GENETIC IMMUNIZATION APPROACHES ON THE CONTROL OF ALLERGIC INFLAMMATION

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Publications

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Summary

The incidence of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and hayfever is increasing over the past few decades. Current treatments for these diseases still mainly provided symptomatic relief. Allergen gene immunization and cytokine gene immunization are novel approaches that have been explored as alternative treatments for allergic diseases. Studies carried out using murine models have shown that allergen gene immunization approach is very effective for anti-allergic reactions; however the major drawback of this approach in human clinical trials is its low immunogenicity.

The first part of the study aimed at enhancing and evaluating the immunogenicity and efficacy of DNA vaccines by the incorporation of additional human CpG motifs (D and K type, designated as DTKT) into the pVAX plasmid backbone. cDNA encoding for a major allergen from the *Blomia tropicalis* mite, Blo t 5, was cloned into the pVAX vector. CpG-modified and unmodified DNA constructs were designated as pVAXBlot5-DTKT and pVAXBlot5 respectively. Blo t 5 was used as the model allergen in this study and the immunogenicity of DNA constructs was evaluated in the Blo t 5-induced murine allergy model. The modified pVAXBlot5-DTKT elicited a higher level of Blo t 5-specific IFN-γ when compared to pVAXBlot5. Both pVAXBlot5-DTKT and pVAXBlot5 attenuated Blo t 5-specific IgE, in addition pVAXBlot5-DTKT elicited higher levels of IgG2c than pVAXBlot5. In addition, allergen gene vaccination also suppressed Blo t 5 specific IFN-γ, IL-17, IL-5, IL-13 and IL-6 induced by intraperitoneal immunization with rBlo t 5 protein adjuvanted with aluminium hydroxide. *In vitro* evaluation of plasmid constructs was carried out

using human peripheral blood mononuclear cells (PBMCs). Co-culture of PBMCs with plasmid DNA revealed that pVAXBlot5-DTKT elicited higher levels of pro-inflammatory cytokines and chemokines such as IL-6, MIP-1α and MIP-1β when compared to pVAXBlot5 and DTKT oligonucleotides stimulation. In addition, pVAXBlot5-DTKT induced up-regulation of the release of chemotactic chemokines and mediators for the inflammatory response such as PDGF-AA, GRO-α, NT-4, osteoprotegerin, VEGF-D, GM-CSF, IL-1β, MCP-1 and ENA-78 from PBMC when compared to pVAXBlot5 at an early time point. *In vivo* evaluation of the various plasmids in rhesus macaques showed that monkeys immunized with both pVAXBlot5 and pVAXBlot5-DTKT showed suppression in Blo t 5-specific IgE when compared to monkeys immunized with the pVAX control. In addition, monkeys immunized with pVAXBlot5-DTKT also produced higher levels of Blo t 5-specific IgG when compared to those immunized with pVAXBlot5. Furthermore, the frequencies of IFN-γ producing cells in response to stimulation with Blo t 5 peptide pools were higher from monkeys immunized with pVAXBlot5-DTKT as compared to those immunized with pVAXBlot5 in the ELISPOT assay.

The second part of the thesis aimed to explore the use of the IL-35 encoding gene immunization approach for the treatment of allergic diseases. IL-35 is a novel cytokine that is produced by $Foxp3^{+}$ regulatory T cells and has been reported to have suppressive functions. However the therapeutic role of IL-35 in Th2 mediated allergic diseases has not been evaluated. Hence in this study, cDNA encoding a single chain IL-35 was cloned into the pVAX vector, designated as pVAX-IL-35, and the suppressive effect of IL-35 was evaluated in two murine models. Firstly, the effectiveness of pVAX-IL-35 was evaluated in the eosinophilic lung inflammation

model induced by a Blo t 5-specific Th2 cell line. Intra-tracheal administration of pVAX-IL-35 was found to be able to attenuate the Blo t 5-specific Th2 cell line induced total cellular infiltration as well as eosinophil, lymphocyte and neutrophil counts in the bronchial alveolar lavage fluids (BALF). In addition, Th2 cytokines IL-4, IL-5 and IL-13 as well as chemotactic chemokines CCL2/MCP-1, CXCL1/KC, CXCL5/LIX, CXCL9/MIG and CXCL10/IP-10 were significantly attenuated in the BALF from the experimental group of mice received pVAX-IL-35 as compared to that from the control pVAX group. Systemic administration of pVAX-IL-35 via the intramuscular injection elicited a long-term suppression of Blo t 5-specific humoral responses in mice upon intranasal Blo t 5 instillation. Mice received pVAX-IL-35 injection produced lower titres of Blo t 5-specific IgE and persistently suppressed Blo t 5-specific IgE and IgG1 production. The long-term suppression by the pVAX-IL-35 injection was also found on the production of total serum IgE in mice which received the Blo t 5 specific Th2 cell transfer and intranasal Blo t 5 challenges. In contrast, there was no difference in the total serum IgM, IgA, IgG1 and IgG2c between mice that received pVAX or pVAX-IL-35. These results indicated that systemic administration of IL-35 by the DNA immunization did not exert a pan immunosuppressive effect on B cells.

Secondly, the effectiveness of pVAX-IL-35 was evaluated in a neutrophilic lung inflammation model induced in Blo t 5-specific T cell receptor (TCR) transgenic mice upon intranasal Blo t 5 sensitization. Intra-tracheal administration of pVAX-IL-35 was also found to effectively reduce total cellular infiltration as well as neutrophil and lymphocyte infiltration in Blo t 5-specfic TCR transgenic mice upon intranasal Blo t 5 instillation. In addition, pro-inflammatory cytokines and chemotactic chemokines

such as IFN-γ, IL-1α, IL-6, IL-17, GCSF, LIF, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, CXCL1/KC, CXCL9/MIG and CXCL10/IP-10 were significantly attenuated in the BALF from mice in the experimental pVAX-IL-35 group as compared to that from the control pVAX group. These data showed that IL-35 could be a promising therapeutic tool against allergic and neutrophilic airway inflammation. Taken together, the new findings from this thesis will make valuable contributions to the development of efficacious approaches in allergen gene as well as cytokine gene deliveries for the control of allergic diseases.

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List of Abbreviations

AHR	airway hyperresponsiveness
AIM-2	absent in melanoma 2
Alum	aluminium hydroxide
APC	antigen presenting cells
BALF	bronchoalveolar lavage fluid
Blo t	*Blomia. tropicalis*
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary DNA
CXCL	chemokine (C-X-C motif) ligand
DAI	DNA dependent activator of interferon
DC	dendritic cell
Der p	*Dermatophagoides. pteronyssinus*
Ebi3	Epstein-Barr-virus induced gene 3
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Flt3L	FMS-like tyrosine kinase 3 ligand
Foxp3	forkhead box P3
GATA3	GATA binding protein 3
GMCSF	granulocyte macrophage colony stimulating factor
Htpa	human tissue plasminogen activator
Hr	hour
ICOS	inducible T cell co-stimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m	intramuscular
i.p	intraperitoneal
IP-10	interferon gamma-induced protein 10
IPS-1	Interferon beta promoter stimulator 1
IRAK	IL-1R associated kinase 1
KC	keratinocyte derived chemokine
LAMP-1	lysosome associated membrane protein 1
LIMP-II	lysosomal integral membrane protein II
MCP-1	monocyte chemotactic protein 1
mDC	myeloid dendritic cell
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
ND	not detectable
NK	natural killer cell
MyD88	myeloid differentiation primary response gene 88
NOD	nucleotide oligomerization domain
OD	optical density

ODN	oligonucleotide
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
pNPP	paranitrophenyl phosphate
Rag1	recombination activating gene 1
RBC	red blood cell
RIG-1	retinoid-inducible gene 1
ROR	retinoid-related orphan receptor
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TAK1	transforming growth factor β-activated kinase 1
TAP	transporter associated with antigen processing
TBK	tank binding kinase
TCR	T cell receptor
Th	T helper cell
TIR	toll/interleukin 1 receptor
TLR	toll like receptor
Treg	T regulatory cell
TRIF	Toll-interleukin-1 receptor domain containing adaptor inducing beta-interferon
ZBP-1	Z-DNA binding protein 1

Chapter 1 .Literature Review

1.1 Allergic airway inflammation

1.1.1 Epidemiology, prevalence and risk factors

The incidence of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and hayfever had increased over the past few years. Population based studies reported the variation in the prevalence of allergic diseases in Asia and rising prevalence of allergic diseases mainly occurred in low and mid-income countries (Bjorksten et al., 2008; Pearce et al., 2007). The major risk factor for asthma and allergic rhinitis was allergic sensitization and it had been estimated that about 300 million people worldwide suffer from asthma (Pawankar et al., 2009). Other risk factors for allergic asthma included environmental, occupational and genetic factors. In atopic individuals, inappropriate immune responses were mounted against otherwise harmless antigens. Although there were differences in the aeroallergens that triggered allergy and asthma in various areas of Asia Pacific, the important source of allergens in Asia was from house dust mites followed by pollens, insects, molds and fungi (Daengsuwan et al., 2003). It was estimated that about 10% of the general population and 90% of asthmatic people developed sensitivity to house dust mites (Chew et al., 1999a).

1.1.2 House dust mites

The main allergenic components from household dust were house dust mites and these mites were complex organisms that secreted a variety of different proteins and other macromolecules. In the temperate regions, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* were the predominant species while in the tropics

Dermatophagoides pteronyssinus and *Blomia tropicalis* were the major allergen sources (Fernandez-Caldas, 1997; van Hage-Hamsten, 1995). Highly diverse allergens had been identified from dust mites with more than 20 groups of allergens been characterized, out of which group 1, 2, 3, 9, 11, 14 and 15 allergens had been reported to have strong IgE binding (Thomas et al., 2002). The group 1 and 2 allergens constituted the greatest proportion of allergenicities in *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae*, besides that they also constituted the major allergens in dustmite extracts (Chapman and Platts-Mills, 1980; Custovic et al., 1996; Lind and Lowenstein, 1983; Platts-Mills and Chapman, 1987).

Blomia tropicalis group 5 allergen (Blo t 5) was the first major allergen from the *Blomia tropicalis* group and was cloned and expressed as a fusion protein with glutathione-S-transferase (Arruda et al., 1997; Caraballo et al., 1996). The 14 kDa Blo t 5 was a very important allergen among the eight allergens evaluated in *Blomia tropicalis,* Blo t 1, Blo t 2, Blo t 3, Blo t 5, Blo t 10, Blo t 11, Blo t 12 and Blo t 13, as it had IgE reactivity of up to 70% in allergic subjects as reflected by skin prick tests and *in vitro* IgE binding assays. In addition, 40-60% of individuals allergic to *Blomia. tropicalis* have Blo t 5 specific IgE (Tsai et al., 2003). Despite sharing a 43% identity in their amino acid sequences, Blo t 5 had been demonstrated via *in vitro* and *in vivo* assays to exhibit low levels of IgE cross-reactivity with its homologue, Der p 5 (Chew et al., 1999b; Kuo et al., 2003; Simpson et al., 2003). Blo t 5 consisted of a 17-residues signal peptide and a 117-residues mature protein in comparison to the homologous Der p 5 that contained a 19-residues signal peptide and a 113-residues mature protein (Arruda et al., 1997; Liaw et al., 2001). The nuclear magnetic resonance structure and IgE epitopes of Blo t 5 had been determined that showed Blo

t 5 to be made up of a helical bundle consisting of three anti-parallel helices (Naik et al., 2008). Four charged residues, Glu76, Asp81, Glu86 and Glu91 had been identified to be located on a major putative linear IgE epitope of Blo t 5 from residues 76-91 and it consisted of the sequence ELKRTDLNILERFNYE (Chan et al., 2008).

1.2 DNA vaccines

DNA vaccines were a promising therapeutic tool as it conferred many advantages over other vaccine approaches. DNA vaccines were inexpensive and easier to purify in large amounts, in addition, there was sustained antigen expression, a cold chain was not required and it was relatively safe. Limitations of major class histocompatibility complex (MHC) restrictions were circumvented as many potential class I and II epitopes were provided by having full length-cDNA (Hirschhorn-Cymerman and Perales). Unlike immunization with protein vaccines, synthesis of proteins encoded by plasmids in host cells resulted in the antigen likely to be correctly glycosylated, folded in the native confirmation and correct post translational modifications which induced the formation of correct neutralizing antibodies. Immunization with plasmid DNA allowed the continued synthesis of protein in the host cells compared to protein vaccines which may be degraded with time. In addition, another interesting feature of DNA vaccine was that it could be engineered to target many diseases by modifications to the plasmid DNA. Genetic immunization involved injecting a DNA plasmid encoding the gene of interest into the muscle or skin of the host whereby it entered the host cells and directed the synthesis of the antigen. The antigen was in turn processed and presented by the host cells or antigen presenting cells (APCs) and an immune response against the antigen was mounted. DNA vaccines had been known to be able to induce both humoral and T cell responses in various animal

models and to be able to mount strong immune responses against infectious diseases such as tuberculosis, Ebola virus and malaria (Hoffman et al., 1997; Lowrie et al., 1997; Strugnell et al., 1997; Wang et al., 1998; Xu et al., 1998).

Immunization with DNA vaccines could manipulate the balance of T helper type 1 (Th1) and T helper type 2 (Th2) immune responses, hence it could be used for the therapy and prevention of many diseases such as cancer, autoimmunity and allergic diseases. Studies had shown that DNA vaccines were able to induce Th1 responses and suppress Th2 responses in immunoglobulin E mediated allergic responses (Hartl et al., 1999; Hsu et al., 1996; Raz et al., 1996). Besides skewing the immune responses to that of a Th1, DNA vaccines were also able to induce Th2 responses to protect against experimental autoimmune encephalitis (Ramshaw et al., 1997). DNA vaccines had been tested and proved to be safe in both small and large animals. HIV-1 encoding DNA plasmid when administered to pregnant, adult and infant chimpanzees had proved to be safe and well tolerated (Bagarazzi et al., 1998). In addition, DNA vaccines for HIV-1 had already entered human clinical trials (Boyer et al., 1999; MacGregor et al., 1998). The safety of DNA vaccine was further emphasized in another study whereby intramuscular administration of a malaria DNA vaccine of up to 2500 μg plasmid DNA was also well tolerated (Le et al., 2000).

The treatment for allergic asthma by pharmaceutical drugs was still mainly asymptomatic (Katial et al., 2011). An alternative treatment for allergy was immunotherapy that could cure allergic asthma, however it consisted of many applications of allergen extracts at increasing dosage over the course of a few years (Edlmayr et al., 2011). More work had to be done to address the issue on the

underlying immunological mechanisms of immunotherapy. However due to the long course of this therapy, it was inconvenient and there were many cases of patient noncompliance. In addition it had the potential to induce anaphylaxis thus requiring close monitoring by clinicians (Bernstein and Epstein, 2011). As such, there was a need for an alternative treatment. DNA-based immunization was a potential candidate to solve this problem in the future.

1.2.1 Optimisation Strategies

However the main disadvantage of DNA vaccines was its low immunogenicity, hence many approaches to enhance the potency of DNA vaccines had been used over the years such as improvement of promoter efficiency by modifying the plasmid backbone, use of more efficient leader sequences (Ulmer et al., 2006), co-delivery of cytokine genes (Chattergoon et al., 2004; Chow et al., 1998; Kim et al., 1997), ubiquitination of the allergen protein (Bauer et al., 2006), incorporating localization and secretory signals (Jiang et al., 2002), use of chemical adjuvants (Jin et al., 2004), increased gene expression (Baldwin et al., 1999; Jiang et al., 2002) and incorporation of immunostimulatory sequences in the plasmid backbone (Kojima et al., 2002; Zhang et al., 2005). Much attention had been placed on the incorporation of immunostimulatory sequences such as CpG motifs into the plasmid backbone.

1.2.2 LAMP-1 targeting sequence

The targeting sequence of the lysosome associated membrane protein-1 (LAMP-1), had been extensively reported to be able to enhance the delivery of antigen to MHC class II rich compartment of APCs by targeting endogenously synthesized protein to the lysosomal/endosomal vesicular pathway in transfected APCs (Chen et al., 1988;

Chen et al., 1985; Wu et al., 1995). The processed antigen would be loaded onto MHC class II molecules, thus enhancing $CD4^+$T cells mediated responses, in contrast, cytoplasmic antigens that lacked the LAMP-1 targeting sequence would be processed and loaded onto MHC class I, hence had less efficacy in inducing $CD4^+$T cells mediated responses (de Arruda et al., 2004). In addition, DNA vaccines that employed the use of LAMP-1 targeting sequence had also been reported to induce robust Th1 responses (de Arruda et al., 2004; Kim et al., 2003; Marques et al., 2003). Besides that, the use of lysosomal targeting approach had been highlighted in another study whereby mice immunized with DNA constructs encoding a mite allergen with signal peptide and lysosomal-targeting sequence showed strong and exclusive Th1 responses (Tan et al., 2006). In contrast, mice immunized with DNA constructs encoding the mite allergen with signal peptide and without lysosomal-targeting sequence showed a mixed Th1/Th2 phenotype, strong IgE production and a large amount of circulating allergen protein encoded by the DNA construct (Tan et al., 2006). This was potentially dangerous as Th2-skewed immune responses may be induced, hence lysosomal-targeting sequences could improve the safety and efficacy of DNA vaccines.

1.2.3 Different Routes of DNA Immunization

The common methods of delivering DNA vaccines had been needle injection into skin or muscles and the use of a gene gun to propel gold particles covered by plasmid DNA using helium or carbon dioxide pressure into tissues (Williams et al., 1991). Gene gun delivery required far lesser quantities of DNA than intramuscular (i.m) injection using needle. Muscle was a preferred route for the delivery of DNA vaccines as it was more efficient in the uptake and expression of plasmid DNA encoded

proteins. Plasmid DNA and encoded proteins had been detected as late as 19 months post injection in the muscle (Wolff et al., 1992). Electroporation had been shown to enhance uptake efficiency of plasmid DNA (Bachy et al., 2001). A study had reported that in muscles injected with DNA, MHC class II positive cells accumulated near transfected mononuclear cells and such accumulation might facilitate antigen transfer to APCs and result in antigen presentation and activation of T cells (Gronevik et al., 2003).

The different sites of DNA immunization also influenced the Th bias of the immune response generated. It had been shown by many studies that intramuscular injection elicited Th1-like responses while DNA administration to the skin elicited more Th2-like immune responses (Fan et al., 1999; Fuller and Haynes, 1994; Sin et al., 1999). Injection into the muscle transfected mainly myocytes while intradermal injection resulted in the transfection of mainly keratinocytes and skin fibroblasts. Delivery of DNA vaccine intradermally and using the gene gun targeted the skin where there were two populations of dendritic cells (DC), namely the interstitial dendritic cells and the epidermal Langerhans cells (Banchereau and Steinman, 1998). However i.m injection targeted the muscle where there were no Langerhans cells. In addition there were fewer dendritic cells as compared to the skin. Both needle injections as well as the use of gene gun were physical injuries that caused local irritation and elicited danger signals which would result in the recruitment of APCs.

Muscle cells may not be as inert as previously thought. Increasing evidence had shed light on the possible role of muscle cells as APCs. Myoblasts had been shown to be capable of presenting antigen and stimulating secretion of IL-2 by T cell hybridomas

using class I and class II MHC transfectant clones of myoblast cell line (Rock and Shen, 2005). The presentation of both exogenous and endogenous antigen by myoblasts stimulated antigen specific $CD4^+$ T cells (Goebels et al., 1992; Rock and Shen, 2005). Another study also showed that *in vivo* plasmid transfected myocytes could present peptides to $CD4^+$ T cells and that i.m injection of unmethylated CpG motifs induced expression of chemokines such as monocyte chemotactic protein 1 (MCP-1) and MHC class II on myocytes (Stan et al., 2001). In addition, muscle cells served as factories for antigen synthesis to provide the antigen to boost Th1 skewed immune response. Using green fluorescent protein, it had been shown that encoded proteins were synthesized within transfected muscle cells (Gronevik et al., 2003).

1.3 Immune responses

1.3.1 Mechanism of activation of $CD4^+$ and $CD8^+$ T cells / MHC class I and II

The immunogenicity of DNA vaccines was mediated by both $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells gave help to B cells to produce antibodies while $CD8^+$ T cells were involved in cytotoxicity. It had been found that in addition to $CD4^+$ cells, $CD8^+$ cells induced by allergen gene immunization played an important role in conferring protective immunity against allergic responses and also in enhancing the immune response (Hsu et al., 1996). Hence in order to enhance the immunogenicity of DNA vaccines, it was important to target and prime both the $CD4^+$ and $CD8^+$ T cells. The two main classes of MHC were known as MHC class I and MHC class II. MHC class I presented peptides to $CD8^+$ T cells while MHC class II presented peptides to $CD4^+$ T cells. Almost all somatic cells expressed MHC class I while mainly APCs expressed MHC class II. It was commonly known that MHC class I presented epitopes from endogenous proteins while MHC class II presented epitopes from exogenous proteins.

When a virus or bacteria infected the host, the antigens were processed within the cell and the proteasome broke down the full length proteins to peptides which were moved via the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). The peptides associated with MHC class I molecules and β2-microglobulin in the ER and this complex was transported to cell surface whereby it would bind to and activate antigen-specific $CD8^+$ T cells. However a phenomenon termed cross priming or cross presentation had enabled some APCs to accept extracellular proteins into the MHC class I pathway (Rodriguez et al., 1999; Watts, 1997). Extracellular antigen in turn was taken up into the lysosomal compartment of professional APCs whereby it was broken down into peptides and bound to MHC class II molecules. This complex moved to the surface of the APCs whereby it bound to and activated $CD4^+$ T helper cells. The activated $CD4^+$ T helper cells then provided help to B cells, resulting in class-switching.

1.3.2 Possible mechanisms for generation of immune responses via genetic immunization

There were three possible mechanisms for the generation of immune responses via genetic immunization. In the first mechanism, genetic immunization would transfect a cell (eg myocyte or keratinocyte) and protein production occured in the cells. The protein would be broken down and peptides would bind to and be presented on MHC class I molecules, stimulating $CD8^+$ T cells. Soluble protein released by transfected cells might be taken up by bone marrow derived APCs such as dendritic cells, monocytes, macrophages which would process the protein and present it in the context of MHC class II, hence activating $CD4^+$ T cells. In the second mechanism, genetic immunization might directly transfect bone marrow derived APCs residing in

the target tissue hence activating $CD8^+$ T cells. Soluble protein produced by transfected APCs or transfected tissue cells would activate $CD4^+$ T cells. However the second mechanism might not correctly reflect the immune mechanism generated after intramuscular immunization as the muscle environment was relatively devoid of APCs. In the third mechanism, bone marrow APCs infiltrated the muscle after immunization and they took up antigens that were produced by transfected tissue cells such as myocytes or keratinocytes. Some of these antigen after internalization were able to cross over to the MHC class I pathway, hence activating both $CD4^+$ and $CD8^+$ T cells (Cohen et al., 1998).

1.3.3 Cross-Presentation and Cross-priming

Cross presentation/cross priming was the phenomenon whereby extracellular antigens were taken up by APCs and were presented on MHC class I instead of on MHC class II molecules. There were two main pathways by which cross-priming could occur. I.m injection led to transfection of myocytes, resulting in subsequent protein synthesis. Myocytes might serve as a reservoir of antigen for professional APCs via antigen transfer which was shown by a study (Fu et al., 1997). Cross priming usually occurred in specialized APCs such as dendritic cells (DCs) (Heath et al., 2004; Heath and Carbone, 2001). DCs had been found to play a vital role in cross presenting antigen *in vivo*, hence priming $CD8^+$ T cell responses. Ablation of $CD8^+$ T cell responses had been found in mice whereby $CD11c^+$ DCs were depleted (Jung et al., 2002). In agreement to this, another group used parental$\rightarrow F_1$ bone marrow chimeric mice to show that bone marrow derived APCs were required for induction of MHC class I-restricted CTLs after i.m vaccination (Corr et al., 1996). Professional APCs were sparse in the muscle environment and APCs such as macrophages and immature DCs

were normally recruited to muscle after danger signals were elicited by injection. Professional APCs such as dendritic cells would uptake the plasmid DNA by phagocytosis or pinocytosis and express the protein which was presented on MHC class I molecules, hence priming $CD8^+$ T cells (Condon et al., 1996). Transfer of protein, processed peptide and uptake of apoptotic cells by professional APCs could also lead to cross priming of $CD8^+$ T cells.

There were two pathways of cross-presentation, in the first pathway, the extracellular antigen was uptaken into the phagosome and transported into the cytosol where it was broken down into oligopeptides by proteasomes. These oligopeptides were then transported by TAP to the ER or phagosomes where they bound to MHC class I molecules. In the second pathway, endosomal proteases such as cathepsin S cleaved the antigen that had been taken up by phagosomes into peptides and these peptides bound to MHC class I molecules in the endocytic compartment. Cross-presentation led to the priming of $CD8^+$ T cells (Rock and Shen, 2005). There were studies that reported that cross priming may be the main mechanism that induced $CD8^+$ T cell responses (Cho et al., 2001; Corr et al., 1999). Supporting evidence for the importance of cross priming came from studies in which DNA vaccines were found to be less effective in mice that were deficient in receptors for type I interferons as vital role of type I interferons had been highlighted for cross priming (Le Bon et al., 2003; Van Uden et al., 2001).

1.3.4 Strategies to induce $CD4^+$ T cells

$CD4^+$ T cells were important in the induction of immune responses as they not only activated B cells to produce antibodies, they also activated $CD8^+$ T cells via the

cytokines they secreted. It had been shown that addition of a LAMP-1 targeting sequence could prime $CD4^+$ T cells and increase safety and efficacy of DNA vaccines (Tan et al., 2006). Another group used lysosomal integral membrane protein-II (LIMP-II) to target proteins directly to the lysosomes from the ER (Vega et al., 1991). Besides using targeting sequences to the lysosomal and endosomal pathway, other groups had looked at the fusion of invariant chain to proteins as invariant chain blocked the binding of MHC class II molecules to peptides in the ER that had entered via TAP. MHC class II molecule, being attached to the invariant chain then left ER and subsequently fused with endosomes whereby it bound to peptides and presented the epitopes at the cell surface (Malcherek et al., 1998; Sanderson et al., 1995).

1.3.5 Strategies to induce $CD8^+$ T cells

There were two main pathways to induce $CD8^+$ T cells, the first pathway was via the conventional MHC class I pathway whereby peptides in the ER bound to MHC class I molecules with the subsequent activation of $CD8^+$ T cells via the interaction of MHC class I/peptide complexes at the cell surface. The second pathway was via cross-priming whereby protein was taken in by the phagosomes into the cytosol, broken down by the proteasome and lastly entered the ER via the TAP. In this way, exogenous proteins were taken in by the cell and crossed over to the MHC class I pathway. Methods to increase the activation or induction of $CD8^+$ T cells include enhancing protein degradation by ubiquitination and enhancing delivery to the proteasome or proteasome rich cellular compartment (Barry et al., 1995; Fu et al., 1998; Hung et al., 2003; Rodriguez et al., 1997). Other groups had used the minigene approach that encoded a single T cell epitope in DNA vaccines and protection against virus had been induced (Del Val et al., 1991). Cytosolic peptides were normally

generated by ubiquitinaton and degradation of endogenous proteins that entered the ER via TAP. In the ER, the degraded peptides bound to MHC class I, hence minigene enhanced MHC class I presentation by the means of having a small translation peptide that facilitated entry into ER via TAP. In addition, attachment of a signal peptide could also boost the immunogenicity of T cell epitopes by directly targeting it to the ER, hence bypassing the need for TAP (Bacik et al., 1994). Besides targeting at the conventional MHC class I pathway, there were other groups that had shifted their focus to target at the MHC class I cross-priming pathway as cross-priming had been speculated to be the main mechanism of activation of $CD8^+$ T cells. Some studies had showed that heat shock proteins could induce $CD8^+$ T cell responses by being molecular chaperones that transport immunogenic peptides into APCs (Blachere et al., 1997). Activation of $CD8^+$ T cell responses could also be brought about by the binding of heat shock proteins to DCs receptors as well as by targeting proteins to T helper secretory pathway (Oran and Robinson, 2003; Qiu et al., 2000; Singh-Jasuja et al., 2000; Todryk et al., 1999).

1.4 Cells associated with allergic responses

1.4.1 T lymphocytes

The origin of all T cells was from haematopoietic stem cells in the bone marrow, maturation of T cells took place in the thymus and the initial thymocytes did not express CD4 or CD8. These double negative cells then developed to become $CD4^+CD8^+$ double positive cells and eventually to single positive thymocytes that migrated from the thymus to peripheral tissues. Generally, there were three subpopulations of T cells, namely the T helper (Th), T-cytotoxic and T regulatory cell (Treg) cells. Activation of Th cells occured when APCs presented peptide antigens

via the MHC class II molecules that were expressed on the surface of APCs. Activated Th cells differentiated into one of several subtypes such as Th1, Th2, Th17 and Th9 which produced different cytokine profiles to induce diverse immune responses. $CD4^+$ T cells were generally Th cells that recognized MHC class II bound to peptides while $CD8^+$ T cells were generally T-cytotoxic cells that recognized MHC class I bound to peptides. Development of Treg cells occured in the thymus from $CD4^+$ thymocytes and they were referred to as natural Treg, in addition, naïve $CD4^+$ T cells could also convert in the periphery to induced Treg under the effect of certain tolerogenic stimuli. Specific Treg markers were CD4 and CD25 and they were involved in the maintenance of tolerance of immune response (Kong et al., 1989; Sakaguchi et al., 1995; Sakaguchi et al., 2008).

1.4.2 T helper 1 and T helper 2 cells

Two functional subsets of T_H clones termed T_H1 and T_H2 in mouse had been described more than 2 decades ago (Mosmann et al., 1986). These two distinct effector cells were divided by virtue of their cellular functions and lymphokine profiles. Th1 cells produced IFN-γ, IL-2 and lymphotoxin while Th2 cells produced IL-4, IL-5, IL-9 and IL-13. Besides distinct cytokine profiles, Th1 cells were usually associated with the induction of IgG2a or IgG2c antibodies in mice while Th2 cells usually induced the production of IgE antibodies. The type of immune response induced was dependent on the routes of administration of the DNA vaccine (reviewed earlier) and also on the nature of the encoded antigen. T helper lineage fates of Th1 or Th2 were determined by differential transcription factors' activation and expression. The lineage related transcription factor would induce characteristic cytokine secretion while at the same time suppressing cytokine secretion of other lineages. T-box

transcription factor (T-bet) was the master regulator of the Th1 lineage and it was highly expressed in polarized Th1 cells and NK cells and expression of T-bet correlated with IFN-γ expression (Lighvani et al., 2001). Expression of IFN-γ also induced expression of T-bet in an autocrine loop (Lighvani et al., 2001). Besides that, Th2 cells' cytokine profile also changed to that of a Th1 with IFN-γ production and suppression of IL-4 and IL-5 expression when there is overexpression of T-bet (Lighvani et al., 2001). Induction of T-bet expression and activity was mediated by IL-12 via STAT4 or by IFN-γ via signal transducer and activator of transcription-1 (STAT1) (Lighvani et al., 2001). Transcription factor GATA binding protein 3 (GATA-3) was the Th2 master regulator. IL-4 producing Th2 cells' differentiation was driven by IL-4 via STAT6 although GATA-3 was also capable of inducing its expression via an autocrine loop independently of STAT6 (Scheinman and Avni, 2009; Zhu et al., 2001). Polarity of Th1 cells was switched to a Th2 phenotype when GATA3 was overexpressed in Th1 cells. Similarly, cells tend to adopt a Th1 phenotype in the absence of polarizing cytokines when GATA-3 was deleted in peripheral $CD4^+$ T cells. Regulation of T cell lineage commitment was brought about by crosstalk between T-bet and GATA-3 that exerted opposing effect on the transcription of a number of common genes, hence affecting the ultimate commitment to either Th1 or Th2 lineage.

1.4.3 Role of T helper 2 cells in allergic inflammation

Allergic asthma had been associated with activation of T_H2 cells in the airways since two decades ago. Canonical Th2 cytokines included IL-4, IL-5 and IL-13 that were involved in the allergic inflammation, eosinophilia as well as in the mediation of IgE responses. IL-4, IL-5 and IL-13 were clustered with GM-CSF in close proximity on

chromosome 5q and the expression of these cytokines was well coordinated in murine and human T_H2 cells (Cousins et al., 2002; Locksley, 2009). IL-4 played a vital role in Th2 polarization, in addition other Th2-polarizing factors such as MCP-1 and OX40 ligand had been described (Ohshima et al., 1998). Upregulation of IL-4 production had been shown to be brought about by OX40 ligation and this in turn promoted Th2 polarization with the subsequent production of IL-4, IL-5 and IL-13 (Ohshima et al., 1998). IL-13 and IL-4 were both capable of inducing class switch of B cells to IgE production, activating macrophages and monocytes and increasing mucus production in the airways via globlet cell hyperplasia (Wills-Karp, 2004). IL-4 and IL-13 also played a vital role by increasing the expression of cell adhesion molecules in vascular endothelial cells which facilitated the migration of eosinophils (Meager, 1999). The role of IL-13 as a key regulator of the extracellular matrix had been pointed out in studies that reported IL-13 to mediate tissue fibrosis in asthma, besides that, the expression of MHC class II on monocytes was also upregulated by IL-13 (Wynn, 2003).

IL-5 was the key cytokine that was involved in the recruitment of eosinophils to the lung from the bone marrow, In addition, IL-5 also played a vital role in the proliferation, differentiation of eosinophils as well as the release of eosinophils from the bone marrow (Broide, 2002; Cho et al., 2004; Lopez et al., 1988; Rothenberg, 1998). The importance of IL-5 was highlighted in a study using IL-5-deficient mice where peribronchial fibrosis was markedly reduced upon sensitization and repetitive challenge with ovalbumin compared to wild type mice (Cho et al., 2004). Previous studies had reported that in a placebo controlled human study, the expression of extracellular matrix proteins such as procollagen III, lumican and tenascin in

bronchial mucosal reticular basement membrane of mild atopic asthmatic patients was significantly decreased upon the administration of anti-IL-5 antibody when compared to placebo group (Flood-Page et al., 2003). In addition, eosinophilia, TGF-β1 mRNA expressing eosinophils and levels of TGF-β in bronchoalveolar lavage fluid were also reduced with the administration of anti-IL-5 antibody (Flood-Page et al., 2003). The evidence stated above clearly highlighted the association between IL-5 and eosinophilia and demostrate the detrimental role of IL-5 and eosinophilia in allergic asthma.

Recently, thymic stromal lymphopoietin, IL-25 and IL-33 were innate immune cytokines that were discovered to have potent Th2 inducing abilities and important mediators in the immunopathogenesis of allergy and asthma. Thymic stromal lymphopoietin was secreted from epithelial cells in the presence of allergens and it had been found to activate DCs resulting in the upregulation of surface OX40 ligand and subsequent Th2 differentiation (Ito et al., 2005; Liu, 2007). The elevation of thymic stromal lymphopoietin in asthma that activated DCs to induce Th2 inflammatory responses had been described in humans and also in murine models whereby inflammation and airway hyperresponsiveness had been reduced by blocking thymic stromal lymphopoietin (Kato et al., 2007; Liu, 2007; Ray et al., 1996). IL-25 also known as IL-17E had been associated with the Th2 type immunity regulation as airway hyperresponsiveness (AHR) and inflammation were attenuated in murine models of allergic asthma when IL-25 was blocked (Ballantyne et al., 2007; Wang et al., 2007). IL-25 was described to be produced by Th2 cells, mast cells, epithelial cells in mouse and by eosinophils, basophils in humans (Wang et al., 2007). The actions of IL-33 were diverse, firstly IL-33 induced production of type 2 cytokines

from eosinophils and T cells, next IL-33 also stimulated type 2 cytokines release from basophils and mast cells via the activation of both IgE-dependent and independent pathways. Eosinophil-mediated differentiation of airway macrophages in the airways towards the alternatively activated macrophage phenotype was also enhanced by IL-33 in an IL-13 dependent manner (Kondo et al., 2008; Kurowska-Stolarska et al., 2008; Stolarski et al., 2010). IL-33 had also been found to induce eosinophils to produce IL-13, chemokine (C-C motif) ligand -17 (CCL-17) and TGF-β (Stolarski et al.). Differentiation of $CD117^+$ progenitors to eosinophils was found to be a result of IL-33 in an IL-5 dependent manner (Stolarski et al., 2010).

