermeable biotinylaion reagent. The biotinylated proteins were precipitated with streptavidin-agarose beads and the proteins were eluted in an SDS-sample buffer. The proteins were analyzed by Western bloting with an anti-CD14 polyclonal antibody. As a control, a duplicate copy of the blot was probed using an anti-β-actin monoclonal antibody (Fig. 4.4A). Without surface biotinylation, CD14-TIR1 and CD14-TIR2 were not detected in the precipitate lysates showing that CD14-TIR1 and CD14-TIR2 were not specifically precipitated with strepavidin beads (Fig. 4.4A). With surface biotinylation, CD14-TIR1 and CD14-TIR2 were detected in the cell lysates with a molecular weight of 75 KDa (Fig. 4.4A). The deletion mutants loopdelCD14-TIR1 and β13delCD14TIR1 showed similar levels of expression as CD14-TIR1 (Fig. 4.4A). The total proteins loaded for each lysate were similar as shown by the similar levels of β-actin detected in the blot (one on a parallel blot) (Fig. 4.4A,). It shows that these deletions had no significant effect on CD14 expression. But these mutations reduced CD14-CD14 interaction and response to LPS (Fig. 4.2 and Fig 4.3) The CD14-TIR1 single residue mutants, i.e L290A, P294A, E298A, L299A, P300A, V302A, L305A and L307A were similarly expressed in 293T cells and detected by western bloting. As a negative control, the cells were also transfected with the pcDNA3.1 vector. No signal was detected in the lysates of control 293T cells (Fig. 4.4B, upper panel). Biotinylated CD14-TIR1 mutants, i.e. L290A, P294A, E298A,

L299A, P300A, V302A, L305A and L307, were all detected in the respective cell lysates as a 75 KDa band. All CD14-TIR1 mutants were efficiently expressed on the cell surface. No significant difference was observed between the different mutants and the original CD14-TIR1 (Fig. 4.4B).

The expression of full-length CD14 on transfected 293T cells was also examined by surface biotinylation and Western blotting. WtCD14 and CD14 mutants (i.e.L290A, P294A, E298A, L299A, P300A, V302A, L305A and L307A) were expressed in 293T cells and later subjected to biotinylation and Western blotting. As shown in Fig. 4.4C (upper panel), the expression levels of the CD14 mutants are largely compatible to that of WtCD14 despite some minor variations. The protein band detected on this blot is 55 KDa in size which is equivalent to full-length CD14. As a negative control, CD14 was not detected in the lysates of pcDNA3.1-transfected cells. Protein loading for each sample was indicated based on the level of β-actin which is apparently similar (Fig. 4.4B and 4.4C, lower panels). These results suggest that the reduced NF-κB activation and/or IL-8 induction by some CD14 mutants, especially L290A and L307A, is not due to significantly differential expression of these mutants. However, double mutation of two leucine (L290 and L307) in CD4-TIR1 (Fig. 4.4B) and full-length CD14 (Fig. 4.4C) resulted in the complete loss of surface expression compared to the respective single leucine mutants. These double mutants have significantly reduced CD14-TIR1/CD14-TIR2 mediated NF-κB activation (Fig. 4.2B) and LPS responsiveness (Fig. 4.3). Double mutation of L290 and L307 probably affected the structural integrity of the region, resulting in loss of expression levels of both CD14-TIR1 and the full-length CD14.

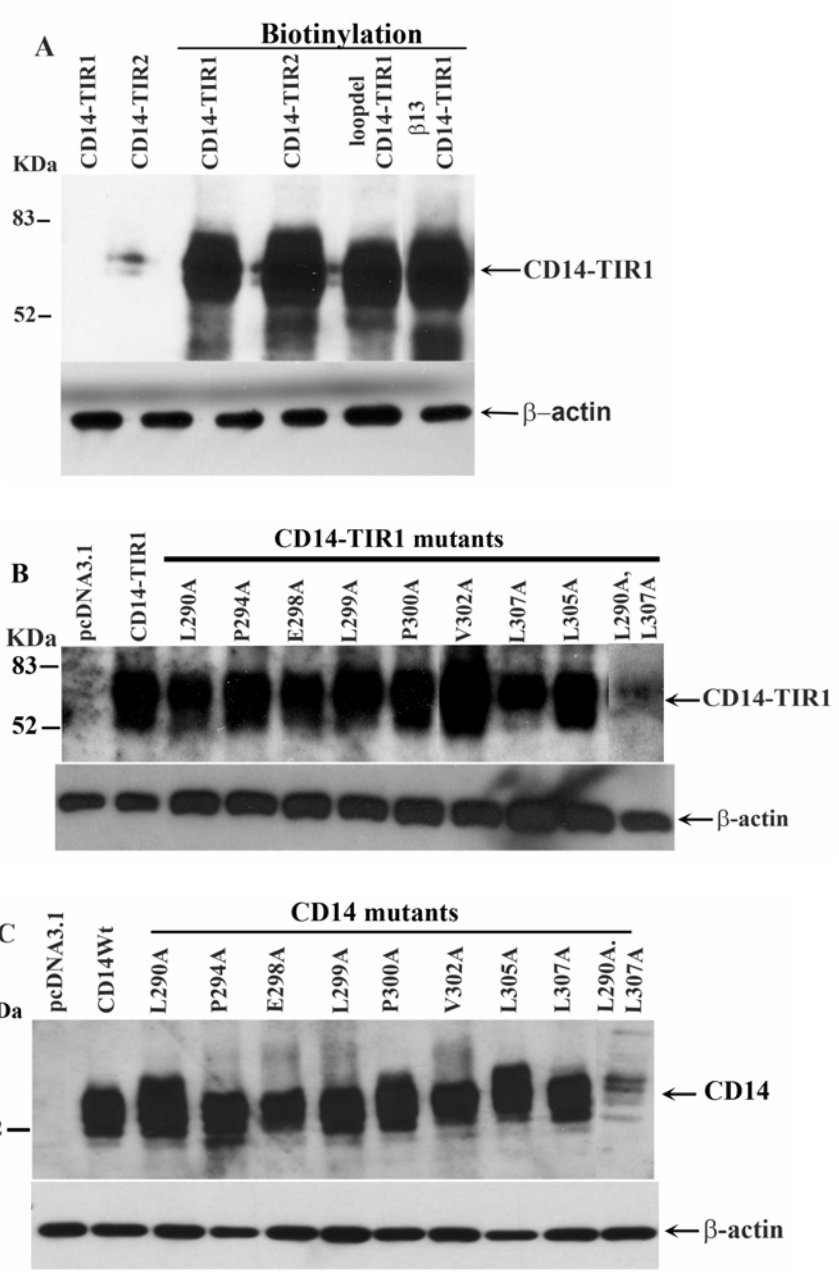


Figure 4.4 Cell-surface expressions of CD14 and CD14 mutants. (A) 293T cells were transfected with either pCD14-TIR1, pCD14-TIR2 or the CD14-TIR1 deletion mutants (loopdelCD14-TIR1 and β13delCD14-TIR1) in 6-well plates (3 μg/well). (B) 293T cells were transfected with pcDNA3.1, pCD14-TIR1, or vectors for the CD14-TIR1 single residue mutants (L290A, P294A, E298A, L299A, P300A, V302A, L305A and L307A) and double mutants (L290A,L307A) in 6-well plates (3 μg/well). (C) 293T cells were similarly transfected with pcDNA3.1, WtCD14, or CD14 single residue mutants (3 μg/well). After 36 hr, the cells were surface biotinylated with membrane-impermeable biotin, and then lysed with lysis buffer. Biotynylated membrane proteins were precipitated from the lysates by incubation with streptavidin-agarose beads. After washing, the beads were boiled in SDS sample buffer under reducing conditions. The eluted proteins were separated on 10% (w/v) SDS-PAGE gels and analyzed by Western blotting using an anti-CD14 polyclonal antibody and a similar blot was probed using an anti-β-actin monoclonal antibody.

4.5 Detection of CD14 homotypic interaction on the cell surface by cross-linking studies

Having shown that CD14 form homotypic interactions using the TIR1/TIR2-based two-hybrid assay, identified specific residues involved in CD14-CD14 interactions, and demonstrated that mutation of these residues altered CD14 signaling for NF-κB activation and IL-8 production, the roles of these residues in CD14-CD14 interaction were further verified using a conventioned cross-linking method. 293T cells were transfected to express WtCD14 or the CD14 mutants (L290A, P294A, E298A, L299A, P300A, V302A, L305A or L307A). pcDNA3.1-transfected 293T cells were used as a control. Cell surface proteins were cross-linked using dithiobis (sulphosuccinimidyl propionate), a membrane-impermeable bifunctional cross-linking reagent. It has an amine reactive N-hydroxysulfosuccinimide (sulfo-NHS) ester at each end of the molecule with a cleavable 12-A° spacer arm containing a disulfide bridge. Thus proteins that are in close proximity on the cell surface are cross-linked with this reagent. The cells were then lysed and total proteins were seperated on SDS-PAGE in the presence or absence of the reducing agent β-mercaptoethanol that breaks the disulfide bridge and allows cross-linked proteins to be separated and migrate as separate bands on SDS-PAGE. Without a reducing reagent, cross-linked proteins migrate together on SDS-PAGE as dimers or oligomers.

Under reducing conditions a 55 KDa band corresponding to CD14 monomers was detected in 293T cells that were tranfected to express WtCD14 and the CD14 mutants (Fig. 4.5A). The levels of expression were similar. Under non-reducing conditions, besides the 55 KDa band, a heterogenous group of high molecular weight CD14-containing complexes (> 80 kDa) were also detected in 293T cells that were transfected

to express WtCD14 (Fig. 4.5B). Thus CD14 may exist as monomer on the cell surface, but dimers or higher oligomers also exist. The existance of CD14 dimers was also indicated in crystallographic structure and it showed that these dimers were made up of the same CD14 monomer subunits. The subunit was indicated to be the β13 and the loop of β12 and β13 (Kim et al., 2005). The CD14-containing high molecular weight materials were also found in the lysates of CD14 mutants that exhibited normal NF-κB activation and IL-8 induction. But it is poorly detected in cell lysates of CD14 mutants that exhibited reduced capacity in mediating LPS-stimulated signaling in transfected 293T cells (i.e. L290A, L307A, P294A and P300A) (Fig. 4.5B). After inter-molecular linkages are disrupted, the high molecular weight materials returned to the 55 kDa form (Fig 4.5A). These proteins were not detected in cells tranfected with pcDNA3.1 showing the specificity of the anti-CD14 antibody (Fig. 4.5A and B). These results suggest that amino acids L290, L307 and possibly P294 and P300 contribute to CD14 dimerization or homotypic ineraction.

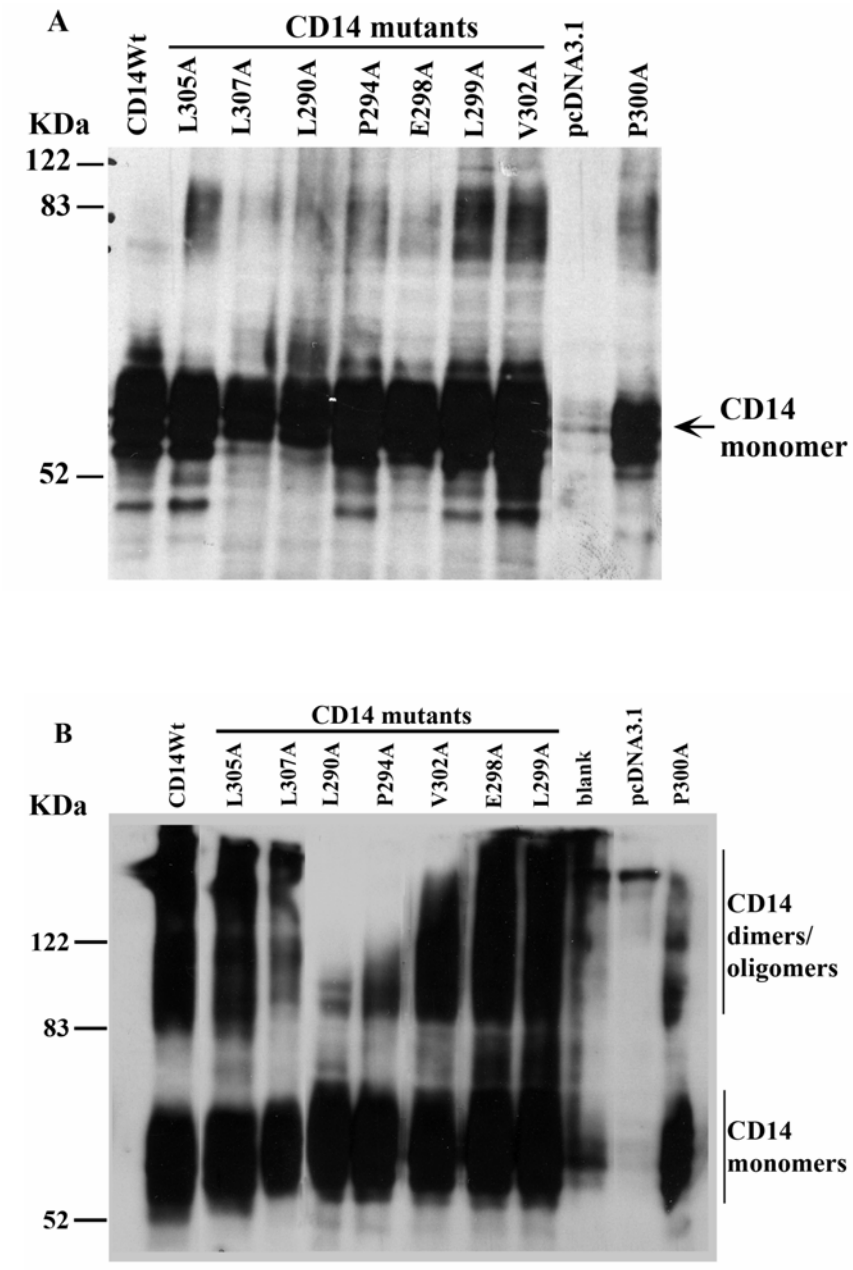


Figure 4.5 Detection of CD14-CD14 dimers. 293T cells were singly transfected either with WtCD14 or CD14 mutants (i.e. L290A, P294A, E298A, L299A, P300A, V302A, L305A and L307A) in 6-well plates (3 µg each). After 36 hr, the cells were washed and exposed to the membrane-impermeable cross-linking reagent. Cell lysates were prepared using a lysis buffer and separated on 10% (w/v) SDS-PAGE gels under reducing **(Fig. 4.5A)** and non-reducing **(Fig. 4.5B)** conditions. The proteins were detected by Western bloting using a polyclonal antibody against human CD14. The molecular weight standards were indicated on the left.

4.6 Conclusion

In this study, the TIR1/TIR2-based two-hybrid assay has been used to investigate CD14-CD14 interactions and it showed that the CD14 EC domain form homo-dimers or homotypic interactions. The β13 sheet and the 'loop' between β12 and β13 were predicted, based on the CD14 crystal structure, to mediate CD14-CD14 interactions. By mutagenesis studies, in combination with the two-hybrid assay, allowed the identification of amino acids L290 and L307 in this region was found to be important in CD14-CD14 inteactions. These two residues are also required for TLR4-mediated LPS signalling of NF-κB activation. These findings have provided new insights into the mechanism by which CD14-CD14 interaction regulates LPS signalling and new assays for the development to therapeutic agents against septic shock.

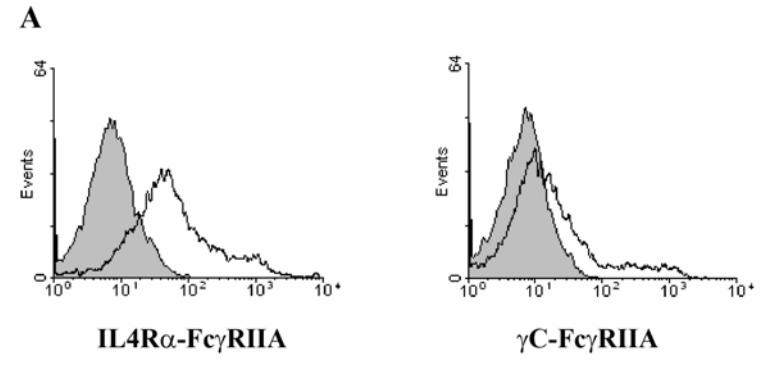
Chapter 5 Investigation of FcγR activation

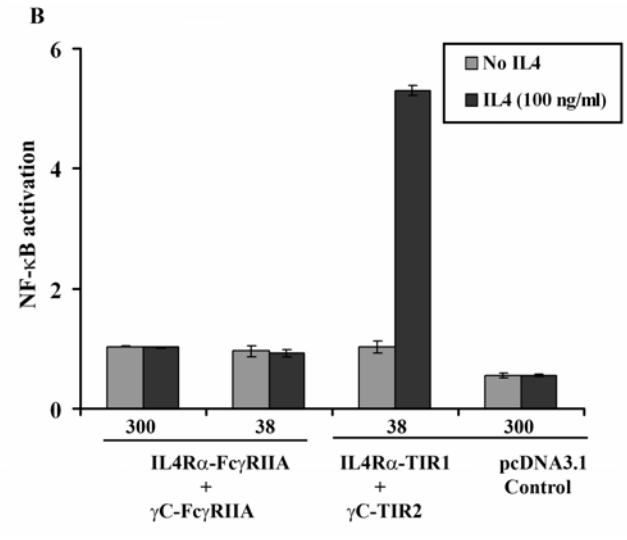
5.1 Investigation of FcγRIIA signalling through IL-4 induced dimerization: a modified two-hybrid assay

In chapters 3 and 4, the TIR1/TIR2-based two-hybrid assay has proved to be a feasible method in detecting protein-protein interactions on cell membrane. The IL-4-induced IL-4Rα and γC dimerization was used to demonstrate the feasibility of using TIR1 and TIR2 to detect protein-protein interactions. Here, the IL-4/ IL-4R system was also used to investigate whether FcγRs can be activated upon induction of dimerization. In this case, the transmembrane and cytoplasmic domains of TLR1 in the IL4Rα-TIR1 and γC-TIR1 chimeras were replaced by that of FcγRIIA to generate the IL4Rα-FcγRIIA and γC-FcγRIIA chimeras. The rationale is that, IL-4 induces the EC domains of IL-4Rα and γC to dimerize and this should cause dimerization of the FcγRIIA cytoplasmic domain. Whether FcγRIIA dimerization elicits FcγRIIA signaling is monitored by examining NF-κB activation and IL-8 production.

The expression of the pIL4Rα-FcγRIIA and pγC-FcγRIIA chimeras on transfected 293T cells was examined by flow cytometry using the anti-IL4Rα and anti-γC antibodies as described in chapter 3 (section 2) and both chimeric receptors were detected on the cell surface (Fig. 5.1A). To examine whether FcγRIIA dimerization activates FcγRIIA signaling, the two chimeras were co-expressed on 293T cells and the cells were stimulated with IL-4. NF-κB activation was measured. As shown in Figure 5.1B, co-expression of pIL4Rα-FcγRIIA and pγC-FcγRIIA in 293T cells did not induce NF-κB activation irrespective of the plasmid dosages (38 or 300 ng/well). Addition of IL-4 also failed to induce NF-κB activation in the transfected cells. As a positive control, while co-expression of pIL4Rα-TIR1 and pγC-TIR2 (38 ng/well) did not cause NF-κB

activation, it was induced by IL-4 (Fig. 5.1B). This seems to suggest that FcγRIIA dimerization might not be sufficient to cause FcγRIIA signaling and induce NF-κB activation. It is unclear from this experiment whether other siganling events may be elicited in the IL-4 induced FcγRIIA dimerization. Similarly, while IL-8 production was induced with IL-4 when 293T cells were transfected with p IL4Rα-TIR1 and γC-TIR2, it was not induced when the cells were transfected with IL4Rα-FcγRIIA and γC-FcγRIIA (Fig. 5.1C)





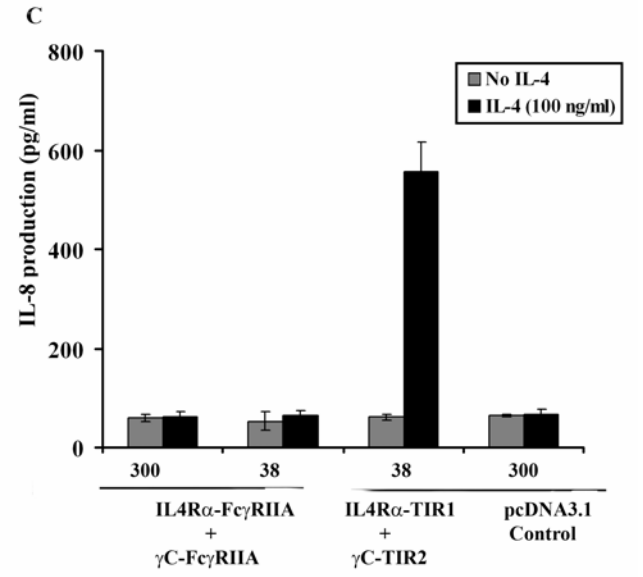


Figure 5.1 Examination of FcγRIIA signaling through IL-4-induced dimerization. (A) pIL4Rα-FcγRIIA and pγC-FcγRIIA were individually expressed in 293T cells at 150 ng/ml and the expression of these hybrid receptors were examined by flow cytometry using specific monoclonal antibodies for IL4Rα and γC. The filled profiles are signals obtained from 293T cells transfected with the pcDNA3.1 vector. Open profiles represent signals detected on 293T cells transfected with the expression vectors. (B) The pIL4Rα-FcγRIIA/pγC-FcγRIIA and pIL4Rα-TIR1/pγC-TIR2 vector pairs were co-expressed in 293T cells as indicated and NF-κB activation was determined using the luciferase assay. As a control, the pcDNA3.1 vector was used to transfect 293T cells. (C) Cell free supernatants were assayed for IL-8 production from the same experiments by ELISA. Results are the average of triplicate wells ±SD.

5.2 Full-length FcγRIIA can mediate NF-κB activation and IL-8 production in transfected 293T cells in response to IgG-opsonized DH5α but not IgG-beads

Since IL-4, which was expected to induce FcγRIIA dimerization in this experimental setting in 293T cells, did not induce NF-κB activation, it was asked whether wild type FcγRs were able to activate NF-κB upon expression in 293T cells. Full-length (FL) FcγRIIIA and FcRγ were also cloned and expressed in 293T cells as comparison. The surface expression of FcγRIIA and FcγRIIIA on transfected 293T cells was examined by flow cytometry. FcγRIIIA was undetectable on the cells unless FcRγ was co-expressed (Fig. 5.2). However, FcγRIIA expression on transfected 293T cells was independent of FcRγ co-expression (Fig. 5.2). It was previously reported that FcRγ expression facilitated cell surface expression of some Fc receptors and FcγRIIIA was not detected on the surface of cells derived from FcRγ knock out mice (Takai et al., 1994). When FcγRIIIA was expressed in COS-1 cells, only trace amounts of the receptor was detected on the cell surface if FcRγ was not co-expressed (Park et al., 1993). The structural requirement of FcRγ for cell surface expression of FcγRIIIA was demonstrated in transfected COS-1 cells (Lanier et al., 1991; Kim et al., 2003). These data indicated that specific sequences in the transmembrane domain of FcRγ including cysteine Cys7 and aspartic acid Asp11 govern its interaction with FcRγIIIA which regulates FcγRIIIA surface expression (Kim et al., 2003).

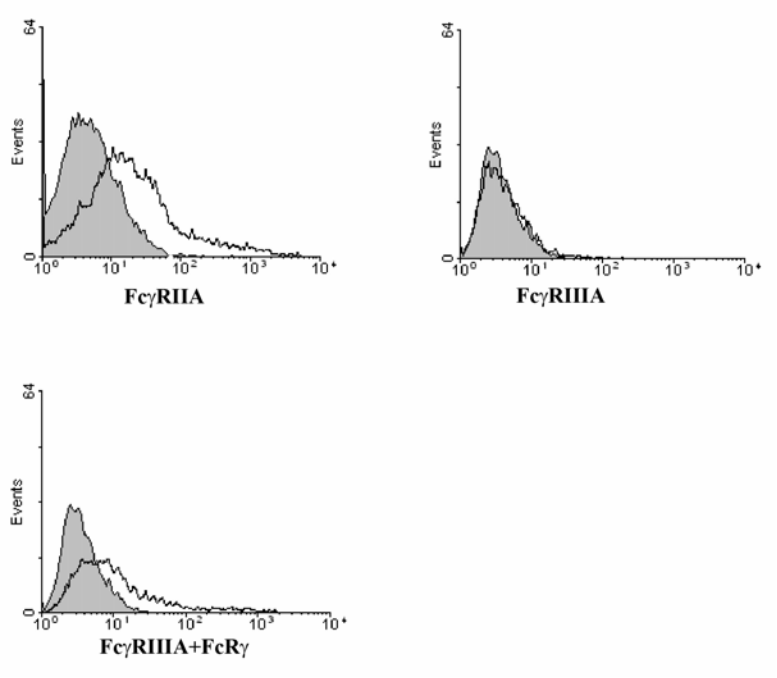


Figure 5.2 Surface expression of wild type (WT) FcγRIIA and FcγRIIIA on transfected 293T cells. 293T cells were transfected with expression vectors for wild type FcγRIIA and FcγRIIIA or co-transfected with vectors for FcγRIIIA and FcR γ chain. All plasmids were used at 150 ng/well in 24-well plates. These receptors were detected by flow cytometry using monoclonal antibodies specific for FcγRIIA and FcγRIIIA. The filled profiles are signals obtained from 293T cells transfected with pcDNA3.1. Open profiles represent specific signals detected on 293T cells transfected with the FcγRIIA and FcγRIIIA expression vectors.

Next, FcγRIIA and FcγRIIIA signaling, in the transfected 293T cells were examined by stimulating these cells with IgG-beads. A previous study showed that IgG-beads (which represent IgG-coated bacteria) can induce TNF-α secretion from macrophages (Loegering and Lennartz, 2004). In our system, when 293T cells were transfected to express FcγRIIA, it did not induce significant NF-κB activation in response to IgG-beads (Fig. 5.3). Co-expression of FcγRIIIA and FcRγ chain also did not induce NF-κB activation. In contrast, 293T cells transfected to express FcγRIIA showed increased NF-κB activation and produced IL-8 upon IgG-DH5α stimulation (Fig. 5.4A and B). It is unclear why the IgG-DH5α, but not the IgG-beads, could acitvate FcγRIIA signaling? An explanation could be that *E.coli* DH5α being more complex, has other molecules on

the cell surface that possibly interact with receptors on 293T cells. There may also be a co-receptor involved in eliciting NF-κB activation. Cross-talk between receptors is biologically relevant; as cells in vivo are not stimulated by one receptor in isolation (i.e. 293T cell transfected single receptor) but rather by many surface receptors working together to induce gene expression through integration of multiple signaling pathways.