1.4.4 T helper 17 cells

IL-17-producing T cells were first reported by Infante-Duarte *et al* to be another Th population besides the classical Th1 and Th2 populations (Infante-Duarte et al., 2000). Differentiation of Th17 was reported to be dependent on STAT3 as *Stat3*-deficient $CD4^+$ T cells was found to have a reduced expression of IL-17 and increased expression of IFN-γ (Yang et al., 2007). Hence Th17 was a distinct subset from the Th1 and Th2 subsets as its' differentiation had been found to be not regulated by T-bet, STAT1, STAT4 and STAT6 which were transcription factors of the Th1 and Th2 subsets (Harrington et al., 2005; Park et al., 2005). The IL-17 family consisted of IL-17A, B, C, D and E (Kolls and Linden, 2004). Besides the production of IL-17A and IL-17F, IL-21 and IL-22 were also produced by Th17 cells (Korn et al., 2007; Liang et al., 2006; Nurieva et al., 2007; Zheng et al., 2007; Zhou et al., 2007). IL-17 was not exclusively produced by Th17 cells, $CD8^+$ T cells, NK T cells and γδ T cells also secreted IL-17 (Ivanov and Linden, 2009; Lockhart et al., 2006; Michel et al., 2008). Not only do Th17 cells produced IL-21, member of the IL-2 family, in large quantities,

$ICOS^+CXCR5^+CCR7^+$ T follicular helper cells were also capable of releasing IL-21 (Chtanova et al., 2004). IL-17 receptor family consisted of IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE (Gaffen, 2009). IL17A and IL17F could form homodimers, besides that they could also form heterodimer IL17F/IL17A which signaled through the receptor that was made up of IL17RA and IL17RC in humans (Toy et al., 2006; Wright et al., 2008). The first transcription factor for the differentiation of Th17 cell had been identified to be retinoic-related orphan receptor γt (RORγt) (Ivanov et al., 2006) while the human homologue had been identified to be RORC2 (Ivanov et al., 2007). Recently, another nuclear receptor RORα had also been found to be highly expressed by Th17 cells (Yang et al., 2008b). Expression of both RORγt and RORα had been reported to be regulated by STAT3 (Laurence et al., 2007; Yang et al., 2007; Yang et al., 2008b). Overexpression of RORα had been found to upregulate the mRNA expression of IL-17, IL-17F and IL-23R, in addition Th17 differentiation had been enhanced by the overexpression of RORα in RORγ-deficient cells (Yang et al., 2008b). RORγ and RORα double deficiency blocked the differentiation of Th17 and onset of experimental autoimmune encephalomyelitis disease (Yang et al., 2008b). It was known that IL-6 downstream signaling activated both STAT3 and STAT1 (Ihle and Kerr, 1995), however in Th17 cells, STAT1 activation was suppressed while STAT3 was activated (Kimura et al., 2007).

TGF-β and IL-6 had been reported to be the prerequisites for Th17 cell differentiation in mouse (Veldhoen et al., 2006) and commitment to the Th17 lineage was reinforced by IL-23 (Yang et al., 2007). Antigen presenting cells like DCs and macrophages upon activation produced IL-23. The receptor for IL-23 was heterodimeric and consisted of IL-12Rβ1 and IL-23R (Kastelein et al., 2007). *In vitro* experiments had

revealed that IL-23 was required for Th17 to secrete high levels of IL-22, which might hint that IL-22 was an effector cytokine produced by Th17 cells that were commited to the Th17 lineage and were fully differentiated (Bettelli et al., 2008; Liang et al., 2006; Zheng et al., 2007). TGF-β, despite playing a vital role in the generation of murine Th17 cells, was not a necessary factor in driving human Th17 differentiation. Differentiation of human Th17 cells was driven by IL-6 and other cytokines IL-1, IL-21 or IL-23 (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007). IL-1β had been shown to be crucial in early differentiation of Th17 cells in both human and mouse, in addition conversion of $Foxp3^+$ T cells into IL-17 producing cells was also reported to be dependent on IL-1β (Acosta-Rodriguez et al., 2007a; Chung et al., 2009). IL-17 had been found to be involved in the activation of nuclear factor-κB (NFκB) and mitogen-activated protein kinase pathways (Gaffen, 2008). It had been proposed that differentiation of Th17 followed a three-step model whereby induction of differentiation of Th17 was first brought about by the action of both TGF-β and IL-6, the frequency of Th17 cells was then enhanced by IL-21 while IL-23 ensured that differentiated Th17 cells were stabilized and committed to their lineage (Bettelli et al., 2008). STAT4 might play an important role in the expansion of Th17 cells driven by IL-23 (Mathur et al., 2007). Human IL-17 producing cells could be identified by the co-expression of both C-C chemokine receptor 4 (CCR4) and CCR6 or by expression of CCR2 and absence of CCR5 (Acosta-Rodriguez et al., 2007b; Sato et al., 2007). Expression of TGF-βR1, IL-6R, IL-23R and chemokine receptors, CCR4 and CCR6, on the cell surface of Th17 cells was reported and the chemokine receptors were involved in the homing to inflammation sites (Lane et al.).

TGF-β had been reported to play a role in the generation of Treg cells via increasing the expression of Forkhead box p3 (Foxp3) (Chen et al., 2003). However, the addition of IL-6 in an environment of TGF- β had been found to stimulate the differentiation of Th17, at the same time impairing Treg generation via the suppression of Foxp3 expression (Bettelli et al., 2006). The reciprocal relationship between Treg and Th17 had been emphasized by reports of retinoic acid, a factor not only involved in the induction of Treg cells, but also capable of inhibiting IL-6 induced generation of Th17 cells (Mucida et al., 2007). Consistent with earlier reports, Foxp3 had also been shown to impair the function of RORγt thus inhibiting IL-17 production, this inhibition was brought about by higher concentrations of TGF-β (Zhou et al., 2008). Direct interaction of Foxp3 and RORγt had also been reported to result in inhibition of IL-17A mRNA transcription (Ichiyama et al., 2008). Foxp3 protein was also being reported to hinder physically the association between RORγt and IL-17 promoter (Zhang et al., 2008; Zhou et al., 2008).

Besides the antagonistic action between Treg and Th17, Th1 and Th2 were also implicated in suppressing the differentiation of the Th17 lineage. Studies had reported that T-bet, a Th1 transcription factor as well as master regulator, abolished and inhibited Th17 differentiation (Guo et al., 2009; Yang et al., 2008c). Other reports had shown that GATA3, a Th2 transcription factor, downregulated STAT3, STAT4 and RORγt, all of which were Th17 directing transcription factors, hence inhibiting the production of IL-17 (van Hamburg et al., 2008). In addition, IL-27, a member of the IL-12 family, was also reported to inhibit Th17 differentiation and was supported by observations that development of Th17 cells was suppressed *in vitro* when naïve primary T cells were treated with IL-27 (Stumhofer et al., 2006). Mice deficient in the

IL-27 receptor also exhibited a robust Th17 response with fatal $CD4^{+}$T cell- mediated pathology when chronically infected with *Toxoplasma gondii* (Stumhofer et al., 2006). In comparison, Th1 cytokine such as IL-2 was more efficient than Th2 cytokines such as IL-4 in antagonizing Th17 differentiation (van Hamburg et al., 2008). While the aforementioned factors were inhibitors of Th17 development, interferon-regulatory factor 4 and dioxin, a ligand of aryl hydrocarbon receptor, were positive regulators of Th17 differentiation (Brustle et al., 2007; Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008).

1.4.5 Role of T helper 17 cells in allergic inflammation

Allergic diseases conventionally had been thought to be only Th2 mediated with the characteristic infiltration of increased numbers of eosinophils, Th2 lymphocytes, activated mast cells and airway remodeling (Bousquet et al., 2000; Pascual and Peters, 2005). However, asthma was not only characterized by eosinophilia, neutrophilia was also a characteristic feature of another subtype of asthma. Studies on neutrophilic asthma had shed light on the involvement of IL-17, the hallmark cytokine produced by Th17 cells (Hellings et al., 2003). Another study also reported the increase in neutrophils and keratinocyte derived chemokine (KC) protein and mRNA when anti-IL17 antibody was used (Park et al.).Th17 cell numbers were also increased in the lungs of mice that had house dust mite allergy (McGee et al., 2010). IL-17 cytokine, IL-25, also had been reported to initiate and enhance Th2 responses (Angkasekwinai et al., 2007; Ballantyne et al., 2007; Tamachi et al., 2006). The association of IL-17 with asthma had been strengthened with reports of IL-17 being upregulated in the bronchoalveolar lavage fluid (BALF), lung cells, sputum and peripheral blood of asthmatic patients (Barczyk et al., 2003; Molet et al., 2001). Studies on hIL-17 had

also reported that hIL-17 could stimulate synovial fibroblasts, human bronchial fibroblasts and bronchial epithelial cells to produce cytokines such as IL-6, IL-8, GCSF resulting in neutrophil hematopoiesis and granulopoiesis (Fossiez et al., 1996; Louten et al., 2009; Molet et al., 2001; Park and Lee). Other studies also showed that the neutrophil attracting function of IL-17 was mediated via the increased production of IL-8 (Cannons et al.) by lung structural cells (Laan et al., 1999; Park and Lee; Ye et al., 2001).All this evidence suggested that IL-17 played a major role in the recruitment and activation of neutrophils during an inflammatory response via the induction of chemokines and cytokines.

1.4.6 Protective effect of T helper 17 cells

Protective effect of Th17 immune responses had been described for defense against fungal infection and supported by evidence whereby IL-17RA deficient mice were more susceptible to infection by *Candida albicans* which could be due to the delay in the recruitment and activation of neutrophils in the infected organs (Huang et al., 2004). IL-17 had been reported to play a vital role in the clearance of pathogens such as *Candida albicans* and *Toxoplasma gondii* (Huang et al., 2004; Kelly et al., 2005). *Candida albicans* specific IL-17A producing CD4 memory T cells were also identified in human subjects (Acosta-Rodriguez et al., 2007a). Th17 cells also helped to control *M.tuberculosis* infections by the induction of chemokines such as chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10 and CXCL11 that served as a chemoattractant for Th1 cells that were rapidly recruited into the lung tissue and infection was controlled (Khader et al., 2007). Hence Th17 cells were proposed to be able to link innate and adaptive immunity via the induction of chemokines that helped

to recruit other Th cells to the infected areas, hence helping to control the infection (Bettelli et al., 2008).

Although many have normally associated production of IL-17 with Th17 cells, the main lymphocyte subset that produced IL-17 in naïve mice at a steady state was the γδ T cells (Stark et al., 2005). Although Th17 had been suggested to be involved in the pathogenesis of proinflammatory diseases, they were not the main IL-17 producers during allergic airway disease, instead IL-17 producing γδT cells were found to be the main producers of IL-17 which was vital and essential in the amelioration of allergic airway inflammation (Murdoch and Lloyd). This shedded new light on the importance of IL-17 in allergic diseases, hence IL-17 might not necessarily be harmful, the cell type that produced IL-17 might actually determine if IL-17 exert a harmful or protective effect. Besides, the importance of γδT cells in maintaining normal airway tone was highlighted in another study (Lahn et al., 1999). γδT cells were a prominent cell type at mucosal surfaces such as the airway epithelium and other studies had suggested that γδT cells were capable of immunoregulation and preventing host tissue from incurring damage during immune responses (Born et al., 2000; Wands et al., 2005). In addition, in murine models of OVA-induced allergic asthma, airway hyperresponsiveness (AHR) was upregulated in the absence of γδT cells (Lahn et al., 1999).

Besides that, the role of IL-17 in allergic asthma had also been highlighted in the development of allergic asthma (Schnyder-Candrian et al., 2006). Surprisingly, coadministration of recombinant murine IL-17 with OVA during challenge reduced metacholine response significantly and this suppression was removed by the use of

IL-17 blocking antibody (Schnyder-Candrian et al., 2006). Besides that, pulmonary CC-chemokine expression and eosinophilia were also reduced by IL-17 (Schnyder-Candrian et al., 2006). This evidence showed that IL-17 may not only play a detrimental role in the initiation of allergic asthma, the protective role of IL-17 in the effector phase could not be underestimated. Other supporting evidence revealed that neutralization of IL-22, another cytokine produced by Th17 cells, resulted in increased eosinophilia (Schnyder et al.). It had also been shown that IL-17A and IL-22 exerted their effects on DCs as transfer of DCs preincubated with IL-22 and IL-17A into naive mouse conferred protection against eosinophilia upon further OVA challenge (Schnyder et al.).

1.4.7 Plasticity of T helper 17 cells

Relationship between Th17 and Th1 had been ambiguous and had been supported by studies in which IFN-γ and IL-17 double producing T cells were generated when $CD4^+$T cells were subjected to a Th17 polarizing environment (Evans et al., 2007; Yang et al., 2009). In addition, other evidence had suggested that Th1 cell recruitment to inflammation sites was dependent on an early Th17 response and this Th17 response also facilitated Th1 memory development (Khader et al., 2007). Consistent with the earlier reports, *in vitro* generated human Th17 cells consisted of IL-17^+, IFN-γ^+(Th17-IFN-γ) and IL-17^+/IFN-γ^+ cells and these subsets also expressed IL-17F, IL-22, CCL20, IL-26, lymphotoxin α and IL-1A transcripts at higher levels at Th1-IFN-γ^+ cells (Boniface et al.). Surprisingly, IL-17 and IFN-γ double producing cells were unstable and had a tendency to convert to IFN-γ single producing cells (Boniface et al.). Plasticity of Th17 cells was also highlighted whereby IL-12 skewed Th17 cell subsets to become "Th1-like" IFN-y^+ cells, in contrast Th1 cells remained stable even

under Th17 promoting environment (Boniface et al.). Another study had reported that the adoptive cell transfer of a Th17 polarized population into mice could convert to IFN-γ producers that could produce either both IFN-γ and IL-17 or IFN-γ only in the lung (Ashino et al.).

1.5 CpG oligonucleotides

1.5.1 Distinct classes of CpG Oligonucleotides

CpG dinucleotides were present at a frequency of 1 in every 16 dinucleotides in bacterial genomes while in vertebrate DNA, there was CG suppression and CpG dinucleotides were present at one-fourth the frequency in bacterial genome (Gardiner-Garden and Frommer, 1987). Hence unmethylated CpG motifs were being regarded as "danger signals". Impaired responses of Toll like receptor (TLR) 9 deficient mice to CpG DNA implied that TLR9 was essential for the recognition of CpG (Hemmi et al., 2000). CpG oligonucleotides (ODN) were known to bind to TLR9 and hence activated a series of signaling cascade that could lead to the differentiation, maturation, and proliferation of effector cells like T cells, NK cells, B cells and dendritic cells to secrete chemokine, cytokines and immunoglobulins (Ig) (Gursel et al., 2002; Klinman et al., 1996; Sparwasser et al., 1998; Stacey et al., 1996). As previously described by other groups, there existed two main structurally distinct classes of synthetic CpG ODN that could activate human immune cells which were namely, the D and K type (Gursel et al., 2002; Hartmann et al., 2003; Verthelyi et al., 2001). "K" type ODN (also known as B type) consisted of phosphorotiorate backbone that encoded multiple CpG motifs such as TCGTT and/or the TCGTA motif and they were capable of inducing the differentiation of plasmacytoid dendritic cells (pDC) to produce TNF-α. B cells were also stimulated by "K" type ODN to proliferate and

secrete IgM (Hartmann and Krieg, 2000; Krug et al., 2001). “D” type ODNs (also known as “A”type) consisted of a purine/pyrimidine/CG/purine/pyrimidine motif, capped by a poly G tail at the 3’ end. “D” type ODNs could stimulate pDC to secrete IFN-α and induced the maturation of monocytes into mature dendritic cells (Coban et al., 2005). It had been proposed that the inability of “K” ODN to induce strong IFN-α secretion could be due to their strong ability of inducing the maturation of pDC (Verthelyi and Zeuner, 2003). Matured pDC tend to not produce high levels of IFN-α (Verthelyi and Zeuner, 2003).

The immunomodulatory effects of CpG ODN varied across species and the optimal sequence motif, number of CpG motifs and flanking sequences might vary in different species which could be due to the evolutionary divergence in TLR9 molecules in different species (Rankin et al., 2001). As such, the optimal CpG motifs for mouse might not be effective in humans as the amino acid sequence of murine and human TLR9 differed by 24% (Hemmi et al., 2000). In humans, B cells and pDC were the major cell types that expressed TLR9 while in mice, TLR9 expression was on immune cells of the myeloiod lineage such as macrophages, myeloid dendritic cells (mDC). One of the earliest studies that involved the transfection of human monocytes with CpG-DNA showed that the immunogenicity of DNA vaccines could be enhanced by CpG motifs (Sato et al., 1996). Human CpG motifs had been inserted into plasmid backbone to increase its immunogenicity and the results had shown that CpG (“K” or “D”) modified plasmid DNA upregulated surface marker expression and increased the production of IL-6 from peripheral blood mononuclear cells (PBMC) (Coban et al., 2005). In addition, monocytes were also stimulated to develop into mature dendritic cells by CpG modified plasmid DNA. Interestingly, although other

groups had incorporated up to 90 CpG motifs, this group proved that insertion of as few as 3 to 5 human CpG motifs was adequate to boost the immunogenicity of plasmid DNA (Coban et al., 2005). Differences between free CpG ODN and "K"-CpG modified plasmid DNA was also demonstrated whereby "K"-CpG modified plasmid DNA was a strong inducer of IFN-γ and IFN-α unlike "K" ODN. "D" ODN was a good inducer of IFN-γ and IFN-α unlike "D"-CpG modified plasmid DNA (Coban et al., 2005).

The differential effects of "D" and 'K" type ODN on human immune cells was investigated by another group that showed that uptake of "D" ODN was greater than that of "K" ODN (Gursel et al., 2002). "D" ODN was also found to occupy punctuated vesicles while "K" ODN had a more diffuse distribution. In agreement with previous reports, "K" ODN was found to activate $CD19^+$ B cells, triggering proliferation and an increase in IgM and IL-6 production. "D" ODN, on the contrary had a weaker activation of $CD19^+$ B cells (Gursel et al., 2002). "D" type ODN activated NK cells and stimulated significant IFN-γ production while "K" ODN did not induce any significant IFN-γ production despite a modest activation of NK cells (Gursel et al., 2002). The differential effects of "K" and "D" type ODN were also shown on purified monocytes while "K" ODN stimulated the proliferation of monocytes and production of IL-6, "D" ODN did not elicit such effects. Instead "D" but not "K" ODN induced the maturation of monocytes into $CD83^+/CD86^+$ DCs (Gursel et al., 2002). In addition, there appeared to be competitive effects between "K" and "D" ODN (Gursel et al., 2002).

Differences in backbone modified CpG-DNA had been reported, CpG ODNs with phosphorothiorate backbone (PS-ODN) when injected intra-peritoneally into mice resulted in an increase in spleen weight and splenocytes based on the CG sequence (Kim et al., 2009). Migration of macrophages into the peritoneal cavity was also increased, hence although PS-ODN led to strong activation of immune responses and production of PS-ODN specific antibodies, but this could result in harmful side effects *in vivo* (Kim et al., 2009).In contrast, CpG ODNs with phosphodiester backbone did not cause any increase in spleen weights, cell number or IgM production hence it might be a more useful and safe tool to enhance innate immune responses (Kim et al., 2009).

1.5.2 Factors affecting immunogenic effects of CpG oligodeoxynucleotides

Structure of CpG ODN, route of administration of CpG ODN and time of administration of CpG ODN were factors that affected the immunogenic effect of CpG ODN. It had been demonstrated that conjugation of a large oligonucleotide to CpG DNA through 5'ends would decrease the immunostimulatory activity of CpG DNA. In comparison, a small oligonucleotide conjugated to 5'end of CpG DNA or oligonucleotide of any size linked to 3' end of CpG DNA did not affect the immunostimulatory effect of CpG DNA (Kandimalla et al., 2002). Another group had shown that the type of antigen and mode of immunization affected the effect of CpG motifs *in vivo*. They had also reported that CpG ODN co-adminstered with DNA was better at downregulating Th2 responses that CpG ODN co-administered with protein (Hochreiter et al., 2001). It was also reported that CpG ODN when administered alone had decreased immunostimulatory effect. To achieve protective immune response, co-administration of CpG ODN with the antigen was essential (Corral and Petray, 2000)

and this effect could be maximized by conjugation of CpG ODN and the allergen (Mo et al., 2006; Suzuki et al., 2007). It was frequently reported that gene gun bombardment induced a Th2 response while intramuscular immunization induced a Th1 response. However another study had reported that addition of CpG ODN to a vaccine could induce a Th1 response and upregulate IL-12 mRNA in draining lymph nodes regardless of the mode of immunization (Liu et al., 2005). The timely administration of CpG ODN was emphasized by another group. They reported that mice immunized with CpG ODN followed by HSV-2 glycoprotein D vaccine exhibit decreased Th1 response. In contrast, when CpG ODN was administered 48h after immunization with vaccine, there was an induction of strong Th1 response characterized by elevated IgG2c and IFN-γ (Tengvall et al., 2005).

1.5.3 Applications of CpG oligodeoxynucleotides

CpG ODN had been shown by many groups to be able to cause a strong adjuvant effect and to induce a Th1 type immune response against different antigens in different animal models via different routes of administration (summarized in table 1). In addition, CpG ODN was also used as treatment tools to reduce airway remodeling and to reverse established Th2 responses in murine models (Jain et al., 2002; Weeratna et al., 2001). CpG ODN also showed promising results against parasites (Zimmermann et al., 2008). CpG ODN not only showed promising results in small animals but also in large animals. CpG ODN were added to Hepatitis B surface antigen with alum and immunized into orang utans. The rate of seroconversion rose to 100% after the second administration of the vaccine and protective antibody titres were also significantly enhanced (Davis et al., 2000). Despite such promising results, there were also groups that had negated the usefulness of CpG ODN. A group showed

that while a strong Th1 immune response was induced in mice co-immunized with *Toxoplasma gondii* soluble antigen and CpG ODN but the mice were not protected when challenged with the virulent strain (Saavedra et al., 2004). This finding was in agreement with another study which reported that although the addition of CpG ODN to vaccines enhanced the production of IFN-γ by lymph nodes but it did not help in the reduction of bacteria load in the lungs or improve histopathology after challenge (Hsieh et al., 2004). However CpG ODN had proven to be effective in humans as they had already entered clinical trials. CpG ODN 7909 had been tested in clinical trials. Melanoma patients were vaccinated with CpG7909 co-administered with melanoma antigen A, analog peptide and incomplete Freund's adjuvant and they showed strong antigen-specific T cell responses (Speiser et al., 2005). This finding was supported by another study in which CpG7909 also showed promising results as an adjuvant in melanoma patients (Molenkamp et al., 2007). Similarly, another phase I study had reported that another CpG ODN, 1018 ISS was a strong adjuvant that induced elevated antibody levels when co-administered with vaccines against hepatitis B virus (Halperin et al., 2003).

Safety of CpG ODN had been demonstrated by some groups. "A" type (also known as "D") CpG ODN was used in a phase I/IIa clinical trial as an adjuvant in allergen-specific immunotherapy in humans. The results of the clinical trial showed that "A" type CpG ODN together with house dust mite allergen when administered subcutaneously was safe with 100% of patients having complete alleviation of allergy symptoms after ten weeks of immunotherapy (Senti et al., 2009). In rhesus macaques, co-administration of CpG ODN with anthrax vaccine adsorbed increased the speed, avidity and immune response than anthrax vaccine alone (Klinman et al., 2004).

Protective IgG anti-PA antibodies were also increased in macaques immunized with the CpG ODN and anthrax vaccine and these serum antibodies, when passively transferred to mice, also conferred protection in nearly half of recipient mice against anthrax challenge (Klinman et al., 2004). In addition, CpG ODN co-adminstration with anthrax vaccines were well tolerated in macaques with no serious local or systemic adverse effects reported (Klinman et al., 2004).

1.5.4 CpG oligonucleotides and Toll like receptor 9

The TLR family provided an important line of defense against pathogens in innate immunity. Toll-like receptor consisted of leucine-rich repeats, a transmembrane domain and a cytoplasmic Toll/interleukin-1 receptor (TIR) homology domain. The TLR family encoded 11 members. While there were TLRs that resided at the cell surface such as TLR2, TLR4, TLR5, and TLR11 that detected lipopolysaccharide, flagellin and profilin respectively, other receptors that detected single stranded DNA such as TLR9 was localized primarily in the endoplasmic reticulum, endosome and lysosome (Akira and Takeda, 2004; Latz et al., 2004; Wagner, 2004). TLR dimerization upon ligand ligation activated two pathways, one that involved myeloid differentiation primary response gene 88 (MyD88) and resulted in the production of type I interferons and proinflammatory cytokines due to activation of NFkB. The other pathway encompassed TIR domain containing adaptor inducing beta interferon (TRIF), with the subsequent production of type I interferons (Wagner and Bauer, 2006).

The intracellular signalling pathway of TLR9 had been debated upon. While it had been suggested that in the translocation of TLR9 from the ER to the early endosomes,

the golgi complex had been bypassed (Latz et al., 2004), other evidence had shown that TLR9 did not bypass the golgi complex, instead golgi export was critical for TLR9 signalling (Chockalingam et al., 2009). TLR9 had been found to be subjected to the action of golgi-resident protease furin during intracellular movement (Chockalingam et al., 2009). Confocal microscopy studies had revealed that colocalization of TLR9, MyD88 and CpG ODN occurred in late endosomes and maturation of endosomes was essential for signaling (Ahmad-Nejad et al., 2002; Takeshita et al., 2001). Endosomal acidification had been reported to be vital for TLR9 signalling. Low pH might constitute a preferred environment for the binding of TLR9 to some DNA as TLR9 signalling had been reported to be hindered with endosomal acidification blockers. CpG ODN localization in the endosome mediated the recognition by TLR9 and upon CpG-ODN binding in the endolysomes, TLR9 had been reported to undergo conformational change (Hacker et al., 1998; Latz et al., 2007; Rutz et al., 2004; Yi et al., 1998). TLR9 movement from the endoplasmic reticulum to endolysosomal compartments was subjected to the control of a 12 membrane spanning endoplasmic reticulum protein known as UNC93B via direct interaction with TLR9 (Tabeta et al., 2006). pDC could uptake CpG DNA via clathrin-dependent endocytic pathways that subsequently bound to TLR9 present in lysosomal compartments (Sasai et al., 2010). Upon binding of CpG DNA to TLR9, MyD88 adaptor molecule with the TIR domain and death domain would be recruited with the subsequent activation of IL-1R associated kinase 1 (IRAK1), IRAK4 and interferon regulatory factor 7. Tumor necrosis factor receptor associated factor 6 was then recruited and transforming growth factor β-activated kinase 1, mitogen-activated protein kinase, and eventually NF-κB pathway was activated (Krieg, 2002). Activation of the innate immune system upon CpG DNA binding to TLR9 brought

about maturation of dendritic cells, proliferation of B cells, induction of proinflammatory cytokines, chemokines and immunoglobulins. TLR9 activity had also recently been found to be regulated by rapamycin sensitive PI(3)k-mTOR-p70S6 pathway.

K" ODN had been reported to bind to TLR9(Takeshita et al., 2001; Verthelyi et al., 2001). In contrast to "K" ODNs, "D" ODNs appeared not to bind to TLR9, another receptor was believed to be involved in the transportation of "D" ODNs to endosomes (Gursel et al., 2002). As "D" type ODN had poly G tail, scavenger receptor A had been proposed to play a role in the binding of "D" ODN as it had been to be able to bind poly G stretches. In addition ligands of the scavenger receptor A were found to decrease the activity of poly G containing CpG ODN on splenic dendritic cells (Lee et al., 2000). Recently, scavenger B1 receptor had been reported to be the mediator for CpG-induced calcium mobilization in B cells but a negative regulator for TLR9 dependent B cell activation, production of IL-6, IL-10 and IgM were downregulated (Zhu et al., 2009). CpG DNA had been reported by earlier studies to bind to TLR9 and TLR9 binding had been shown to be vital for the immune effects of CpG DNA as TLR9 deficient mice showed a refractory response to CpG DNA (Hemmi et al., 2000). Transfection of either murine or human TLR9 gene also enabled previously non-responder cells to respond to CpG ODN (Bauer et al., 2001). The optimal CpG motif for murine TLR9 was GACGTT while that for human TLR9 was GTCGTT.(Bauer et al., 2001)

Plasmid DNA had been shown to stimulate the activation of TLR-9 positive pDCs or conventional dendritic cells (cDC) and induced cytokine production and upregulation

of co-stimulatory molecules. pDC and cDC interaction elicited the production of IL-12 in response to TLR9 stimulation. TLR9 had been found to stimulate production of IL-15 as well as expression of CD40 on cDC, the IL-15 elicited stimulated the expression of CD40L on pDC. CD40-CD40L ligation between cDC and pDC elicited the production of IL-12 from cDC (Kuwajima et al., 2006). However, there were also studies reported by other groups that casted doubt on the importance of TLR9 (Spies et al., 2003). The role of TLR9 remained unclear as *in vivo*, plasmid DNA vaccination of TLR9 or MyD88 deficient mice still could result in clonal expansion of SIINFEKL-specific $CD8^+$ T cells and priming of OVA-specific cytotoxic T lymphocyte (Spies et al., 2003). Another group also reported similar findings whereby they found that DNA immunization elicited similar levels of antigen-specific antibody and stimulation of antigen specific IFN-γ secreting cells in $TLR9^{-/-}$ as well as in $TLR9^{+/+}$ mice (Babiuk et al., 2004). However involvement of TLR9 had been shown in a study whereby TLR9 inhibitory sequences had been found to result in the impediment of TLR9 mediated activation in human and mouse cells (Duramad et al., 2005).

Recently CpG ODN had been shown to be able to induce IL-33 via TLR9 engagement (Shimosato et al.). It had been proposed that CpG ODN, via the induction of IL-33, could result in an inhibition of ST2/IL-33 activation, hence IL-33 could be able to counteract the effects of proinflammatory cytokines such as the IL-1 family (Shimosato et al.). Besides binding to CpG ODN, TLR9 was also vital in the defense against bacterial and viral infection such as *Streptococcus pneumonia* (Lee et al., 2007) and *herpes simplex virus type I* (Krug et al., 2004; Lee et al., 2007), resulting in the production of IFN-α by pDC. The phosphodiester 2' deoxyribose backbone was also

found to be able to determine TLR9 activation (Haas et al., 2008). Besides, sequence specificity had been found to be exclusive when phosphorothiorate ODN were used, this specificity was not observed when the ODN was changed to a phosphodiester backbone (Roberts et al., 2005). In contrast, another study demonstrated that TLR9 bound to phosphorothiorate DNA devoid of CpG motifs (Vollmer et al., 2004). Surprisingly, TLR9 had been found to be able to bind to non-CpG as well as CpG ODN, however binding of CpG ODN skewed immune responses to that of a Th1 while non-CpG ODN skewed immune responses to that of a Th2 (Vollmer et al., 2004). It was proposed that dependant on the type of stimulus (CpG or non-CpG ODN), Th1 or Th2 effects could be mediated by TLR9 (Vollmer et al., 2004).

The regulatory network that underlied CpG dependent gene expression *in vivo* was investigated by microarray analysis of mRNA expression level in the spleen of mice that had been injected intraperitoneally with immunostimulatory CpG DNA (Klaschik et al., 2009). After stimulation, gene expression changes were first detected at 30min , peaked at 3h and declined after that, major inducers were found to be TNF, IL1B, IL1A and IFNG while minor inducers included IL6, NFKB1, IL15, TNFSF10, IL18 and MYC (Klaschik et al., 2009). TNF, IL1B, IL1A and the minor inducers triggered a fast but short-lived upregulation of genes which was in stark contrast to IFNG that activated a long-lived network of genes at 3h post CpG stimulation (Klaschik et al., 2009). The major inducers activated genes that elicited a proinflammatory response (Klinman et al., 1996). Suppressors such as IL10, MYC, NF-kB light chain gene enhancer in B cells inhibitor, α(NFKBIA), suppressor of cytokine signaling I(SOCS1), SOCS3, IL1RN and FOS were found to target at inducers , resulting in the decrease in expression of upregulated genes (Klaschik et al., 2009).

1.5.5 TLR9 independent pathways

Recognition of host DNA might not be totally dependent on TLR as it had been reported that DNA-specific TLR signaling was not essential for IFN production (Okabe et al., 2005). In the past decade, evidence had hinted at the presence of DNA sensors besides TLR9 that played a role in innate immune responses. Infection of cells lacking TLR9 with herpes simplex virus I or Listeria monocytogenes did not affect induction of type I IFN (Lund et al., 2003; Stetson and Medzhitov, 2006). In addition, type I IFN production was also not affected when TLR9 deficient cells were transfected with synthetic double stranded DNA (Stetson and Medzhitov, 2006). A growing body of evidence had suggested the presence of an innate immune activation pathway that was induced by DNA, yet independent of TLR (Ishii and Akira, 2005) as it had been shown that a TLR9 independent pathway mediated the increased expression of co-stimulatory molecules as well as induction of interferon-inducible genes by double stranded DNA (Li et al., 2005; Yasuda et al., 2005). In addition to that, it had also been proven that double stranded right handed helical form of DNA resulted in type I IFN and chemokines production via activation of mouse and human stromal and dendritic cells (Ishii et al., 2006).

Recently, DNA mediated signalling pathways had been identified and they included stimulator of interferon genes (STING) pathway, RNA polymerase III/ retinoid-inducible gene 1 (RIG-1)/ IFN-β promoter stimulator 1 (IPS-1) dependent pathway and nucleotide oligomerization domain (NOD)-like receptor pathway. STING was an endoplasmic reticulum associated molecule found to be crucial for the induction of type I interferons via cytosolic DNA stimulation in macrophages and dendritic cells

(Ishikawa and Barber, 2008; Ishikawa et al., 2009; Jin et al., 2008; Sun et al., 2009b; Zhong et al., 2008). Interestingly, single stranded DNA failed to induce robust type I interferon production via the STING pathway, implying that double stranded DNA structure was essential in activating the STING pathway. In addition, STING was involved in the induction of type I interferon but not IL-1β production in response to DNA stimulation, IL-1β production was dependent instead on absent in melanoma 2 (AIM2) (Ishikawa et al., 2009). In pDCs, STING pathway as well as DEAH/RNA helicase A existed as DNA sensors (Ishikawa et al., 2009). However, STING did not appear to bind DNA, instead other molecules such as DNA dependent activator of interferon (DAI), also known as Z DNA binding protein 1 (ZBP-1), IFI16 (PYHIN protein) functioned as cytosolic DNA receptors (Takaoka et al., 2007; Unterholzner et al., 2010). Besides that, high mobility group box proteins had also been implicated in regulating DNA mediated innate immune signalling and were speculated to be essential in mediating the complete activation of the TLR, AIM-2 and STING pathways (Yanai et al., 2009).

B-DNA was discovered to activate Tank binding kinase 1 (TBK1)-dependent pathways, subsequently activating NF-κB dependent pathway as well as induction of type I interferon production (Ishii et al., 2008; Miyahira et al., 2009). Upstream of TBK1 existed STING hence STING was essential for the regulation of TBK1 activity in response to DNA. When MyD88 and TRIF deficient mouse embryonic fibroblasts (MEFs) were transfected with B-DNA, there was still upregulation of expression of *Ifnb* , *Cxcl10* and *Ccl2* mRNA expression, which was similar to that induced in wild type MEF. This demonstrated that TLR signalling did not constitute to the induction of type I interferons and chemokines (Ishii et al., 2006) Similarly, MEFs lacking

RIG–I also upregulated *Ifnb*, *Cxcl10* and *Ccl2* mRNA expression, this suggested that RIG-I was not involved in the innate immune recognition of B-DNA (Ishii et al., 2006). Microarray analysis of B-DNA stimulated cells revealed that B-DNA upregulated interferon-inducible antiviral genes that were TBK1-dependent such as gene encoding Vig-1(Rsad2), Mx1 and Mx2 genes encoding chemokines (Cxcl11 and Cxcl10), GTPases, RNA helicases (D11Lgple and Ifih1), TLR3, genes encoding Z-DNA binding proteins (Zbp1 and Adar) and Irf7 (Ishii et al., 2006). Expression of interferon-inducible genes by immune and non immune cells was found to be induced by double stranded DNA and double stranded synthetic polynucleotides. Single stranded DNA on the other hand was unable to elicit such effects (Ishii et al., 2001; Suzuki et al., 1999).

As mentioned earlier, IL-1β production in response to DNA was found to be AIM-2 dependent and involved caspase I mediated production of proinflammatory cytokines like IL-1β. This production of IL-1β proceeded via the activation of the NOD-like receptor pathway and subsequent inflammasome mediated signalling (Yanai et al., 2009). AIM-2, a cytoplasmic dsDNA sensor belonged to the PYD/HIN-200 (PYHIN) family which also consisted of other members like IFIX, IFI16 and MNDA (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). Besides the STING pathway that triggered type I interferon production and the AIM-2 inflammasome/ Nod-like receptor pathway that mediated proinflammatory cytokine production, there was also a DNA mediated RNA polymerase III/ RIG-1/IPS-1 signalling pathway which was activated by AT rich dsRNA (Ablasser et al., 2009; Chiu et al., 2009).