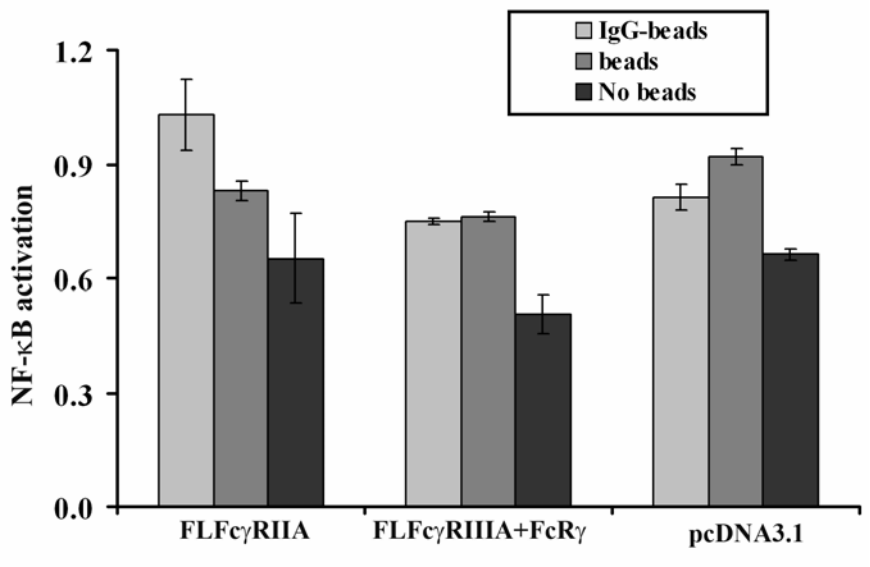


Figure 5.3 Full-length (FL) FcγRIIA mediates NF-κB activation in response to IgG-beads. 293T cells were transfected to express FcγRIIA or co-transfected to express FcγRIIIA and FcRγ. All plasmids were used at 150 ng/well. After 24 hr, the transfected cells were stimulated with IgG-beads or beads for 6 hr in serum-free media or untreated (No beads). The beads:cell ratios were 100:1. As controls, the pcDNA3.1 vector was used to transfect 293T cells (300 ng/well). All results are representative of at least three similar experiments and are presented as means ±SD.

However, 293T cells that were co-transfected to express FcγRIIIA and FcRγ did not induce NF-κB activation and IL-8 produciton (Fig. 5.4A and B). It is not clear whether the functional discrepancy between FcγRIIA and FcγRIIIA was due to the different levels of expression of these receptors on the 293T cells (Fig. 5.2) or it was due to intrinsic differences between the two receptors in their signaling properties (Indik et al., 1995; Kim et al., 2001).

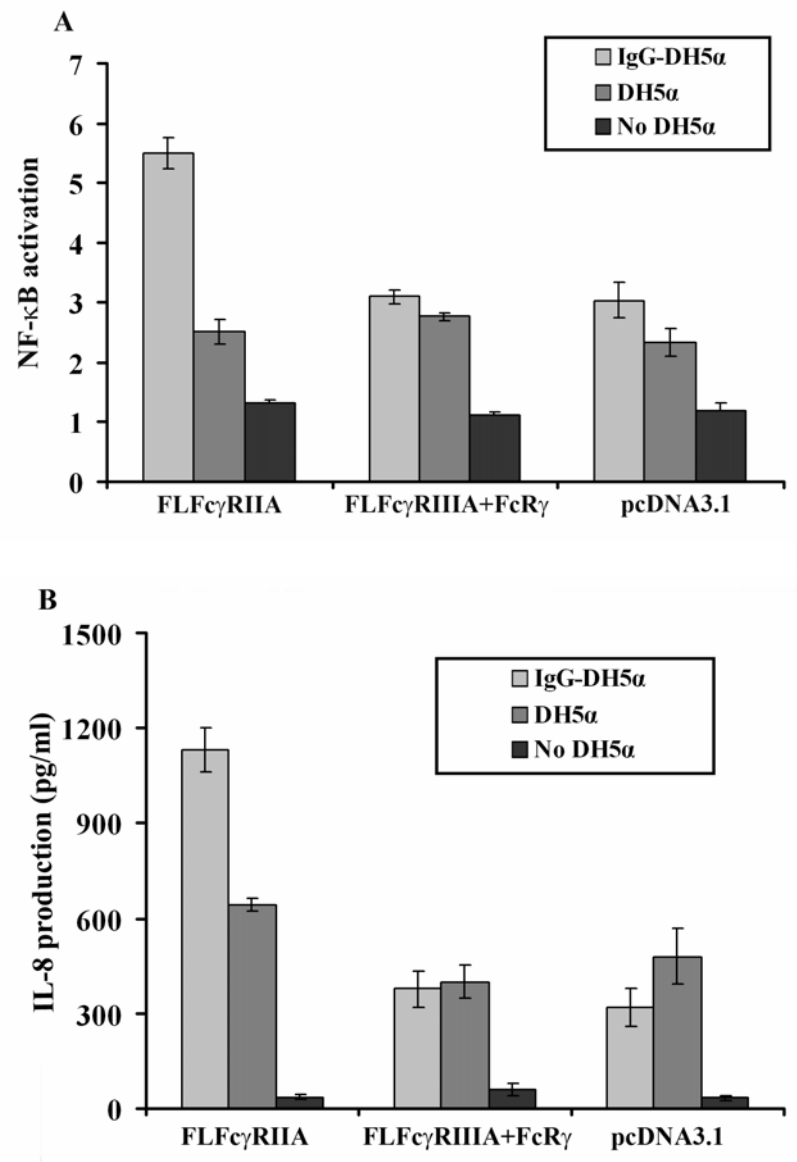


Figure 5.4 Full-length (FL) FcγRIIA mediates NF-κB activation and IL-8 production in response to IgG-opsonized DH5α (IgG-DH5α). 293T cells were transfected to express FcγRIIA or co-transfected to express FcγRIIIA and FcRγ. All plasmids were used at 150 ng/well. After 24 hr, the transfected cells were stimulated with DH5α or IgG-DH5α for 6 hr in serum-free media or untreated (No DH5α). The bacteria:cell ratios were 100:1. As controls, the pcDNA3.1 vector was used to transfect 293T cells (300 ng/well). After 6 hr, NF-κB activation was determined using the luciferase reporter assay. All results are representative of at least three similar experiments and are presented as means ±SD.

Nonetheless, 293T cells can clearly support FcγRIIA signaling of NF-κB activation and the inability of IL-4, expected to induce IL4Rα-FcγRIIA and γC-FcγRIIA dimerization to elicit NF-κB activation implied that FcγRIIA dimerization might not be sufficient to

induce NF-κB activation. Further oligomerization may be required which can be achieved by immune complexes such as IgG-DH5α.

5.3 FcγRs mediate the production of different cytokines from macrophages in response to IgG of different degrees of aggregation

The results obtained with 293T cells led us to investigate whether different FcγRs may be activated by different immune complexes reflecting different degrees of IgG aggregation, i.e. dimerization is not sufficient to activate these receptors. In this case, macrophages were used and FcγR activation was assessed by the induction of cytokine production from macrophages. Human GM-CSF macrophages were generated by culturing human monoctyes with recombinant human GM-CSF for 6 days. Colony stimulating factors (CSFs) such as granulocyte macrophage-CSF (GM-CSF) and macrophage-CSF (M-CSF) are hematopoietic growth factors that play important roles in the survival and differentiation of monocytes/macrophages (Burdach et al., 1998; Motoyoshi, 1998). Two phenotypically distinct types of macrophages can be generated from human monocytes when GM-CSF or M-CSF was used (Akagawa et al., 2006).

Three different types of FcγRs (FcγRI, FcγRII and FcγRIII) are expected to be expressed on monocytes and macrophages (van de Winkel and Anderson, 1991). This was examined on the cultured macrophages in this study by flow cytometry. As shown in Fig. 5.5, all three FcγRs were detected on these macrophages. The expression of CD14 was also detected on these macrophages (Fig. 5.5).

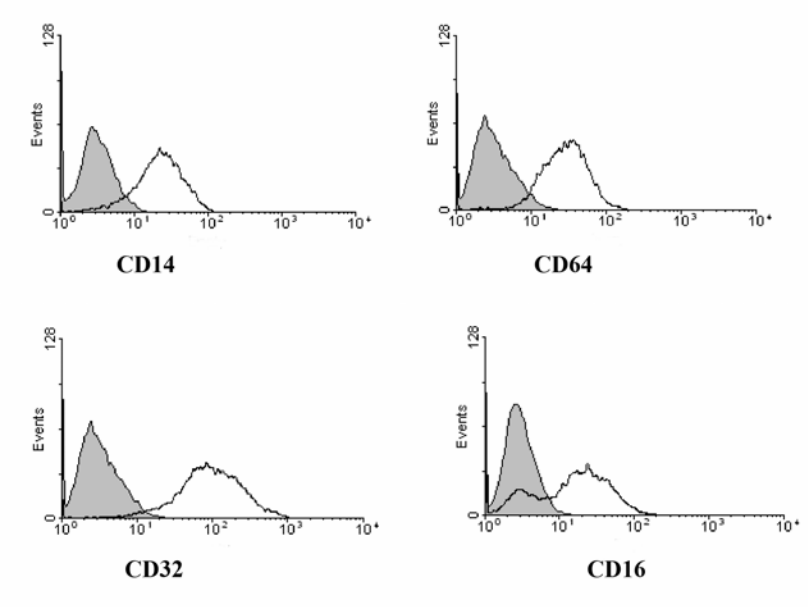


Figure 5.5 FcγR expression on macrophages. Monocytes were cultured for 6 days with 20 ng/ml GM-CSF to yield macrophages. Macrophages (3 x 10^5) were surface-stained with the indicated antibodies. Filled profiles were signals obtained with isotype IgG and open profiles were signals detected with the respective specific antibodies.

It has been reported that when FcγRs are cross-linked with immune complexes on monocytes/macrophages, it induces the production of IL-1 (Chou et al., 1985; Remvig et al., 1990), IL-6 (Krutmann et al., 1990; Ling et al., 1990), TNF-α (Debets et al., 1988; Debets et al., 1990; Polat et al., 1993), IL-8 (Marsh et al., 1995), IL-10 (Berger et al., 1996), and GM-CSF (Ishiguro et al., 1991; Herrmann et al., 1992). In this study, macrophages were stimulated with different forms of aggregated IgG and the productions of different cytokines were examined using a cytokine antibody array blots which are dotted in duplicate with anti-cytokine antibodies. Macrophages were treated with soluble IgG, heat-aggregated IgG (HA-IgG), IgG-coated latex beads (IgG-beads) or IgG-coated plates (ImIgG). The culture media were collected and used to probe human cytokine antibody arrays. Data from representative arrays are shown in Fig. 5.6

and are also summarized in Table 5.1. Analysis of the cytokine array data indicated that some cytokines were differently induced by the different forms of IgG.

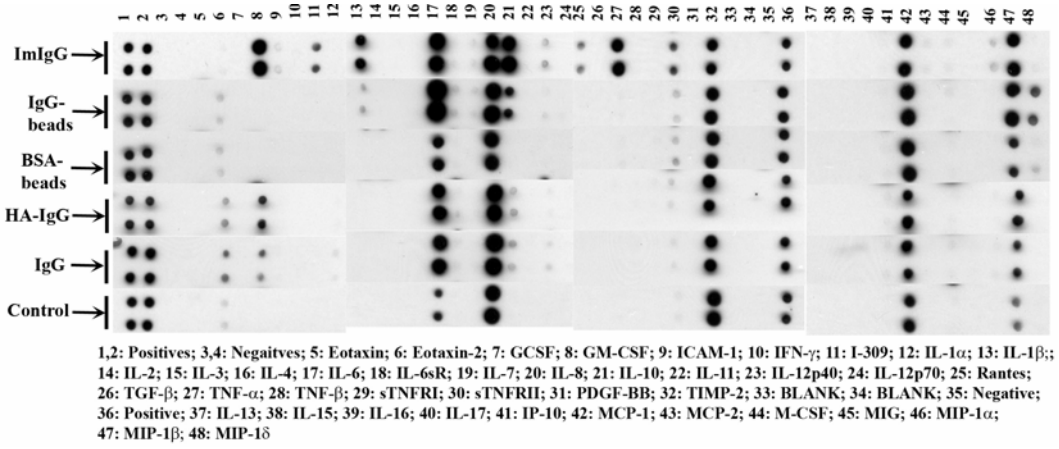


Figure 5.6 Cytokine inductions from macrophages by IgG of different forms. Human cytokine arrays were incubated with media of macrophages which were stimulated with different forms of IgG. The arrays were obtained from RayBiotech, Inc. (Cat# H0128003, Human Inflamamtion Antibody Array III). Autoradiographs of the arrays were scanned and differences in the density between different arrays were referred to the intensity of the positive control spots (1 & 2). The relative signal intensities for detectable cytokines have been arbitrarily evaluated and summarized in Table 5.1. This is representative of two experiments.