1.6 Dendritic cells

Innate immunity encompassed a variety of cells such as DCs, NK cells, γδ T cells, basophils, eosinophils, mast cells and neutrophils of which the most competent antigen presenting cells are the DCs which played a vital role in the regulation of adaptive immunity (Pulendran et al., 2010; Steinman and Nussenzweig, 2002). Dendritic cells are complex cell populations that are diverse in their antigen recognition, anatomic location, migratory capacity and processing machinery. Dendritic cells were involved in T cell differentiation and activation by taking up exogenous antigens, transmiting the signals to immune system by moving to draining lymph nodes and lastly presenting the processed antigens to T cells, inducing their proliferation and effector functions. Dendritic cells played essential roles in modulating innate and adaptive immune responses to infections as well as in the maintainenance of immune tolerance to self tissue (Banchereau and Steinman, 1998). Dendritic cells could be characterized based on their maturation status, immature DC were very efficient at capturing antigen but yet were inefficient at stimulating T cells, besides that, surface expression of costimulatory molecules and intracellular MHC class II was low. Immature DCs were speculated to promote tolerogenic responses due to inefficient T cell priming as well as expansion of regulatory T cells (Hawiger et al., 2001; Jonuleit et al., 2000; Mahnke et al., 2003). In contrast, mature DC were inefficient at capturing antigen but yet were very efficient at stimulating T cells and promoting immunogenic responses, in addition surface expression of costimulatory molecules and MHC class II were high (Daro et al., 2002). Dendritic cells as mentioned earlier also played a vital role in cross priming and in the generation of $CD8^{+}$T cell responses.

A variety of DC subsets existed both in mice and humans and they could be characterized by their phenotypic characteristics, functions and localizations (Pulendran et al., 2008). Steady state DCs were those present before infection such as type I interferon-producing pDC, cDC in lymphoid tissue and 'migratory' dendritic cells like the Langerhans cells (Shortman and Naik, 2007). Dendritic cells such as monocyte-derived 'inflammatory DCs', tumor necrosis factor producing and inducible nitric oxide synthase expressing DCs were those that developed after infection or inflammation (Geissmann et al., 1998; Naik et al., 2006; Randolph et al., 1999b; Serbina et al., 2003; Wu and Liu, 2007). Conventional DC possessed dendritic form and functioned in the steady state while pre-DC such as plasmacytoid pre-DC required further activation and development. Plasmacytoid pre-DC had spherical shapes with the absence of dendrites, low expression of co-stimulatory molecules and inefficient T cell priming. Once activated, plasmacytoid pre-DC assumed dendritic cell form with dendrites and high expression levels of co-stimulatory molecules. In addition they were major producers of type I interferons and were able to present antigens as well as cross-present (Asselin-Paturel et al., 2001; Grouard et al., 1997; Irla et al., 2010; Naik et al., 2005a; Sapoznikov et al., 2007). Importantly, the type I interferons produced by pDC could also activate cDC. However pDCs were not very capable in the priming of naïve $CD4^+$T cells but it could bring about the induction of secondary responses by increasing the number of memory $CD4^+$ T cells. In addition, priming and survival of antigen-specific $CD8^+$T cells could also be facilitated by pDCs (Di Pucchio et al., 2008; Swiecki et al., 2010). Involvement of pDC in differentiation of Th1, Th2, Th17, Th22 and Treg cells had been reported in some studies, with supporting evidence of the Tregulatory role of pDC being highlighted in another study whereby elevated levels of inducible T cell co-stimulator (ICOS) ligand

had been found on non-activated human pDCs and this interacted with a subset of ICOS expressing Foxp3^{+}T reg cells, hence inducing its proliferation and IL-10 production (Colonna et al., 2004; Ito et al., 2008).

Besides TLR, DCs possessed a wide range of receptors such as RIG-I like receptors, NOD-like receptors and C-type lectins , which upon binding to microbial structures, triggered the activation of DCs (Iwasaki and Medzhitov, 2010; Kawai and Akira, 2009). Besides TLR9, another MYD88 dependent DNA sensor had been identified to be cytosolic DExD/H-box helicases (Kim et al., 2010). In order to obtain sufficient number of dendritic cells for studies, *in vitro* generation was necessary as the number of DCs *in vivo* was low. Common methods to derive dendritic cells *in vitro* included using GMCSF or Flt3 ligand to derive dendritic cells from bone marrow (Boonstra et al., 2003; Brasel et al., 2000; Naik et al., 2005b). Bone marrow derived DCs using GMCSF and Flt3 ligand were representations of different DC subsets *in vivo,* such as Flt3 ligand when cocultured with bone marrow generated three distinct subsets, as pDC, CD8^{+} cDC and CD8^{-} cDC that were similar to steady state DCs *in vivo* in terms of function and phenotype (Naik et al., 2005b).

1.7 IL-35

IL-35 was a heterodimeric cytokine from the IL-12 family that had been found to have potent T regulatory activity. IL-35 was composed of Epstein-Barr-virus-induced gene 3 (Ebi3) and interleukin-12 alpha which had been found to be expressed at high levels in mouse Foxp3^{+} Treg cells but not in resting or activated CD4^{+} cells (Collison et al., 2007b). Ebi3 was first identified in Epstein-Barr virus infected B cells more than a decade ago (Devergne et al., 1996). Interleukin-12 alpha (IL-12p35) had a

constitutive expression unlike IL12p40 that had a inducible expression. In addition, IL-35 had also been found to be an inhibitor of Th17 cell differentiation *in vitro* and this finding was confirmed in a mouse model of collagen induced arthritis where IL-17 production was found to be suppressed with the simultaneous upregulation of IFN-γ synthesis (Niedbala et al., 2007). IL-17 was the signature cytokine produced by Th17 cells along with IL-17F and IL-22 and the Th17 lineage determining factor was RORγt. The inhibitor role of IL-35 in Th17 differentiation had been further exemplified in this study whereby $Ebi3^{-/-}$ splenocytes were reported to express increased levels of IL-17 and IL-22 upon antigen stimulation and the mRNA expression level of RORγt was upregulated (Yang et al., 2008a). Besides playing a role in attenuating established collagen-induced arthritis in mice, IL-35 had also been reported to play an immunosuppressive role in inflammatory bowel disease murine model whereby wild type T_{reg} cell recipients recovered from disease, had weight gain and decreased histopathology (Niedbala et al., 2007). In contrast, $Ebi3^{-/-}$ or $IL12\alpha^{-/-}$ T reg cells recipients continued to lose weight and had an increased mortality rate (Niedbala et al., 2007). Treg cells were also reported to constitutively express IL-35 and in the presence of wild type Treg cells, the proliferation of T_{eff} cells was markedly decreased in a lymphopenic, recombination activating gene1 $(Rag1)^{-/-}$ environment. However this reduction in the proliferation of T_{eff} cells was abolished in the presence of either $Ebi3^{-/-}$ or $IL12\alpha^{-/-}$ T reg cells (Collison et al., 2007b). IL-35 had also been reported to have a suppressive effect on $CD4^{+}CD25^{-}$ effector cells *in vitro* by another group (Niedbala et al., 2007). This immunosuppressive or immunomodulator effect of IL-35 may be linked to its high expression in placental trophoblasts so as to prevent rejection of the fetus (Devergne et al., 1997; Niedbala et al., 2007). The suppressive effect of IL-35 had been shown to be mediated by IL-35

induced Treg cells, IL-35 producing inducible costimulator positive Treg cells or IL-10 producing $CD39^{+}CD4^{+}$ T cells upon IL-35 treatment (Collison et al., 2010; Kochetkova et al., 2010; Whitehead et al., 2011).

Table 1 Applications of CpG oligonucleotides

Author/ Ref	Animal Model	Vaccine	Route of administration	Sequence of CpG oligonucleotide (ODN)	Findings
(Lopez et al., 2006)	Horses	Killed equine influenza virus vaccine co-administered with CpG ODN	Intra-muscular	5'-TCGTCGTT GTCGTTTTGT CGTT-3'	Vaccine/CpG ODN group had higher antigen-specific antibody levels compared to vaccine only group.
(Zhang et al., 2007)	Pigs	Swine *Pasteurella multocida* living vaccine coadminstered with CpG ODN	Intra-muscular	5'-GGGTGCA TCGATGCAG GGGGG-3'	Induction of higher antigen-specific antibody titres in vaccine/CpG ODN group compared to vaccine only and PBS control group. Induction of Th1 response evident from upregulation of IFN-γ , TNFα and lower IgG1/IgG2 ratio in vaccine/CpG ODN group.
(Teshima et al., 2006)	Mice	Vaccine encoding ovalbumin conjugated to CpG ODN	Oral	5'-TCCATGA CGTTCCTGA CGTT-3'	Induction of a strong Th1 response evident from upregulation of production of IFN-γ and IgG2a
(El-Malky et al., 2005)	Mice	Vaccine encoding *Toxoplasma* lysate antigen coadministered with CpG ODN	Intra-muscular	CpG-1826 5'-TCCATGA CGTTCCTGA CGTT-3'	A Th1 immune response induced before challenge that persisted after challenge.Mice were also protected against infection
(Wedlock et al., 2005)	Cattle	Vaccine encoding *M.bovis* culture filtrate protein(CFP) coadministered with CpG ODN and commercial	Sub-cutaneous	5'-TCGTCGTT GTCGTTTTGT CGTT-3'	Induction of Th1 responses. Upregulation of IFN-γ and antibody titres

		adjuvants			
(Shi et al., 2005)	Mice	Vaccine encoding *H.pylori* whole cell sonicate co-administered with CpG ODN	Intranasal Oral	5'-TCCATGA CGTTCCTGA CGTT-3'	When the vaccine was administered intranasally, protective effect was induced that protected 70% of mice against infection. A Th1 response was induced with upregulation in IgG2a and IFN-γ producing T cells. In contrast, oral administration of the vaccine failed to protect the mice.

1.8 Rationales and specific aims of the study

1.8.1 Rationales of study

Allergic asthma was one of the most common and serious health problems worldwide. The prevalence of allergy had shown an increase in the past years with house dust mite allergy being the major triggering factor. Approximately 30% of the Singapore's population had dust mite sensitization, out of which about 10-15% of schooling children suffered from allergic asthma and/or rhinitis; about 80% of them were triggered by dust mite allergy (Holt and Thomas, 2005). *Blomia tropicalis* was a clinically important mite species that played an important role in densely populated tropical and subtropical areas and about half of total mite allergy globally were caused by Blo t (Holt and Thomas, 2005). In addition in the tropics, the most vital major allergen was Blo t 5; with as many as 80% of asthmatic/rhinitis children in Singapore being sensitized by Blo t 5 allergen (Arruda et al., 1997; Kuo et al., 2003; Kuo et al., 1999). Hence Blo t5 was the chosen allergen to work with in this study.

Concept of DNA vaccines surfaced in early nineties (Ulmer et al., 1993). DNA vaccines consisted of a plasmid DNA backbone that could encode antigens from

allergens, pathogens and tumors. DNA vaccines were a promising therapeutic tool as it conferred many advantages over other vaccine approaches. DNA vaccines were inexpensive and easier to purify in large amounts, in addition, there was sustained antigen expression, a cold chain was not required and it was relatively safe. However although DNA vaccines had elicited strong immune responses in rodent disease models, most DNA vaccines exhibited low potency in non-human primates and humans (Ulmer et al., 2006). The safety and tolerability of vaccines had been tested in humans via clinical trials and it proved to be very safe, hence the next issue to address was the enhancement of potency of DNA vaccines.

CpG-containing DNA motifs in the plasmid backbone of DNA vaccines played the role of molecular adjuvants that could activate the TLR9 pathway. In addition, the immunogenicity of DNA vaccines could also be mediated by a TLR-independent pathway involving the signalling molecule, TBK1 (Ishii et al., 2008). Hence in this study, multiple copies of optimized species-specific CpG motifs suitable for human use would be inserted into the plasmid backbone of DNA vaccines to enhance the priming of the immune responses induced by DNA vaccines by targeting at the innate arm of immunity. In addition to the insertion of additional CpG motifs, LAMP-1 targeting strategy was also employed to improve the efficiency of antigen processing and presentation, hence promoting the priming of Th1-skewed cellular responses. LAMP-1 targeting sequence linked to antigen gene sequences had been reported to be an effective way to target newly synthesized antigens *in vivo* to vacuolar lysosomal/endosomal compartments that contained MHC class II (HuangFu et al., 2006; Lu et al., 2003; Marques et al., 2003; Tan et al., 2006). It had also been demonstrated that the potential risk of allergen sensitization had been reduced by

targeting the allergen to lysosomal/endosomal compartments, hence preventing the systemic distribution of conformationally intact allergen (Tan et al., 2006).

Besides allergen gene delivery, cytokine gene delivery was also a promising option to alleviate allergic diseases as shown by cytokines such as IL-12, IL-10, TGF-β, type I and II IFNs (Fu et al., 2006; Hansen et al., 2000; Hogan et al., 1998; Huang et al., 2001; Oh et al., 2002; Urban et al., 1993). Regulatory T cells were vital in the maintenance of immune homeostasis, in recent years, increasing attention had been placed on the reciprocal regulation of effector and regulatory T cell subsets so as to come up with novel approaches to treat inflammatory diseases. Studies had shown that TGF-β and IL-10 contribute to the suppressive effects of T_{reg} cells (Bettini and Vignali, 2009). However biological effects of these cytokines had been found to be highly pleiotropic *in vivo,* hence it might not be advisable to administer such therapeutic cytokines systematically as the potential pleiotropic effects of such cytokines might result in toxicities *in vivo*. Recombinant IL-10 when delivered systemically had little success in a number of small clinical trials (Herfarth and Scholmerich, 2002; Tilg et al., 2002). TGF-β could induce the differentiation of proinflammatory Th17 cells in synergy with IL-6 (McGeachy et al., 2007). Hence although IL-10 and TGF-β were both cytokines produced by T_{reg} cells, they were not ideal therapeutic tools. Recent studies had revealed the novel cytokine IL-35 that was produced by $Foxp3^{+}$ T_{reg} cells also had suppressive functions (Collison et al., 2007a). IL-35 had also been found to be an inhibitor of Th17 cell differentiation *in vitro* and this finding was confirmed in an mouse model of collagen induced arthritis where IL-17 production was found to be suppressed with the simultaneous upregulation of IFN-γ synthesis (Niedbala et al., 2007). Although IL-35 had been shown to exert an

immunosuppressive effect on naïve $CD4^+$ T cells, it was still unknown if IL-35 could also exert immunosuppressive effects on other cell populations such as Th2 cells that cause allergic diseases. The therapeutic role of IL-35 in Th2 mediated disease, for example, allergic asthma, had not been evaluated. Hence in this study we explored the use of IL-35 as a cytokine gene therapy approach.

1.8.2 Specific aims of the study

1) **To evaluate the immunogenicity and efficacy of DNA vaccines with the incorporation of CpG motifs into the plasmid backbone**

   I) *In vitro* analyses of the stimulatory effects of CpG motifs modified plasmid DNA using different murine dendritic cells subsets and splenocytes

   II) *In vivo* analyses of the efficacies of CpG motifs modified plasmid DNA in a murine allergy model

   III) *In vitro* analyses of the stimulatory effects of CpG motifs modified plasmid DNA using human peripheral blood mononuclear cells

   IV) *In vivo* analyses of the immunogenicity of CpG motifs modified plasmid DNA in non-human primates

2) **To explore the use of cytokine gene delivery against airway inflammation and allergic responses**

   I) Effects of IL-35 in the Blo t 5 specific Th2 cells induced airway inflammatory murine model

   II) Effects of IL-35 in a neutrophilic airway inflammatory murine model

Chapter 2 Materials and Methods

2.1 Construction of pVAXBlot5

The Blo t5target construct was generated by fusing the sslamp to the 5'end of Blo t 5 and the targeting sequence to the 3'end of Blo t 5 by using the forward primer for sslamp and the reverse primer for the targeting sequence. The leader sequence (sslamp) was first fused to the 5' end of Blo t5 construct by PCR using forward primer for sslamp (5'-TGGAGGATCCACAAATGAAGTTCGCCATCGTTCTTATTG-3') and reverse primer for Blo t5 (5'-TGGATCTAGATTATTGGGTTTGAATATCCTTCACTTTT-3') on a template DNA obtained from the lab. The lamp targeting sequence was then fused to sslamp-Blot5 construct by using the forward primer (5'-TGGAGGATCCACAAATGAAGTTCGCCATCGTTCTTATTG-3') and the reverse primer (5'-CCCTTCTAGACTAGATGGTCTGATAGCCGGCGTG-3'). The lamp targeting sequence template was obtained from the lab. The Blo t5target construct was generated by cloning cDNA coding for the mature Blo t5 into a construct of pVAX1 using the restriction enzyme BamHI and XbaI. Amplifications were performed using high fidelity DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) in a DNA thermal cycler (Perkin-Elmer Gene Amp PCR system 9700). Polymerase chain reaction (PCR) parameters were 2 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 55°C, 2 min at 68° C and a final extension of 7 min at 68°C. pVAXBlot5 and pVAXBlot5-DTKT were used for studies done in murine models and on mouse cells.

2.2 Construction of pVAXhBlot5

The humanized Blo t5target construct was generated by fusing the human tissue plasminogen activator (htpa) to the 5'end of Blo t5 and the human lamp targeting

sequence to the 3'end of Blo t5 by using the forward primer for htpa and the reverse primer for the targeting sequence. Human tissue plasminogen activator sequence, humanized Blo t 5, human lamp targeting sequence were amplified from DNA templates in the lab by the forward primer for htpa (htpa-F 5'-AAAAGGATCCGCCACCATGGATGCAATGAAGAGAGGGCTCTGC-3'), reverse primer for htpa (htpa-R 5' TTGGGCTTGTGCTCCTGGAGGGGCGGGACA CAGGGGTCCTG-3'), forward primer for humanized Blo t 5 (hBlot5-F 5'-GGACCCCTGT GTCCCGCCCCTCCAGGAGCACAAGCCCAAGAAGGACG-3'), reverse primer for humanized Blo t 5 (hBlot5-R 5'-ACAGCGATGGGGATCAGCTG GGTCTGAATGTCCTTCACTTTCTGC-3'), forward primer for human lamp targeting (LampT-F 5'-GGAC ATTCAGACCCAGCTGATCCCCATCGCTGTGGG TGGTGCC-3') and reverse primer for human lamp targeting (LampT-R 5'-AAAATCTAGACTAGATAGTCTGGTAGCCTG CGTGACTCC-3'). Human tissue plasminogen sequence was then fused to humanized Blot5 construct by using the forward primer for htpa (htpa-F 5'-AAAAGGATCCGCCACCATGGATGCAATGA AGAGAGGGCTCTGC-3'), and reverse primer for humanized Blo t 5 (hBlot5-R 5'-ACAGCGATGGGGATCAGCTGGGTCTGAATGTCCTTCACTTTCTGC-3').

Humanized lamp targeting sequence was then fused with the htpa-hblot5 construct by using the forward primer for htpa (htpa-F 5'-AAAAGGATCCGCCACCATGGATGC AATGAAGAGAGGGCTCTGC-3') and reverse primer for human lamp targeting (LampT-R 5'-AAAATCTAGACTAGATAGTCTGGTAGCCTGCGTGACTCC-3'). The humanized Blo t5target construct was generated by cloning cDNA coding for the mature Blo t5 into a construct of pVAX1 using the restriction enzyme BamHI and XbaI. Amplifications were performed using high fidelity DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) in a DNA thermal cycler (Perkin-

Elmer Gene Amp PCR system 9700). PCR parameters were 2 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 55°C, 2 min at 68°C and a final extension of 7 min at 68°C. pVAXhBlot5 and pVAXhBlot5-DTKT were used for studies done in non-human primates and on human cells.

2.3 Incoporation of K type CpG oligonucleotide into the pVAX backbone

The DNA plasmid encoding Blo t5 was digested with PMII (New England Biolabs, MA) and incubated at 37°C for 4 hours. It was then treated with calf intestinal alkaline phosphatase (Promega, Madison, Wisconsin, USA) and incubated at 37° C for 15min. Nucleotide removal kit (Qiagen, Duesseldorf, Germany) was then used to remove excess nucleotides according to the manufacturer's instructions. All primers and oligonucleotides were synthesized commercially (Sigma, St Louis). KT1 (5'-ATCGACTCTCGAGCGTTGTTCTTCGTTCGTTCTC-3') and KT2 (5'-GAGAACG AACGAAGAACAACGCTCGAGAGTCGAT-3') were incubated at 95° C for 5 mins to allow annealing to form double stranded KT. Double stranded KT was then digested with PMII (New England Biolabs, MA) and incubated at 37°C. Excess nucleotides were removed using nucleotide removal kit. Digested double stranded KT was then incubated with linearized DNA plasmid together with DNA ligase (Promega, Madison, Wisconsin, USA) overnight at 16°C. The mixture was transformed into E coli strain DH5α. The clones were then selected and amplified using the primer PMII-F (5'-CTAAATACATTCAAATATGTATCCGC-3') and BGH-R. PCR amplifications were performed using high fidelity DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) in a DNA thermal cycler (Perkin-Elmer Gene Amp PCR system 9700). PCR parameters were 2 min at 94°C, 40 cycles of 30 s at 94°C, 30s at 50°C, 2 min at 72°C and a final extension of 7 min at 72°C.

The PCR products were then run under electrophoresis in a 1% agarose gel. Plasmids of suspected clones containing KT were digested with PMII and another restriction enzyme NheI (Promega, Madison, Wisconsin, USA) to confirm the presence of the insert KT.

2.4 Incoporation of D type CpG oligonucleotide into pVAX backbone

The DNA plasmid encoding Blo t5 with KT were digested with MIuI (Promega, Madison, Wisconsin, USA) and incubated at 37° C for 4 hours. It was then treated with klenow polymerase (Promega, Madison, Wisconsin, USA) to create blunt ends. After that, it was further treated with calf intestinal alkaline phosphatase (Promega, Madison, Wisconsin, USA) and incubated at 37° C for 15min. Nucleotide removal kit was then used to remove excess nucleotides according to the manufacturer's instructions. DT1 (5'-GGTGCATCGATGCAGCATCGAGGCAGGTGCATCGATA CAGGGG-3') and DT2 (3'-CCCCTGTATCGATGCACCTGCCTCGATGCTGCAT CGATGCACC-3') were incubated at 95°C for 5 mins to allow annealing to form double stranded DT. Double stranded DT was then digested with MIuI (Promega, Madison, Wisconsin, USA) and incubated at 37°C. Excess nucleotides were removed using nucleotide removal kit. Digested double stranded DT was then incubated with linearized DNA plasmid together with DNA ligase (Promega, Madison, Wisconsin, USA) overnight at 16°C. The mixture was transformed into E coli strain DH5α. Plasmids of suspected clones containing DT was digested with MluI and another restriction enzyme XhoI (Promega, Madison, Wisconsin, USA) to confirm the presence of the insert DT. The clones were then selected and sequenced using the primer MluI-R (5'-GGCGTTACTATGGGAACAT ACGTCAT-3') with either DT1 or DT2.

2.5 DNA sequencing

DNA sequencing was used to verify the sequence of the reading frame of the recombinant constructs. To set up the PCR reaction, 200 ng of recombinant construct was added to 8 µl of ABI-prism Big Dye termination reaction mix (PE applied biosystems) and 10 pmol of either the forward or reverse primer. The PCR mixture was topped up to a final volume of 20 µl with ddH_2O. PCR parameters were 1 min at 96°C, 25 cycles of 10s at 95°C, 15s at 50°C and 4 min at 60°C. After amplification, precipation of the PCR mixture was carried out by the addition of 2 µl of 3 M NaOAc and 2 µl of 0.5 M EDTA, 60 µl of 100% ethanol and incubated on ice for 10 mins. The mixture was then centrifuged at 14000 rpm for 20min, the supernatant was discarded and the precipitate washed with 250 µl of 70% ethanol. It was centrifuged at 14000 rpm for 5min, the supernatant was discarded and the precipitate left to air dry. The precipitate was then sent to an external company for sequencing.

2.6 Preparation of single cell suspension of splenocytes

Spleens were excised from mice and placed in 10 ml of 1x HBSS (Sigma, St Louis) in petri dishes. Spleens of mice were meshed and grinded using slides and red blood cells were lysed with 1.5 ml of RBC lysis buffer (10 mM Tris, 0.83% NH_4Cl, pH 8.2-8.4) for 90 seconds. The cells were then washed three times with large volumes of 1 x HBSS and resuspended in RPMI-1640 (Thermo fisher scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated bovine calf serum (StemCell Technologies, Vancouver, BC, Canada), 1 mM sodium pyruvate (HyClone, South Logan, UT, USA), 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), 5.5 x 10^{-2} mM β-mercapthoethanol (Gibco BRL, life

technologies, Paisley, UK) after which cell count was determined using hematocytometer under the light microscope.

2.7 *In vitro* coculture of CpG-modified plasmids with splenocytes

A total of 3 x 10^6 splenocytes were cocultured with 10 nM ,33 nM, 66 nM and 100 nM of CpG-modified plasmid DNA per well in a 24 well plate (Greiner bio-one, Frickenhausen, Germany) for 24 hours. Plasmid DNA were tested to be endotoxin free. The culture supernatant was collected after 4 hours and 24 hours and the cytokines assayed via Milliplex.

2.8 Cytokine ELISA

50 µl of purified rat anti-mouse IFN-γ (Pharmingen, San Diego CA), TNF-α (R&D systems, Minneapolis, MN), IL-6 (Pharmingen, San Diego CA), IL-10 (R & D systems, Minneapolis, MN), IL-12 (Pharmingen, San Diego CA) antibodies were diluted in coating buffer (0.1 M $NaHCO_3$, pH 8.3) to a concentration of 2 µg/ml, 0.8 µg/ml, 1 µg/ml , 4 µg/ml and 2 µg/ml respectively and coated onto 96 well high binding plate (Costar). The plate was incubated at 4°C overnight and then washed with 0.05%Tween/TBS. 100 µl of blocking buffer (1% BSA (Sigma)/0.05%Tween/TBS) was added to each well and incubated at room temperature for 1 hour, after which the plate was washed again. 50 µl of culture supernatant was added and incubated at 4°C overnight. Standard curves were prepared and added to plate using 50 µl of recombinant mouse IFN-γ, TNF-α, IL-6, IL-10, IL-12 of starting concentration 20 ng/ml, 2.5 ng/ml,10 ng/ml, 5 ng/ml and 10ng/ml respectively with two fold serial dilution in blocking buffer. This allowed the levels of cytokines in the culture supernatant to be quantified. After the overnight incubation,

the plate was washed and 50 µl of biotinylated anti-mouse IFN-γ (2 µg/ml), TNF-α (300 ng/ml), IL-6 (500 ng/ml), IL-10 (0.4 µg/ml), IL-12 (200 ng/ml) diluted in blocking buffer was added to each well. The plate was incubated for 1 hour at room temperature and washed. After which, 50 µl of ExtraAvidin®-alkaline phosphatase (Sigma, St Louis) (1:2000) diluted in blocking buffer was added and incubated at room temperature for 1 hour. The plate was washed after an hour of incubation and 50 µl of phosphatase substrate *p*-nitrophenyl phosphate was added (Sigma, St Louis). The plates were read in a BioTek Synergy 2 reader at a wavelength of 405nm. Type I interferons (IFN-α and IFN-β) were assayed using ELISA kits (PBL biomedical laboratories, NJ) according to the manufacturer's instructions.

2.9 Deriving dendritic cells from bone marrow

Bone marrow cells from C57BL/6 mice were isolated by flushing femurs with 5 ml of RPMI 1640 (Thermo fisher scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated bovine calf serum (StemCell Technologies, Vancouver, BC, Canada), 1 mM sodium pyruvate (HyClone, South Logan, UT, USA), 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), 5.5 x 10^{-2} mM β-mercapthoethanol (Gibco BRL, life technologies, Paisley, UK) The bone marrow cells were centrifuged once and then resuspended in tris-ammonium chloride for 90 seconds to lyse red blood cells. The cells were centrifuged again and then strained through a 70-µm filter before being resuspended in RPMI 1640. For FMS-like tyrosine kinase 3 ligand (Flt3L)-DCs, bone marrow cells were resuspended at 2 x 10^6/ml in medium containing 200 ng/ml murine recombinant Flt3L (Peprotech, Rocky hill, NJ), plated at 5 ml/well in 6 well plates and cultured for 8 days without disturbing. For granulocyte macrophage colony stimulating factor (GMCSF)-DCs,

bone marrow cells were seeded at 5 x 10^5/ml in medium containing 40 ng/ml GMCSF (Peprotech, Rocky hill, NJ), plated at 10ml per petri dish and cultured for 6 days. After 3 days, another 10ml of fresh medium containing 40 ng/ml GMCSF was added. All cells were incubated at 37ºC with 10% CO_2.

2.10 *In vitro* stimulation of dendritic cells

Flt3L-DCs were collected on day 8 and stained with APC-conjugated CD11c (Pharmingen, San Diego CA) in staining buffer (2%FCS/5mM EDTA/PBS/pH7.4) for 20 mins at 4ºC. The stained cells were then washed twice with staining buffer and resuspended at a density of 2.5 x 10^7 cells/ml and sorted based on surface markers. The $CD11c^+$ Flt3L-DCs were collected after cell sorting. GMCSF-DCs were collected on day 6 and subjected to dendritic cell enrichment. Purity of dendritic cells was checked by flow cytometry and found to be more than 95%. GMCSF-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with either 10 nM or 100 nM of plasmid DNA or CpG oligonucleotide. Flt3L-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with either 10 nM or 100 nM of plasmid DNA. Plasmid DNA were tested to be endotoxin free. The supernatant was collected after 24 hours and was assayed for cytokines and chemokines by cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.11 Cytokine/chemokine multiplex assay (Milliplex)

Cytokine/chemokine multiplex assay was carried out according to the manufacturer's instructions (Millipore, Bedford, MA, USA). Briefly, 25 µl of the samples and diluted standards were added into the wells, followed by the addition of 25 µl of premixed beads and 25 µl of assay buffer. The plate was incubated at 4°C overnight with

shaking. The fluid was then removed by vacuum, followed by washing with 200 µl of wash buffer per well twice. Next, 25 µl of detection antibodies was added and incubated for 1h at room temperature with shaking, followed by the addition of 25 µl of Streptavidin-Phycoerythrin. The plate was incubated for 30 mins at room temperature with shaking and the fluid was then removed by vacuum, followed by washing with 200 µl of wash buffer per well twice. Lastly, 150 µl of sheath fluid was added to all wells, followed by shaking for 15 mins and run on Luminex 100^{TM} IS.

2.12 Cytokine and chemokine array

The cytokine and chemokine array was performed according to the manufacturer's instructions (Raybio® mouse cytokine antibody array G series 1000 array III and IV, Raybio® human cytokine antibody array G series 2000, RayBiotech Inc). Briefly, the glass chip was first assembled into the incubation chamber and frame. 100 µl of 1x blocking buffer was added to the glass chip and incubated for 30 mins at room temperature. The blocking buffer was then removed from the glass slide after 30 mins and 100 µl of culture supernatant was added and incubated for 2 hours. The samples were then removed from the wells and washed 3 times with 150 µl of 1x wash buffer I in each well at room temperature. The glass chip with frame was then placed into a box with 1 x wash buffer I and washed for 20 mins with gentle shaking, the wash buffer I was then removed with the addition of 1 x wash buffer I and washed at room temperature twice for 5 mins with gentle shaking. After removal of the wash buffer II, 70 µl of diluted biotin-conjugated antibodies was added to each well and incubated at room temperature for 2 hours. The glass slide with frame was then washed with wash buffer I and II as mentioned above. 70 µl of diluted fluorescent dye conjugated strepavidin was added and incubated for 2 hours, it was then washed with wash buffer

I twice and the slide's signals were scanned and read with Axon GenePix 4000b scanner.

2.13 Flow cytometric analysis of the stimulatory effect of modified plasmids on bone marrow derived dendritic cells using GMCSF

Bone marrow derived cells were cultured with 40 ng/ml GMCSF (Peprotech, Rocky hill, NJ) for 6 days. The bone marrow derived dendritic cells were enriched by gradient separation on day 6 and co-cultured with 10 nM and 100 nM of modified or unmodified plasmids for 24 hours. Flow cytometry was conducted to examine the expression of dendritic cell marker CD11c as well as the surface expression of MHC class II and costimulatory molecules CD86, CD80, CD40 on plasmids activated dendritic cells. Briefly, the cells were stained with either APC- conjugated CD11c (Pharmingen, San Diego CA), PE-conjugated CD40 (eBioscience, San Diego CA) and FITC-conjugated CD80 (Southern Biotech, USA) or with APC- conjugated CD11c (Pharmingen, San Diego CA), PE-conjugated CD86 (Pharmingen, San Diego CA) and FITC-conjugated MHC class II (Pharmingen, San Diego CA) in staining buffer (1% BSA/PBS) for 20 mins at 4ºC. The stained cells were then washed twice with staining buffer and resuspended in 1% paraformaldehyde/PBS. Flow cytometry was performed using FACScalibur (Becton Dickson Immunocytometry Systems, San Jose, CA).

2.14 Flow cytometric analysis of the stimulatory effect of CpG oligonucleotides on bone marrow derived myeloid dendritic cells

Bone marrow derived cells were cultured with 40 ng/ml GMCSF (Peprotech, Rocky hill, NJ) for 6 days. The bone marrow derived dendritic cells were enriched by

gradient separation on day 6 and co-cultured with 10 nM and 100 nM of modified plasmids or CpG oligonucleotides for 24 hours. Flow cytometry was conducted to examine the expression of dendritic cell marker CD11c as well as the surface expression of MHC class II and costimulatory molecules CD86, CD80, CD40 on plasmids and CpG ODN activated dendritic cells. Briefly, the cells were stained with either APC-conjugated CD11c (Pharmingen, San Diego CA), PE-conjugated CD40 (eBioscience, San Diego CA) and FITC-conjugated CD80 (Southern Biotech, USA) or with APC-conjugated CD11c (Pharmingen, San Diego CA), PE-conjugated CD86 (Pharmingen, San Diego CA) and FITC-conjugated MHC class II(Pharmingen, San Diego CA) in staining buffer (1% BSA/PBS) for 20 mins at 4ºC .The stained cells were then washed twice with staining buffer (1% BSA/PBS) and resuspended in 1% paraformaldehyde/PBS. Flow cytometry was performed using FACScalibur (Becton Dickson Immunocytometry Systems, San Jose, CA).

2.15 TLR 9 inhibition experiment

Flt3L-DCs were collected on day 8 and stained with APC-conjugated CD11c (Pharmingen, San Diego CA) , biotin-conjugated B220 (Pharmingen, San Diego CA) and FITC-conjugated CD11b antibodies (Pharmingen, San Diego CA) in staining buffer (2%FCS/5mM EDTA/PBS/pH7.4) for 20 mins at 4ºC. The stained cells were then washed twice with staining buffer and stained with PE-conjugated strepavidin antibodies (Pharmingen, San Diego CA) for 20 mins at 4 ºC. The stained cells were then washed twice with staining buffer and resuspended at a density of 2.5 x 10^7 cells/ml and cell sorted. The Flt3L-DCs were sorted for $CD11c^+B220^+CD11b^-$ fraction and $CD11c^+B220^-CD11b^+$ fraction. Purity of the $CD11c^+B220^+CD11b^-$ and $CD11c^+B220^-CD11b^+$ fractions were found to be more than 95%. These fractions

were then seeded at 1 x 10^5 cells per well in 96-well plates and cocultured with 100 nM of plasmid DNA for 24 hours in the presence of 0.1 μM, 1 μM or 2 μM of TLR9 inhibitory ODN. The most effective dose of TLR9 inhibitory ODN was selected to be 2 μM and the experiment repeated with the addition of 2 μM of control ODN (described below). The culture supernatant was collected after 24 hours and assayed for IFN-α, IFN-β (PBL biomedical laboratories, NJ) and cytokines using cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.16 TLR9 inhibitory and control oligonucleotides

Toll-like receptor 9 inhibitory oligonucleotide sequences used were IRS 2088, 5'-TCCTGGCGGGGAAGT-3'. Control oligonucleotide sequence was 5'-TCC TGCAGGTTAAGT-3'. Control ODN had the same length (15 mer) and base composition as TLR9 inhibitory ODN with no CpG motif and apparent activity. Uppercase letters represent phosphorothiorate linkages (Duramad et al., 2005).

2.17 Coculture of plasmid DNA pulsed dendritic cells with Blot5- specific T cells

Bone marrow cells from female C57BL/6 mice were isolated by flushing femurs with 5 ml RPMI 1640 supplemented with 10% heat-inactivated bovine calf serum (StemCell Technologies, Vancouver, BC, Canada), 1 mM sodium pyruvate (HyClone, South Logan, UT, USA), 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 5.5 x 10^{-2} mM β-mercapthoethanol (Gibco BRL, life technologies, Paisley, UK). The bone marrow cells were centrifuged once and then resuspended in tris-ammonium chloride for 90 seconds to lyse red blood cells. The cells were centrifuged again and then strained through a 70-μm filter before being

resuspended in RPMI 1640. Bone marrow cells were resuspended at 2 x 10^6/ml in medium containing 200 ng/ml murine recombinant Flt3L (Peprotech, Rocky hill, NJ), plated at 5 ml/well in 6 well plates and cultured for 8 days without disturbing. Flt3L-DCs were collected on day 8 and stained with APC conjugated CD11c (Pharmingen, San Diego CA), PE-conjugated B220 (Pharmingen, San Diego CA) and FITC-conjugated CD11b antibodies (Pharmingen, San Diego CA). The $CD11c^+CD11b^+B220^-$ Flt3L-DCs were collected after cell sorting and pulsed with 100 nM of plasmid DNA for 24 hours. Purity of $CD11c^+CD11b^+B220^-$ Flt3L-DCs was found to be more than 95%. After 24 hours, the dendritic cells were washed twice with HBSS and pulsed with purified $CD4^+$T cells (described below) from spleen of female Blo t5 T cell receptor (TCR) transgenic mouse at a 1:2 ratio. After 24hours of coculturing dendritic cells with plasmid DNA, the cells were washed twice with HBSS and pulsed with $CD4^+$ T cells from spleens of Blo t 5 T cell receptor transgenic mice at a 1:2 ratio. 72 hours later, fresh medium containing IL-2 was added. 7 days after the coculture of DCs and T cells, the T cells were washed and subjected to ficoll separation to remove the dead cells. After which the T cells were restimulated again with freshly prepared plasmid DNA pulsed dendritic cells. The culture supernatant was collected after 72 hours and assayed for IL-17 and IFN-γ using cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.18 Purification of $CD4^+$ T cells

After the removal of dead cells from splenocytes obtained from Blo t 5 TCR transgenic mice, the cells were stained with biotinylated anti-CD4 mAb in staining buffer (0.5% BSA/2mM EDTA/PBS, pH7.2) for 10 mins at 4°C. The cells were then washed with staining buffer and streptavidin-microbeads were added and incubated at

4°C for another 10min. The stained cells were washed twice and and suspended in staining buffer. Cell separation was performed using AutoMACS (Miltenyi Biotec GmbH, Gladbach, Germany) by running the "double positive selection" program. The purity of the $CD4^+$ T cells was checked by flow cytometry and showed more than 95% purity.

2.19 Coculture of human peripheral blood mononuclear cells with plasmid DNA and CpG oligonucleotides

Whole blood was obtained from volunteers and diluted one time with HBSS (Sigma, St Louis). The diluted blood was layered gently on top of 5 ml of Ficoll-Hypaque (GE healthcare, UK) and subjected to density gradient separation. The PBMC obtained were washed three times with HBSS and cultured in Aim V medium (Gibco, Invitrogen, CA, USA) with 1 nM, 10 nM and 100 nM of plasmid DNA or CpG ODN for 5 hours and 24 hours. The culture supernatants were collected and assayed via cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.20 Mice

6-8 weeks female C57BL/6 mice from the Centre for Animal Resources, National University of Singapore were used in the experiments. All experiments were performed according to Institutional Guidelines for Animal Care and Handling (IACUC). Blo t 5 specific TCR transgenic mice were generated in the laboratory (manuscript in preparation).

2.21 Immunization regimen

Female C57BL/6 Mice were immunized with 50 μg of plasmid DNA (pVAX, pVAXBlot5, pVAXBlot5-DTKT) in 50 μl of PBS into the quadriceps of mouse with electrophoration as described in (Wolfowicz et al., 2003) on days 0 and 14. Briefly, the quadriceps of the anesthetized mice was shaved and plasmid DNA were injected using a 1 ml syringe and 27G needle in the anterior tibalis followed by electrophoration with an ECM 830 apparatus (BTX, Genetronics, San Diego, CA). Electrophoration was carried out with 0.5 cm 2-needle array inserted 2 mm deep into the muscle that delivered 4 pulses of 20 ms, 80V each with a 200 ms interval. To study the early priming response, primed mice were sacrificed on day 21 and spleens were harvested for *in vitro* culture study.

For the allergy model, female C57BL/6 mice were intraperitoneally immunized with 10 μg of rBlo t 5 protein adjuvanted with aluminium hydroxide (alum) on day 0. Three weeks later, mice were given another intraperitoneal immunization with 5 μg of rBlo t 5 protein adjuvanted with alum and they were sacrified one week later. Spleens were harvested for *in vitro* study. Mice were bled weekly and sera were analysed for Blo t 5 specific IgG1, IgG2c and IgE by ELISA.

In another study, mice were immunized with 50 μg of plasmid DNA (pVAX, pVAXBlot5, pVAXBlot5-DTKT) in 50 μl of PBS per mouse with electrophoration on days 0 and 14. Mice were immunized intraperitoneally with 10 μg of Blo t 5 protein adjuvanted with alum and 5 μg of Blo t 5 protein adjuvanted with alum on days 21 and 42 respectively. Mice were sacrificed on day 49 and spleens were harvested for *in vitro* culture study. Mice were bled weekly and the sera were analyzed for Blo t 5 specific IgG1, IgG2c and IgE by ELISA.

2.22 Splenic cell culture

Splenocytes were cultured in complete RPMI-1640 medium and incubated at 37°C in the presence of 5% CO_2. To determine the cytokine production of freshly isolated splenic cells, 100 µl of cells (4 x 10^6 cells/ml) were stimulated in the presence of 100 µl of Blo t 5 protein (20 µg/ml) per well in a 96 well U bottomed plate. To establish the short term T cell culture, splenocytes were cultured in 24 well (4 x 10^6 cells/ml) or 6 well (2 x 10^7 cells/well) plates in the presence of 10 µg/ml rBlo t 5 protein for 7 days. On days 3 and 5, cells were supplemented with fresh medium containing 10 U/ml of recombinant mouse IL-2 (R&D Systems, Minneapolis, MN, USA). To determine the cytokine profile of short term culture T cells, 50 µl of T cells (2 x 10^6 cells/ml) collected after Ficoll-Pague centrifugation (described below) and 50 µl of APCs (6 x 10^6 cells/ml) were added to 96-well U bottomed plate, followed by 100 µl of rBlo t 5 protein (20 µg/ml). The supernatant were collected at various timepoints for cytokine and chemokine profiling via Milliplex or ELISA.

2.23 Preparation of antigen presenting cells

Mitomycin C treated splenocytes of naïve mice were used as antigen presenting cells. Mitomycin C (Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in PBS to a final concentration of 0.5 mg/ml and sterile filtered with 0.22 µM filter. Spleens of mice were meshed and grinded using slides and washed twice with 1x HBSS, after which, splenocytes were suspended in PBS at a concentration of 5 x 10^7 cells/ml. Mitomycin C was added to a final concentration of 50 µg/ml and the tube was wrapped in aluminium foil and incubated for 20 mins in a 37°C water bath. After

the 20 mins incubation, the cells were washed 3 times with 30 ml of 1x HBSS and suspended in RPMI-1640 medium.

2.24 Separation of dead cells from short term cultured splenocytes by Ficoll-Paque centrifugation

Cells were collected and suspended in 5 ml of 1x HBSS in 15 ml tube after 7 days of primary cell culture with antigen. Three milliliters of Ficoll-Paque (Amersham Biosciences Corp. Piscataway, NJ) was added to the bottom of the tube without interfering with the interface of the two layers, followed by centrifugation of the cells at 800x g. The live cells were then carefully collected from the interface, washed 3 times with 1x HBSS and suspended in RPMI 1640.

2.25 Construction of pVAX-IL-35

The pVAX vector (Invitrogen Corporation) was used for the expression of murine IL-35. The Epstein-Barr virus induced gene 3 (EBI3) was amplified from the first strand cDNA prepared from lipopolysaccharide activated bone marrow derived dendritic cells. EBI3 sense: 5′-AAAAGGATCCATCCAAGGAACAGAGCCACAGAGCATG TCC-3′ and EBI3 antisense: 5′-AGAACCACCACCAAGCTTGGGCTTATGGGGTG CAC-3′. *BamHI* and *HindIII* sites were introduced into EBI3 sense primer and antisense primer respectively. The p35 subunit of IL-12 (IL-12p35) was amplified from the first strand cDNA prepared from splenocytes. IL-12p35 sense: 5′-GGTGGTGGTTCTGAATTCAGGGTCATTCCAGTCTCTGGACCTG-3′ and IL-12p35 antisense: 5′-A AAAGCTAGCTCAGGCGGAGCTCAGATAGCCCATCACC CTGTT G-3′. *EcoRI* and *NheI* sites were introduced into IL-12p35 sense primer and antisense primer respectively. Two complementary oligonucleotides encoding for 4

repeats of GGGS linker were designed and annealed. Linker sense: 5′-A ACCAAGCTTGGTGGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCTGGTGGT GGTTCTGGATCCGGTT-3′ and linker antisense: 5′-AACCGAATTCAGAACCAC CACCAGAACCACCACCAGAACCACCACCAGAACCACCACCAAGCTTGGTT -3′. *HindIII* and *EcoRI* sites were included in the linker sense and linker antisense primers. Amplified EBI3, IL-12p35 and the double stranded linker were digested with respective restriction enzymes and ligated into *Bam*HI and *Nhe*I sites of pVAX. The recombinant single-chain IL-35 in the pVAX vector (pVAX-IL-35) was verified by DNA sequencing.

2.26 Intratracheal instillation of pVAX-IL-35 on the suppression of Blo t 5 specific Th2 cell line induced airway inflammation

Long-term cultured Blo t 5-specific Th2 cells were routinely harvested 7 days after re-stimulation with rBlo t 5 protein and washed with sterile PBS. Six to 8 week-old female C57BL/6 mice were intratracheally administered with 400 μg of either pVAX or pVAX-IL-35 plasmid DNA on day 0. One day later, mice were received the transfer of 2×10^6 of Blo t 5 specific Th2 cells intravenously. A daily dose of 50 μg of rBlo t 5 protein was given intranasally to mice on days 2, 3 and 4. Mice were euthanized by carbon dioxide and the BALF was collected on day 5. Cellular infiltrates were assessed by differential cell count of 500 cells based on standard hematological characteristics of macrophages, lymphocytes, neutrophils and eosinophils. Cytokines/chemokines in the BALF were assessed by the cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.27 Intramuscular injection of pVAX-IL-35 on the suppression of Blo t 5 specific Th2 cell line induced humoral responses

Six to 8 week-old female C57BL/6 mice were intramuscularly injected with 50 μg of either pVAX or pVAX-IL-35 plasmid DNA in 50 μl of PBS into the quadriceps of mouse with electrophoration as described (Wolfowicz et al., 2003) on days 0 and 14. Briefly, the quadriceps of the anesthetized mice were shaved and plasmid DNA was injected using a 1 ml syringe and 27G needle in the anterior tibalis followed by electrophoration with an ECM 830 apparatus (BTX, Genetronics, San Diego, CA). Electrophoration was carried out with 0.5 cm 2-needle array inserted 2 mm deep into the muscle that delivered 4 pulses of 20 ms, 80V each with a 200 ms interval. The mice received intravenous transfer of 2×10^{6} of Blo t 5 specific Th2 cells on day 17 and then given the first course of intranasal instillation of 50 μg of Blo t 5 protein daily on days 18, 19 and 20. The second course of intranasal instillation of 50 μg of rBlo t 5 protein was given daily on days 77, 78 and 79. Sera were collected on days 30, 37, 44, 51, 84 and 91 for the assay of Blo t 5 specific IgE and IgG1 by ELISA. Concentrations of total IgG1, IgG2c, IgM, IgA and IgE were also determined from sera by ELISA.

2.28 Intramuscular injection of pVAX-IL-35 on the suppression of Th2 cytokines

Six to 8 week-old female C57BL/6 mice were intramuscularly injected with 50 μg of either pVAX or pVAX-IL-35 plasmid DNA with electroporation in 50 μl of PBS into the quadriceps of mouse with electrophoration as described (Wolfowicz et al., 2003) on days 0 and 14. Briefly, the quadriceps of the anesthetized mice were shaved and plasmid DNA was injected using a 1ml syringe and 27G needle in the anterior tibalis

followed by electrophoration with an ECM 830 apparatus (BTX, Genetronics, San Diego, CA). Electrophoration was carried out with 0.5 cm 2-needle array inserted 2 mm deep into the muscle that delivered 4 pulses of 20 ms, 80V each with a 200 ms interval. The mice received intravenous transfer of 2×10^6 of Blo t 5 specific Th2 cells on day 17 and then given the first course of intranasal instillation of 50 μg of rBlo t 5 protein daily on days 18, 19 and 20. 1 week after the intranasal instillation, the mice were sacrificed and spleens were excised. For intracellular staining, one milliliter of cell suspension (2×10^6 /ml) was added to each well and stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 4 hours after which 3 μM of monesin (Sigma-Aldrich, St Louis, MO) was added and further cultured for 8 hours. After staining with FITC-conjugated anti-Vβ3 TCR (Pharmingen, San Diego CA) and PerCP-conjugated anti-CD4 (Pharmingen, San Diego CA), the cells were fixed and permeabilized with Cytofix/Cytoperm™ solution and further stained with PE-conjugated anti-IL-4 (Pharmingen, San Diego CA)

2.29 Intratracheal instillation of pVAX-IL-35 on the suppression of airway inflammation in Blo t 5 TCR transgenic mice

Six to eight week-old female Blo t 5 TCR transgenic mice were intratracheally administered with 400 μg of either pVAX or pVAX-IL-35 plasmid DNA on day 0. A daily dose of 50 μg of rBlo t 5 protein was given intranasally to mice on days 1, 2 and 3. Mice were euthanized by carbon dioxide and the BALF was collected on day 4. Cellular infiltrates were assessed by differential cell count of 500 cells based on standard hematological characteristics of macrophages, lymphocytes, neutrophils and eosinophils. Cytokines / chemokines in the BALF were assessed by the cytokine / chemokine multiplex assays (Millipore, Bedford, MA, USA).

2.30 Detection of antigen-specific mouse immunoglobulin responses

The levels of antigen specific IgG1 and IgE were determined by ELISA. rBlo t 5 protein was diluted in coating buffer (0.1 M $NaHCO_3$, pH 8.3) to a concentration of 5 μg/ml, 50 μl of rBlo t 5 protein (5 μg/ml) was used to coat each sample well of 96-well ELISA plate (Sigma, St Louis) while Ig κ light chain antibody (BD Pharmingen, USA) was used to coat the standard wells and incubated at 4°C overnight. After the overnight incubation, the plates were washed with 0.05%Tween/TBS and blocked with 100 μl of blocking buffer (1% BSA (Sigma) /0.05%Tween/TBS) for 1 hour. The plate was washed, sera was added and incubated at 4°C overnight. Standard curves were prepared and added to plates using 50 ng/ml of IgG1 and IgE (BD Pharmingen, USA), subjected to two fold serial dilution. After the incubation ,plates were washed and incubated with biotin-conjugated monoclonal rat anti-mouse IgE (LO-ME-3) or IgG1(LO-MG1-2)(Serotec Ltd, Oxford, England) for 1hour, after which the plate was washed and 50 μl of ExtrAvidin-alkaline phosphatase (Sigma, St Louis, MO, USA) was added. After the 1 hour incubation, the plates were washed 6 times and 50 μl of phosphatase substrate *p*-nitrophenyl phosphate (Sigma, St Louis) were added to the plates. The plates were measured at 405nm on BioTek Synergy 2 reader. Anti-mouse Igκ light chain antibody (187.1) and commercial standards of purified IgG1 (107.3) and IgE (38.2) (BD PharMingen, San Diego, CA, USA) were used to generate the standard curves. For IgG2c detection, ELISA kit (Bethyl laboratories, Montgomery, TX, USA) was used according to the manufacturer's instructions. ELISA unit was defined as the OD_{405nm} reading corresponding to the signal generated with 1 pg/ml from the standard curve in a sandwich ELISA.

2.31 Detection of total mouse immunoglobulin responses

The levels of total antibodies were determined by ELISA. IgE, IgG1, IgA and IgM capture antibody (IgE antibody clone no: R35-72, IgG1 clone no: A85-3, IgA antibody clone CC10-3 BD Pharmingen, USA, IgM antibody clone no LO-MM3, Serotec) were diluted in coating buffer (0.1 M $NaHCO_3$, pH 8.3) to a concentration of 2 μg/ml and 50 μl used to coat each well of 96-well ELISA plates (Sigma, St Louis) and incubated at 4°C overnight. After the overnight incubation, the plates were washed with 0.05%Tween/TBS and blocked with 100 μl of blocking buffer (1% BSA (Sigma)/0.05%Tween/TBS) for 1 hour. The plate was washed, sera was added and incubated at 4°C overnight. Standard curves were prepared and added to plates using 50 ng/ml of IgG1 and IgE, 200ng/ml of IgA (BD Pharmingen, USA), 250ng/ml of IgM (Serotec), subjected to two fold serial dilution. After the incubation, plates were washed and incubated with biotin-conjugated monoclonal rat anti-mouse IgE , IgG1, IgA(BD Pharmingen, USA) or IgM (Serotec) antibodies for 1hour, after which the plate was washed and 50 μl of ExtrAvidin-alkaline phosphatase (Sigma, St Louis) was added. After the 1 hour incubation, the plates were washed 6 times and 50 μl of phosphatase substrate *p*-nitrophenyl phosphate (Sigma, St Louis) were added to the plates. For IgG2c detection, ELISA kit (Bethyl laboratories, Montgomery, TX, USA) was used according to the manufacturer's instructions. The plates were read at a wavelength of 405 nm with BioTek Synergy 2 reader.

2.32 Collection of bronchoalveolar lavage fluid and cytopsin preparation for differential cell count

Mice were under deep anaesthesia by an intraperitoneal injection of a strong dose of a mixture containing 1.25 mg/ml midazolm, 2.5 mg/ml fluasione and 0.079 mg/ml

fentanyl citrate. Using a 20G cannula, the trachea was incised and cannulated by tracheotomy. The lung was then lavaged with 0.8ml of cold Hank's balanced salt solution (HBSS) (Sigma, St Louis) without calcium and magnesium 3 times. The BALF collected was used for cytokine/chemokine multiplex assays (Millipore, Bedford, MA, USA). The same process was repeated 2 more times. The cells were then centrifuged at 3000 rpm for 10 min, the supernatant discarded and the cells resuspended in HBSS. The cells were then centrifuged again at 3000 rpm for 10 min and the cells resuspended in RPMI 1640 (Hyclone, Thermo Scientific). The cells were counted under the light microscope and the absolute cell number determined. Cytospin was carried out by centrifugation of 1 x 10^5 cells in 100 µl at 600 rpm for 10 mins using Cytospin 3 (Shandon Lipshaw) (Cytospin, Runcorn, Cheshire, U.K.). After centrifugation, the cells were stained with Liu stain (Liu, 1953). Differential cell counts based on standard hematological characteristics were carried out under the light microscope and cells were differentiated into lymphocytes, macrophages, neutrophils and eosinophils.

2.33 Rhesus macaques and immunization regimen

Fifteen 3 to 4 year old Chinese-origin rhesus monkeys, weighing 4-5 kg were bred and maintained in Shanghai Medical College, China. All animal studies were carried out in accordance with the standards set forth in the *Guide for the Care and Use of Laboratory Animals* (published by the National Academy of Science, National Academy Press, Washington, DC, USA). Rhesus monkeys were immunized with 2 mg of plasmid DNA thrice at 4 weeks interval. 4 and 8 weeks after the last immunization, the rhesus monkeys were subcutaneously immunized with 500 µg rBlo t 5 protein adjuvanted with alum. Sera were collected from the rhesus macaques

regularly. Titers of Blo t 5 -specific total IgG, IgG1, IgG3 and IgE were determined by ELISA.

2.34 Enzyme-linked immunospot assay

The monkey IFN-γ and IL-4 ELISPOT kits (U-Cytech, Utrecht, Netherlands) were used according to the manufacturer's instructions. Plates were coated overnight with 50 µl of anti IFN-γ coating antibodies or anti-IL-4 coating antibodies per well at 4°C. Blocking buffer (200 µl/well) was then added, and plates were incubated for 1hour at 37 C. Rhesus monkey PBMC were plated at a density of 4×10^5 cells/well and stimulated for 48hours at 37°C in 5% CO_2 with 1 µM of Blo t 5 peptide pools. After a 1hour incubation at 37°C with 100 µl of biotinylated anti-IFN-γ detection antibodies or biotinylated anti-IL-4 detection antibodies, 50 µl of streptavidin-HRP was added to each well, and plates were incubated for 1hour at 37°C. After this incubation, 100 µl of AEC substrate solution was added to each well, and plates were incubated at room temperature in the dark. When clear spots developed, reactions were stopped by rinsing the wells with deionized water. The spots were counted using an immunospot image analyzer.

2.35 Statistical analyses

All experimental results were presented as either mean ± SD or mean ± SEM and analyzed by either the student t test or the two-tailed Mann-Whitney *U* test. A value of $p < 0.05$ was considered significant.

Chapter 3 Evaluation of the Immunogenicity and Efficacy of DNA Vaccines with the Incorporation of CpG Motifs into the Plasmid Backbone

3.1 Introduction

DNA vaccines were promising tools that had been extensively researched on in the past decade. DNA vaccines conferred many advantages over conventional protein vaccines such as it can be customized with the antigen of interest and it could be produced easily in huge amounts. However the major fallback of DNA vaccines was its low immunogenicity in humans and primates than in mice especially in the induction of humoral responses, hence research efforts have been directed at enhancing the immunogenicity of DNA vaccines.

CpG motifs were molecular adjuvants that target at Toll-like receptor 9. There were two main classes of CpG oligonucelotides (ODN), K (also referred to as B) and D (also referred to as A). Characteristics of K type ODN were a phosphorothiorate TCGT or TCGA motif that induced the stimulation of B cells and proliferation of monocytes, resulting in the subsequent secretion of IgM and IL-6 (Coban et al., 2005). K types ODN also stimulated the activation and maturation of plasmacytoid dendritic cells (pDC) leading to production of TNF-α, IL-6, low levels of type I interferon and increased expression of MHC II, CD80 and CD86 (Kerkmann et al., 2003). D type ODN on the other hand, consisted of a central CpG flanked by a purine and pyrimidine on both sides (Coban et al., 2005). In addition, there were 3 to 4 complementary bases on both sides of the CpG motif and a 3' poly G tail (Coban et al., 2005; Verthelyi and Klinman, 2003). D type ODN induced the production of high levels of IFN-α, IFN-γ from pDC and NK cells respectively (Coban et al., 2005).

In this study, both human K and D type CpG motifs were inserted into the plasmid backbone containing the Blo t 5 allergen gene.To study if the incorporation of human CpG motifs into the plasmid backbone could increase the activation of antigen presenting cells, *in vitro* coculture of CpG modified plasmid DNA with murine dendritic cells were carried out. To answer the question if additional human CpG motifs confer increased immunogenicity, immune responses of CpG modified plasmid DNA immunized mice were evaluated. To further evaluate if human CpG modified plasmid DNA have any possible applications in humans, *in vitro* coculture of plasmid DNA with human peripheral blood mononuclear cells and *in vivo* immunization of rhesus macaques were carried out.

3.2 Results

3.2.1 Construction of pVAXBlot5 and pVAXhBlot5

The Blo t 5 construct was generated with the Blo t 5 leader sequence at the 5' end of mature coding sequence of Blo t 5 and the LAMP-1 targeting sequence at the 3' end. The Blo t 5 construct was then cloned into pVAX-1 at the *BamHI* and *XbaI* sites and designated pVAXBlot5 (Fig 3.1a). The humanized Blo t 5 construct was generated by fusing the leader sequence of human tissue plasminogen activator (htpa) to the 5' end of mature coding sequence of human codon optimized Blo t 5 and the human LAMP-1 targeting sequence at the 3' end. The humanized Blo t 5 construct was then cloned into pVAX-1 at the *BamHI* and *XbaI* sites and designated pVAXhBlot5 (Fig 3.1b) pVAXBlot5 was used in murine models and on mouse cells while pVAXhBlot5 was used in non-human primates and on human cells.

3.2.2 Insertion of D and K type CpG motifs into the plasmid backbone of pVAXBlot5

Schematic diagrams of the CpG modified plasmids were shown in Fig 3.2. K type CpG motif (KT) was first inserted into the plasmid backbone at the *PmlI* site followed by insertion of D type CpG motif (DT) at the *MluI* site. To determine if D and K type CpG motifs had been inserted into the plasmid backbone of pVAXBlot5, restriction enzyme digestion and polymerase chain reactions were carried out. K type CpG motif was inserted into the *PmlI* site, successful insertion of K type CpG motif would give 1 band (~3500bp) upon digestion with PmlI and NheI as *PmlI* site would not be cleaved upon insertion of K type CpG motif (Fig 3.3). D type CpG motif was inserted in *MluI* site. Polymerase chain reaction was carried out using the reverse primers BGH-R at 3' untranslated region and specific forward primer DT-1, successful insertion of D type

CpG motif was represented by an amplified PCR product of approximately 1300bp (Fig 3.4). To further verify if D type CpG motif had been inserted at the *MluI* site, restriction enzyme digestion was carried out using MluI and XhoI. Insertion of D type CpG motif at the *MluI* site would render the site uncleavable hence successful insertion of D type CpG motif would give 1 band (~3500bp) upon digestion (Fig 3.5).

3.2.3 pVAXBlot5 and pVAXBlot5-DTKT induced IL-6 and IFN-γ production from splenocytes in a dose dependent manner

Various doses (10 nM, 33 nM, 66 nM, 100 nM) of pVAXBlot5 and pVAXBlot5-DTKT were cocultured with murine splenocytes and found to induce the production of proinflammatory cytokines IL-6 and Th1 signature cytokine IFN-γ in a dose dependent manner (Fig 3.6), pVAXBlot5 induced the increased production of IFN-γ from 840 pg/ml to 2964 pg/ml after 24 hours of coculture when the dose of pVAXBlot5 increased from 10 nM to 100 nM (Fig 3.6a). Similarly, production of IL-6 also increased from 1109 pg/ml to 2704 pg/ml as dose of pVAXBlot5 increased from 10 nM to 100 nM (Fig 3.6b). IFN-γ production from pVAXBlot5-DTKT stimulated splenocytes increased from 754 pg/ml to 2410 pg/ml after 24 hours of coculture when the dose of pVAXBlot5-DTKT increased from 10 nM to 100 nM (Fig 3.6a). Similarly, production of IL-6 also increased from 770 pg/ml to 1516 pg/ml as dose of pVAXBlot5 increased from 10 nM to 100 nM (Fig 3.6b). There was a trend of higher production of IFN-γ and IL-6 from pVAXBlot5 stimulated splenocytes than pVAXBlot5-DTKT stimulated splenocytes. Thus, pVAXBlot5 and pVAXBlot5-DTKT induced production of cytokines and chemokines from splenocytes in a dose dependent manner.

3.2.4 Plasmid DNA induced higher cytokine and chemokine production from antigen presenting cells when compared to free CpG oligonucleotides.

To determine the effect of plasmid DNA on antigen presenting cells, dendritic cells were generated from murine bone marrow using GMCSF and cocultured with either plasmid DNA or free CpG ODN for 24 hours. Coculture with dendritic cells derived from bone marrow using GMCSF (GMCSF-DC) showed that similar levels of cytokines were elicited with pVAXBlot5 or pVAXBlot5-DTKT coculture (Fig 3.7). Plasmid DNA induced significantly higher cytokines and chemokines production from GMCSF-DC as compared to free CpG ODN (Fig 3.7). pVAXBlot5-DTKT induced significantly higher IL-12p70 (110 ± 7.3 pg/ml *vs* 54 ± 1.2 pg/ml), IFN-γ (73 ± 1.8 pg/ml *vs* 16 ± 1.8 pg/ml), IL-6 (9194 ± 257 pg/ml *vs* 3411 ± 129 pg/ml), TNF-α (1003 ± 143 pg/ml *vs* 157 ± 15 pg/ml), MIP-1α (7616 ± 485 pg/ml *vs* 2574 ± 341 pg/ml), MIP-2 (9160 ± 832 pg/ml *vs* 1685 ± 91 pg/ml) compared to DTKT ODN at 100 nM (Fig3.7). A similar trend was seen at 10 nM. Thus, pVAXBlot5 and pVAXBlot5-DTKT induced comparable levels of cytokines and chemokines production from GMCSF-DC. Plasmid DNA elicited significantly elevated levels of cytokines and chemokines production from GMCSF-DC as compared to free CpG ODN.

3.2.5 Modified and unmodified plasmid DNA induced comparable levels of upregulation of surface markers on antigen presenting cells

To compare the potency of pVAXBlot5 and pVAXBlot5-DTKT on the upregulation of surface markers on antigen presenting cells, dendritic cells were first generated from bone marrow using GMCSF. Plasmid DNA was cocultured with GMCSF-DC for 24hours and pVAXBlot5-DTKT was found to elicit similar levels of upregulation

of surface markers CD80 (116.7 ± 12 MFI *vs* 110.1 ± 3 MFI), CD86 (1359 ± 50 MFI *vs* 1590 ± 102 MFI) and MHC class II (604 ± 40 MFI *vs* 506 ± 31 MFI) when compared to pVAXBlot5 at 10 nM (Fig 3.8b-d). A similar trend was observed at 100 nM (Fig 3.8f-h). Higher upregulation of CD40 was observed for pVAXBlot5 stimulated GMCSF-DC as compared to pVAXBlot5-DTKT stimulated GMCSF-DC at 10 nM (92.3 ± 2.7 MFI *vs* 60.3 ± 4.7 MFI) as well as at 100 nM (135.9 ± 6.8 MFI *vs* 91.8 ± 4.8 MFI) (Fig3.8a, e). Thus pVAXBlot5 and pVAXBlot5-DTKT elicited comparable upregulation of surface markers, CD80, CD86 and MHC class II on GMCSF-DC.

3.2.6 CpG modified plasmid elicited higher upregulation of surface markers on bone marrow derived dendritic cells using GMCSF than free CpG oligonucleotides

To compare the potency of CpG modified plasmid DNA and free CpG oligonucleotides (ODN) on upregulation of surface markers, both CpG modified plasmids and free CpG ODN were cocultured with bone marrow derived myeloid dendritic cells using GMCSF (GMCSF-DC). At a concentration of 10 nM, DTKT modified plasmids induced significantly higher upregulation of CD40 (60.3 ± 4.7 MFI *vs* 21.4 ± 3.2 MFI), CD80 (116.7 ± 12 MFI *vs* 69.4 ± 1.7 MFI), CD86 (1359 ± 49 MFI *vs* 302 ± 11 MFI) and MHC class II (604 ± 40 MFI *vs* 327 ± 14 MFI) on GMCSF-DC compared to the mixture of D type and K type CpG ODN (DTKT ODN) at 10 nM (Fig 3.9a-d). However, there was no difference in the upregulation of surface markers at 100 nM (Fig 3.9e-h). CpG modified plasmids were more potent than the free CpG ODN in the upregulation of surface markers on GMCSF-DC.

3.2.7 pVAXBlot5 and pVAXBlot5-DTKT elicited proinflammatory cytokines and type I interferon production from bone marrow derived dendritic cells using Flt3 ligand (Flt3L-DC)

Dendritic cells could be generated from bone marrow using either GMCSF or Flt3 ligand. To evaluate the responses of Flt3L-DC to plasmid DNA stimulation, Flt3L-DC were cocultured with plasmid DNA for 24 hours. Dose dependent increase in production of IL-6, TNF-α, IFN-α, IFN-β was observed when Flt3L-DC was stimulated with plasmid DNA (Fig 3.10). Production of TNF-α increased from 252 ± 7.6 pg/ml to 293 ± 39.6 pg/ml, IL-6 increased from 1678 ± 9.8 pg/ml to 2302 ± 2.3 pg/ml, IFN-α increased from 1043 ± 14 pg/ml to 1700 ± 140 pg/ml and IFN-β increased from 102 ± 1.5 pg/ml to 226 ± 4.7 pg/ml as the dose of pVAXBlot5-DTKT increased from 10 nM to 100 nM (Fig 3.10). A similar trend was observed for pVAXBlot5. There were no observable differences in cytokine and chemokine production between pVAXBlot5 and pVAXBlot5-DTKT stimulated Flt3L-DC. Plasmid DNA elicited cytokine and chemokine production from Flt3L-DC in a dose dependent manner.

3.2.8 Cytokine and chemokine profiling of GMCSF-DC and Flt3L-DC when cocultured with CpG modified plasmid DNA

To further evaluate the differences between GMCSF-DC and Flt3L-DC in response to CpG modified plasmid DNA stimulation, supernatants of GMCSF-DC and Flt3L-DC pulsed with pVAXBlot5-DTKT were assayed via a protein array whereby the supernatants were incubated on a glass chip with mobilized capture antibodies. Biotin conjugated antibodies and strepavidin were added and the glass chip scanned. The scanned images were presented in Fig 3.11. Fold differences of cytokines and

chemokines between supernatants of stimulated Flt3L-DC and GMCSF-DC were represented in Fig 3.12 and 3.13. Flt3L-DC was found to be more responsive to pVAXBlot5-DTKT stimulation as it produced higher levels of G-CSF, IL-1α, KC, MCP-5, VCAM-1 , VEGF, MCP-1, PF-4, TCA-3 (Fig 3.14a, b) while GMCSF-DC produced elevated levels of MIP-1α (Fig 3.14c). Both GMCSF-DC and Flt3L-DC produced comparable levels of IL-12p40 (Fig 3.14c). Thus there was a difference in the cytokine and chemokine profile of GMCSF-DC and Flt3L-DC in response to pVAXBlot5-DTKT stimulation.

3.2.9 Differential responses of subsets of Flt3L-DC to plasmid DNA and free CpG ODN stimulation

Flt3L-DC was known to consist of two distinct subsets distinguished by differential expression of B220 and CD11b. To investigate the responsiveness of these different subsets of dendritic cells to plasmid DNA stimulation, Flt3L-DC was sorted based on the surface markers CD11c, CD11b and B220. $CD11c^{+}CD11b^{+}B220^{-}$ DCs were first cocultured with increasing doses (1 nM, 10 nM, 100 nM) of either plasmid DNA or free CpG ODN .There was a dose dependent increase in the production of cytokines and chemokines by $CD11c^{+}CD11b^{+}B220^{-}$ DCs when cocultured with plasmid DNA. Production of IFN-γ increased from 9 ± 0.3 pg/ml to 24 ± 1 pg/ml, IL-6 (1362 ± 112 pg/ml to 5738 ± 45 pg/ml), TNF-α (27 ± 1.6 pg/ml to 127 ± 9.8 pg/ml), IL-12p40 (183 ± 15 pg/ml to 673 ± 33 pg/ml), MIP-1α (304 ± 3.6 pg/ml to 1385 ± 109 pg/ml), MIP-1β (690 ± 52.5 pg/ml to 2634 ± 172 pg/ml), IP-10 (872 ± 105 pg/ml to 2491 ± 307 pg/ml), KC (61 ± 8.7 pg/ml to 283 ± 6.1 pg/ml) and MIP-2 (118 ± 0.1 pg/ml to 385 ± 8.7 pg/ml) when the dose of pVAXBlot5-DTKT increased from 1 nM to 100 nM (Fig 3.15). pVAXBlot5 stimulated $CD11c^{+}CD11b^{+}B220^{-}$ DCs also showed a dose

dependent increase in production of cytokines and chemokines (Fig 3.15). There were no observable differences in the cytokine and chemokine production between unmodified and modified plasmid DNA.

Similar to that noticed of $CD11c^{+}CD11b^{+}B220^{-}$ DCs, plasmid DNA also elicited a dose dependent production of IL-6, IP-10 and IL-12p40 from $CD11c^{+}CD11b^{-}B220^{+}$ DCs. Production of IL-6 from $CD11c^{+}CD11b^{-}B220^{+}$ DCs increased from 102 ± 10.7 pg/ml to 214 ± 26 pg/ml, IL-12p40 (11 ± 1.2 pg/ml to 22.2 ± 1.4pg/ml), IP-10 (156 ± 4.4 pg/ml to 421 ± 136 pg/ml) when the dose of pVAXBlot5-DTKT increased from 1 nM to 100 nM (Fig 3.16). A similar trend was seen with pVAXBlot5 (Fig 3.16). The observable differences between the two dendritic cells subsets were that $CD11c^{+}CD11b^{+}B220^{-}$ DCs produced elevated amounts of IL-6 (5738 ± 45 pg/ml *vs* 214 ± 26 pg/ml), IL-12p40 (673 ± 33 pg/ml *vs* 22.2 ± 1.4 pg/ml) and IP-10 (2491 ± 307 pg/ml *vs* 421 ± 136 pg/ml) than $CD11c^{+}CD11b^{-}B220^{+}$ DCs in response to 100 nM of pVAXBlot5-DTKT (Fig 3.15b, 3.15d, 3.15g, 3.16a-c). A similar trend was seen at 1 nM and 10 nM of pVAXBlot5-DTKT coculture as well as with pVAXBlot5 stimulation. Hence subsets of Flt3L-DC responded differentially to plasmid DNA stimulation with greater production of cytokines and chemokines by $CD11c^{+}CD11b^{+}B220^{-}$ DCs.