The cultured GM-CSF macrophages constitutively produce IL-8, MCP-1 and TIMP-2 (tissue inhibitor of metaloprotinase-2) and, to less extents, IL-6 and MIP-1β (macrophage inflammatory protein 1 β). Soluble IgG increased IL-6 production and also induced low levels of eotaxin-2, MIP-1β and GM-CSF (Fig. 5.6). HA-IgG showed similar induction of these cytokines as free soluble IgG except that it induced a more prominent GM-CSF production than soluble IgG. IgG-beads showed further increased IL-6, IL-10 and MIP-1β induction than soluble and HA-IgG, but it did not induce GM-CSF. Overall, all these IgG forms are poorer cytokine inducer than ImIgG (Fig. 5.6). ImIgG effectively induced GM-CSF, IL-1α, IL-1β, IL-10 IL-12p40, RANTES, TNF-α, soluble TNFRII (tumour necrosis factor receptor II) and MIP-1β. Based on this

experiment, IL-1β, TNF-α and RANTES were only effectively induced by ImIgG among all these IgG forms. Previous reports showed that ImIgG could not induce IL-1β from monocytes unless the cells were primed with LPS (Arend et al., 1991). Interestingly, LPS-priming is apparently not required for ImIgG to induce IL-1β from GM-CSF macrophages. MIP-1δ (macrophage inflammatory protein 1 δ) is only induced by IgG-beads. These are also shown in Table 5.1.

Table 5.1 Production of selected cytokines in response to IgG of different forms by macrophages

Human Cytokine	ImIgG	IgG-Beads	BSA-beads	HA-IgG	IgG	Control
IL-6	6	6	3	4	4	2
TNF-α	4	-	-	-	-	-
sTNFRII	2	1	1	-	-	-
GM-CSF	5	-	-	2	1	-
IL-1β	3	-	-	-	-	-
IL-1α	2	-	-	-	-	-
IL-8	6	6	6	4	4	4
IL-10	5	2	-	1	1	-
IL-12p40	1	-	-	-	-	-
Rantes	2	-	-	-	-	-

This table shows relative cytokine induction by different forms of IgG as detected using the human cytokine arrays (Fig. 5.5). The values were obtained arbiturily based on visual assesments of the array spots.

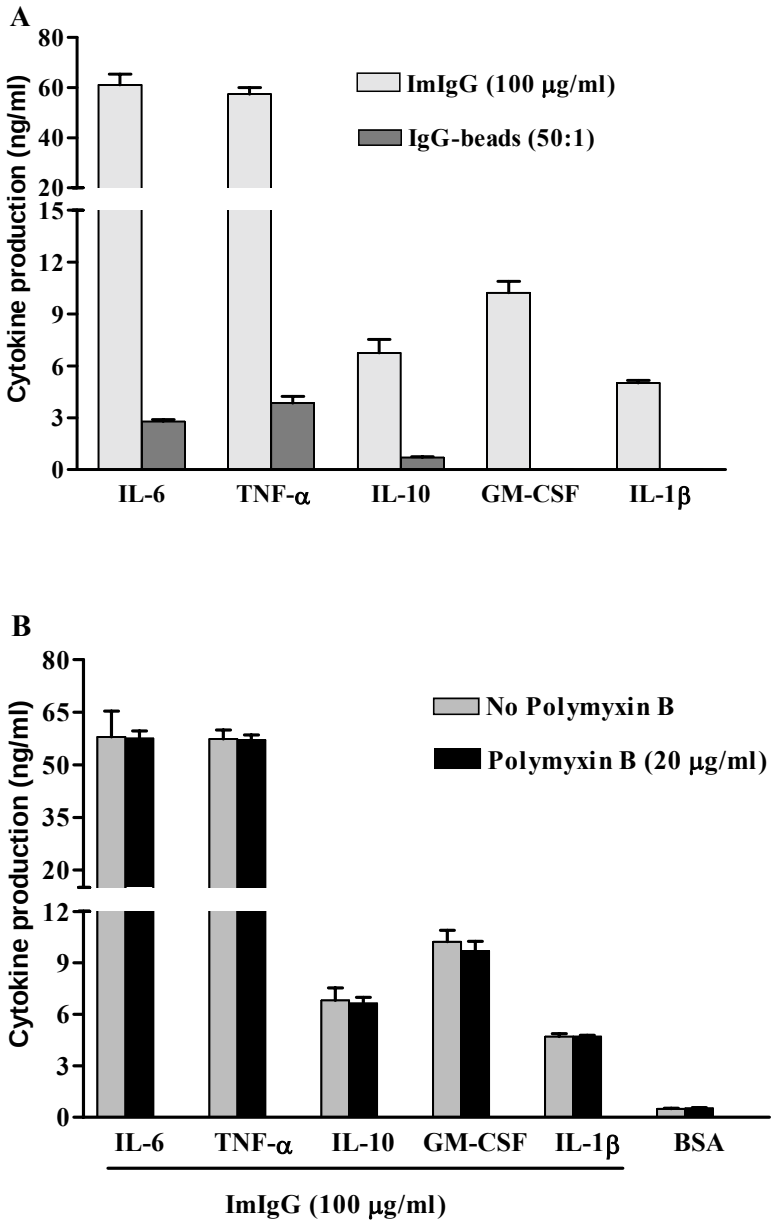


Figure 5.7 Induction of selected cytokines by macropahges in response to ImIgG and IgG-bead. (A) 1 x 10^5 macrophages were stimulated with ImIgG (100 μg/ml) or IgG-beads (beads:cell ratio=50:1). Cell-free supernatants were used to measure the production of different cytokines by ELISA. (B) 1 x 10^5 macrophages were stimulated with ImIgG (100 μg/ml) in the presence or absence of polymyxin B (20 μg/ml)). After 24 hr, cell-free supernatants were assayed for different cytokines by ELISA. As a negative control, BSA-coated plates were used to stimulate macrophages.

Although the cytokine arrays are useful for examining cytokines expression in a more global manner, it does not yield quantitative data. Therefore, ELISA was used to

quantify the expression of some cytokines that were differentially induced by the different forms of IgG. Macrophages were stimualted with ImIgG or IgG-beads before ELISA for IL-6, TNF-α IL-10, GM-CSF and IL-1β.

As shown in Figure 5.6A, while IL-6 and TNF-α was induced by ImIgG and IgG-beads, ImIgG induced 50 folds more IL-6 and TNF-α than IgG-beads (Fig. 5.6A). Similarly, while IL-10 was induced by both ImIgG and IgG-beads, ImIgG induced 4~5 folds more IL-10 than IgG-beads (Fig. 5.7A). Some inconsistent results were obtained with cytokine arrays and ELISA. TNF-α induction by IgG-beads was detected by ELISA (Fig. 5.7A) but not the array (Fig. 5.6). The IL-6 levels induced by ImIgG as compared with IgG-beads showed strong differences depending on whether antibody array or ELISA was employed. By ELISA, ImIgG induced 50 folds more IL-6 than IgG-beads whereas, based on the array methods IgG-beads induced more IL-6 than ImIgG. Consistent levels of GM-CSF, IL-1β and IL-10 were detected with ELISA (Fig. 5.7A) and the antibody arrays (Fig. 5.6).

GM-CSF macrophage mimics inflammatory macrophages (Hamilton et al., 1980). This is refelcted in some cytokine production by the cells without stimulation. The interesting observation was that these cells produce high levels of GM-CSF in response to ImIgG but not IgG-beads (Fig. 5.7A). IgG-beads also failed to induce IL-1β which was induced by ImIgG. It is possible that ImIgG provides more prolonged stimulation to macrophages than other forms of IgG and this elicit additional signaling pathways which are not activated by IgG-beads. The ability of ImIgG to induce GM-CSF from GM-CSF cultured macrophages means that ImIgG primes macrophages for inflammatory responses.

Since contaminating LPS may induce similar cytokines from macrophages, this possibility was assessed using the LPS inhibitor, polymyxin B, a peptide antibiotic that binds and neutralizes LPS (Cooperstock, 1974). 20 μg/ml polymyxin B showed no inhibition of ImIgG-induced cytokine production (Fig. 5.7B). As another control, macrophages were stimulated with BSA-coated plates to eliminate non-specific protein stimulation. Macrophages cultured in BSA-coated plates showed none of these cytokines induction (Fig. 5.7B). In addition, complement-induced cytokine production was also unlikely in these experiments because heat-inactivated FCS was used in all experiments. In summary, data from both cytokine array and ELISA suggest that (i) ImIgG is more potent in cytokine induction than IgG-beads and (ii) IgG-beads do not significantly induce certain cytokines, such as IL-1β and GM-CSF, which were induced by ImIgG.

5.4 Role of different FcγRs in the induction of IL-6, TNF-α and IL-10 by ImIg and IgG-beads

IL-6, TNF-α, and IL-10 production vary significantly depending on whether ImIgG or IgG-beads were used to stimulate macrophages (Fig. 5.7A). ImIgG is apparently more potent than IgG-beads. From these experiments, the types of FcγR involved in cytokine induction are not clear. This was examined by pre-incubation of macrophages for 1 hr at 4°C with FcγR-blocking antibodies (20 μg/ml) before stimulation with ImIgG or IgG-beads for another 12 hr. As shown in Fig. 5.8A, IL-6 produciton was markedly inhibited by the FcγRI and FcγRIII antibodies, compared to control mouse IgG. In both cases, > 50% inhibition was observed (Table 5.2). The anti-FcγRIIIA antibody was particularly inhibitory which reduced IL-6 induction by ImIgG to 68% (Table 5.2). Blocking of FcγRII showed less inhibition. When macrophages were pre-incubated with a

combination of all three FcγR blocking antibodies, IL-6 production was reduced to 80% (Table 5.2).

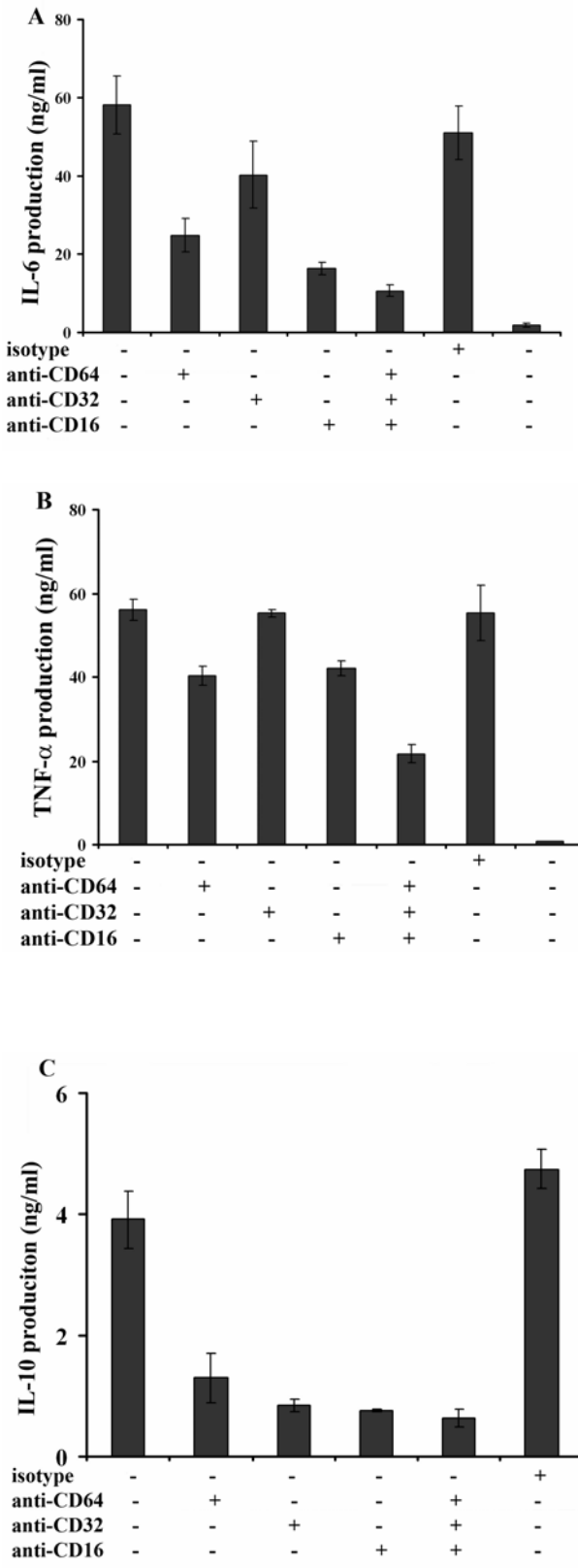


Figure 5.8 Roles of different FcγRs in ImIgG-induced IL-6, TNF-α and IL-10 production.
Macrophages were pre-incubated with FcγR blocking antibodies (20 μg/ml) for 1 hr on ice and then stimulated with ImIgG (100 μg/ml)) for another 12 hr. Cell-free supernatants for IL-6, TNF-α and IL-10 were measured by ELISA. Results are presented as means ±SD of triplicate experiments. One representative of three independent experiments is shown.