3.2.10 TLR9 inhibition of dendritic cells attenuated the production of cytokines and chemokines

DNA immunization had been found to elicit similar levels of antigen specific antibodies and interferon-γ secreting cells in $TLR9^{-/-}$ as well as in $TLR9^{+/+}$ mice (Babiuk et al., 2004). To evaluate the involvement of TLR9, $CD11c^{+}CD11b^{-}B220^{+}$

DCs were cocultured with plasmid DNA in the presence of increasing doses (0.1 μM, 1 μM and 2 μM) of a TLR9 inhibitory oligonucleotide. Production of IL-6, IL-12p40 and IP-10 were almost completely abolished in the presence of TLR9 inhibitory oligonucleotides (Fig 3.17). The dose of TLR9 inhibitory oligonucleotide was fixed at 2 μM and the experiment repeated with the addition of an oligonucleotide with an irrelevant sequence as a control. The addition of 2 μM of TLR9 inhibitory oligonucleotide sharply attenuated the production of IP-10 from $CD11c^{+}CD11b^{-}B220^{+}$ DCs (748 ± 327 pg/ml to 8 ± 3 pg/ml), IL-6 (381 ± 166 pg/ml to 1.8 ± 1.2 pg/ml), IL-12p40 (30 ± 7.4 pg/ml to 1.4 ± 0.3 pg/ml), IFN-α (2352 ± 10 pg/ml to 10.4 ± 10.4 pg/ml) (Fig 3.18). However IFN-β production was not abolished. In comparison, the use of a control oligonucleotide did not abolish the cytokines and chemokine levels, IP-10, IL-6, IL-12p40, IFN-α remained high at 484 ± 353 pg/ml, 375 ± 229 pg/ml, 25 ± 4.3 pg/ml and 1461 ± 9 pg/ml respectively (Fig 3.18). A similar trend was observed for pVAXBlot5.

Similarly $CD11c^{+}CD11b^{+}B220^{-}$ DCs were cocultured with plasmid DNA in the presence of increasing doses (0.1 μM, 1 μM, 2 μM) of a TLR9 inhibitory oligonucleotide. TLR9 inhibitory oligonucleotide attenuated cytokines and chemokines production from $CD11c^{+}CD11b^{+}B220^{-}$ DCs in a dose dependent manner. Production of IL-6 from pVAXBlot5-DTKT stimulated $CD11c^{+}CD11b^{+}B220^{-}$ DCs decreased from 530 pg/ml to 6.3 pg/ml, IP-10 (1093 pg/ml to 104 pg/ml), IL-12p40 (96 pg/ml to 2.1 pg/ml), KC (63 pg/ml to 5.7 pg/ml), MIP-1α (198 pg/ml to 6.5 pg/ml), MIP-1β (442 pg/ml to 12.2 pg/ml), TNF-α (8.1 pg/ml to 1.6 pg/ml) and MIP-2 (130 pg/ml to 47.7 pg/ml) when the dose of TLR9 inhibitory oligonucleotide increased from 0.1 μM to 2 μM (Fig 3.19). The addition of 2 μM of TLR9 inhibitory

oligonucleotide sharply attenuated the production of TNF-α from pVAXBlot5-DTKT stimulated $CD11c^{+}CD11b^{+}B220^{-}$ DCs (245 ± 118 pg/ml to 5.4 ± 3.8 pg/ml), IL-6 (8473 ± 2735 pg/ml to 10.8 ± 4.6 pg/ml), IL-12p40 (1079 ± 550 pg/ml to 4 ± 2 pg/ml), IP-10 (3317 ± 826 pg/ml to 125 ± 21 pg/ml), KC (289 ± 5.7 pg/ml to 6.5 ± 0.8 pg/ml), MIP-1α (3472 ± 2087 pg/ml to 15.7 ± 9.2 pg/ml), MIP-1β (4854 ± 2220 pg/ml to 9.6 ± 2.6pg/ml), IFN-α (146.4 ± 14.4 pg/ml to 38.3 ± 0.7 pg/ml) and MIP-2 (332 ± 53 pg/ml to 74.4 ± 26.7 pg/ml) (Fig 3.20).. However IFN-β production was not abolished. This inhibition was TLR9 specific as the use of a control oligonucleotide did not abolish the cytokines and chemokines TNF-α (164 ± 74 pg/ml), IL-6 (6961 ± 1460 pg/ml), IL-12p40 (1026 ± 429 pg/ml), IP-10 (2634 ± 702 pg/ml), KC (262 ± 1.5 pg/ml), MIP-1α (2336 ± 1247 pg/ml), MIP-1β (4616 ± 1618 pg/ml), MIP-2 (237 ± 48.6 pg/ml) and IFN-α (60.8 ± 5.9 pg/ml) (Fig 3.20). A similar trend was observed for pVAXBlot5 stimulated $CD11c^{+}CD11b^{+}B220^{-}$ DCs. Hence TLR9 was involved in the activation of dendritic cells by plasmid DNA as inhibition of TLR9 attenuated sharply the cytokines and chemokines production.

3.2.11 Plasmid DNA pulsed dendritic cells presented antigen to Blo t 5 specific $CD4^{+}$ T cells and polarized them to a similar T helper phenotype that produced IL-17 and IFN-γ

To evaluate if there was any difference in the phenotype of antigen specific T cells primed by modified and unmodified plasmid DNA pulsed dendritic cells, Blo t 5 specific $CD4^{+}$ T cells were activated first round with plasmid DNA stimulated dendritic cells in the presence of recombinant Blo t 5 protein for 7 days, after which it was restimulated with freshly prepared plasmid DNA stimulated dendritic cells in the presence of recombinant Blo t 5 protein. It was observed that Blo t 5 specific $CD4^{+}$ T

cells activated by pVAXBlot5-DTKT pulsed dendritic cells produced lesser IFN-γ compared to the cells activated by pVAXBlot5 pulsed dendritic cells (5463 ± 783 pg/ml *vs* 8557 ± 898 pg/ml) (Fig 3.21a). In addition, there was a trend of attenuation of IL-17 produced by Blo t 5 specific $CD4^+$ T cells activated with pVAXBlot5-DTKT pulsed dendritic cells as compared to those cells activated by pVAXBlot5 pulsed dendritic cells (677 ± 121 pg/ml vs 1054 ± 506 pg/ml) (Fig 3.21b). Production of IFN-γ and IL-17 was via interaction between plasmid DNA pulsed dendritic cells and Blo t 5 specific $CD4^{\pm}$ T cells as plasmid DNA pulsed dendritic cells and Blo t 5 specific $CD4^+$ T cells did not secrete much IFN-γ and IL-17 when they were cultured alone (Fig 3.21). Plasmid DNA pulsed dendritic cells presented antigen to Blo t 5 specific $CD4^{\pm}$ T cells and polarized them to a similar T helper phenotype that produced IL-17 and IFN-γ. There was a trend of attenuation of IL-17 and IFN-γ production by pVAXBlot5-DTKT pulsed dendritic cells activated Blo t 5 specific T cells when compared to pVAXBlot5 pulsed dendritic cells activated Blo t 5 specific T cells.

3.2.12 pVAXBlot5-DTKT immunized mice produced the highest magnitude of Blo t 5 specific immune responses.

To determine the magnitude of Blo t 5 specific immune responses elicited by pVAXBlot5 and pVAXBlot5-DTKT immunization, C57BL/6 mice were intramuscularly immunized with plasmid DNA and the levels of T cell cytokines were measured to compare the immunogenicity of modified and unmodified plasmid DNA (Fig 3.22a). pVAXBlot5-DTKT immunized group responded with a trend of higher levels of Blo t 5 specific IFN-γ (538 ± 89 pg/ml *vs* 379 ± 101 pg/ml), IL-6 (73 ± 13.4 pg/ml *vs* 64.5 ± 13.2 pg/ml), IP-10 (420 ± 73.4 pg/ml *vs* 290 ± 70.4 pg/ml), IL-17

(21.3 ± 8.1 pg/ml *vs* 16.9 ± 9.7 pg/ml), MIG (302 ± 41.8 pg/ml *vs* 171 ± 75.4 pg/ml) and GMCSF production (233 ± 52.5 pg/ml *vs* 130 ± 27 pg/ml) when compared to pVAXBlot5 immunized group (Fig 3.23). In addition, pVAXBlot5-DTKT immunized group also produced significantly higher levels of IL-6 (73 ± 13.4 pg/ml *vs* 30.2 ± 4.1 pg/ml), IP-10 (420 ± 73.4 pg/ml *vs* 207 ± 20 pg/ml), MIG (302 ± 41.8 pg/ml *vs* 134 ± 28.3 pg/ml) and a trend of elevated levels of IFN-γ (538 ± 89 pg/ml *vs* 406±101 pg/ml), IL-17 (21.3 ± 8.1 pg/ml *vs* 9.3 ± 3 pg/ml), GMCSF (233 ± 52.5 pg/ml *vs* 115 ± 17.8 pg/ml) when compared to pVAX-1 vector immunized group (Fig 3.23). Overall, pVAXBlot5-DTKT immunized groups produced the highest magnitude of Blo t 5 specific immune responses.

3.2.13 Mixed cytokine profile in mice immunized with Blo t 5 protein adjuvanted with aluminium hydroxide (Allergy model)

Naïve C57BL/6 mice receiving two intraperitoneal injections of 10 μg and 5 μg of rBlo t 5 protein adjuvanted with aluminum hydroxide (alum) respectively at 3 weeks apart (Fig 3.22b) showed a mixed T cell cytokine profile in a secondary culture *in vitro* and produced significantly higher Blo t 5 specific IFN-γ (2288 ± 286 pg/ml *vs* 395 ± 258 pg/ml), IL-17 (934 ± 220 pg/ml *vs* 42.5 ± 35.6 pg/ml), IL-5 (353 ± 53.9 pg/ml *vs* 38.2 ± 24.5 pg/ml), IL-13 (168 ± 30 pg/ml *vs* 16.3 ± 7.7 pg/ml), IL-6 (519 ± 104 pg/ml *vs* 103 ± 49.5 pg/ml), MCP-1 (1732 ± 279 pg/ml *vs* 499 ± 219 pg/ml) and a trend of higher MIG production (1165 ± 361 pg/ml *vs* 186 ± 72 pg/ml) than mice immunized with PBS adjuvanted with alum indicating that Th1, Th2 and Th17 phenotypes were induced in mice immunized with rBlo t 5 protein adjuvanted with alum (Fig 3.24). Allergen adjuvanted with alum would induce Th1, Th2 and Th17

phenotypes in immunized mice. Such a strategy was previously used to prime Th2 dominant immune responses.

3.2.14 IL-17 and IFN-γ producing cells were distinct subsets in Blo t 5 specific T cells

To evaluate if the IL-17 and IFN-γ produced in the T cell cultures of mice immunized with rBlo t 5 protein adjuvanted with alum were from the same cell, intracellular staining was carried out on Blo t 5 specific T cells. It was found out that IL-17 and IFN-γ were produced from distinct cell populations (Fig 3.25) and $CD4^+$ T cells produced slightly higher levels of IFN-γ compared to IL-17 (5.9 ± 2.4% *vs* 3.3 ± 0.8%) (Fig 3.25c)while $CD8^+$ T cells produced much elevated levels of IFN-γ compared to IL-17 (47.7 ± 1.6% *vs* 8.1 ± 2.1%) (Fig 3.25d). Hence the majority of IFN-γ produced could possibly be from Blo t 5 specific $CD8^+$ T cells. IL-17 and IFN-γ producing cells were distinct subsets in Blo t 5 specific T cells from Blo t 5 protein adjuvanted with alum immunized mice.

3.2.15 Blo t 5 specific antibody responses in mice immunized with Blo t 5 protein adjuvanted with aluminium hydroxide (Allergy model)

Mice immunized with Blo t 5 protein adjuvanted with alum showed significantly higher Blo t 5 specific IgE levels than the control group (1876 ± 450 ELISA unit *vs* ND on day 14, 1521 ± 40 ELISA unit *vs* 261 ± 58 ELISA unit on day 28, $p < 0.05$) (Fig 3.26c). In addition, higher Blo t 5 specific IgG1 levels were also produced by mice immunized with Blo t 5 protein adjuvanted with alum compared to control mice from days 14 to 28 ($p < 0.05$) (Fig 3.26a) Mice immunized with Blo t 5 protein adjuvanted with alum also produced elevated levels of Blo t 5 specific IgG2c when

compared to control mice (Fig 3.26b). Mice immunized with Blo t 5 protein adjuvanted with aluminium hydroxide produced Blo t 5 specific IgG1, IgG2c and IgE.

3.2.16 Allergen gene immunization effectively suppressed T effector cytokines induced by Blo t 5 protein adjuvanted with alum immunization

This study had shown that naïve mice immunized twice intraperitoneally with rBlo t 5 protein adjuvanted with alum develop a mixed T cell cytokine profile (Fig 3.24). To determine if DNA vaccination had any prophylactic suppressive effect on this mixed T cell cytokine profile, C57BL/6 mice were immunized with allergen gene DNA constructs (pVAXBlot5, pVAXBlot5-DTKT) and then challenged by intraperitoneal immunizations with rBlo t 5 protein adjuvanted with alum (Fig 3.22c). Control mice were not immunized with DNA constructs and they were only given the intraperitoneal immunization of rBlo t 5 protein adjuvanted with alum. Splenocytes from pVAXBlot5 immunized mice showed a 20 fold and 136 fold increase in IFN-γ production (137 ± 47 pg/ml) as compared to pVAX-1 immunized mice and control mice respectively at 72 hours ($p < 0.05$) (Fig 3.27a). In comparison, cells from pVAXBlot5-DTKT immunized mice showed a greater increase in IFN-γ production (210 ± 70 pg/ml), about 30 fold and 209 fold increase as compared to pVAX-1 immunized mice and control mice respectively at 72 hours (Fig 3.27a). Cells from pVAXBlot5 and pVAXBlot5-DTKT immunized mice continued to show a significant increase in IFN-γ production when compared to pVAX-1 immunized mice and control mice at 96 hours (Fig 3.27a). Low levels of IL-13, IL-17, IL-5 and IL-6 were also produced by mice in all the groups at 72 hours and 96 hours (Fig 3.27). Besides cytokine production, DNA immunization also induced chemokine production. pVAX-1, pVAXBlot5, pVAXBlot5-DTKT immunized mice also showed a 4.8, 5.3 and 6.2

fold increase in MCP-1 production compared to control mice at 96 hours ($p < 0.05$) (Fig 3.27f). Cells from pVAXBlot5 and pVAXBlot5-DTKT immunized mice produced significantly higher levels of MIG when compared to pVAX-1 and control mice at 72hours. There was a trend of higher production of IFN-γ, IL-6 and MCP-1 elicited by pVAXBlot5-DTKT immunized mice when compared to pVAXBlot5 immunized mice in the primary culture.

Upon secondary stimulation with rBlo t 5 protein, plasmid DNA immunized mice showed a significant attenuation in Blo t 5 specific IFN-γ (Fig 3.28a) and IL-17 (Fig 3.28b) when compared to control mice. It was interesting to note that pVAXBlot5 and pVAXBlot5-DTKT immunized group showed a greater attenuation in IL-17 compared to pVAX immunized group ($p < 0.05$) (Fig 3.28b). In addition, the pVAXBlot5 and pVAXBlot5-DTKT immunized groups showed significant reduction in production of Blo t 5 specific IL-5, IL-13, IL-6 (Fig 3.28c, 3.28d, 3.28e), when compared to both pVAX-1 immunized group and control group ($p < 0.05$). pVAXBlot5-DTKT immunized group showed a greater suppression in IL-5, IL-6, IL-13 and IL-17 when compared to pVAXBlot5 group out of which suppression in IL-13 was statistically significant ($p < 0.05$) (Fig 3.28). Notably, pVAX immunized group failed to show suppression in Blo t 5 specific IL-5, IL-13 and IL-6 when compared to the control mice. Besides attenuation in cytokine production, plasmid DNA immunized groups also showed a trend in reduction in MCP-1 and MIG (Fig 3.28f, 3.28g) when compared to control mice, pVAXBlot5-DTKT immunized group in particular showed a significant reduction in MCP-1 (Fig 3.28f) level when compared to control mice ($p < 0.05$). Allergen gene immunized mice suppressed Blo t 5 specific IFN-γ, IL-17, IL-5, IL-13 and IL-6 induced by intraperitoneal immunization with rBlo

t5 protein adjuvanted with alum. pVAXBlot5-DTKT immunized mice showed a trend of greater suppression in Blo t 5 specific IL-5, IL-6, IL-13 and IL-17 when compared to pVAXBlot5 immunized mice.

3.2.17 Allergen gene immunization effectively suppressed antigen specific IgE production induced by intraperitoneal immunization with allergen adjuvanted with alum

To evaluate the modulatory effect of pVAXBlot5 and pVAXBlot5-DTKT on the humoral responses induced by rBlo t 5 protein adjuvanted with alum immunization, Blo t 5 specific IgG1, IgG2c and IgE were measured from the sera collected weekly. Control mice only received intraperitoneal immunizations with rBlo t 5 protein adjuvanted with alum. Mice immunized with pVAXBlot5-DTKT produced the highest titres of Blo t 5 specific Th1 signature antibody IgG2c subclass (Fig 3.29b) and IgG1 subclass (Fig 3.29a) among the groups. Notably, pVAXBlot5-DTKT induced significantly higher levels of Blo t 5 specific IgG1 than pVAXBlot5 (5028 ± 835 (ELISA unit $x10^3$) *vs* 2208 ± 605 (ELISA unit $x10^3$) on day 28, 11148 ± 3830 (ELISA unit $x10^3$) *vs* 2894 ± 534 (ELISA unit $x10^3$) on day 35, 18319 ± 2904 (ELISA unit $x10^3$) *vs* 6528 ± 1483 (ELISA unit $x10^3$) on day 49, $p < 0.05$) (Fig 3.29a). Allergen gene immunized mice (pVAXBlot5 and pVAXBlot5-DTKT groups) also persistently produced significantly higher levels of Blo t 5 specific IgG2c from days 21-49 as compared to pVAX group ($p < 0.05$) (Fig 3.29b). Both pVAXBlot5 and pVAXBlot5-DTKT immunized groups showed an attenuation in Blo t 5 specific IgE antibody when compared to pVAX empty vector group on days 35 and 42 but only pVAXBlot5-DTKT group continued to show a significant suppression in Blo t 5 specific IgE till day 49 ($p < 0.05$) (Fig 3.29c). pVAXBlot5-DTKT immunized mice

produced higher levels of Blo t 5 specific IgG1 and IgG2c when compared to pVAXBlot5 immunized mice. Mice immunized with Blo t 5 constructs effectively suppressed Blo t 5 specific IgE production induced by intraperitoneal immunization with rBlo t 5 protein adjuvanted with alum.

3.2.18 CpG modified plasmid DNA induced higher proinflammatory cytokine and chemokine production from human peripheral mononuclear cells compared to unmodified plasmid DNA

To evaluate the effect of pVAXhBlot5 and pVAXhBlot5-DTKT on human cells, various doses (1 nM, 10 nM, 100 nM) of pVAXhBlot5, pVAXhBlot5-DTKT and DTKT ODN were cocultured with human peripheral mononuclear cells (PBMC) for 5 hours and 24 hours. Dose dependent secretion of proinflammatory cytokines, TNF-α, IL-6 and activation chemokines MIP-1α, MIP-1β were observed for plasmid DNA stimulated PBMC at both the 5 hour and 24 hour timepoints. At an early timpoint of 5 hours, pVAXhBlot5-DTKT stimulated PBMC produced significantly higher levels of IL-6 (494 ± 46 pg/ml *vs* 76.9 ± 30.4 pg/ml), MIP-1α (1014 ± 74 pg/ml *vs* 324 ± 122 pg/ml) and MIP-1β (577 ± 127 pg/ml *vs* 198 ± 32.7 pg/ml) when compared to pVAXhBlot5 stimulated PBMC at 100 nM (Fig 3.30).At a later timepoint of 24 hours, pVAXhBlot5-DTKT stimulated PBMC continued to produce a trend of higher production of TNF-α (54.2 ± 12.8 pg/ml *vs* 42.8 ± 7.8 pg/ml), IL-6 (478 ± 150 pg/ml *vs* 385 ± 172 pg/ml), MIP-1α (1275 ± 288 pg/ml *vs* 689 ± 110 pg/ml) and MIP-1β (881 ± 116 pg/ml *vs* 511 ± 125 pg/ml) when compared to pVAXhBlot5 stimulated PBMC (Fig 3.31). Thus pVAXhBlot5-DTKT induced higher production of cytokines and chemokines from human PBMC than pVAXhBlot5. These distinct differences were not observed using mouse cells.

3.2.19 Protein array analysis of differentially expressed regulatory cytokines and chemokines profile of plasmid DNA stimulated human peripheral mononuclear cells

To extensively study the differences in cytokine and chemokine production among pVAXhBlot5-DTKT, pVAXhBlot5 and DTKT ODN stimulated human peripheral mononuclear cells, supernatants of pVAXhBlot5-DTKT, pVAXhBlot5 and DTKT ODN stimulated human peripheral blood mononuclear cells were analysed via protein array and it was found that there was differential regulation of different proteins at different timepoints. At an early timepoint of 5 hours, pVAXhBlot5-DTKT induced significant upregulation of PDGF-AA, GRO-α, NT-4, IL-1β, osteoprotegerin, VEGF-D, GMCSF, MCP-1 and ENA-78 (Fig 3.32a) and downregulation of ErbB3, IP-10, angiogenin, IGFBP-2 and MCP-2 (Fig 3.32b) compared to pVAXhBlot5 ($p<0.05$). At a later timepoint of 24 hours, pVAXhBlot5-DTKT induced significant upregulation of angiogenin, Flt3 ligand, I-309, IL-10, MCP-1, MCP-4, MIP-3α, PARC, TARC and TECK (Fig 3.33a) with downregulation of IL-12p40 as compared to pVAXhBlot5 ($p < 0.05$) (Fig 3.33b). When compared to DTKT ODN at an early timepoint of 5 hours, pVAXhBlot5-DTKT induced significant upregulation of ENA-78, Flt3 ligand, GMCSF, IL-1β, IL-6, MIP-3α, PDGF-BB, GRO-α, MIP-1α, NT-4 and osteoprotegerin (Fig 3.34a) with the downregulation of leptin R, IP-10 and angiogenin (Fig 3.34b) ($p < 0.05$). At a later timepoint of 24 hours, GCP-2, MCP-3, MCP-4, MDC, MIP-3α, PARC, TNF-α, ENA-78, IL12p40, IL-8, oncostatin M, TIMP-1, uPAR, Siglec-5, GMCSF, sTNFRII, I-309, IL-10, IL-1β, IL-6, GRO-α, MIP-1α, MMP-9 and Activin A were upregulated in the pVAXhBlot5-DTKT group when compared to DTKT ODN ($p < 0.05$) (Fig 3.35a, 3.35b). PDGF-AA and angiogenin

were downregulated ($p < 0.05$) (Fig 3.35c). Interestingly, pVAXBlot5-DTKT induced higher upregulation of IL-1β when compared to both pVAXhBlot5 (4 fold) (Fig 3.32a) and DTKT ODN (30 fold) (Fig 3.33a) at 5 hrs. Hence pVAXhBlot5-DTKT upregulated higher cytokines and chemokines production from human PBMC as compared to pVAXhBlot5 and DTKT ODN. There was a dynamic change in the upregulation profile of cytokines and chemokines profile at early (5 hours) and later (24 hours) timepoints.

3.2.20 Evaluation of immunogenicity of Blo t 5 encoded plasmid DNA by production of Blo t 5 specific antibodies in non human primates

To evaluate if CpG modified pVAXhBlot5-DTKT had any enhanced immunogenic effect *in vivo,* non-human primates were immunized with pVAX-1, pVAXhBlot5 and pVAXhBlot5-DTKT and subsequently received subcutaneous immunizations with rBlo t 5 protein adjuvanted with alum (Fig 3.22d). Sera was collected monthly and assayed for allergen specific antibodies. Production of Blo t 5 specific total IgG and IgG1 in non-human primates vaccinated with plasmid encoding allergen gene (pVAXBlot5 and pVAXBlot5-DTKT) were earlier than the group vaccinated with pVAX-1. At week 14, pVAXhBlot5 and pVAXhBlot5-DTKT immunized groups produced significantly higher levels of Blo t 5 specific total IgG when compared to pVAX-1 group ($p < 0.05$) (Fig 3.36a). Blo t 5 specific IgG1 production also showed similar kinetics as Blo t 5 specific total IgG. pVAXhBlot5-DTKT was found to induce significantly higher levels of Blo t 5 specific IgG1 than pVAX-1 (89 ± 43 (OD x DF) *vs* 3 ± 2 (OD x DF) at week 14, 230 ± 33 (OD x DF) *vs* 56 ± 31 (OD x DF) at week 18, $p < 0.05$) (Fig 3.36b). There was a trend of higher production of Blo t 5 specific total IgG and IgG1 in pVAXhBlot5-DTKT group as compared to pVAXhBlot5 group

at week 18 (Fig 3.36a, b). All three groups elicited comparable levels and kinetics of Blo t 5 specific IgG3 production (Fig 3.36c). Both pVAXhBlot5 and pVAXhBlot5-DTKT immunized groups showed an attenuation in Blo t5 specific IgE compared to pVAX-1 immunized group at week 18 (Fig 3.36d). Immunogenicity of allergen gene constructs was demonstrated by faster kinetics and levels of Blo t 5 specific IgG production.

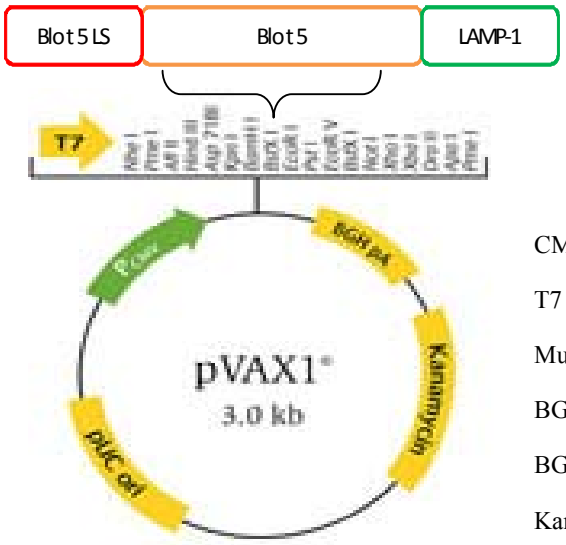
3.2.21 Blo t 5 specific IFN-γ and IL-4 producing cells in plasmid DNA immunized non-human primates

To evaluate the cellular responses induced by plasmid DNA immunization, Blo t 5 specific IFN-γ and IL-4 producing cells were examined by ELISPOT. Monkeys' PBMC were stimulated with three Blo t 5 peptide pools. There was a trend of higher numbers of IFN-γ producing cells elicited in allergen gene vaccinated groups in response to all three Blo t 5 peptide pools when compared to pVAX-1 immunized group (Fig 3.37). pVAXhBlot5-DTKT immunized monkeys produced higher number of IFN-γ producing cells per million PBMC upon stimulation with Blo t 5 peptide pool 1 when compared to pVAXhBlot5 and pVAX groups (Median of 4.2 spots *vs* 2.5 spots and 0.83 spots respectively) (Fig 3.37a). Higher number of IFN-γ producing cells per million PBMC was also produced from pVAXhBlot5-DTKT immunized group in response to Blo t 5 peptide pool 2 stimulation when compared to pVAXhBlot5 and pVAX groups (Median of 3 spots vs 2.5 and 0 spots respectively) (Fig 3.37b). pVAXhBlot5-DTKT and pVAXhBlot5 immunized groups also attenuated the number of IL-4 producing cells per million PBMC upon stimulation with Blo t 5 peptide pool 2 and 3 when compared to pVAX group (Fig 3.38b, 3.38c). Notably, 3 out of 5 non-human primates immunized with pVAXhBlot5-DTKT did not

induce any IL-4 producing cells in response to Blo t 5 peptide pool 2 stimulation (Fig 3.38b). Thus pVAXhBlot5-DTKT immunized group yielded the highest number of IFN-γ producing cells and lowest number of IL-4 producing cells in response to Blo t 5 peptide pools stimulation.

Figures

a) pVAXBlot5



CMV promoter : Bases 137-724

T7 promoter/priming site : Bases 664-683

Multiple cloning site : Bases 696-811

BGH reverse priming site : Bases 823-840

BGH polyadenylation signal : Bases 829-1053

Kanamycin resistance gene : Bases 1226-2020

pUC origin : Bases 2320-2993

b) pVAXhBlot5

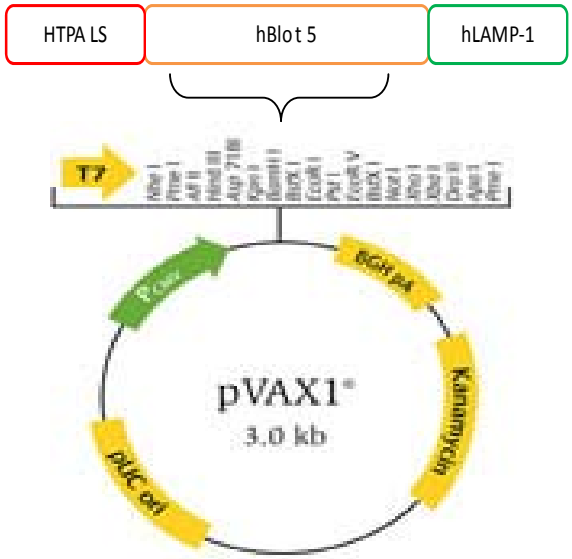


Figure 3.1: Construction of pVAXBlot5 and pVAXhBlot5

The mature coding sequence of Blo t 5 was cloned into the pVAX vector containing Blo t 5 leader sequence (LS) and LAMP-1 targeting region and designated as pVAXBlot5 construct (a) The human codon optimized Blo t 5 gene (hBlot5) was cloned into pVAX vector containing human tissue plasminogen activator leader sequence (HTPA LS) and human LAMP-1 targeting region and designated as pVAXhBlot5 construct (b). The sequence was verified by sequencing and these plasmids were subsequently used for the insertion of the CpG motifs.

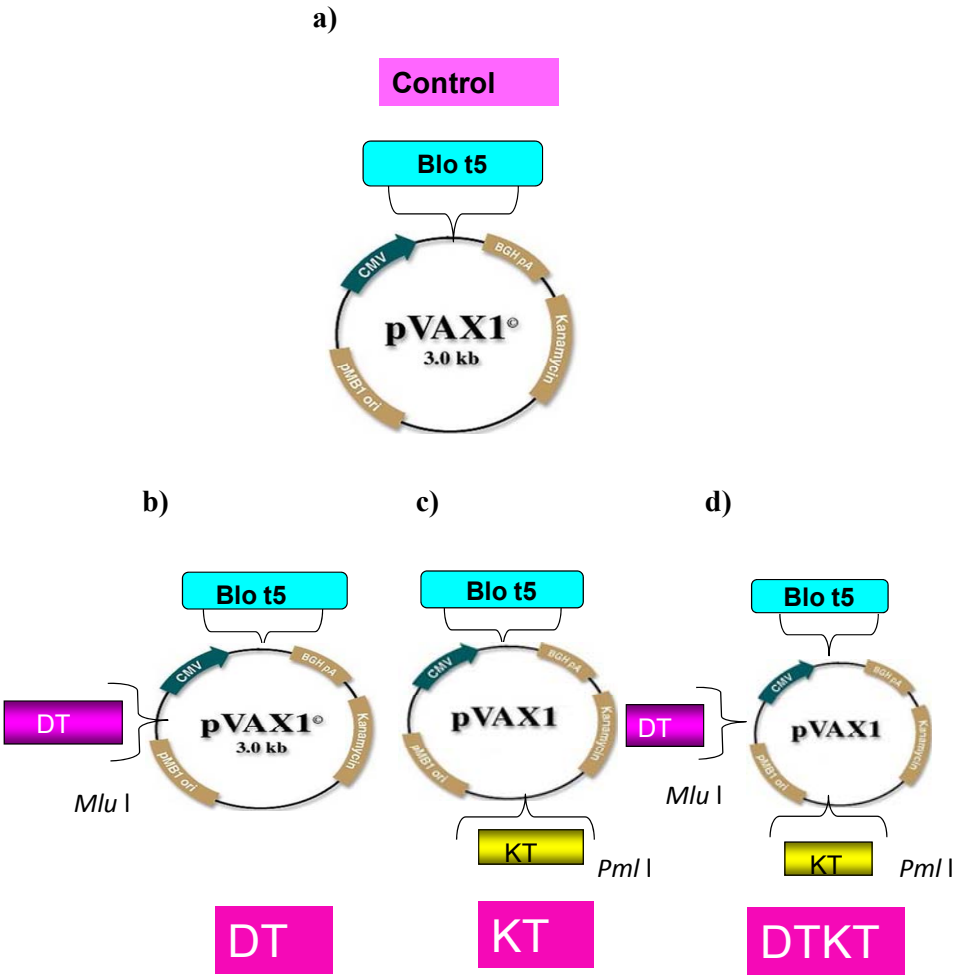


Figure 3.2: Schematic diagram of the CpG modified plasmid DNA constructs

D type and K type CpG motifs were inserted into the *MluI* and *PmlI* sites sequentially in the plasmid backbone of pVAXBlot5 and pVAXhBlot5, giving rise to double insertion of D type and K type CpG motif designated as DTKT (d).

Lane 1: 1 kb DNA ladder
Lane 2-5: Clones 1 to 4
Lane 6: negative control

Figure 3.3: Incoporation of K type CpG oligonucleotide (KT) into the *PmlI* site in the plasmid pVAXBlot5

To confirm the presence of KT in the selected clones, plasmids of selected clones were extracted and digested with PmlI and NheI. Presence of the insert KT will result in only 1 band (~3500bp) after digestion. Negative control consists of 3 bands. Clones 1 to 4 all contain the insert KT. These positive clones containing the insert KT were then used for the insertion of DT so as to create clones with double insertion of DT and KT.

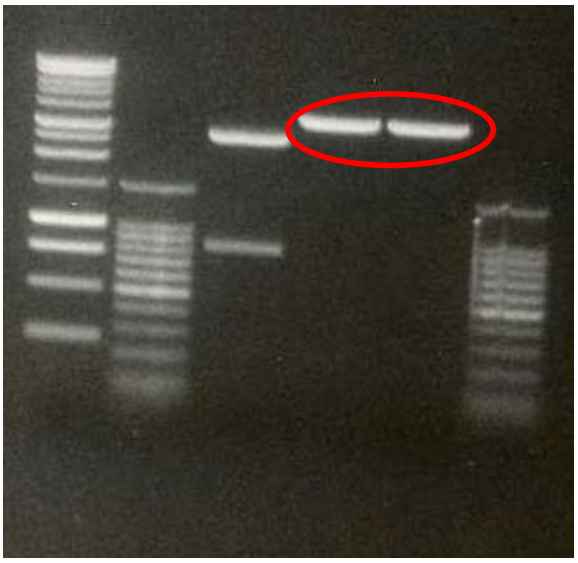
Lane 1: 1kb DNA ladder

Lane 2: 100bp DNA ladder

Lane 3-8: clones 1-6

Figure 3.4: Incoporation of D type CpG oligonucleotide into *MIuI* site of pVAX-Blo t5

After PCR amplification with the primers BGH-R and DT1, 10 µl of the PCR products were run in a 1% agarose gel at 100V for 1 hr. Clones 1 and 6 are suspected to contain the insert DT (~1300bp).



Lane 1: 1 kb DNA ladder
Lane 2, 6:100 bp DNA ladder
Lane 3: negative control
Lane 4: clone 1
Lane 5: clone 6

Figure 3.5: Restriction enzyme digest to confirm the presence of D type CpG oligonucleotide (DT) in the suspected clones 1 and 6

To confirm the presence of DT in clones 1 and 6 ,the plasmids of the clones were extracted and digested with *MIuI* and *XhoI*. Presence of insert DT will result in 1 band (~3500bp) after digestion. Both clones 1 and 6 contain the insert DT. Sequences of the clones were verified by DNA sequencing.