Table 5.2 ImIgG-induced IL-6, TNF-α and IL-10 production upon antibody blocking of different FcγRs

Cytokines	Mouse IgG	Anti-CD64	Anti-CD32	Anti-CD16	Anti-CD64+CD32+CD16
IL-6	0%	51%	21%	68%	80%
TNF-α	0%	27%	0%	24%	61%
IL-10	0%	73%	82%	84%	87%

TNF-α production was reduced to 61% when macrophages were pre-incubated with all three FcγR-blocking antibodies (Fig. 5.8B) (Table 5.2). Individually, each FcγR antibody only caused slight inhibition of TNF-α production. It shows that IL-6 and TNF-α are differently induced by ImIgG. FcγR synergy is not key to ImIgG-induced TNF-α production. For IL-6 induction, FcγRIII and, to a lesser extent, FcγRI play more important roles than FcγRII. ImIgG-elicited IL-10 production appeared to require all three FcγRs as the blocking of any of these FcγRs markedly inhibited ImIgG-induced IL-10 production (Fig. 5.8C) (Table 5.2).

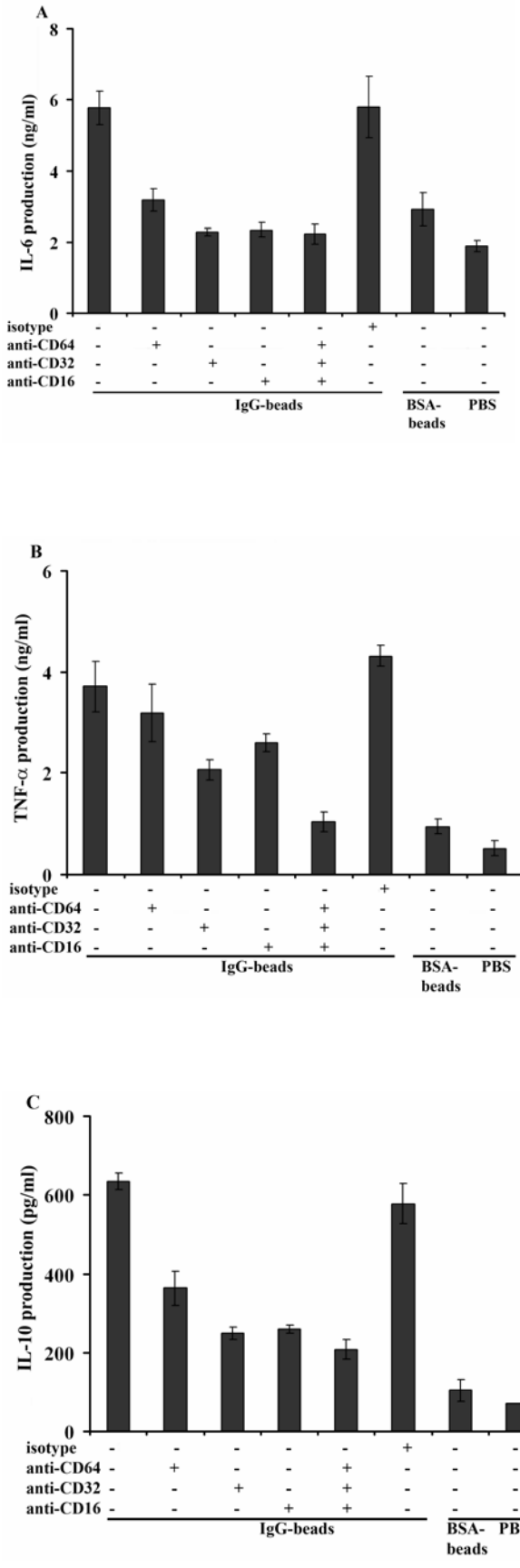


Figure 5.9 The roles of specific FcγR in IgG-beads-induced IL-6, TNF-α and IL-10 production.
Macrophages were pre-incubated with blocking antibodies (10 μg/ml) for 1 hr on ice and then stimulated with IgG-beads for another 12 hr. Cell-free supernatants were measured for IL-6, TNF-α and IL-10 by ELISA. Results are presented as means of ±SD of triplicate experiments. One representative of three independent experiments is shown.

Table 5.3 IgG-bead-induced IL-6, TNF-α and IL-10 production upon antibody blocking of different FcγRs

Cytokines	Mouse IgG	Anti-CD64	Anti-CD32	Anti-CD16	Anti-CD64+CD32+CD16
IL-6	0%	45%	61%	60%	62%
TNF-α	0%	26%	52%	40%	76%
IL-10	0%	37%	57%	55%	64%

The roles of different FcγRs in IgG-bead induction of cytokines are also assessed using the FcγR-blocking antibodies. As shown in Figure 5.9A, IL-6 induction by IgG-beads was similarly and significantly reduced by each of the three antibodies. Pre-incubation with either the FcγRII or FcγRIII antibody reduced IL-6 to levels similar to that observed in PBS addition to macrophages. Compared with IL-6, TNF-α production was less inhibited by individual antibodies (Fig.5.9B). However, incubation of macrophages with a combination of all three FcγR antibodies reduced TNF-α production approximately 76% (Table 5.3) to the backround levels induced by BSA-beads and PBS. IL-10 production by IgG-beads appears to be less sensitive to FcγR blocking (Fig. 5.9C). While all antibodies showed inhibition, the degree of inhibition was much less compared with ImIgG-induced IL-10 production (Table 5.2).

5.5 Specific FcγR requirement for IL-1β and GM-CSF induction by ImIgG.

As shown in Fig.5.6 and Fig. 5.7A, ImIgG but not other forms of IgG induced signifcant IL-1β and GM-CSF production. The FcγR which is involved in the induction of these two cytokines is here also examined. Macrophages were pre-incubated for 1 hr

at 4°C with specific FcγR antibodies (20 μg/ml) before stimulation with ImIgG for another 12 hr. IL-1β and GM-CSF in culture supernatants were measured by ELISA.

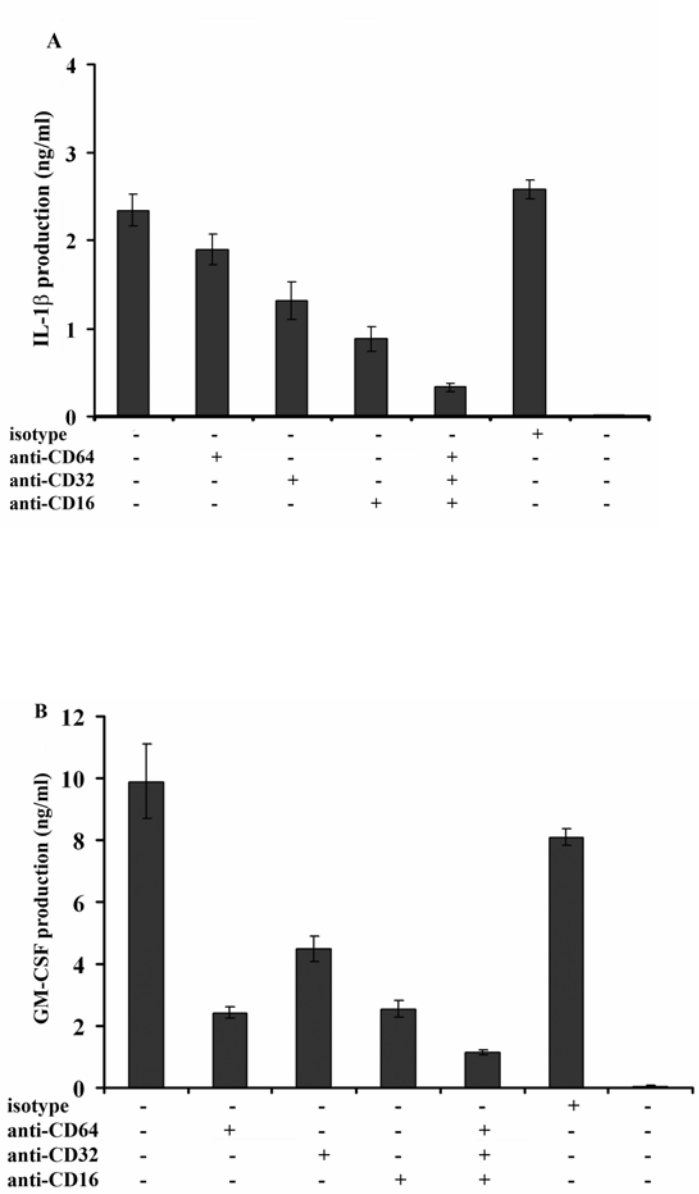


Figure 5.10 Roles of different FcγRs in ImIgG-induced IL-1β and GM-CSF production. Macrophages were pre-incubated with blocking antibodies (10 μg/ml) for 1 hr on ice and then stimulated in ImIgG (100 μg/ml) for another 12 hr. Cell-free supernatants were measured for IL-1β and GM-CSF by ELISA. Results are the means ±SD of triplicate wells. One representative of three independent experiments is shown.

Table 5.4 ImIgG-induced IL-1β and GM-CSF production upon antibody blocking of different FcγRs

Cytokines	Mouse IgG	Anti-CD64	Anti-CD32	Anti-CD16	Anti-CD64+CD32+CD16
IL-1β	0%	27%	49%	66%	87%
GM-CSF	0%	70%	44%	69%	87%

IL-1β secretion was not significantly inhibited by the FcγRI blocking antibody (Fig. 5.10A). However, it was markedly inhibited by the FcγRIII antibody (66%, Table 5.4). The FcγRII antibody also showed ~50% inhibition. When macrophages were pre-incubated with a combination of all three FcγR antibodies, IL-1β production was almost completely blocked, i.e reduced to 87% (Table 5.4).

GM-CSF secretion was inhibited by all three FcγR blocking antibodies (Fig. 5.10B) especially the FcγRI and FcγRIII antibodies which showed ~70% inhibition. When macrophages were pre-incubated with a combination of three FcγR antibodies, it almost completely blocked GM-CSF production (Table 5.4). These results suggest a more prominent role for FcγRIII in mediating GM-CSF and IL-1β production. While FcγRI is as potent as FcγRIII in mediating GM-CSF induction, it is the weakest among the three FcγRs in IL-1β induction (Table 5.4).

5.6 IL-8 production is not sensitive to the blocking of any of the three FcγRs

FcγR cross-linking with ImIgG was known to activate IL-8 production effectively from monocytes (Marsh et al., 1995). Similarly, FcγR cross-linking with either ImIgG or IgG-beads also effectively induced IL-8 production from macrophages (Fig. 5.11).

BSA-beads induced some IL-8 production which is un-related to IgG or FcγRs. As shown in Fig. 5.6, GM-CSF macrophages constitutively produced IL-8 even in PBS-treated plates. This may be due to the effects of GM-CSF which was used to culture these cells.

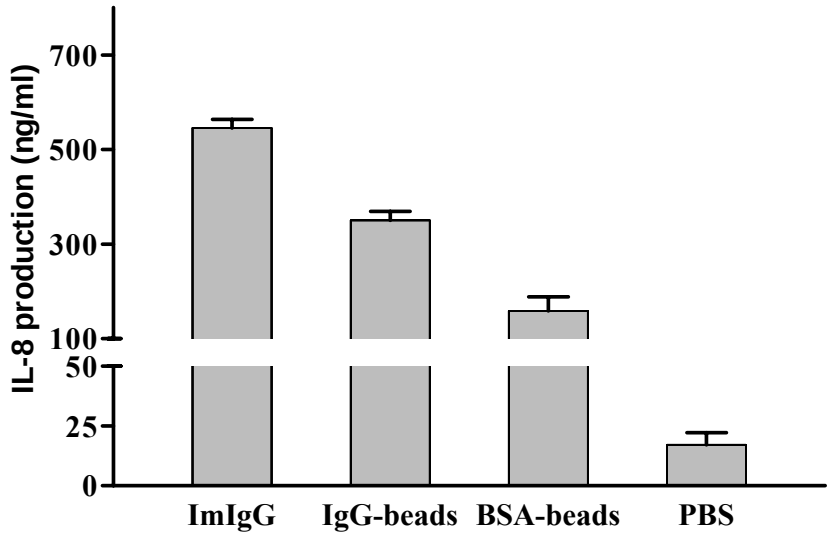


Figure 5.11 ImgG and IgG-bead induction of IL-8 from macrophages. Macrophages (1×10^5) were stimulated with either ImIgG (100 μg/ml), IgG-beads or BSA-beads (50:1) for 12 hr. As a control, PBS-treated plates (PBS) were used. IL-8 was measured by ELISA. Results are presented as means ±SD of triplicate wells. One representative of three independent experiments with similar results is shown.

Nonetheless, the FcγR which is involved in ImIgG and IgG-bead induction of IL-8 production was similarly examined using the blocking antibodies. As shown in Fig. 5.12A and B, elicited IL-8 production by ImIgG and IgG-beads was not inhibited by the FcγRI, FcγRII or FcγRIII antibody individually or in combination. This suggests the involvement of other unidentified receptors in mediating IgG induction of IL-8 production from macrophages. In summary, IL-8 production may be induced by ImIgG

or IgG-beads from macrophages through any of the three known FcγRs examined or potentially other receptors that regulate IgG. Another explanation is that IL-8 is easily induced from macrophages.

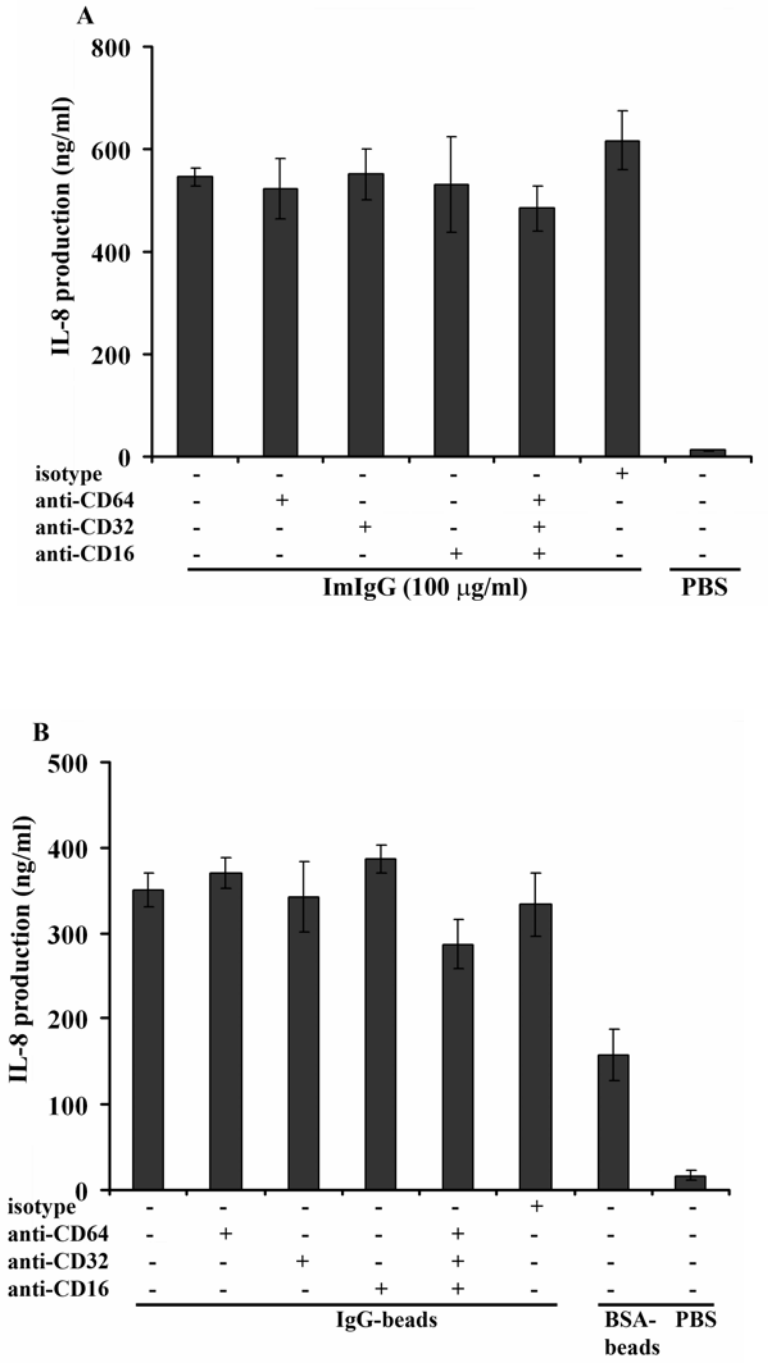


Figure 5.12 Roles of different FcγRs in ImIgG and IgG-beads-induciton of IL-8 from macrophages. Macrophages were pre-treated with FcγR-blocking antibodies (20 μg/ml) for 1 hr on ice and then stimulated with either ImIgG (100 μg/ml) (A) or IgG-beads (50:1) (B) for another 12 hr. IL-8 was measured by ELISA. BSA-beads were used as a negative control (B). Results are presented as means ±SD of triplicate wells. One representative of three independent experiments is shown.

Table 5.5 FcγR involvement in IgG-elicited IL-8 production.

IL-8	Mouse IgG	Anti-CD64	Anti-CD32	Anti-CD16	Anti-CD64+CD32+CD16
ImIgG	0%	0%	11%	14%	20%
IgG-beads	0%	0%	0%	0%	14%

5.7 Conclusion

Based on the above results, the following conclusions can be drawn:

i) Dimerization is not sufficient to induce FcγRIIA signaling for NF-κB activation and cytokine induction. To induce FcγR signaling, higher degrees of FcγR oligomerization is probably required.

ii) ImIgG is a more potent stimulus of cytokine production compared to IgG-beads or HA-IgG. IgG-beads can not induce IL-1β and GM-CSF production which was induced by ImIgG.

iii) FcγRIII and, to lesser extents, FcγRI play important roles in ImIgG-elicited IL-6 production. In the case of TNF-α induction, all three FcγRs are potent mediators. In contrast, ImIgG requires synergy of all three FcγRs for effective IL-10 induction. FcγIII plays prominent roles in IL-1β and GM-CSF induction by ImIgG. While FcγR1 is as potent as FcγIII in GM-CSF induction, it is the weakest mediator of all three FcγRs in IL-1β induction.

FcγRII and FcγRIII play significant roles in IgG-beads-elicited IL-6 and IL-10 production. All FcγRs appears to be potent inducer in IgG-beads-elicited TNF-α production.

iv) ImIgG or IgG-beads-induction of IL-8 is not sensitive to the blocking of any of the three FcγRs, suggesting the involvement of other receptors. Alternatively, IL-8

induction from GM-CSF macrophages may not need substantial involvement of IgG or its receptors

The biological implications of these findings are that different FcγRs may have distinct roles in FcγR mediated effector functions. Understanding of the roles of specific FcγRs may help to further understand the progression of FcγR-mediated autoimmune diseases.

Chapter 6 Discussion

6.1 Development of a TLR-based two-hybrid assay for the detection of protein-protein interactions

While the yeast two-hybrid assays have enabled the identification and investigation of numerous cytoplasmic protein interactions, the application of such assays in the investigation of the interactions between membrane-bound receptors, between receptors and the protein ligands, and between secreted proteins, are limited. In the present study, we evaluated an assay for detecting protein-protein interactions based on the mechanism of TLR2-mediated NF-κB signaling is activated (Ozinsky et al., 2000). TLR2 transduces signals when it dimerizes with TLR1, which causes the dimerization of cytoplasmic TIR domains of TLR1 and TLR2 (i.e. TIR1 and TIR2). The sensitivity and specificity of this TIR1/TIR2-based two-hybrid assay have been evaluated on whether this assay can reflect known interactions between IL-4 and/or its two receptor subunits (Nelms et al., 1999), i.e. IL4Rα and γC. Our results (Fig 3.3) showed that the TIR1/2-based two-hybrid assay could detect the lack of interaction between IL4Rα and γC when fused to TIR1 and TIR2 as hybrid receptors and stimulated with IL-4. The assay also demonstrated direct IL-4 interaction with IL4Rα (Fig. 3.4A) and the lack of direct IL-4 interaction with γC (Fig. 3.4B). These results are largely consistent with current knowledge of interactions between IL-4 and its receptors (Duschl and Sebald, 1996; Russell et al., 1993). IL-4 primarily binds with IL4Rα. Subsequently, this intermediate IL-4/IL-4Rα complex recruits the γC to form an IL-4Rα/ γC heterodimer. These results highlight the potential of TIR1/TIR2-based two-hybrid assay in the detection of receptor-receptor and receptor-ligand interactions. The relatively small TIR1 and TIR2 reporter domains approximately 20 KDa permit easy expression of relatively large test

proteins using plasmid vectors. Therefore, this assay can be conveniently employed in detecting interactions between extracellular proteins and/or the EC domains of membrane proteins.

The sensitivity and specificity of this novel two-hybrid assay has been demonstrated, but this is apparently only achieved when the assay is carried out in a well controlled manner. For example, co-expression of IL4Rα-TIR1 and γC-TIR2, which were not expected to interact unless stimulated with IL-4, led to constitutive NF-κB activation when both hybrid receptors were excessively over-expressed (Fig. 3.3). Over-expression of IL4-TIR1 and γC-TIR2 also led to constitutive NF-κB activation (Fig. 3.4B). There is no evidence that such interactions occur under physiological conditions. The fact that NF-κB activation was only observed at high plasmid dosage suggests that it probably results simply from over-expression of the TIR1 and TIR2 hybrid receptors. When the plasmids were used at reduced dosages, i.e. 38 ng/well, such NF-κB activation also decreased in a dose dependent manner.

Over-expression of the IL4-TIR1/TIR2 (Fig 3.5A), IL4Rα-TIR1/TIR2 (Fig. 3.5B), and γC-TIR1/TIR2 (Fig. 3.7A) hybrid pairs also led to potent NF-κB activation. However, when the dosage of these plasmids were reduced to levels at which co-expression of the IL4Rα-TIR1/γC-TIR2 and IL4-TIR1/γC-TIR2 pairs elicited insignificant NF-κB activation, NF-κB activation by IL4-TIR1/TIR2 (Fig. 3.5A), IL4Rα-TIR1/TIR2 (Fig. 3.5B), and γC-TIR1/TIR2 (Fig.3.7A) hybrid pairs was sustained. The observed NF-κB activation by IL4-TIR1/TIR2, IL4Rα-TIR1/TIR2, and γC-TIR1/TIR2 hybrid pairs suggest that the NF-κB activation associated with these 3 hybrid pairs was probably not simply due to over-expression but a result of homotypic interactions between the pairs of IL-4, IL4Rα and γC. The homotypic interactions between pairs of IL-4, IL4Rα and γC based on TIR1/TIR2 hybrid assay was verified by immunoprecipitation studies. This

technique also confirmed homotypic interactions between IL-4, IL4Rα and γC pairs. The observed homotypic interactions of IL-4, IL4Rα and γC are not in conflict with the known mechanism of IL-4 mediated receptor interaction.

This assay detected interactions between the EC domains of IL4Rα and γC largely in the context of their natural microenvironments except that their TM and Cyt domains were replaced by TIR domains. In contrast, homotypic interaction of IL-4 and interactions between IL-4 and its receptor EC domains were examined under artificial conditions where the IL-4 was anchored to the cell surface of the hybrid receptors. The anchoring of IL-4 on the cell surface could potentially enhance or inhibit its lateral homotypic interaction and its interaction with the EC domains of IL4Rα and γC. Therefore, it is less certain whether IL-4 would similarly form homodimers in its native soluble form. Nevertheless, IL-4 has apparently, retained its binding properties and specificity after it has been expressed in the form of hybrid receptors.

A weakness of the TIR1/TIR2-based two-hybrid assay is that the assay is over-sensitive. This can be over come in two ways: Firstly, the NF-κB activation due to over-expression can be titrated down to background level with the reduction of plasmid dosages as low as 38 ng/well. In this condition, this TIR1/TIR2-based two-hybrid assay then can report the true interaction of the two different test proteins. Secondly, background NF-κB activation derived from the over-expression of this two-hybrid assay can be reduced through selective mutations in any of the TIR1 or TIR2 domain of the two-hybrid proteins. An additional shortcoming of this TIR1/TIR2-based two-hybrid assay was its poor quantitative quality in reporting interactions between two hybrid molecules. The assay relies on the activation of endogenous NF-κB, which is required for multiple essential cellular activities. NF-κB activation was apparently tolerated in the transfected 293T cells. But its activity was tightly regulated through feedback

mechanisms. For example, NF-κB is activated through the degradation of IκBα, but activated NF-κB rapidly induces IκBα expression (Karin and Ben Neriah, 2000). This means that the time window during which linear correlation between hybrid receptor interactions and the extent of NF-κB activation is likely to be very narrow.

Despite the above weaknesses, the TIR1/TIR2-based two-hybrid assay remains a convenient supporting assay for detecting interactions between receptors, between receptors and ligands, and between soluble secreted proteins. This assay may also be improved to detect interactions between membranes and/or secreted proteins in a high throughput manner.

6.2 Investigation of CD14 dimerization and its role in CD14 signal transduction

In this study, we have applied the TIR1/TIR2-based two-hybrid assay to investigate the interactions between the EC domains of CD14. In chapter 6.1, we have validated the feasibility of this two-hybrid assay by using known interactions between IL-4 and/or its two receptor subunits, i.e. IL4Rα and γC (Wang et al., 2004). Co-expression of IL4-TIR1/TIR2 (Fig. 3.5A), IL4Rα-TIR1/TIR2 (Fig. 3.5B), and γC-TIR1/TIR2 (Fig.3.7A) hybrid pairs at 38 ng/well, NF-κB actiavation was sutained. Similarly, Co-expresssion of CD14-TIR1 and CD14-TIR2 hybrid pairs at 38 ng/well led to NF-κB activation (Fig. 4.1A). In addition, the control IL4Rα-TIR1/CD14-TIR2 and β5-TIR1/CD14-TIR2 hybrid pairs at the same concentration did not induce NF-κB activation (Fig. 4.1B). These results clearly show that the observed NF-κB activation by CD14-TIR1/CD14-TIR2 pairs was not simply due to over-expression of the hybrid receptors but a result of homotypic interactions between CD14-CD14. CD14 may form dimers is reported in a recent crystallography study (Kim et al., 2005). The dimerization may mediated by the

residues located in β13 and in the 'loop' between β12 and β13. Based on this study, two CD14 deletion mutants were generated in CD14-TIR1 by removing amino acids 289-300 (the 'loop' between β12 and β13) or 302-315 (β13) which were named loopdelCD14-TIR1 and β13delCD14-TIR1 respectively. The results showed that deletion of these regions caused the loss of CD14-TIR1/CD14-TIR2-induced NF-κB activation (Fig. 4.2A), indicating an important role of these regions in CD14-mediated cell signaling. The specific amino acids in β13 and in the 'loop' between β12 and β13 of CD14 were further investigated in details. Alanine substitution mutation study showed that L290ACD14-TIR1 or L307ACD14-TIR1 abrogated CD14TIR1/CD14TIR2-mediated NF-κB activation (Fig. 4.2B). Mutant P294ACD14-TIR1 or P300ACD14-TIR1 with CD14-TIR2 caused partial reduciton of NF-κB activation (Fig. 4.2B). These results indicate that L290 in the 'loop' and L307 in β13 are critical to CD14-CD14 dimerization. P294 and P300 in the same 'loop' may also be involved in CD14-CD14 dimerization

While CD14 binds to LPS, it requires the TLR4/MD-2 complex to activate cellular signaling (Shimazu et al., 1999; Akashi et al., 2000; Yang et al., 2000). TLR4-mediated NF-κB activation and IL-8 production was investigated in association with WtCD14 and CD14 mutants. Similarly, TLR4-mediated NF-κB activation (Fig. 4.3A) as well as IL-8 (Fig. 4.3B) production was significantly reduced by L290A or L307A CD14 mutants in response to LPS. This suggests that these two amino acids are important in TLR4-mediated LPS signaling. Another mutant of CD14, i.e P294 and P300 partially reduced TIR1/TIR2-mediated NF-κB activation also reduced TLR4-mediated NF-κB activation as well as IL-8 production. These amino acids may also be important in TLR4-mediated LPS signalling.