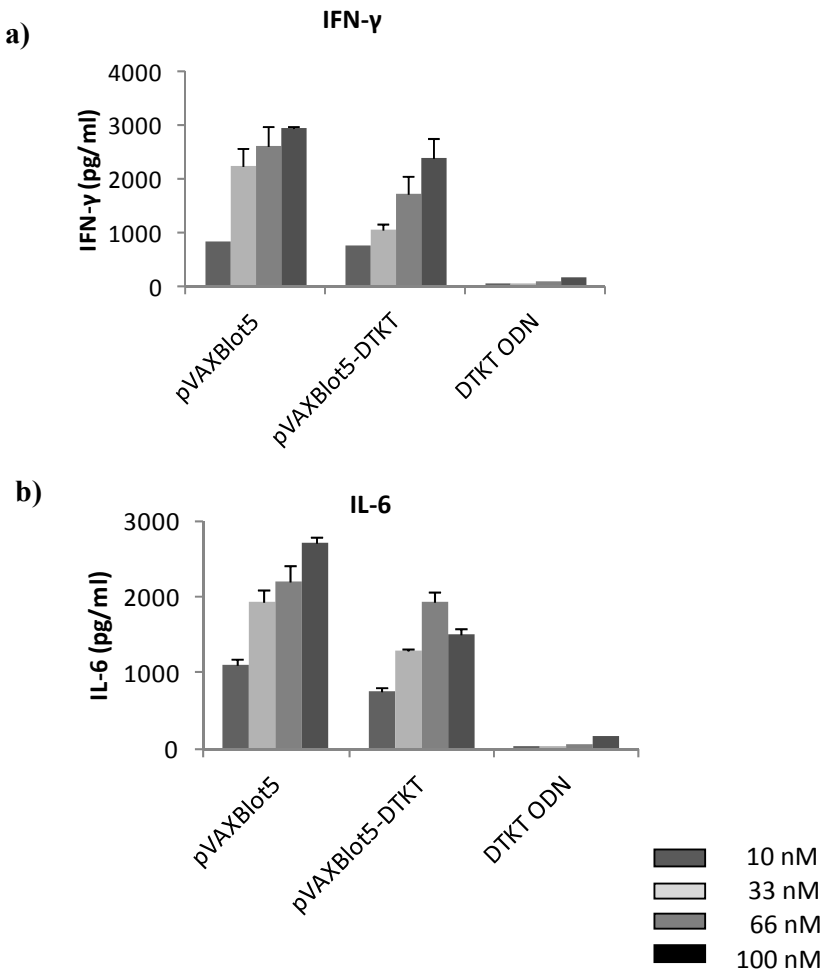


Figure 3.6: Response of mouse splenocytes to plasmid DNA and free CpG oligonucleotides

Mouse splenocytes were incubated with 10 nM, 33 nM, 66 nM and 100 nM of pVAX-Blot5, pVAXBlot5-DTKT and DTKT ODN. IFN-γ and IL-6 in culture supernatant were assessed by Milliplex at 24hours.

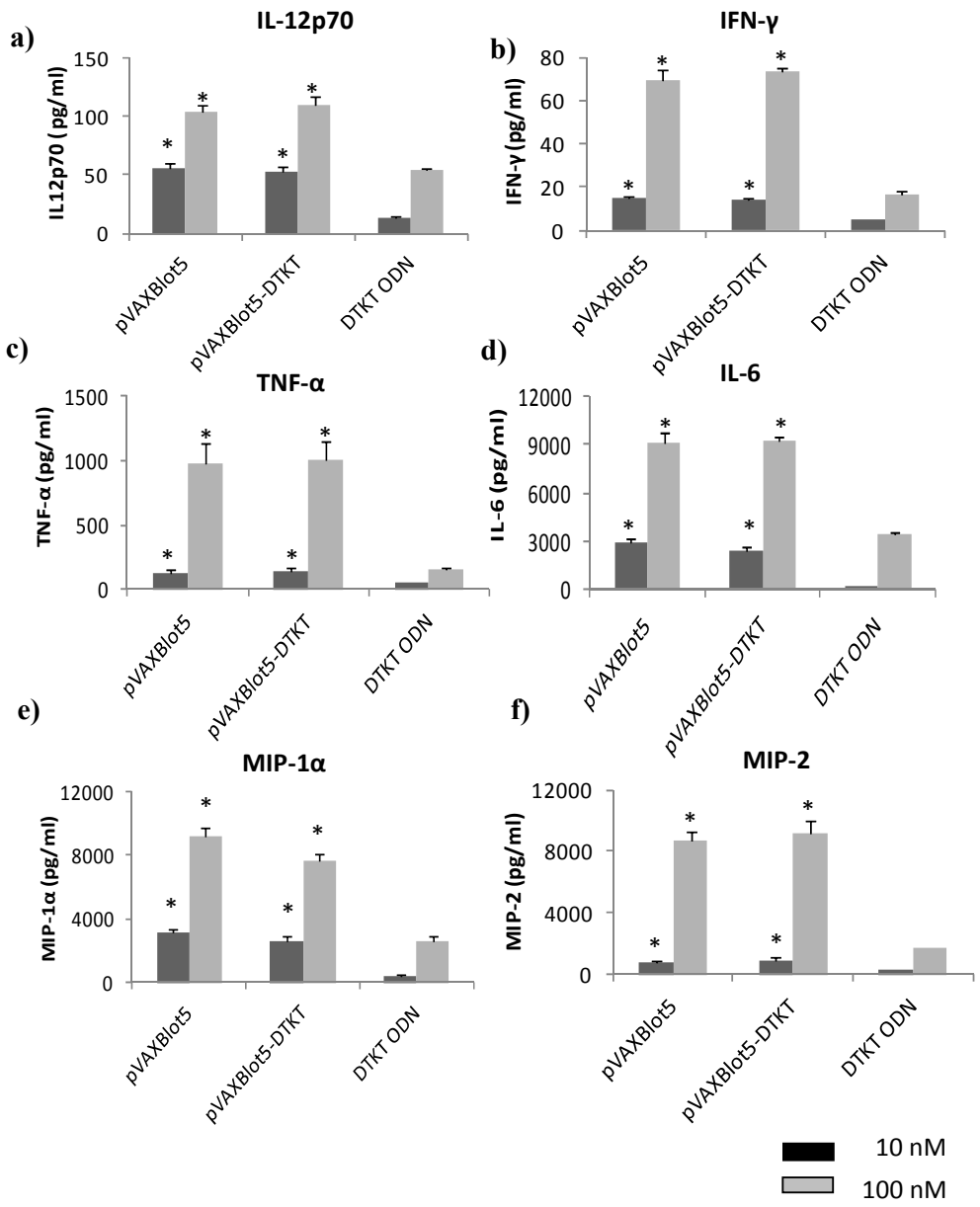


Figure 3.7: Response of dendritic cells (GMCSF-DC) to plasmid DNA and free CpG oligonucleotides stimulation

Bone marrow derived dendritic cells using GM-CSF (GMCSF-DC) were cocultured with 10 nM and 100 nM of plasmid DNA or free CpG ODN for 24 hours. The culture supernatant collected after 24hours was assayed for IL-12p70, IFN-γ, IL-6, TNF-α, MIP-1α and MIP-2. Data illustrated was the mean±SEM of two independent experiments. * stands for statistically significant differences when compared to DTKT ODN.$p < 0.05$.

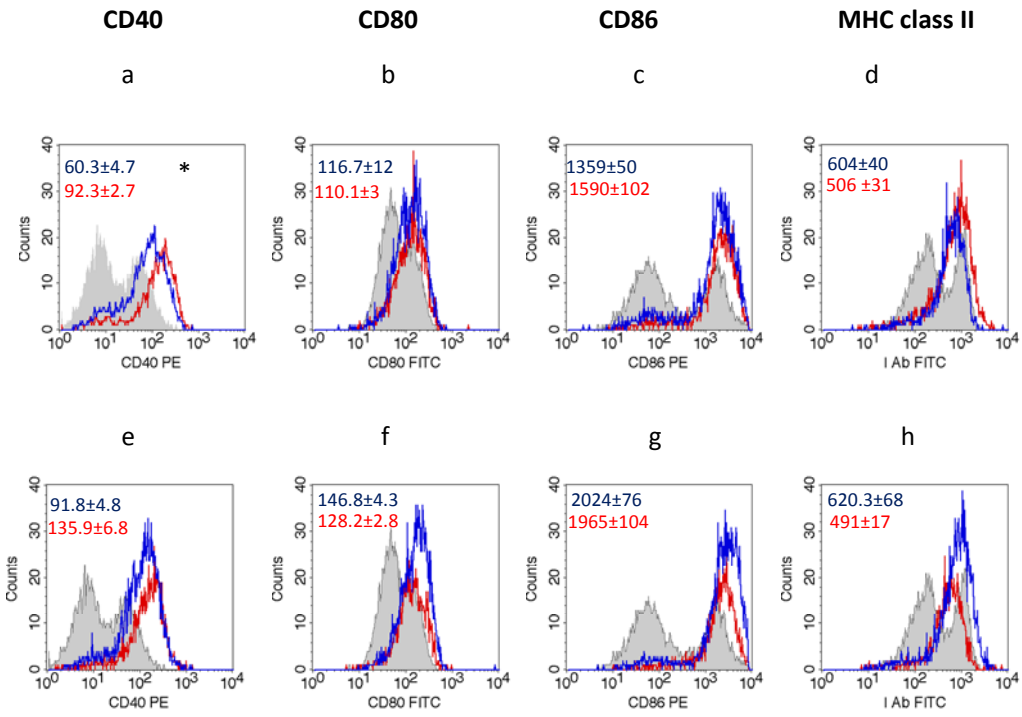


Figure 3.8: Modified and unmodified plasmid DNA elicited similar levels of upregulation of surface markers on bone marrow derived dendritic cells using GMCSF

Bone marrow derived dendritic cells using GMCSF were incubated with 10 nM(a-d) and 100 nM(e-d) of the modified plasmid, pVAXBlot5-DTKT (blue line) and the unmodified plasmid, pVAXBlot5 (red line). Grey areas represent dendritic cells without stimulation. Cells were harvested at 24hours and stained with mAb against CD11c, CD86, CD80, CD40 and MHC class II. Data analyzed was gated on $CD11c^{+}$ cells. The geometric mean of fluorescent intensity of surface markers on bone marrow derived dendritic cells using GMCSF when stimulated with pVAXBlot5-DTKT and pVAXBlot5 was indicated in black and red respectively. Figure shown was the representative of a triplicate in an experiment. Numbers were the mean ± SD of triplicates of a representative experiment. Data shown was representative of two independent experiments. Student t test was used, $p < 0.05$

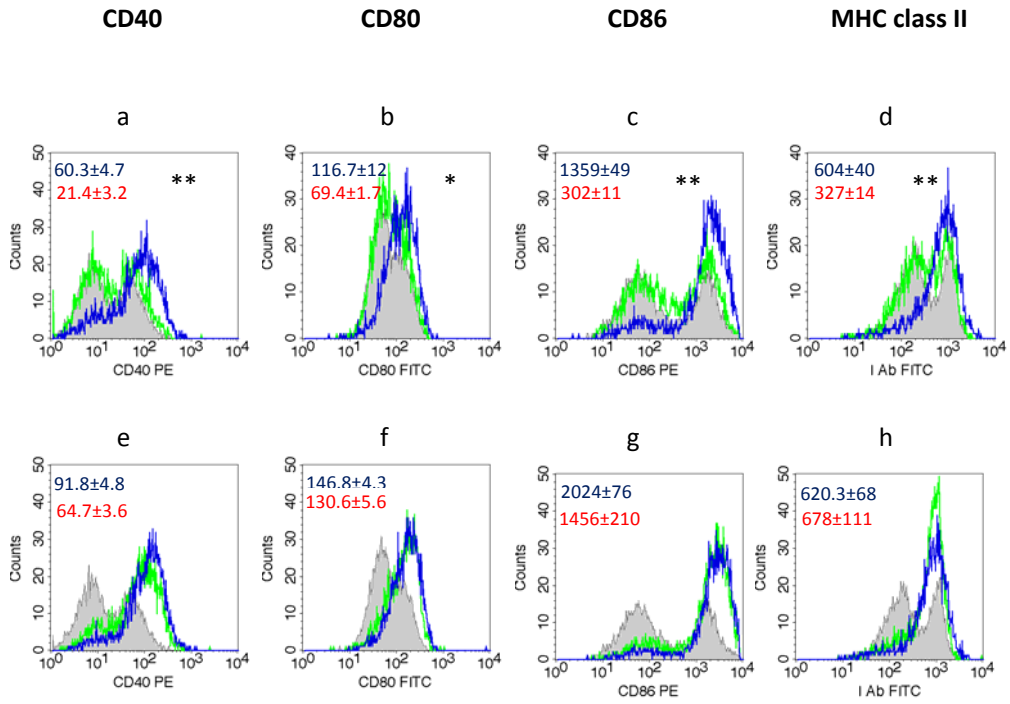


Figure 3.9: Modified plasmid elicit higher upregulation of activation surface markers compared to the corresponding free CpG oligonucleotides

Bone marrow derived dendritic cells using GMCSF were incubated with 10 nM (a-d) and 100 nM (e-h) of the modified plasmid, pVAXBlot5-DTKT (blue line) and the free CpG oligonucleotide, DTKT ODN (green line). Grey areas represent dendritic cells without stimulation. Cells were harvested at 24hr and stained with mAb against CD11c, CD86, CD80, CD40 and MHC class II. Data analyzed was gated on $CD11c^{+}$ cells. The geometric mean of fluorescent intensity of surface markers on bone marrow derived dendritic cells using GMCSF when stimulated with pVAXBlot5-DTKT and DTKT ODN was indicated in black and red respectively. Figure shown was the representative of a triplicate in an experiment. Numbers are the mean ± SD of triplicates of a representative experiment. Data shown was representative of two independent experiments. Student t test was used. * $p < 0.05$, ** $p < 0.005$

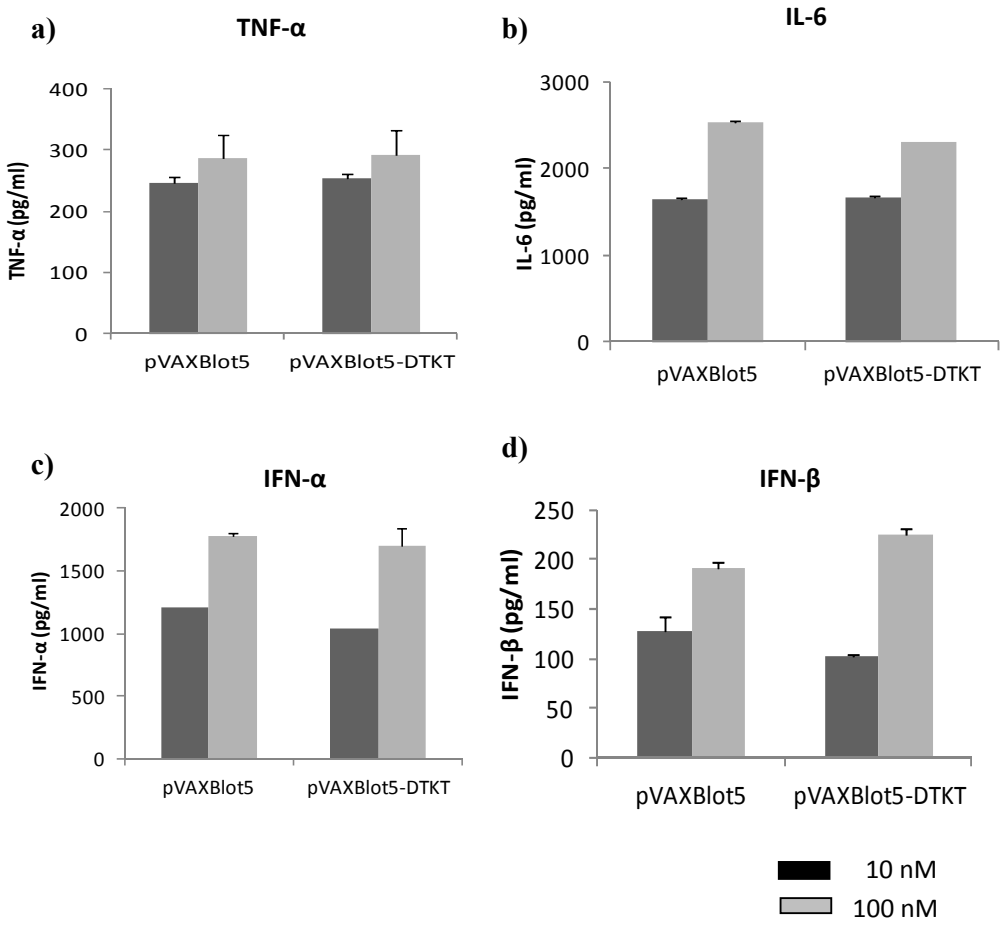


Figure 3.10: Response of dendritic cells (Flt3L-DC) to plasmid DNA stimulation

Bone marrow derived dendritic cells using Flt3L was cocultured with 10 nM and 100 nM of plasmid DNA for 24 hours. The culture supernatant collected after 24hours was assayed for IL-6, TNF-α, IFN-α and IFN-β. Data illustrated was the mean ± SEM of two independent experiments.

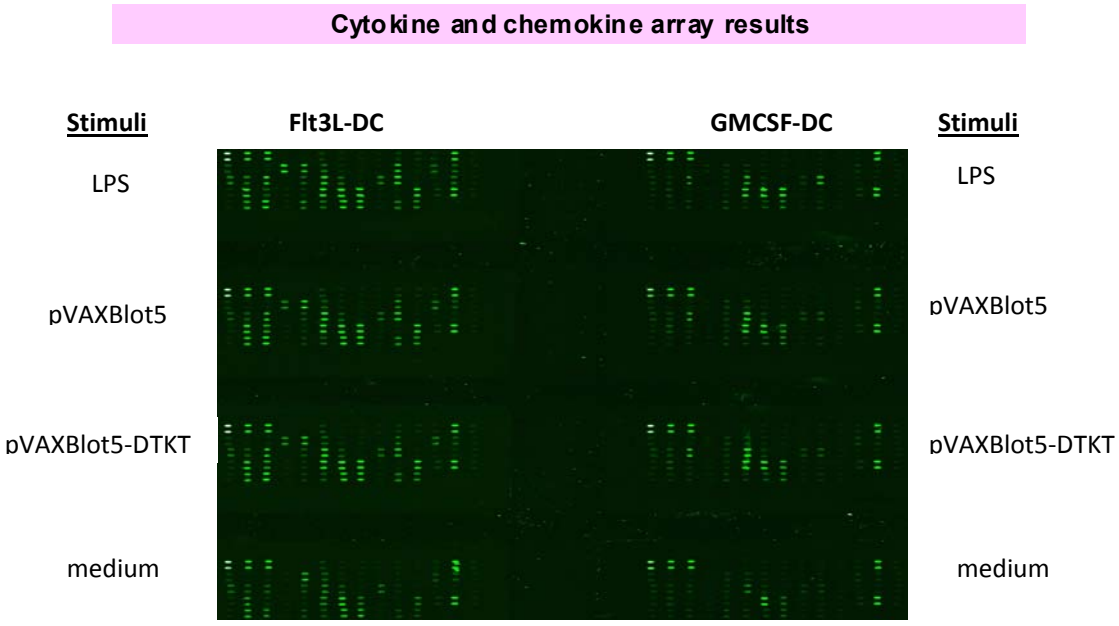


Figure 3.11: Image of protein array

Both GMCSF-DCs and Flt3L-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with 100 nM of plasmid DNA. The supernatant collected after 24 hours was assayed for cytokines and chemokines by the protein array.

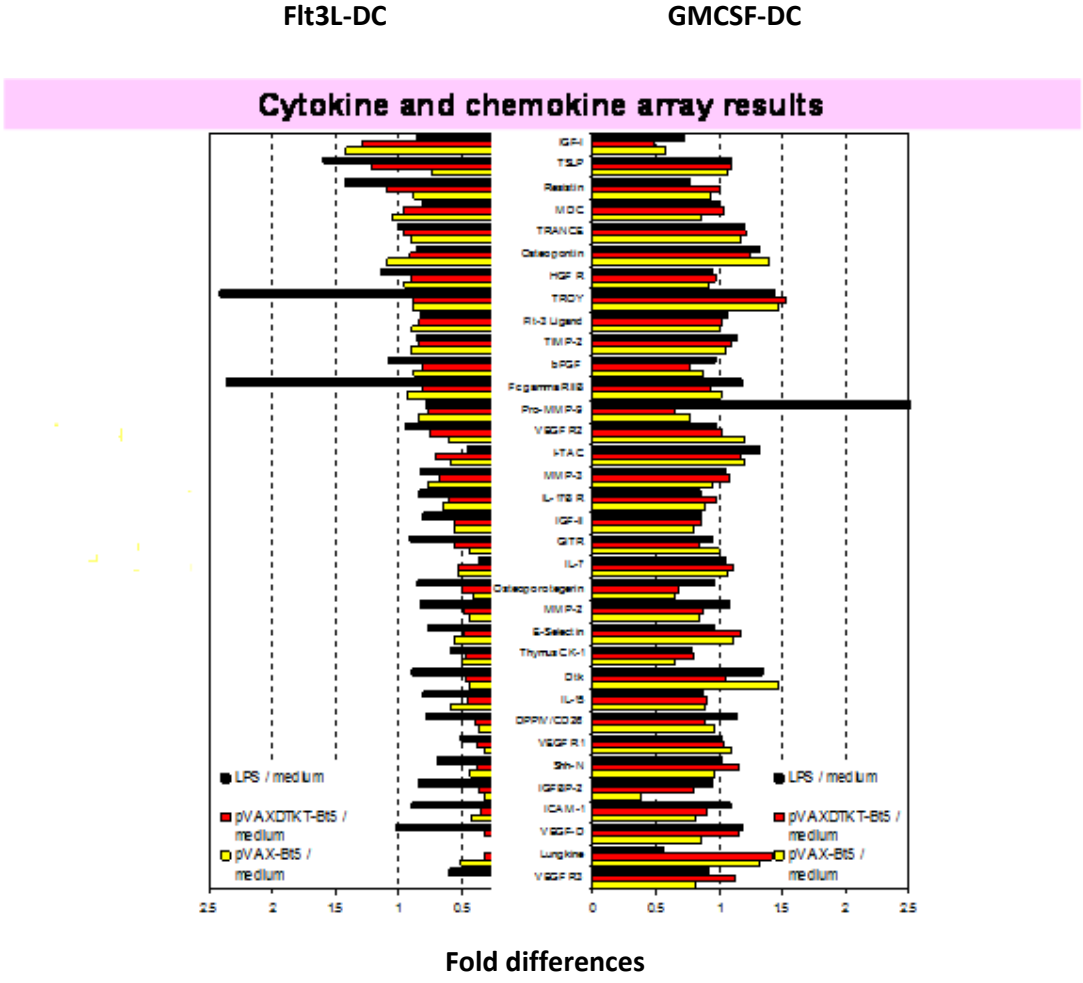


Figure 3.12: Cytokine and chemokine profiling of stimulated Flt3L-DC and GMCSF-DC

Both GMCSF-DCs and Flt3L-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with 100 nM of plasmid DNA. The supernatant collected after 24 hours was assayed for cytokines and chemokines by the protein array.

Flt3L-DC GMCSF-DC

Cytokine and chemokine array results

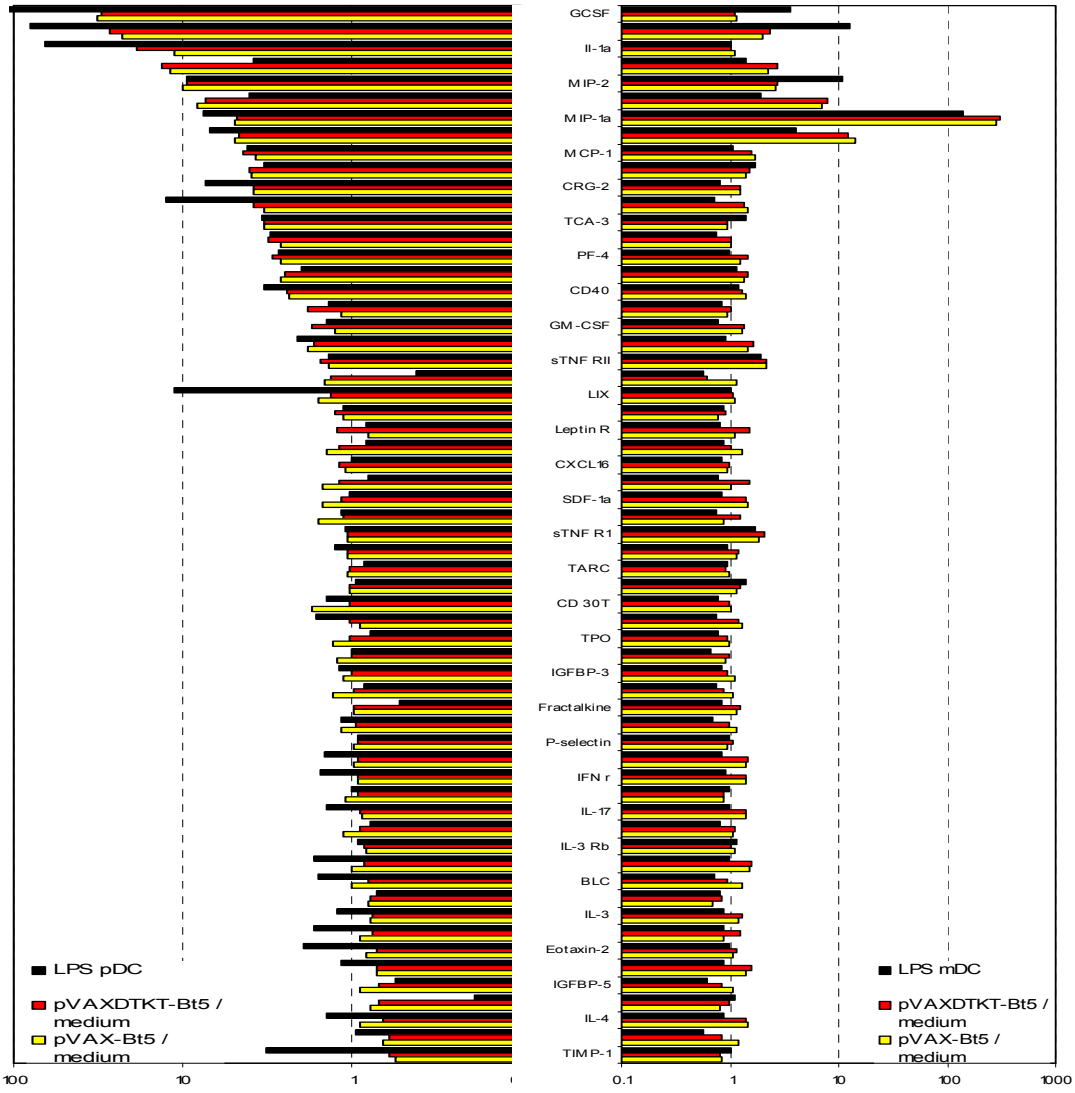


Figure 3.13: Cytokine and chemokine profiling of stimulated Flt3L-DC and GMCSF-DC

Both GMCSF-DCs and Flt3L-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with 100 nM of plasmid DNA. The supernatant collected after 24 hours was assayed for cytokines and chemokines by the protein array.

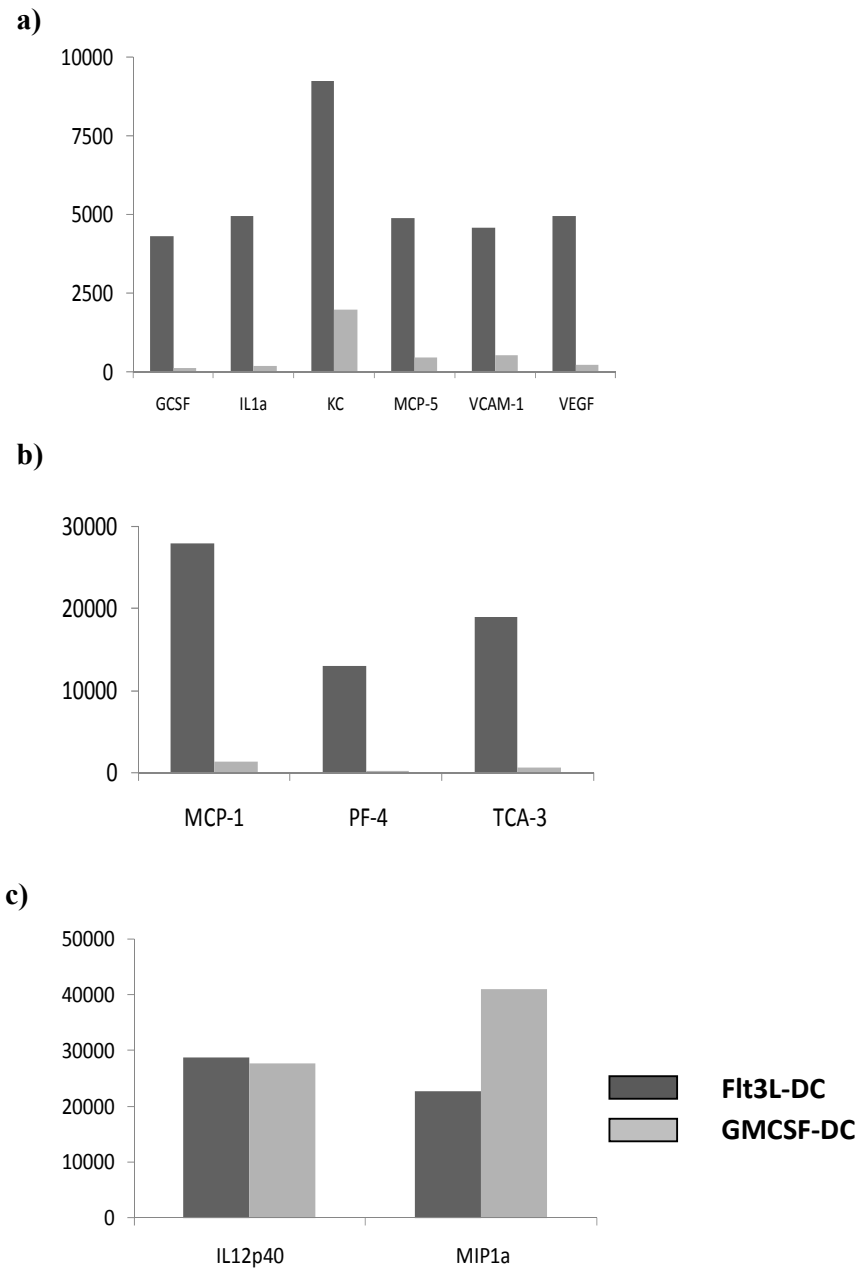
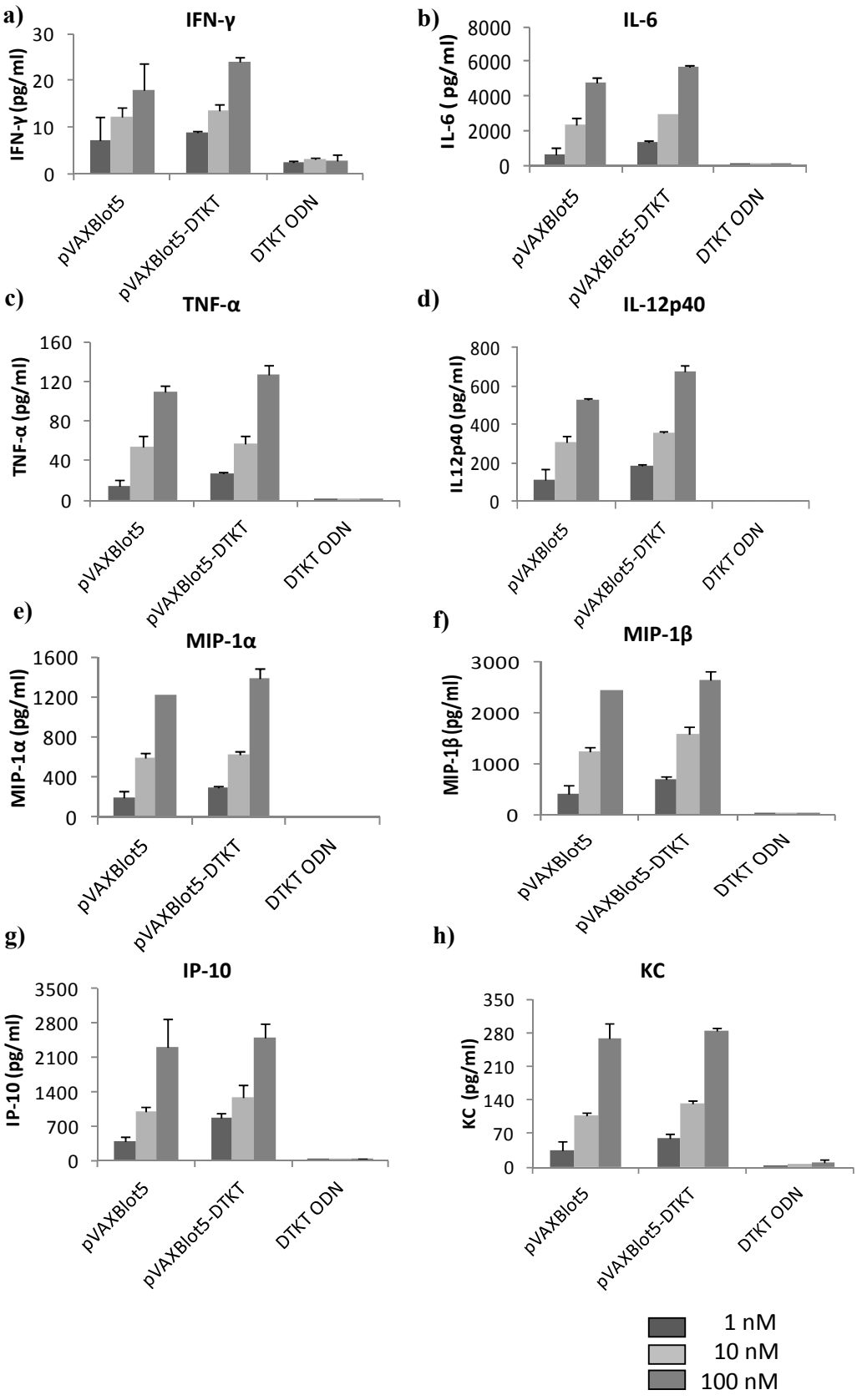


Figure 3.14: Differential protein expressions between Flt3L-DC and GMCSF-DC

Both GMCSF-DCs and Flt3L-DCs were plated at 1 x 10^6 per well in 24 well plate and cocultured with 100 nM of pVAXBlot5-DTKT. The supernatant collected after 24 hours was assayed for cytokines and chemokines by the protein array.


a)
IFN-γ
IFN-γ (pg/ml)
30
20
10
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
b)
IL-6
IL-6 (pg/ml)
8000
6000
4000
2000
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
c)
TNF-α
TNF-α (pg/ml)
160
120
80
40
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
d)
IL-12p40
IL12p40 (pg/ml)
800
600
400
200
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
e)
MIP-1α
MIP-1α (pg/ml)
1600
1200
800
400
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
f)
MIP-1β
MIP-1β (pg/ml)
3000
2000
1000
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
g)
IP-10
IP-10 (pg/ml)
3500
2800
2100
1400
700
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
h)
KC
KC (pg/ml)
350
280
210
140
70
0
pVAXBlot5
pVAXBlot5-DTKT
DTKT ODN
1 nM
10 nM
100 nM

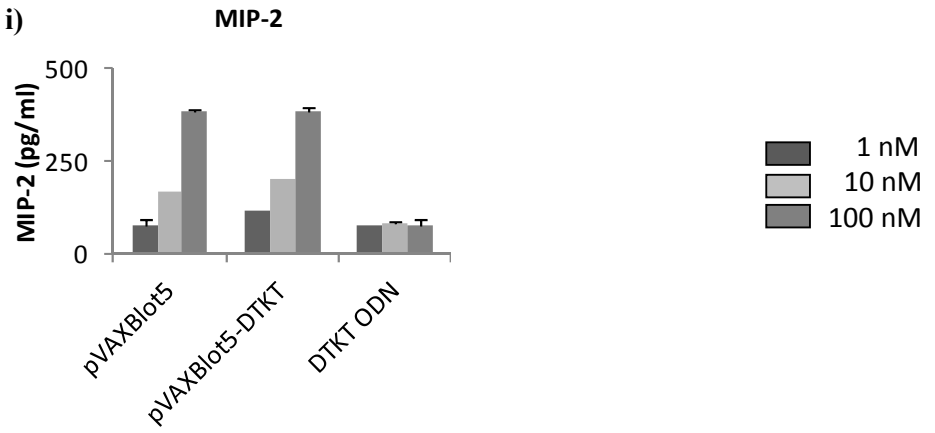


Figure 3.15: Response of CD11c^{+} CD11b^{+}B220^{-} DCs to plasmid DNA and free CpG oligonucleotides stimulation

CD11c^{+} CD11b^{+}B220^{-} DCs were cocultured with 1 nM, 10 nM and 100 nM of plasmid DNA or free CpG ODN for 24 hours. The culture supernatant collected after 24hours was assayed for IFN-γ, IL-6, TNF-α, IL-12p40, MIP-1α, MIP-1β, IP-10, KC and MIP-2. Results shown were the mean ± SEM of duplicates of an experiment. Data shown were the representative of two independent experiments.

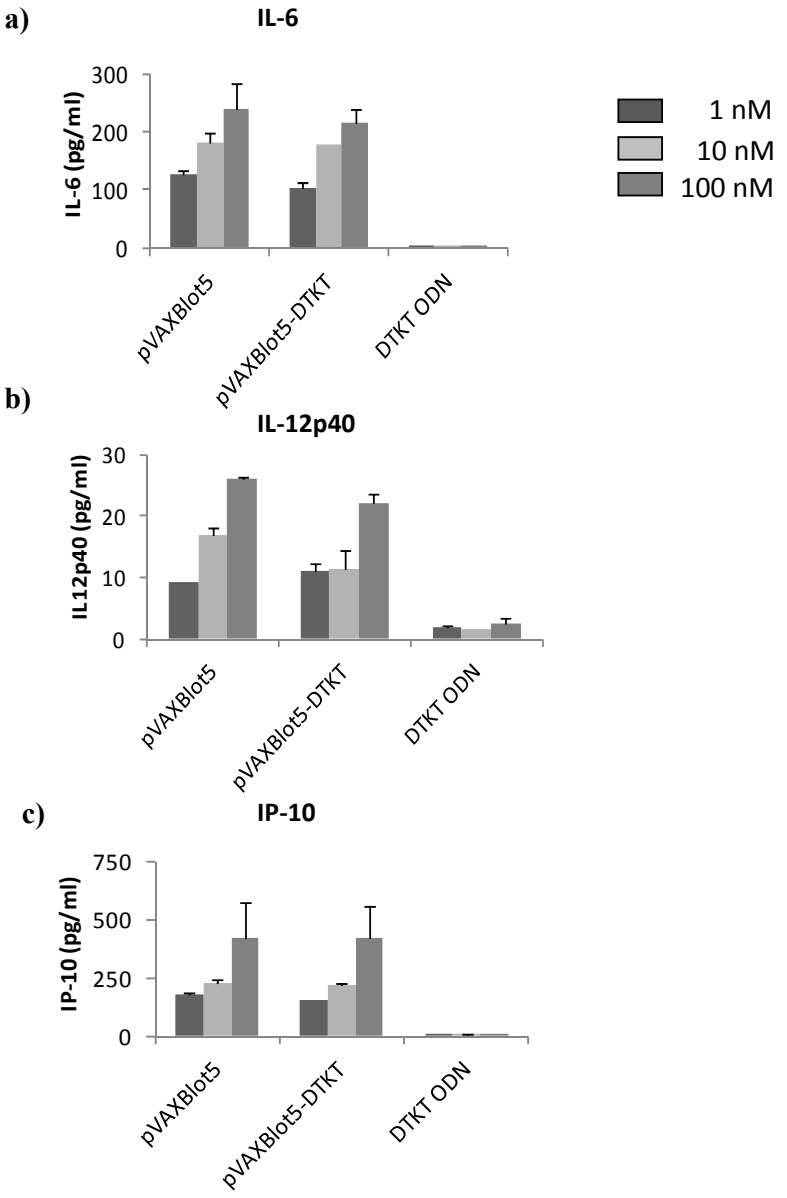


Figure 3.16: Response of $CD11c^{+}$ $CD11b^{-}B220^{+}$ dendritic cells to plasmid DNA and free CpG oligonucleotides stimulation

Bone marrow derived dendritic cells using Flt3 L ($CD11c^{+}B220^{+}$ pDCs) were cocultured with 100nM of plasmid DNA or free CpG ODN for 24 hours. The culture supernatant collected after 24hours was assayed for IL-6, IL-12p40 and IP-10. Results shown were the mean ± SEM of duplicates of an experiment. Data shown were the representative of two independent experiments.

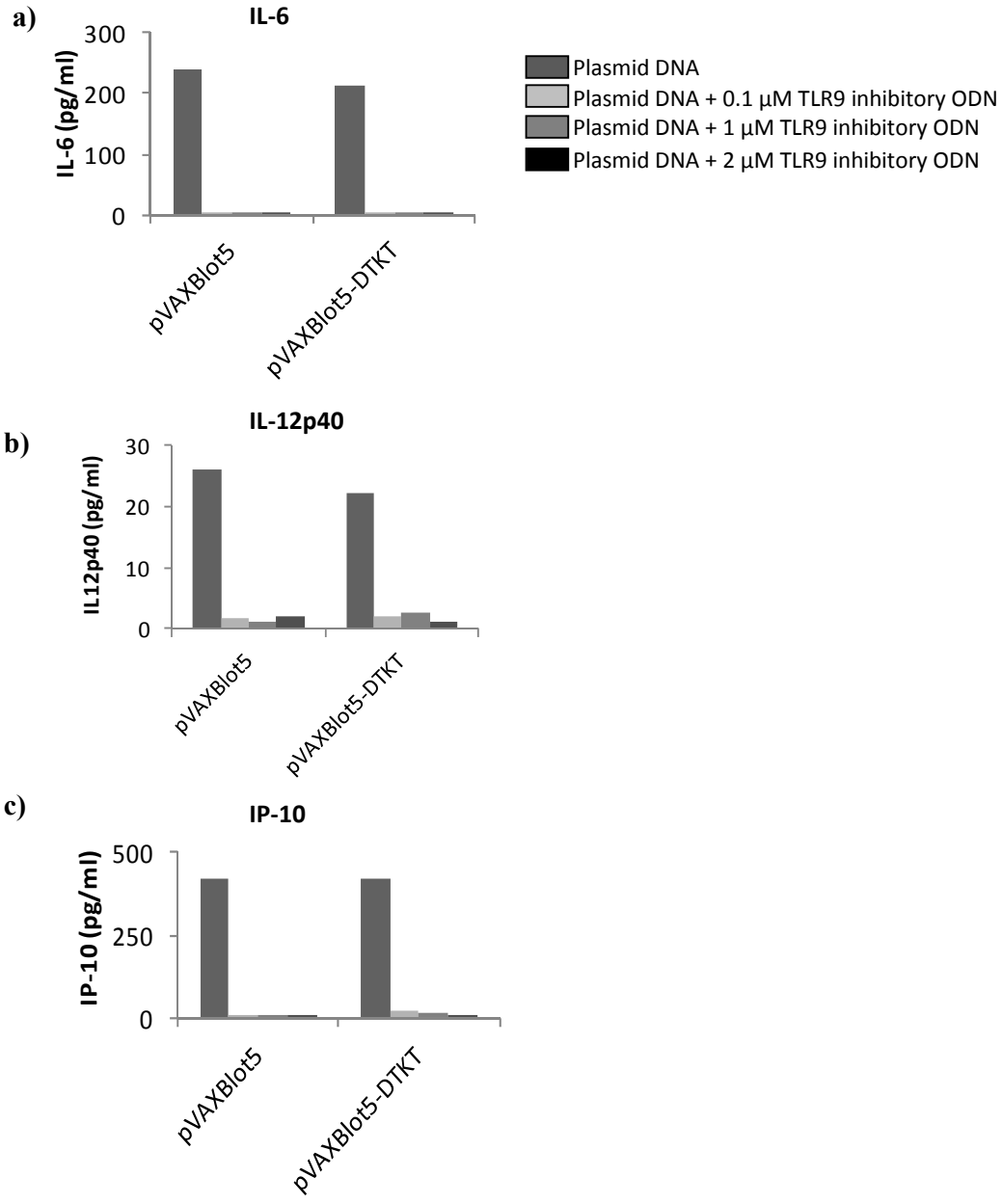


Figure 3.17: Dose dependent TLR9 inhibition of $CD11c^{+}$ $CD11b^{-}B220^{+}$ dendritic cells

$CD11c^{+}CD11b^{-}B220^{+}$ DCs were seeded at 1 x 10^{5} cells in 96 well plate and cocultured with 100 nM of plasmid DNA for 24 hours in the presence of titrated doses of TLR9 inhibitory ODN. The culture supernatant was collected after 24 hours and assayed for IL-6, IL-12p40 and IP-10.

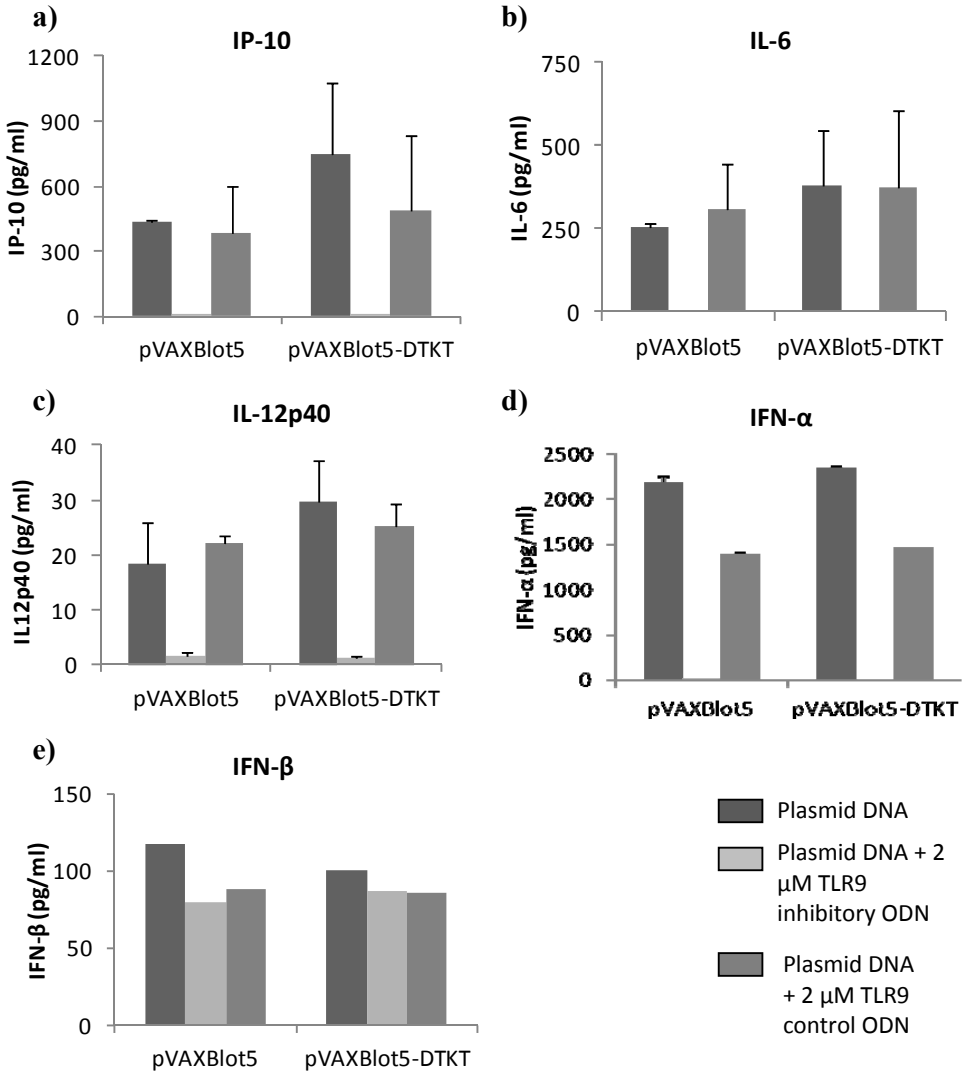


Figure 3.18: TLR9 blocking of $CD11c^{+}$ $CD11b^{-}B220^{+}$ dendritic cells

$CD11c^{+}CD11b^{-}B220^{+}$ DCs were seeded at 1 x 10^{5} cells in 96 well plate and cocultured with 100 nM of plasmid DNA for 24 hours in the presence of either 2 µM of TLR9 inhibitory ODN or control ODN. The culture supernatant was collected after 24 hours and assayed for IP-10, IL-6, IL-12p40, IFN-α and IFN-β. Results were the mean ± SEM from 2 separate experiments.

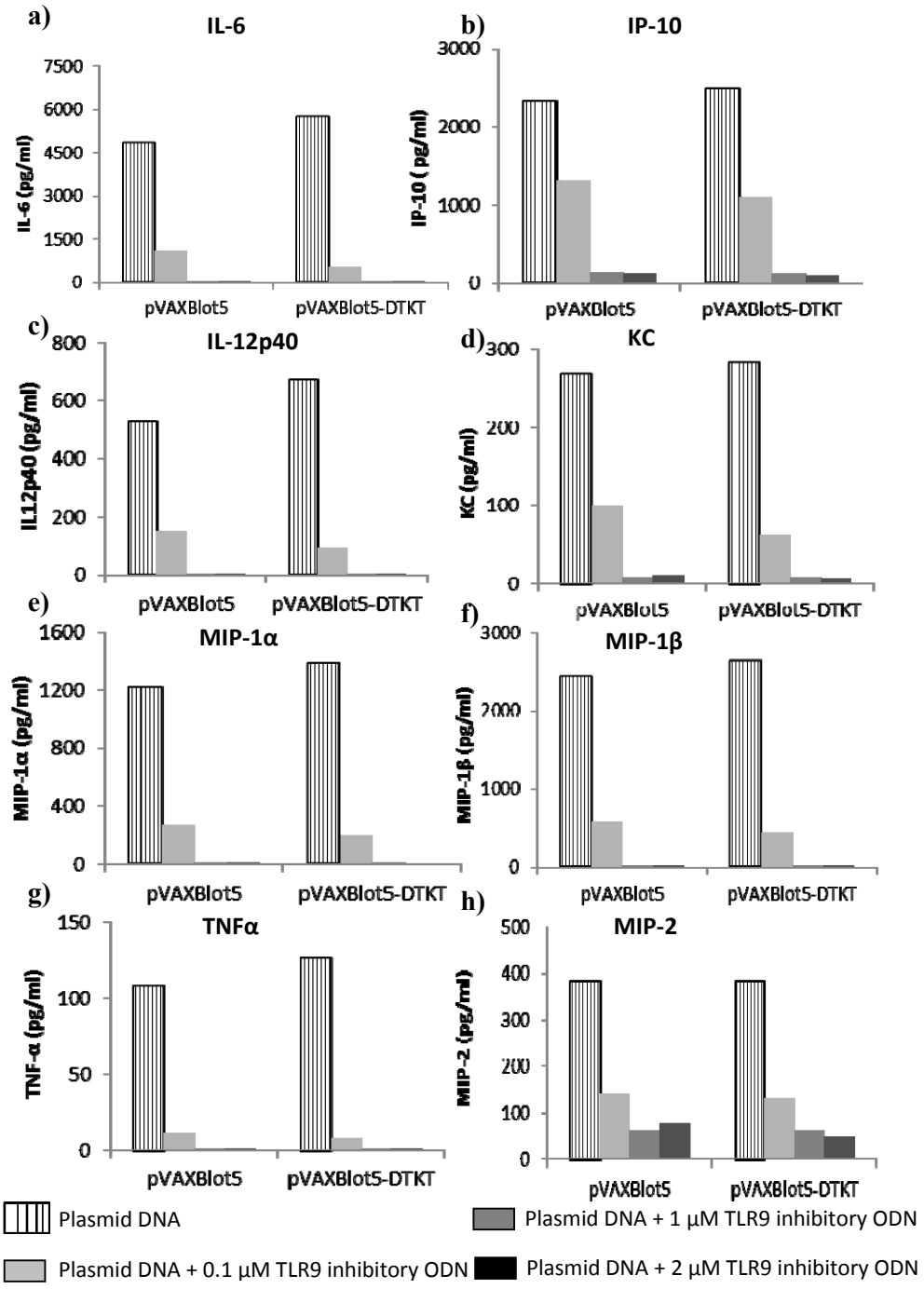


Figure 3.19: Dose dependent TLR9 inhibition of $CD11c^{+}$ $CD11b^{+}B220^{-}$ dendritic cells

$CD11c^{+}CD11b^{+}B220^{-}$ DCs were seeded at 1 x 10^{5} cells in 96 well plate and cocultured with 100 nM of plasmid DNA for 24 hours in the presence of titrated doses of TLR9 inhibitory ODN.The culture supernatant was collected after 24 hours and assayed for IL-6, IP-10, IL-12p40, KC, MIP-1α, MIP-1β, TNF-α and MIP-2.

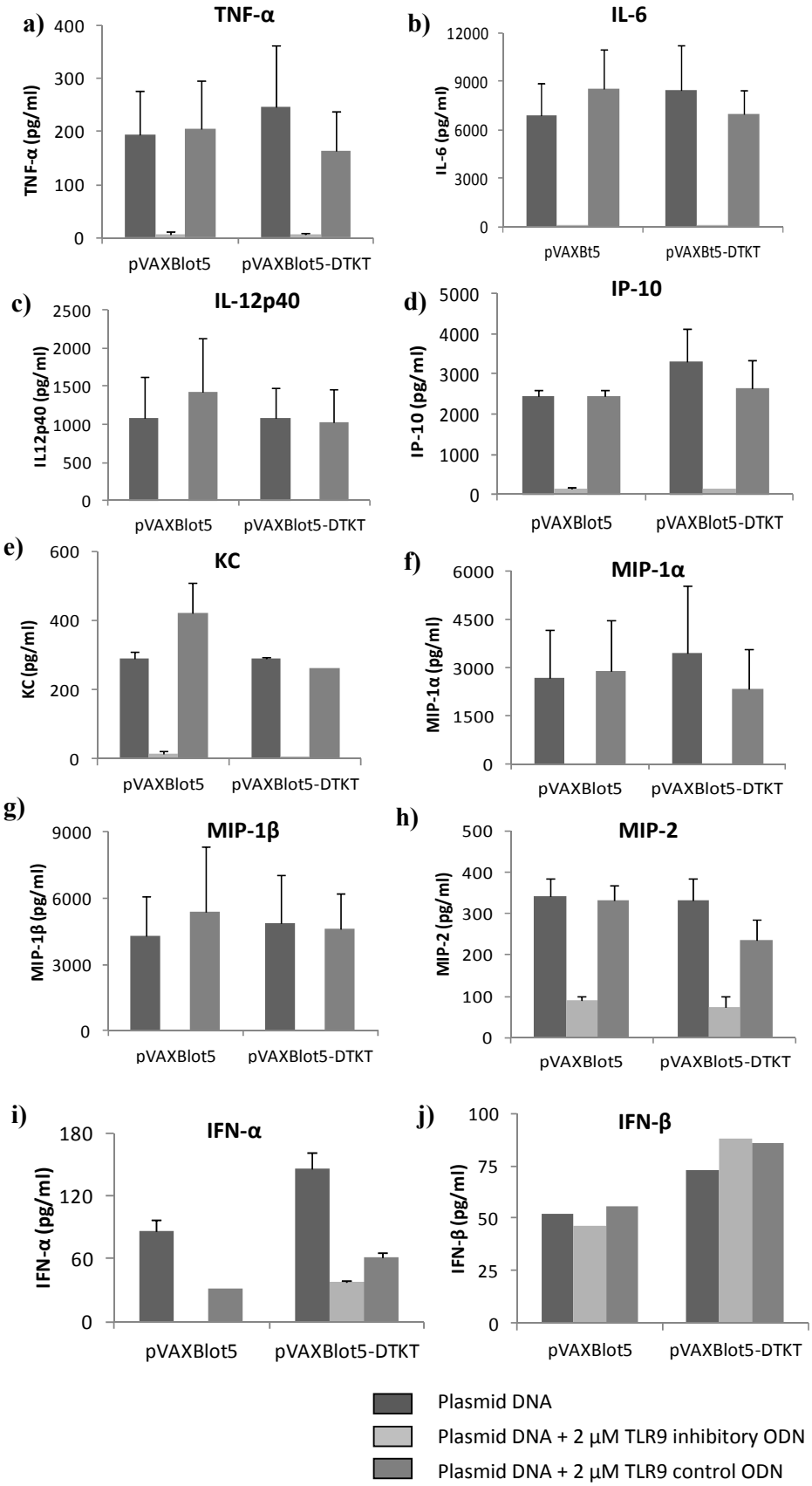

a)
TNF-α
TNF-α (pg/ml)
400
300
200
100
0
pVAXBlot5
pVAXBlot5-DTKT
b)
IL-6
IL-6 (pg/ml)
12000
9000
6000
3000
0
pVAXBt5
pVAXBt5-DTKT
c)
IL-12p40
IL12p40 (pg/ml)
2500
2000
1500
1000
500
0
pVAXBlot5
pVAXBlot5-DTKT
d)
IP-10
IP-10 (pg/ml)
5000
4000
3000
2000
1000
0
pVAXBlot5
pVAXBlot5-DTKT
e)
KC
KC (pg/ml)
600
400
200
0
pVAXBlot5
pVAXBlot5-DTKT
f)
MIP-1α
MIP-1α (pg/ml)
6000
4500
3000
1500
0
pVAXBlot5
pVAXBlot5-DTKT
g)
MIP-1β
MIP-1β (pg/ml)
9000
6000
3000
0
pVAXBlot5
pVAXBlot5-DTKT
h)
MIP-2
MIP-2 (pg/ml)
500
400
300
200
100
0
pVAXBlot5
pVAXBlot5-DTKT
i)
IFN-α
IFN-α (pg/ml)
180
120
60
0
pVAXBlot5
pVAXBlot5-DTKT
j)
IFN-β
IFN-β (pg/ml)
100
75
50
25
0
pVAXBlot5
pVAXBlot5-DTKT
Plasmid DNA
Plasmid DNA + 2 μM TLR9 inhibitory ODN
Plasmid DNA + 2 μM TLR9 control ODN

Figure 3.20: TLR9 inhibition of $CD11c^+$ $CD11b^+B220^-$ dendritic cells

$CD11c^+CD11b^+B220^-$ DC were seeded at 1 x 10^5 cells in 96 well plate and cocultured with 100 nM of plasmid DNA for 24 hours in the presence of either 2 μM of TLR9 inhibitory ODN or control ODN. The culture supernatant was collected after 24 hours and assayed for TNF-α, IL-6, IL-12p40, IP-10, KC, MIP-1α, MIP-1β, MIP-2, IFN-α and IFN-β. Results were the mean ± SEM from 2 separate experiments.

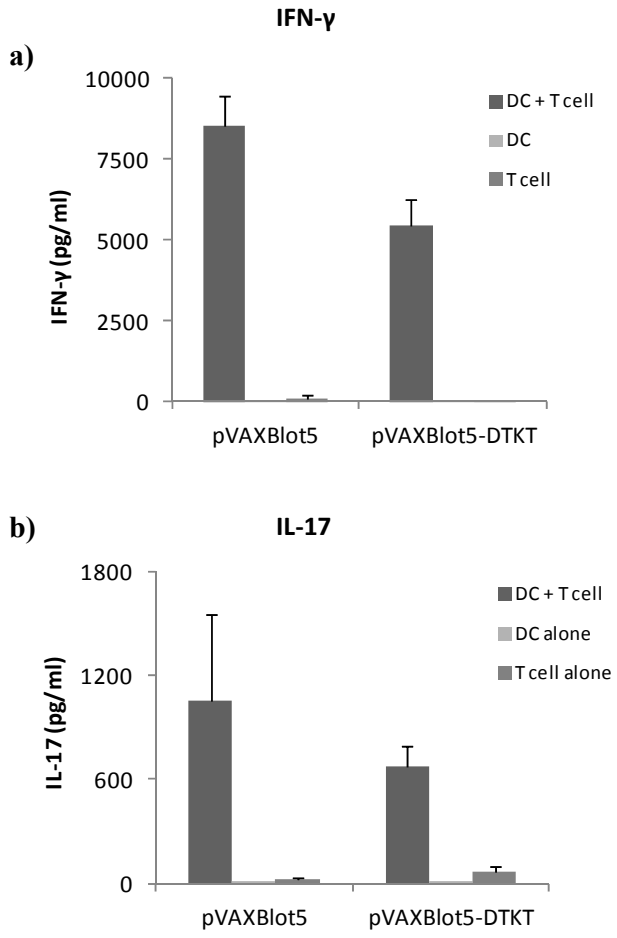


Figure 3.21: Coculture of plasmid DNA pulsed dendritic cells with Blo t 5 specific T cells

Flt3L-DCs were collected on day 8 and stained with antibodies against CD11c, CD11b and B220. The $CD11c^{+}CD11b^{+}B220^{-}$ DCs were collected after cell sorting and pulsed with 100 nM of plasmid DNA for 24hours. After 24hours, the dendritic cells were washed twice with HBSS and pulsed with $CD4^{+}$ Tcells from spleen of Blo t 5 TCR transgenic mouse at a 1:2 ratio. 72hours later, fresh medium containing IL-2 was added. 7 days after the coculture of DC and T cell, the T cells were washed and subjected to ficoll separation to remove the dead cells. After which the T cells was restimulated again with plasmid DNA pulsed dendritic cells. The culture supernatant was collected after 72hours and assayed for IFN-γ and IL-17. Results were the mean ± SEM of 2 independent experiments.

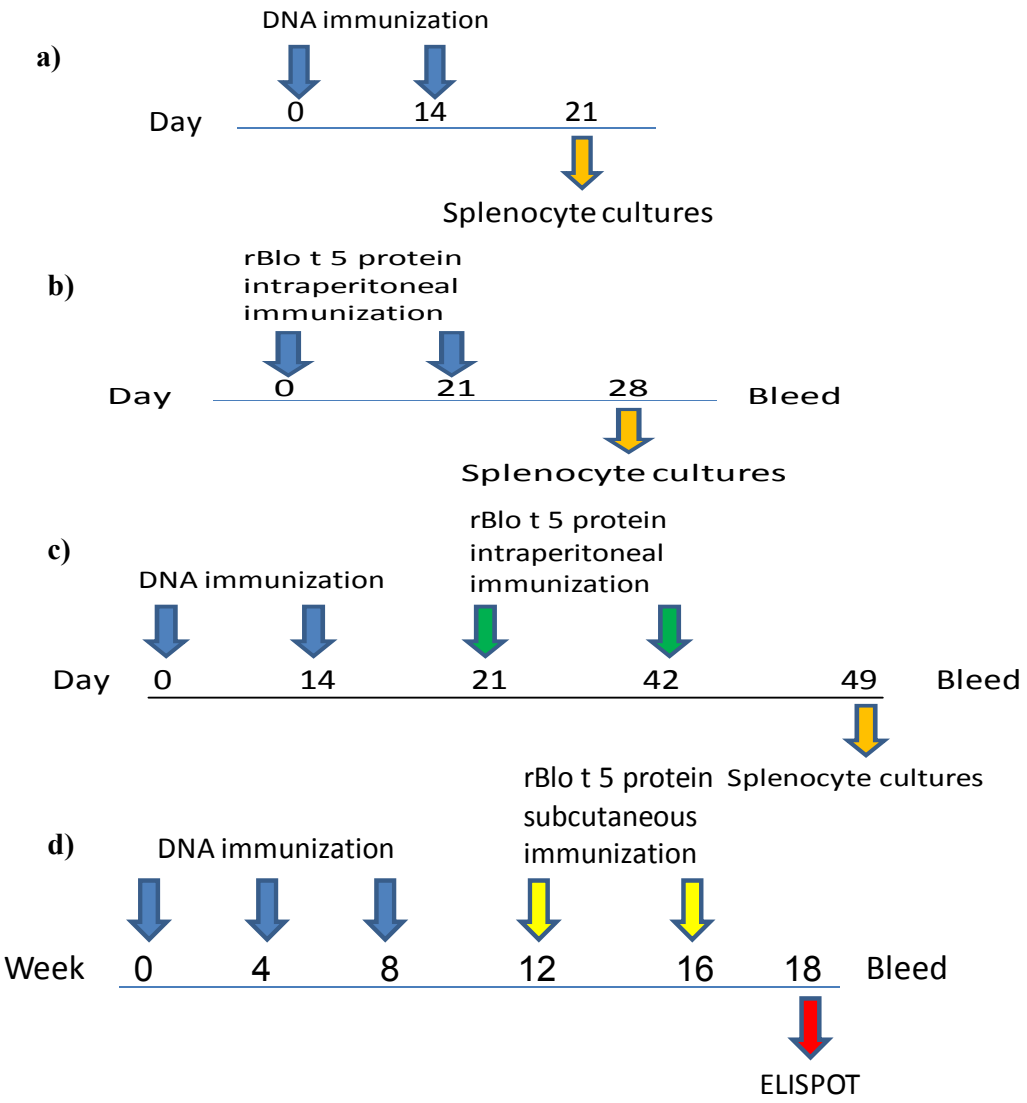


Figure 3.22: Schematic diagrams on the experimental regimens

C57BL/6 mice were immunized via intramuscular route with pVAX, pVAXBlot5, pVAXBlot5-DTKT on days 0 and 14.The mice were sacrified on day 21 for splenocyte culture (a). C57/BL6 mice were immunized via intraperitoneal route with rBlo t 5 protein adjuvanted with aluminium hydroxide on days 0 and 21. Control mice were immunized with PBS adjuvanted with aluminium hydroxide, the mice were bled weekly and sacrified on day 28 for splenocyte culture (b). C57/BL6 mice were immunized via intramuscular route with pVAX, pVAXBlot5, pVAXBlot5-DTKT on days 0 and 14.The mice were then intraperitoneally immunized with rBlo t 5 protein adjuvanted with aluminium hydroxide on days 21 and 42 and they were sacrified on day 49 for splenocyte culture. Mice were bled weekly. (c) Rhesus macaques were immunized via the intramuscular route with pVAX, pVAXhBlot5, pVAXhBlot5-DTKT on weeks 0, 4, 8 and subcutaneously immunized with rBlo t 5 protein adjuvanted with aluminium hydroxide on weeks 12 and 16. ELISPOT analysis was carried out on week 18. Rhesus macaques were bled monthly (d). All sera collected were analysed for Blo t 5-specific immunoglobulin analysis by ELISA.

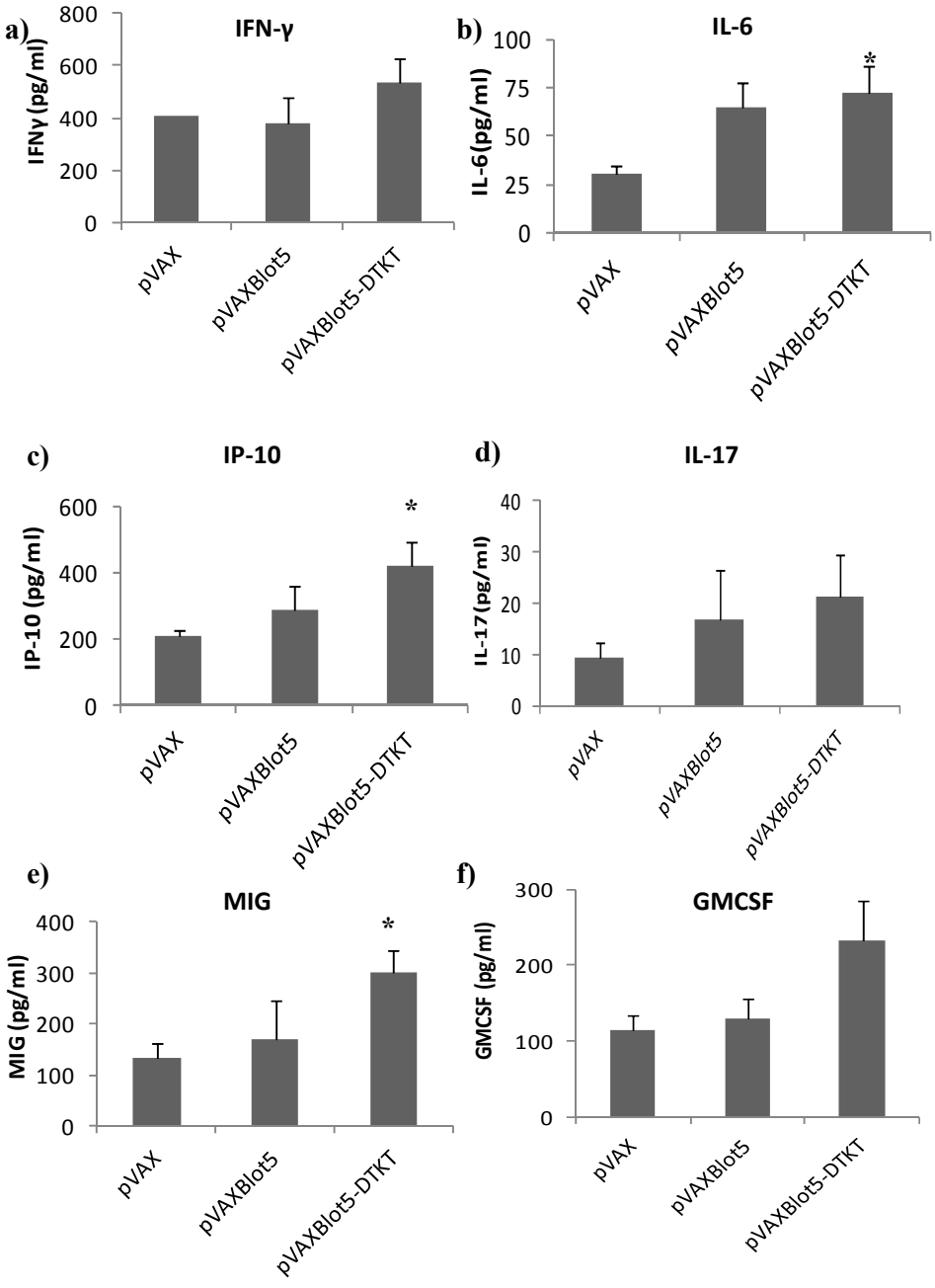


Figure 3.23: Cytokine profile of splenocytes in mice immunized with plasmid DNA

Mice were immunized with 50 μg of plasmid DNA with electrophoration on day 0 and 14. The mice were sacrificed one week after the second intramuscular immunization. Splenocytes of mice were stimulated with 10 μg/ml rBlo t 5 protein and culture supernatant collected after 72 hours. Culture supernatant was assayed for IFN-γ, IL-6, IP-10, IL-17, MIG and GMCSF by milliplex. Data were presented as mean ± SEM (n = 4-5) * statistical significance when compared with pVAX vector group, $p < 0.05$

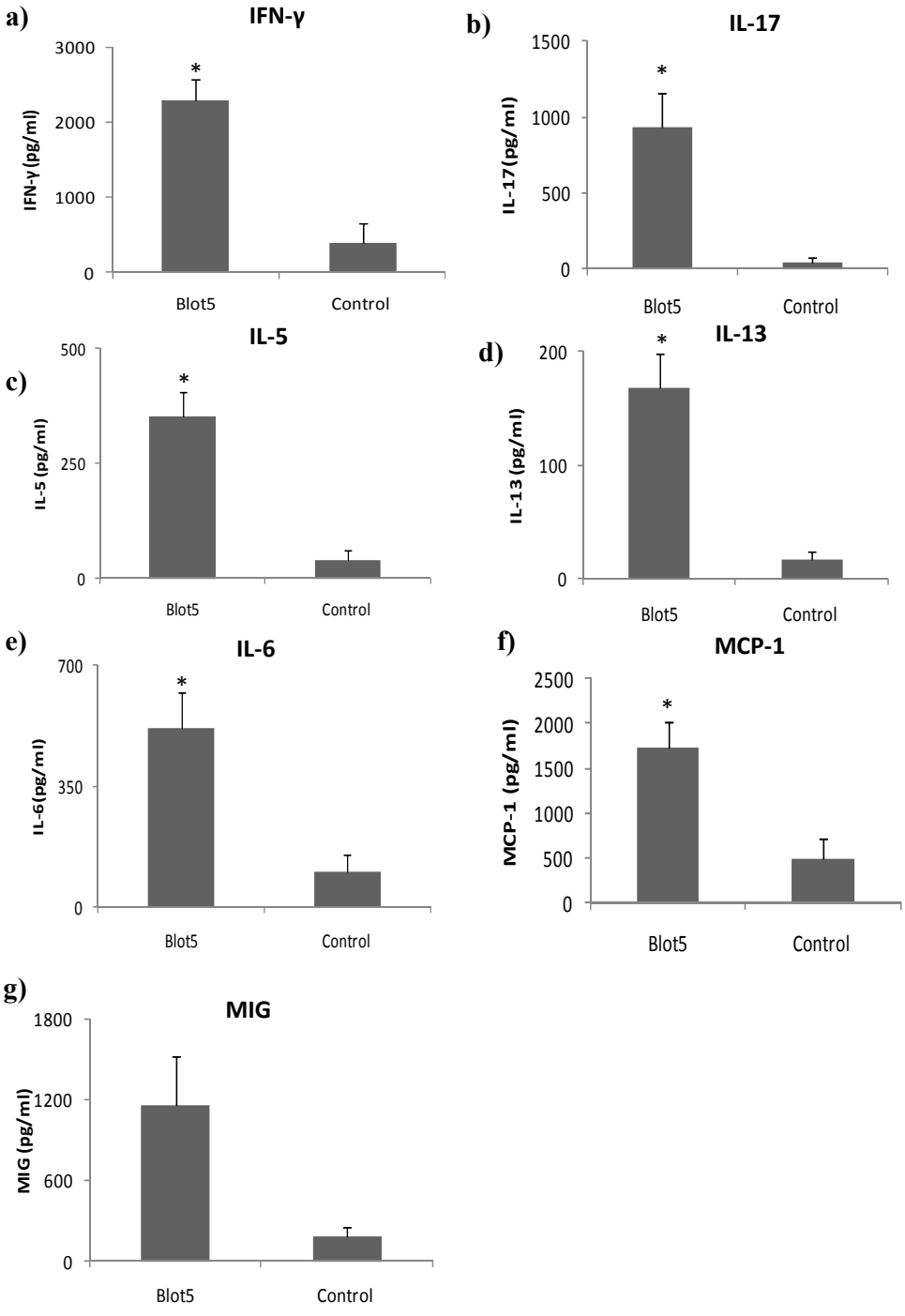

a)
IFN-γ
IFN-γ (pg/ml)
3000
2000
1000
0
*
Blot5
Control
b)
IL-17
IL-17(pg/ml)
1500
1000
500
0
*
Blot5
Control
c)
IL-5
IL-5 (pg/ml)
500
250
0
*
Blot5
Control
d)
IL-13
IL-13 (pg/ml)
200
100
0
*
Blot5
Control
e)
IL-6
IL-6(pg/ml)
700
350
0
*
Blot5
Control
f)
MCP-1
MCP-1 (pg/ml)
2500
2000
1500
1000
500
0
*
Blot5
Control
g)
MIG
MIG (pg/ml)
1800
1200
600
0
Blot5
Control

Figure 3.24: Cytokine profile of cultured T cells of mice intraperitoneally injected with Blo t 5 protein (allergy mode)

Mice were immunized intraperitoneally at 3 weeks apart with rBlo t 5 protein adjuvanted with aluminium hydroxide. Control mice were intraperitoneally immunized with PBS adjuvanted with alum only. Mice were sacrified one week after the second intraperitoneal immunization. Splenocytes of treated and control mice were stimulated with rBlo t 5 protein for 7 days *in vitro*. Viable cells were restimulated with Blo t 5 in the presence of antigen-presenting cells for 72 hours. Culture supernatant was collected and analyzed for IFN-γ, IL-17, IL-5, IL-13, IL-6, MCP-1 and MIG by milliplex. Data were presented as mean ± SEM (n=4-7) * compared with control mice, $p < 0.05$

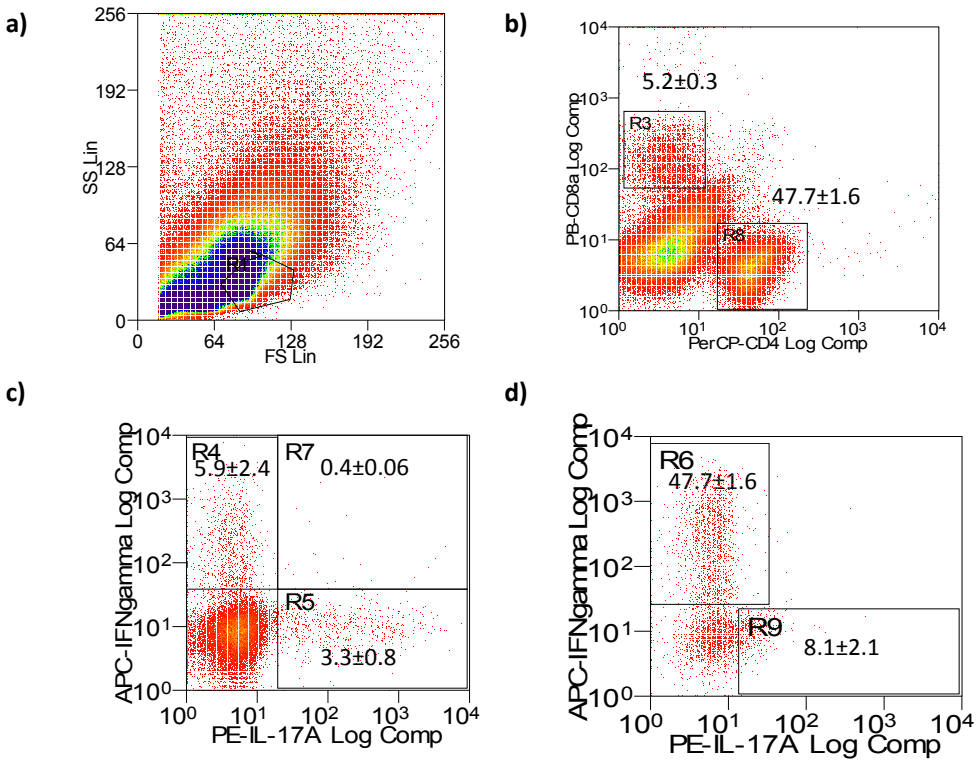


Figure 3.25: Intracellular cytokine staining of mice intraperitoneally immunized with rBlo t 5 recombinant protein adjuvanted with alum.