To exclude the possibility that mutant CD14 had lost its ability to induce TIR1/TIR2 and TLR4-mediated NF-κB activation due to differences in their cell surface expression, we tested both CD14-TIR1 and CD14 mutants for membrane surface expression. The results showed that they expressed on the cell surface. This suggests that deletion or alanine substitution in the 'loop' between β12-13 and β13 does not alter cell surface expression. Double mutation of two leucines (L290 and L307) in these regions ('loop' between β12-13 and β13), affected the structural integrity of CD14, resulting in reduced expression in both the CD14-TIR1 (Fig. 4.4B) and full-length CD14 (Fig. 4.4C). For the same reason, these double mutants also caused significant reduction of CD14-TIR1/CD14-TIR2 mediated NF-B activation (Fig. 4.2B) and LPS rsponsiveness (Fig. 4.3).

On leukocytes CD14 exists as a GPI protein having a molecular weight of approximately 55 Kda (Haziot et al., 1988; Simmons et al., 1989; Haziot et al., 1993). As mentioned earlier, CD14 may form dimer in a crystallography study (Kim et al., 2005). However, there have been no detailed experimental studies to show that CD14 exists as dimer and the molecualr mechanism of dimer in LPS signaling. In our study, the existence of both monomeric and dimeric form of CD14 was showed by cross-linking assay (Fig. 4.5). Under non-reducing conditions, besides the 55 KDa band, a heterogenous group of high molecualr weight CD14 containing complexes (> 80Kda) were also detected in 293T cells that were transfected to express Wt CD14 (Fig. 4.5B). Thus CD14 may exist as monomer on the cell surface but dimers or higher oligomers also exist. The existance of CD14 dimers was also indicated in crystallographic structure and it showed that these dimers were made up of the same CD14 monomer subunits. The subunit was indicated to be the β13 and the loop of β12 and β13 (Kim et al., 2005). Our study also showed that CD14 mutants, i.e. L290 and L307 that reduced

the dimer/oligomer formation (Fig. 4.5B) also exhibited reduction capacity in mediating LPS-stimulated signaling (Fig. 4.3). Some CD14 mutants, i.e. P294 and P300 partially retained dimer formation also exhibited partial reduction in LPS signaling. This implies that dimers or oligomers may be critical for the propagation of external stimuli across the cell membrane and to the CD14 receptor complex and therefore underlie the basis of the signaling.

CD14 contains 10 LRR motif spanning amino acids 67 to 312 (Ferrero et al., 1990). The binding sites for LPS in CD14 have been extensively studied by mutagenesis and by epitope mapping of antibodies that block LPS binding. Four LRR have been identified as LPS binding sites which correspond to N-terminal 152 amino acids of CD14 (Juan et al., 1995c; Juan et al., 1995a; Viriyakosol and Kirkland, 1995; Cunningham et al., 2000). All these studies clearly indicated that the remaining LRR which corresponds to C-terminal amino acids do not play a part in LPS receptor functions. As pointed out by Juan et al (Juan et al., 1995c), the above is unexpected because the LRR have been thought to be important for interactions between proteins and was predicted to contain the activation domain. In addition, a study has shown that a deletion mutant of human CD14 consisting of the N-terminal 152 amico acids has the capacity to mediate the stimulation of different cell types by LPS (Stelter et al., 1999). These mutants were in amino acids 9-13 and 91-101 that are not essential for LPS binding but important for cellular activation. The alanine substituion of these amino acids significanlty reduced LPS induced IL-6 production in U373 astrocytoma and human vascular endothelial cells. Very little investigation has been done on the functional impact of C-terminal region of CD14. However a study has shown that the amino acids in the region 273-275, corresponding to C-terminal of mouse CD14 was required for TLR4-mediated NF-κB activation (Muroi et al., 2002). In addition to this, our results indicate that the amino

acids in the region 290-300 ('loop' between β12-13) and 301-315 (β13) are required for CD14-CD14 dimerization. Mutation of these amino acids significantly reduced TLR4-mediated LPS signalling.

In summary, this study revealed the new mecahnism by which CD14-CD14 interaction regulates LPS signaling and its implication as a therapeutic agents for LPS-induced shock.

6.3 Investigation of FcγRIIA activation

6.3.1 Dimerizaiton is not sufficient for the induction of FcγRIIA signaling

As discussed above (chapter 6.1 and 6.2), the TIR1/TIR2-based two-hybrid assay has proved to be a feasible method in detecting protein-protein interactions. Here, the IL-4/IL-4R system was also used to investigate whether FcγRs can be activated by inducing dimerization with IL-4. The Tm/Cyt domains of TLR1 in the IL4Rα-TIR1 and γC-TIR1 chimeras were replaced with the TM/Cyt domain of FcγRIIA. It is known in human three different classes of FcγRs, i.e. FcγRI, FcγRII and FcγRIII are expressed on monocytes/ macrophages (van de winkel and Anderson, 1991; Hulett and Hogarth, 1994). The reason to choose FcγRIIA rather than other FcγRs in this system, it is the only known FcγR that contains both extracellular IgG-like domains and cytoplasmic signaling domain, ITAM (immunoreceptor tyrosine-based activation motif) in a single polypeptide (Ravetch and Kinet, 1991). Our result in 293T cells co-transfected with IL4Rα-FcγRIIA and γC-FcγRIIA (Fig. 5.1B) showed that dimerzation of FcγRIIA was not sufficient to induce NF-κB activation. FcγRs induce target effects if the receptors are cross-linked by immune complexes (complexes between antigens and IgG molecules) (Ravetch and Bolland, 2001). Previous studies showed that binding of IgG

to both specific antigens (Ags) and FcγR is required for cell activation (MacIntyre et al., 1989). It appears that binding of IgG by the Fab (fragment of antibody) portion to specific Ag increases the affinity of the FcγR for the Fc, end of the same antibody molecule. However in our study, IgG-opsonized DH5α effectively activated FcγRII signaling in 293T cells, but not FcγRIII signaling (Fig. 5.3A and B). The functional discrepancy between these FcγRs may be due to differences in interaction of IgG with FcγRII and FcγRIII, although these receptors exihibit substantial amino acid sequence conservation and similar IgG binding affinities and specificities (Hibbs et al., 1994; Hulett et al., 1995). Another possibility is that FcγRIIA has unique structural, signaling, and biologic features. Unlike, other receptor such as FcαRI, FcγRI and FcγRIIIA, FcγRIIA can signal without the homodimeric FcR γ chain. FcγRIIA contain both the ligand binding site and the ITAM are in the same polypeptide (Ravetch and Kinet, 1991). It has previously been shown that there are indeed functional differences between FcγRIIA and the FcγRI/FcγRIIIA γ chain in their ability to transmit a phagocytic signal (Kim et al., 2001).

6.3.2 ImIgG is a potent inducer of cytokine production

Monocyte/macrophage FcγR mediate a large spectrum of functions including phagocytosis and endocytosis of IgG-coated particles (Anderson et al., 1990; Davis et al., 1995), antibody-dependent cell-mediated cytotoxicity (ADCC) (Graziano and Fanger, 1987), and production of inflammatory cytokines (Sanchez-Mejorada and Rosales, 1998). Previous studies have shown that cross-linking of FcγR on monocytes resulted in at least two potent immunomodulating cytokines, IL-6 (Krutmann et al., 1990) and TNF-α (Debets et al., 1990; Polat et al., 1993). Similarly, results from cytokine-array (Fig.5.6) and ELISA (Fig. 5.7A) showed that IL-6 and TNF-α production

was induced by both ImIgG and IgG-beads. ImIgG induced IL-6 and TNF-α production is relevant to disease states such as rheumatoid arthritis caused by tissue-fixed immune complexes (Feldmann et al., 1996). This disease is frequently accompanied by fever owing to the pyrogenic properties of TNF-α (Dinarello et al., 1986) and IL-6 production (Kishimoto et al., 1995). These cytokines also contribute to the articular damage observed in rheumatoid arthritis. Induction of IL-6 (Helfgott et al., 1989) and TNF-α (Debets et al., 1988) by IgG-beads is relevant to the course of antibody-coated pathogens against bacteria and viral infection. However, our data showed that ImIgG is more potent in cytokine induction compared to IgG-beads (Fig. 5.7A). The reasons for the differential responses in the cytokines production by two different composition of ICs i.e. ImIgG versus IgG-opsonized particles can be explained as follows: i) an elevated amount of IL-6 and TNF-α released by ImIgG stimulated macrophages is relevant to the disease states such as rheumatoid arthiritis, where production of pro-inflammatory cytokines fails to cease, resulting in sustained changes in receptor expression, augmentation of macrophage effector function, and sometimes tissue injury (O'Shea et al., 2002). ii) The relatively lower amount of IL-6 and TNF-α produced by IgG-beads may be due to the different form of IgG (IgG opsonized latex beads) used in this experiment. ImIgG may provide more prolong stimulation to macrophages than other forms of IgG and hence more potent in cytokine induction.

FcγR cross-linking also induces IL-1β (Remvig et al., 1990) and GM-CSF (Herrmann et al., 1992) production. Results from cytokine-array (Fig. 5.6) and ELISA (Fig. 5.7A) assay showed that IL-1β and GM-CSF was only induced by ImIgG. Previous studies reported that ImIgG does not induce IL-1β unless stimulated with low doses of LPS in monocytes (Arend et al., 1991; Kindt et al., 1993). This is in contrast to our study that

ImIgG induces IL-1β production. The possibility of LPS contribution in this experiment was eliminated by using polymyxin B to inhibit LPS response (Fig. 5.7B). However, IL-1β (Buchan et al., 1988; Mullazehi et al., 2006) and GM-CSF (Xu et al., 1989; Alvaro-Gracia et al., 1991) production can be seen in tissue-fixed immune complex mediated chronic disease such as RA. During RA, the dominant cell type in the joint is the macrophage (Kinne et al., 2000). In our experiment, the cell type we used was GM-CSF macrophage which mimicks proinflammatory macrophage (Hamilton et al., 1980). The proinflammatory role of GM-CSF has been demonstrated using various models of inflammation and immunity. It is shown that administration of rGM-CSF accelerates onset and exacerbates the pathology of murine collagen-induced arthritis (CIA) (Campbell et al., 1997) while GM-CSF-deficient mice show decreased susceptibility to CIA (Campbell et al., 1998). GM-CSF-deficient mice also showed exacerbated susceptibility to infection with *Listeria monocytogenes*, which correlates with a poor inflammatory response and failure to maintain a supply of phagocytic cells over the long term (Zhan et al., 1998). Moreover, GM-CSF can prime monocytes/macrophages for cytokine production (Hart et al., 1988; Hamilton, 1993a), led to to the proposal of a cytokine stimulating factor (CSF) network loop in RA where GM-CSF has a central role in maintaining joint inflammation (Hamilton, 1993b). Therefore, it is not surprising that ImIgG can induce IL-1β and GM-CSF in macrophages. The possibility of IgG-beads not inducing IL-1β and GM-CSF could be due to different forms of IgG that was given to stimulate FcγRs.

However, in addition to these pro-inflammatory cytokines, FcγR cross-linking induces anti-inflammatory cytokines, IL-10 (Berger et al., 1996). It showed that TNF-α, IL-6 and IL-10 was produced when two different immune-complexes formed of tetanus

toxoid and polyclonal anti-tetanus toxoid antiserum, as well as heat-aggregated human IgG stimulated human monocytes. In this study, cytokine was induced in dose dependent and antigen/antibody ratio-dependent manner. Additionally, this investigator demonstrated that endogenously synthesized IL-10 limited the immune complex induced secretion of pro-inflammatory cytokines TNF-α and IL-6 (Berger et al., 1996). This anti-inflammatory property is associated with the T helper type 2 (Th2) immune responses. Therefore, this study concluded that immune complexes, besides their well-known ability to cause acute and chronic inflammation, can mediate immunosuppressive effects and influence the balance of Th1/Th2 responses. Similarly, our data showed that IL-10 was induced by both ImIgG and IgG-beads (Fig. 5.6). The presence and the role of IL-10 were investigated in RA condition. IL-10 produced spontaneously by the RA cultures was shown by neutralization experiments to play an important regulatory role in RA in the synovial membrane cultures (Katsikis et al., 1994). Blocking IL-10 in these cultures with a neutralizing monoclonal antibody resulted in two to threefold increase in the levels of TNF-α and IL-1β whereas, exogenous rIL-10 decreases these cytokines in RA culture (Katsikis et al., 1994). Similarly, IL-10 produced by IgG-beads down regulates the pro-inflammatory effects in the course of bacterial infection (Sutterwala et al., 1998).

6.3.3 The role of three classes FcγRs are different for different cytokine production

FcγRs cross-linking is known to induce different cytokine production. The particular FcγRs involved in cytokine induction is not clearly addressed. It would be very interesting to know what type of FcγR is responsible for the induction of cytokine production by macrophages. In this study, it was determined by using blocking antibody

specific for FcγRI, FcγRII and FcγRIII in response to immobilize IgG and IgG-beads. Instead of cross-linking each FcγR separately we took this approach because cross-linking assay would give rise to inadequate binding affinity of antibody to each FcγRs. This would make it difficult to assess the differences of cytokine production induced by FcγRs. Previous study showed that IL-6 was produced when FcγRI was cross-linked with solid-phase bound mouse IgG1. Stimulation of FcγRII with solid-phase bound mouse IgG1 did not significantly induce IL-6 production from monocytes (Krutmann et al., 1990). Our antibody blocking study showed that ImIgG induced IL-6 production was significantly inhibited by FcγRIII and to lesser extent by FcγRI antibodies (Fig. 5.7A). This suggests that either FcγRIII or FcγRI may play important role in IL-6 induction. In the case of IgG-beads induced IL-6 prodcution, either FcγRIII or FcγRII play important role (Fig. 5.9A). A study showed that cross-linking of monocyte FcγR by solid-phase human IgG induces secretion of TNF-α (Debets et al., 1988). This investigator further showed that cross-linking of FcγRI by solid-phase mouse IgG1 induced TNF-α secretion from monocytes (Debets et al., 1990). FcγRII does not induce TNF-α release from monocytes but triggers this effect after exposure of cells to proteolytic enzymes. In addition, immune complex mediated TNF-α production occurred via FcγRII has been shown in transgenic mouse model (Tan et al., 2005). FcγRII-transgenic mice spontaneously developed a feature like rheumatoid arthritis (RA) and systemic lupus erythrematosus (SLE) diseases. Elevated levels of TNF-α production was observed from the same FcγRII-trangenic macrophages. In another study on synovial macrophages from RA patients showed increased expression of FcγRII and FcγIII compared with controls (Blom et al., 2003). Macropahges from these patients showed much higher production of TNF-α and IL-1β after immune complex sitmulation. This study suggests that enhanced expression of FcγRII and FcγRIII is

associated with higher TNF-α and IL-1β production (Blom et al., 2003). Our antibody blocking data showed that each FcγR independently is a potent inducer of TNF-α induction by both ImIgG (Fig. 5.8B) and IgG-beads (Fig. 5.9B). As shown earlier IL-1β and GM-CSF was induced only by ImIgG. Previous study showed that tetanus toxoid human anti-tetanus toxoid IC induces FcγRs on monocytes to release IL-1β (Remvig et al., 1990). Specific FcγR involved in induction of this cytokine has not been shown. Our data suggest that FcγRIII play a more prominent role in IL-1β induction (Fig. 5.9A). FcγRI cross-linking by solid-phase mouse IgG2 have been shown to induce GM-CSF production from monocytes. Neither endotoxin, nor phorbol compounds have been shown to elicit GM-CSF by monocytes (Herrmann et al., 1992). Similar to this report, our data showed it is only induced by human ImIgG and FcγRI play important role for this cytokine nduction (Fig. 5.9B). Over all, our data from antibody blocking study suggest that the different FcγRs determine for different cytokine induction and the requirement of different FcγRs are different. Also our data is not contradicted with previous report the role of different FcγRs for different cytokine production. Only that the requirement of different FcγRs for different cytokines was not clarified.

In addition to these pro-inflammatory cytokines, the production of anti-inflammatory cytokines IL-10 production was observed by both ImIgG and IgG-beads stimulated macrophages (Fig. 5.7). In this regard, a study from knockout mice identified the specific FcγR responsible for IL-10 induction. Macrophages from mice lacking the FcRγ-chain, which is required for assembly and signaling FcγRI and FcγRIII, failed to up regulate IL-10 in response to IgG-opsonized sheep red blood cell (Sutterwala et al., 1998). However, mice lacking either the FcγRII or the FcγRIII were fully capable of up regulating IL-10 production, implicating FcγRI in this process. In this mouse models tested, the ligation of FcγR promoted the production of IL-10 and inhibited the secretion

of IL-12. This reciprocal alteration in the pattern of macrophage cytokine production illustrated an important role for FcγR-mediated clearance in suppressing macrophage pro-inflammatory responses. The findings in our study that ImIgG-elicited IL-10 production was inhibited by all three FcγRs antibody (Fig. 5.8C) suggest that IL-10 induction required all the three FcγRs. In the contrast, IgG-beads induced IL-10 production was inhibited by either FcγRII or FcγRIII antibody (Fig. 5.9C). This suggests that these two receptors play important role in IL-10 induction. In summary, all these data suggest that different cytokines are differently induced by different FcγRs.

6.3.4 IL-8 production is not sensitive to the blocking of any of the three FcγRs

Cross-linking of monocyte FcγR on LPS free human immobilized IgG induced bioactive IL-8 release (Marsh et al., 1995). Our study showed that ImIgG and IgG-beads induced GM-CSF macrophages to produce huge amount of IL-8 (Fig. 5.11). The induction of IL-8 is mediated by FcγRs has clinical relevance, because high levels of IL-8 and activated neutrophils are found in immune complex-mediated diseases such as the joint space in rheumatoid arthritis (Seitz et al., 1991; Verburgh et al., 1993) or the lung in pulmonary idiopathic fibrosis (Lynch, III et al., 1992; Car et al., 1994). The mechanism of IL-8 production in these diseases has been postulated to occur through monocyte and macrophage IL-1β and TNF-α induction fibroblast and epithelial cell IL-8 (Standiford et al., 1990; Rathanaswami et al., 1993). However, in the absence of LPS, monocytes or macrophage FcγR cross-linking does not induce IL-1β (Marsh et al., 1994) and releases relatively low concentrations of TNF-α (Kindt et al., 1993). Because neither rheumatoid arthritis nor idiopathic pulmonary fibrosis is associated with circulating LPS, immune complexes may directly induce local IL-8 production. LPS contamination in our study was excluded by using LPS inhibitor polymyxin B (data not

shown). Thus it is conceivable that ImIgG and IgG beads may induce larger amount of IL-8 from macrophages.

The role of specific FcγRs in induction of IL-8 production was determined by using anti-FcγR antibodies (Marsh et al., 1996). It showed that IL-8 was stimulated directly through FcγRI cross-linking and indirectly through an FcγRIII-stimulated soluble lymphocyte factor. The role of different FcγRs was also demonstrated in transgenic mice (Sylvestre and Ravetch, 1994). These mice were deficient for the homodimeric γ subunit required for signaling through FcγRI, FcεRI and FcγRIII, while control mice had intact γ subunits. Interestingly, IgG-containing immune-complexes did not cause neutrophil recruitment in the transgenic γ subunit deficient mice, but did in the controls (Sylvestre and Ravetch, 1994). Because FcεRI also requires a γ subuint for signal transduction, FcεRI-deficient mice with intact FcR γ chain was subsequently found to recruit neutrophils. This was observed in the control mice as well. Thus, immune complex-mediated neutrophil recruitment is dependent on functional FcγRI and FcγRIII. This study is in contrast to our study where blocking of all the three FcγRs did not inhibit IL-8 production by both ImIgG and IgG-beads (Fig. 5.12).

Importantly, sustained amount of IL-8 release by ImIgG and IgG-beads where combination of any of these FcγRs did not block IL-8 production raises the possibility that lymphocytes in the macrophage preparations are producing some factors that further stimulates macrophage IL-8 release. Although in our experiment, the target cell population of macrophage did have lymphocyte contamination, the percentage was negligible as more than 90% cells are adherent macrophages. It has been described previously, that FcγR-mediated IL-8 release is primarily a monocyte product, since

equivalent numbers of autologous lymphocytes and neutrophils released significantly less amount of IL-8 (Smyth et al., 1991). In regard to this, Marsh et al (Marsh et al., 1996) demonstrated that supernatants from ImIgG or anti-FcγRIII-stimulated lymphocytes induced monocytes to release more IL-8 than lymphocytes incubated on plastic alone. They also showed that THP-1 cells, which do not produce IL-8 in response to FcγR cross-linking, also released IL-8 in response to supernatants from IgG or FcγRIII-stimulated lymphocytes. This result suggests that there may be unknown soluble secretor factors produced by the stimulated lymphocytes which could stimulate IL-8 production by monocytes. However, they could not block this activity using recombinant cytokines or neutralizing anti-cytokine Abs (Marsh et al., 1996). Since none of the FcγR antibody blocks IL-8 release, it can be explain that the macrophage express some other receptors that regulate ImIgG and IgG-beads. Another explanation is that IL-8 is easily induced from macrophages. However, further investigation needs to be done in relation to sustained amount of IL-8 production.

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APPENDIX

MEDIA and SOLUTIONS

Molecular biology

LB-broth (Luria-Bertani medium)

Bacto-tryptone	1.0%
Yeast extract	0.5%
NaCl	0.5%

Adjust pH to 7.5 with 5 M NaOH prior to autoclaving.
Media was sterilized by autoclaving at 15 LB/in^2 for 15 min.
Thermolabile antibiotics were filter-sterilized through 0.2 μm filters and added to the autoclaved LB-broth. A final concentration for ampicillin was 100 μg/ml.

LB agar plates

LB-broth was prepared as above with 15 g/L of Bacto-agar added. Media was sterilized by autoclaving.

50 x TAE buffer (for DNA gel electrophoresis)

Tris base	2 M
Acetic acid	1 M
EDTA	0.1 M
pH 7.8	

10 x DNA loading buffer

Ficoll400	20% (w/v)
EDTA(pH 8.0)	0.1 M
Bromophenol blue	0.25% (w/v)

Ethidium bromide

The stock solution was made at 10 mg/ml and stored in a light-tight bottle. Final working concentration was 0.2 μg/ml.

Cell Biology

1 x PBS

KH_2PO_4	1.76 mM
Na_2HPO_4	10.4 mM
NaCl	137 mM
KCl	2.7 mM

2 x HBS buffer

NaCl	280 mM
KCl	10 mM
$Na_2HPO_4.2H_2O$	1.5 mM
KCl	2.7 mM
D.Glucose anhydrous	12mM
Hepes	50mM

Protein Biology

Cell lysis buffer

Tris-HCl, pH 7.4	50 mM
NaCl	150 mM
EDTA	2mM
EGTA	2mM
KCl	20mM
NP-40	1% (v/v)

Wash buffer

Tris-HCl, pH 7.4	50 mM
NaCl	300 mM
KCl	20mM
EDTA	2mM
EGTA	2mM

Elution Buffer

Tris-HCl, pH 8.0	20 mM
NaCl	500 mM
Imidazole	250 mM
$CaCl_2$	1 mM
$MgCl_2$	1 mM

Protease inhibitors were added to all these buffers just before using:

PMSF	2 mM
Aprotinin	10 μg/ml
Leupeptin	10 μg/ml

Reagents for SDS-PAGE gel electrophoresis

Stacking gel preparation (for 1 mini SDS-PAGE gel)

dH_2O	3.05 ml
0.5 M Tris-HCL, pH 6.8	1.25 ml
10% (w/v) SDS	50 μl
30% Acrylamide/Bis Solution 29:1 (3.3%C)	0.65 ml
10% APS	25 μl
TEMED	5 μl

Separating gel preparation (for 1 mini SDS-PAGE gel)

% of gel	5%	7.5%	10%	12.5%	15%
dH_2O (ml)	5.1	4.85	4	3.17	2.35
1.5 M Tris-HCL, pH 8.8 (ml)	2.25	2.5	2.5	2.5	2.5
10% (w/v) SDS (µl)	90	100	100	100	100
30% Acrylamide/Bis Solution 29:1 (3.3%C) (ml)	1.5	2.5	3.33	4.16	5
10% APS (µl)	45	50	50	50	50
TEMED (µl)	6	5	5	5	5

10 x SDS-PAGE electrophoresis buffer

Tris base 25 mM
Glycine 192 mM
SDS 1% (w/v)
Adjust the pH to 8.3.

SDS-PAGE electrophoresis was carried out at a constant voltage of 100V.

5 x Reducing sample loading buffer

Tris-HCL, pH 6.8 1 M
Glycerol 50% (v/v)
SDS 10% (w/v)
Bromophenol Blue 1% (w/v)
β-mercaptoethanol 0.5 ml in 10 ml of sample loading buffer

Non-reducing sample loading buffer is prepared as above without the addition of β-mercaptoethanol.

10 x Western blot transfer buffer

Tris base 25 mM
Glycine 192 mM

15% (v/v) methanol was added to 1x Western blot transfer buffer before use.

TBST buffer

TBS buffer with 0.05% Tween-20

Blocking Buffer

Non-fat milk 5%
Buffer prepared in TBST