Mice were intraperitoneally immunized with rBlo t 5 protein adjuvanted with alum twice at three weeks apart. One week after the second intraperitoneal injection, the mice were sacrificed. Splenocytes of treated and control mice were stimulated with Blo t 5 protein for 7 days *in vitro*. Viable cells were restimulated with anti-CD3 and CD28 antibodies and cells were stained with anti-CD4, anti-CD8, anti IFN-gamma, anti IL-17A antibodies and analysed via flow cytometry. Data analysed was based on R1 gated cells (b), R1 and R8 gated cells (c), R1 and R3 gated cells (d), mean ± SEM (n=4)

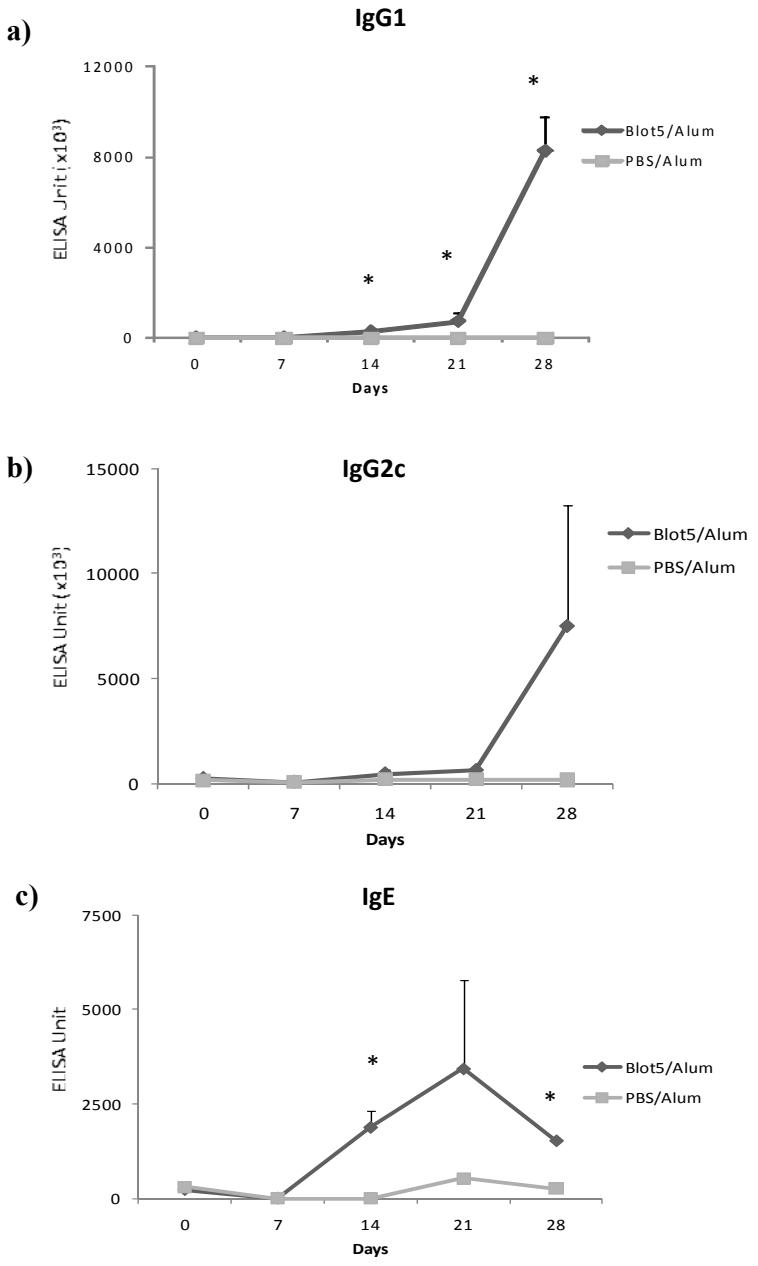
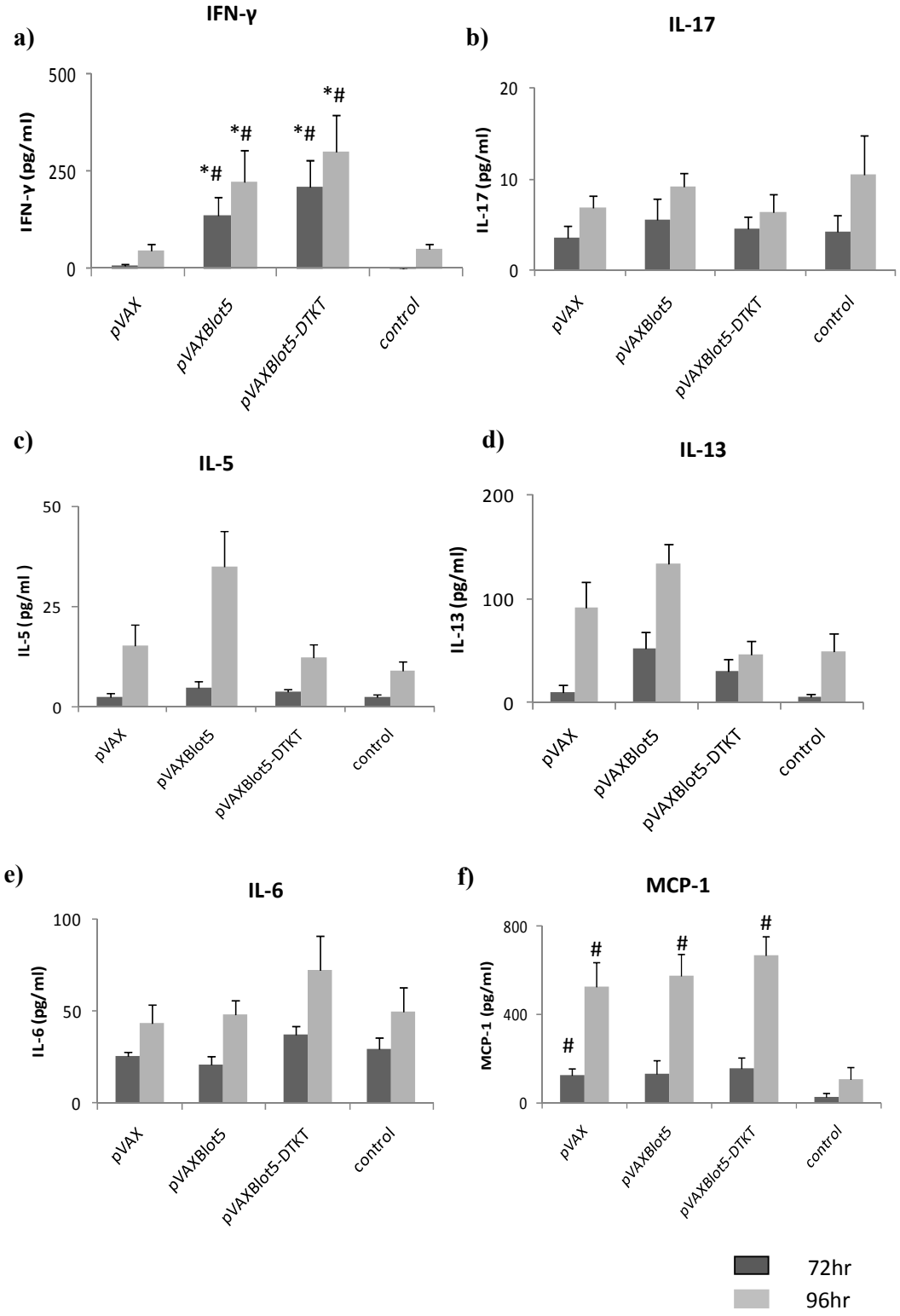


Figure 3.26: Allergen specific antibody responses of mice intraperitoneally injected with rBlo t 5 protein (allergy model)

Mice were immunized intraperitoneally at 3 weeks apart with rBlo t 5 protein adjuvanted with aluminium hydroxide. Control mice were intraperitoneally immunized with PBS adjuvanted with alum only. Sera were collected weekly. Titers of Blo t 5 -specific IgG1, IgG2c and IgE were determined by ELISA. Data were presented as mean ± SEM (n = 4-7). * compared with control mice, $p < 0.05$.


a)
IFN-γ
IFN-γ (pg/ml)
500
250
0
*#
*#
*#
*#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
b)
IL-17
IL-17 (pg/ml)
20
10
0
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
c)
IL-5
IL-5 (pg/ml)
50
25
0
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
d)
IL-13
IL-13 (pg/ml)
200
100
0
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
e)
IL-6
IL-6 (pg/ml)
100
50
0
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
f)
MCP-1
MCP-1 (pg/ml)
800
400
0
#
#
#
#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
72hr
96hr

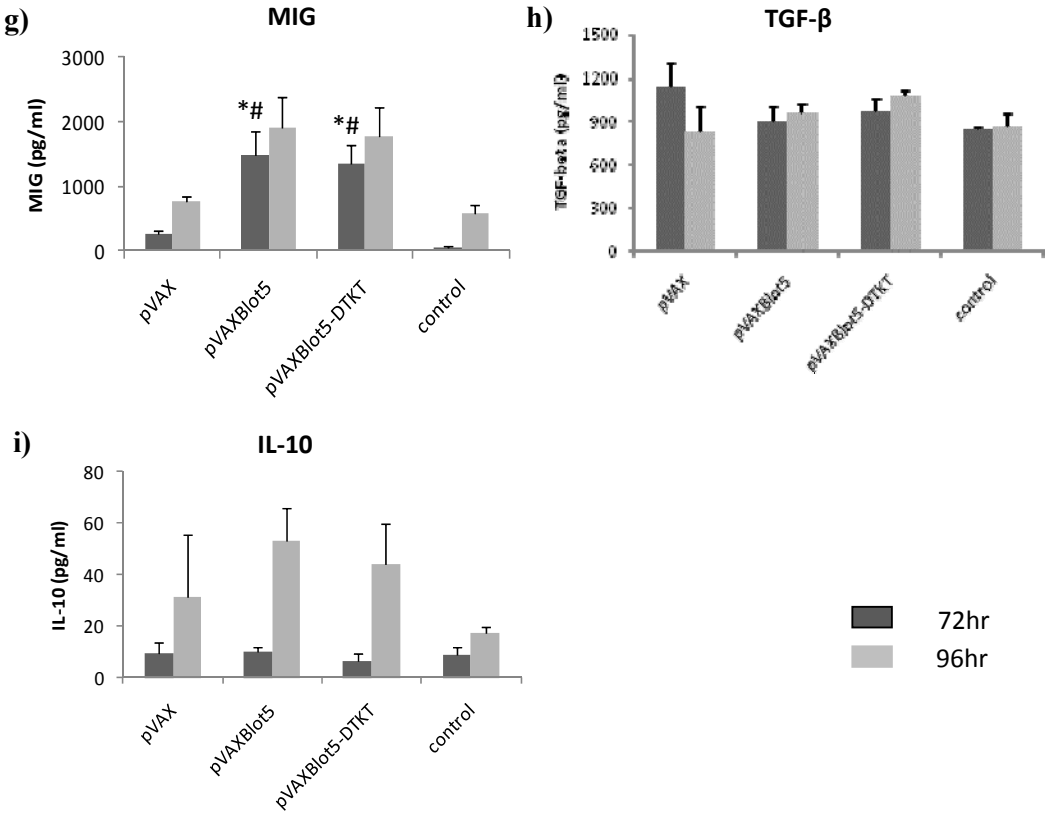
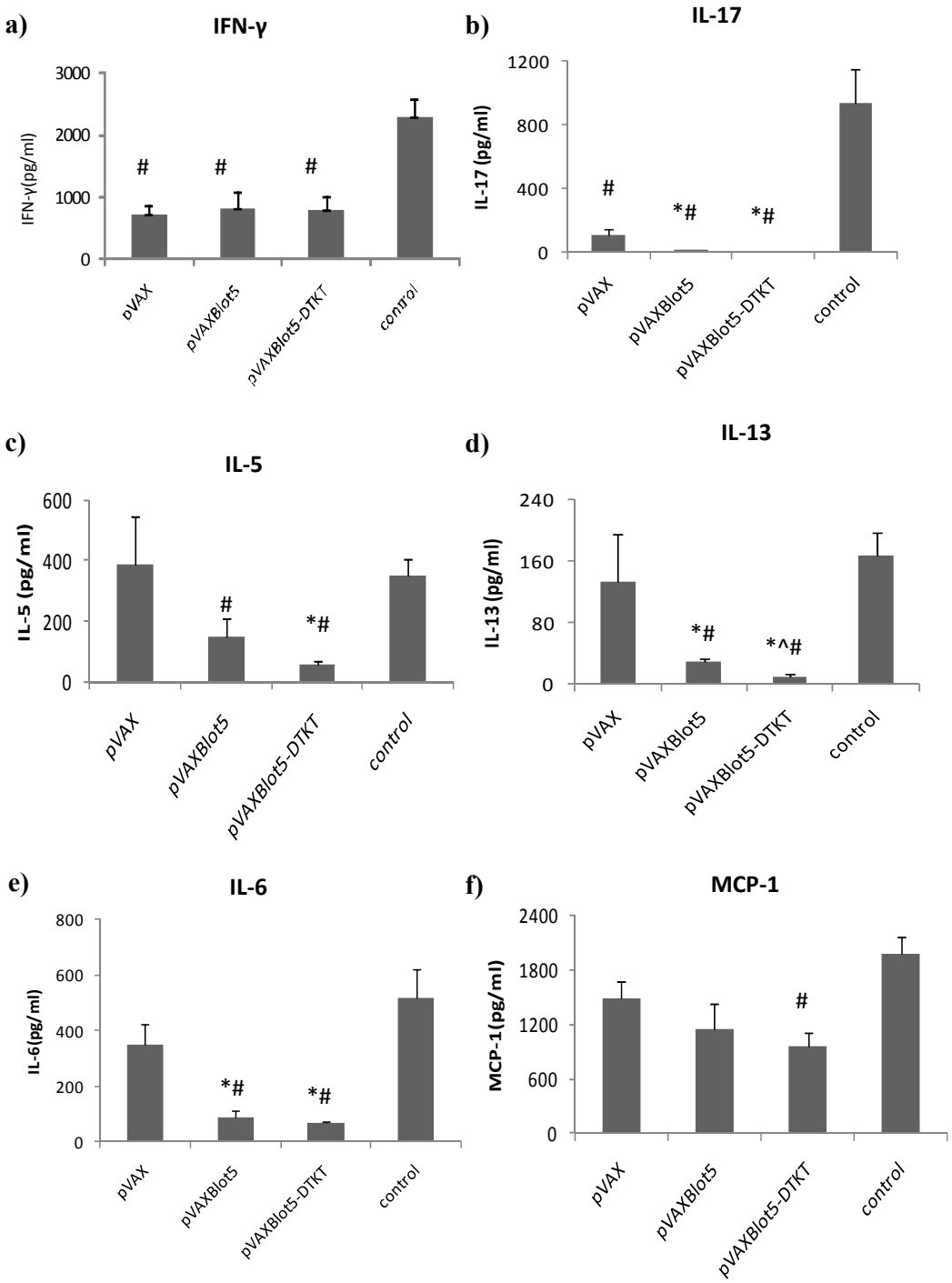


Figure 3.27: Cytokine and chemokine profile of splenocytes in mice immunized with plasmid DNA and intraperitoneally injected with rBlo t 5 protein

Mice were immunized with 50 μg of plasmid DNA with electrophoration at day 0 and 14. 1 and 4 weeks after the second immunization, the mice were intraperitoneally injected with rBlo t 5 protein adjuvanted with alum. The mice were sacrificed one week after the second intraperitoneal injection. Splenocytes were stimulated with 10 μg/ml rBlo t 5 protein. Seventy-two and ninety-six hours later, supernatants were collected and analyzed for IFN-γ, IL-17, IL-5, IL-13, IL-6, MCP-1, MIG, TGF-β and IL-10 by milliplex. Control mice were not immunized with plasmid DNA. Data were presented as mean ± SEM (n = 7-8) # compared with control group, *compared with pVAX vector group, $p < 0.05$.


a)
IFN-γ
IFN-γ(pg/ml)
3000
2000
1000
0
#
#
#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
b)
IL-17
IL-17 (pg/ml)
1200
800
400
0
#
*#
*#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
c)
IL-5
IL-5 (pg/ml)
600
400
200
0
#
*#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
d)
IL-13
IL-13 (pg/ml)
240
160
80
0
*#
*^#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
e)
IL-6
IL-6(pg/ml)
800
600
400
200
0
*#
*#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control
f)
MCP-1
MCP-1(pg/ml)
2400
1800
1200
600
0
#
pVAX
pVAXBlot5
pVAXBlot5-DTKT
control

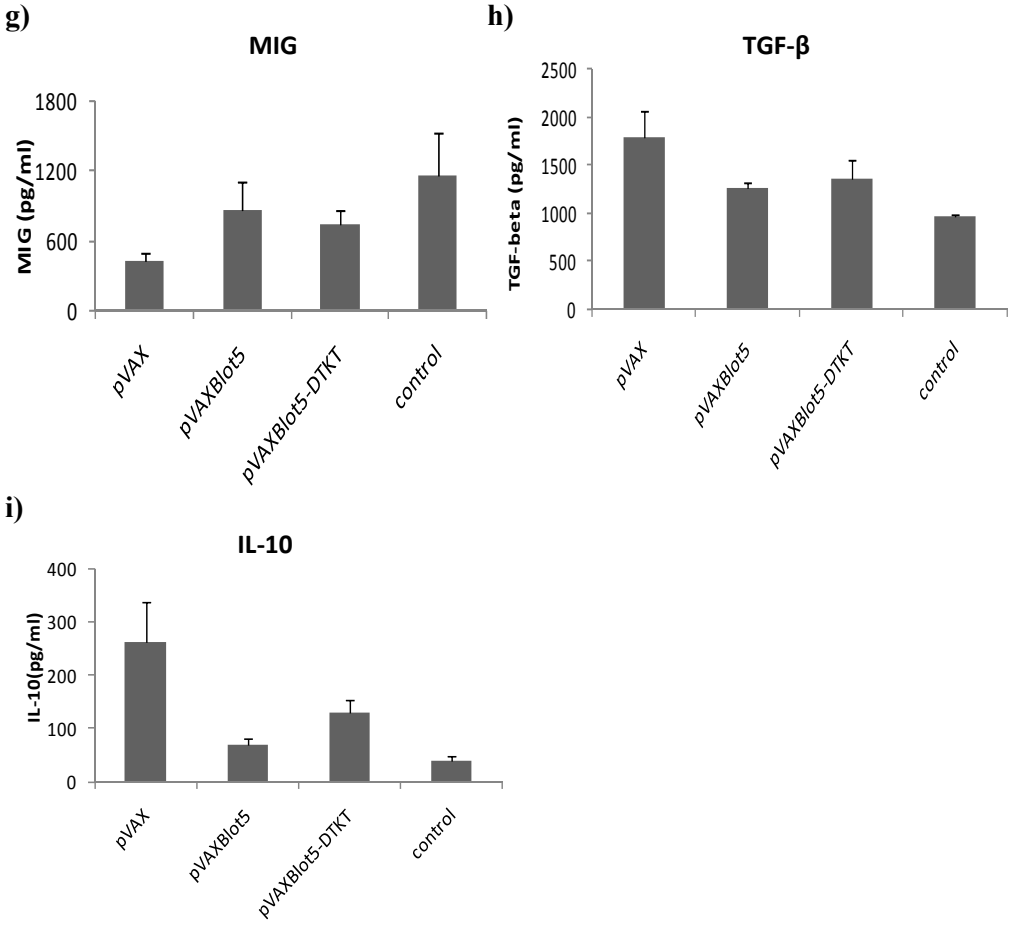


Figure 3.28: Cytokine and chemokine profile of cultured T cells of mice immunized with plasmid DNA and intraperitoneally injected with rBlo t 5 protein

Splenocytes of treated and control mice were stimulated with rBlo t 5 protein for 7 days *in vitro*. Viable cells were restimulated with rBlo t 5 protein in the presence of antigen-presenting cells for 72 hours. Culture supernatant were collected and analyzed for IFN-γ, IL-17, IL-5, IL-13, IL-6, MCP-1, MIG, TGF-β and IL-10. Control mice were not immunized with plasmid DNA. Data were presented as mean ± SEM (n = 7-8) # compared with control mice, * compared with pVAX vector group, ^ compared with pVAXBlot5 group, $p < 0.05$.

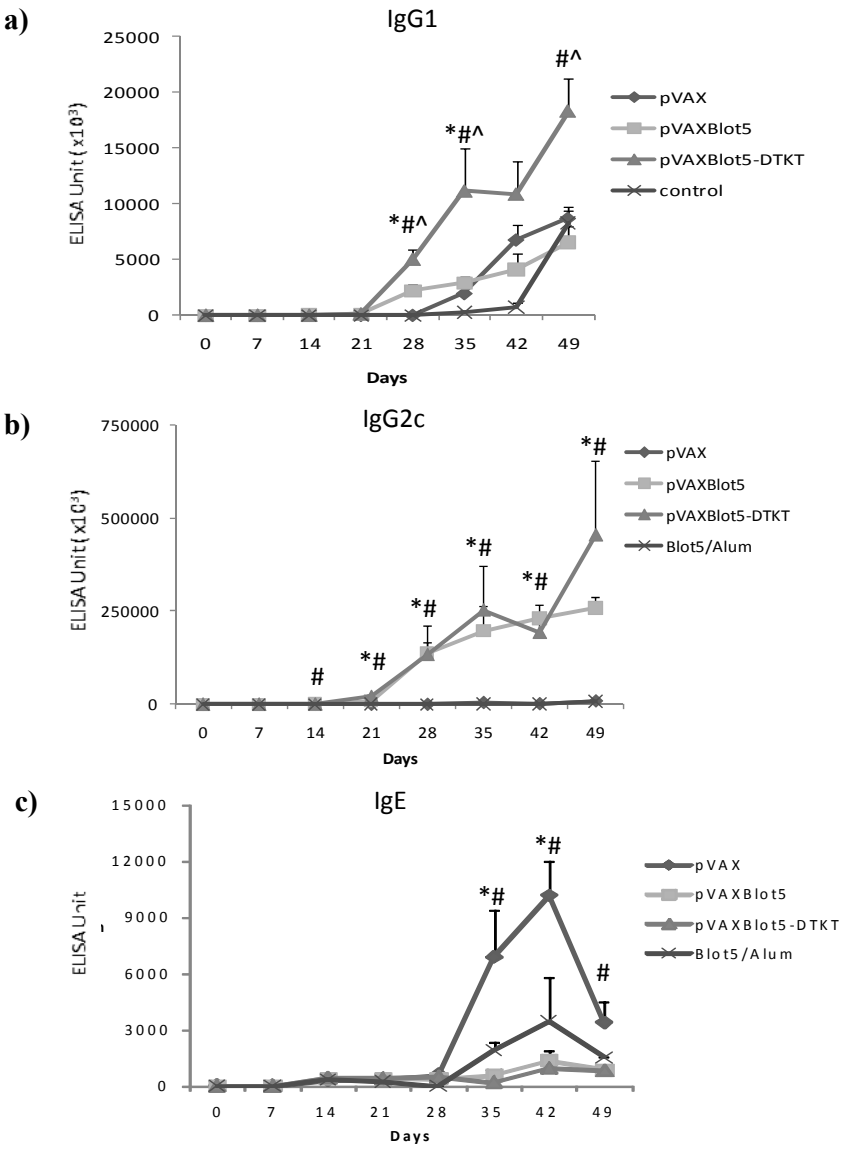


Figure 3.29: Allergen specific antibody responses in mice immunized with plasmid DNA and intraperitoneally injected with rBlo t 5 protein

Mice were immunized with 50 µg of plasmid DNA with electrophoration at day 0 and 14. 1 and 4 weeks after the second immunization, the mice were intraperitoneally injected with rBlo t 5 protein. The mice were sacrificed one week after the second intraperitoneal injection. Sera were collected weekly. Titers of Blo t 5 -specific IgG1, IgG2c and IgE were determined by ELISA. * stands for statistical significance between pVAXBlot5 and pVAX; # stands for statistical significance between pVAXBlot5-DTKT and pVAX, ^ stands for statistical difference between pVAXBlot5-DTKT and pVAXBlot5, $p < 0.05$.

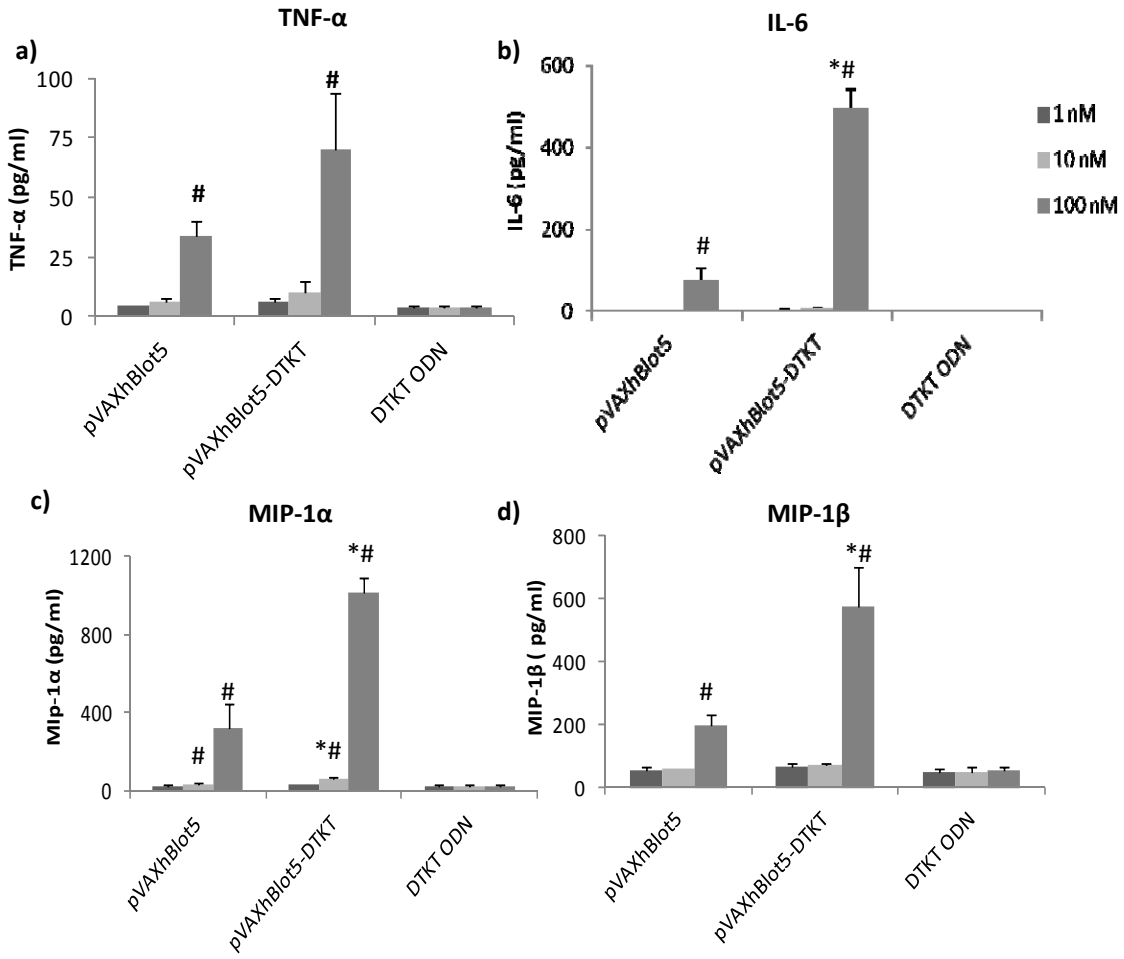


Figure 3.30: Differential response of human PBMC to plasmid DNA or free CpG oligonucleotides at 5 hours

Human PBMCs were cocultured with various doses (1 nM, 10 nM, 100 nM) of plasmid DNA or free CpG ODN and the culture supernatants were collected at 5 hours. The culture supernatants were assayed for TNF-α, IL-6, MIP-1α and MIP-1β via milliplex. Data illustrated were the mean ± SEM from three donors. # stands for statistical significance compared to DTKT ODN. * stands for statistical significance compared to pVAXhBlot5, $p < 0.05$.

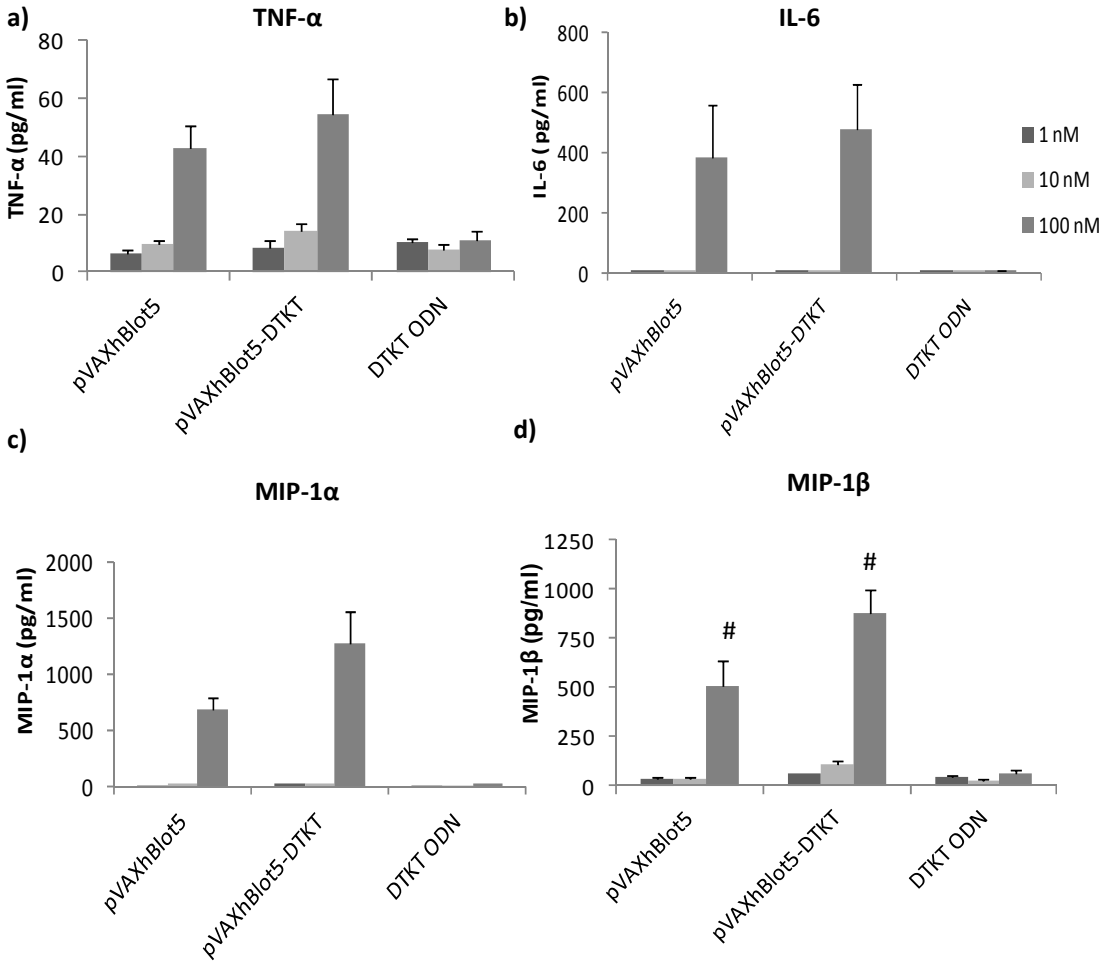


Figure 3.31: Differential response of human PBMC to plasmid DNA or free CpG oligonucleotides at 24 hours

Human PBMCs were cocultured with various doses (1 nM, 10 nM, 100 nM) of plasmid DNA or free CpG ODN and the culture supernatant was collected at 24 hours. The culture supernatants were assayed for TNF-α, IL-6, MIP-1α and MIP-1β via milliplex. Data illustrated were the mean ± SEM from three donors. # stands for statistical significance compared to DTKT ODN. * stands for statistical significance compared to pVAXhBlot5, $p < 0.05$.

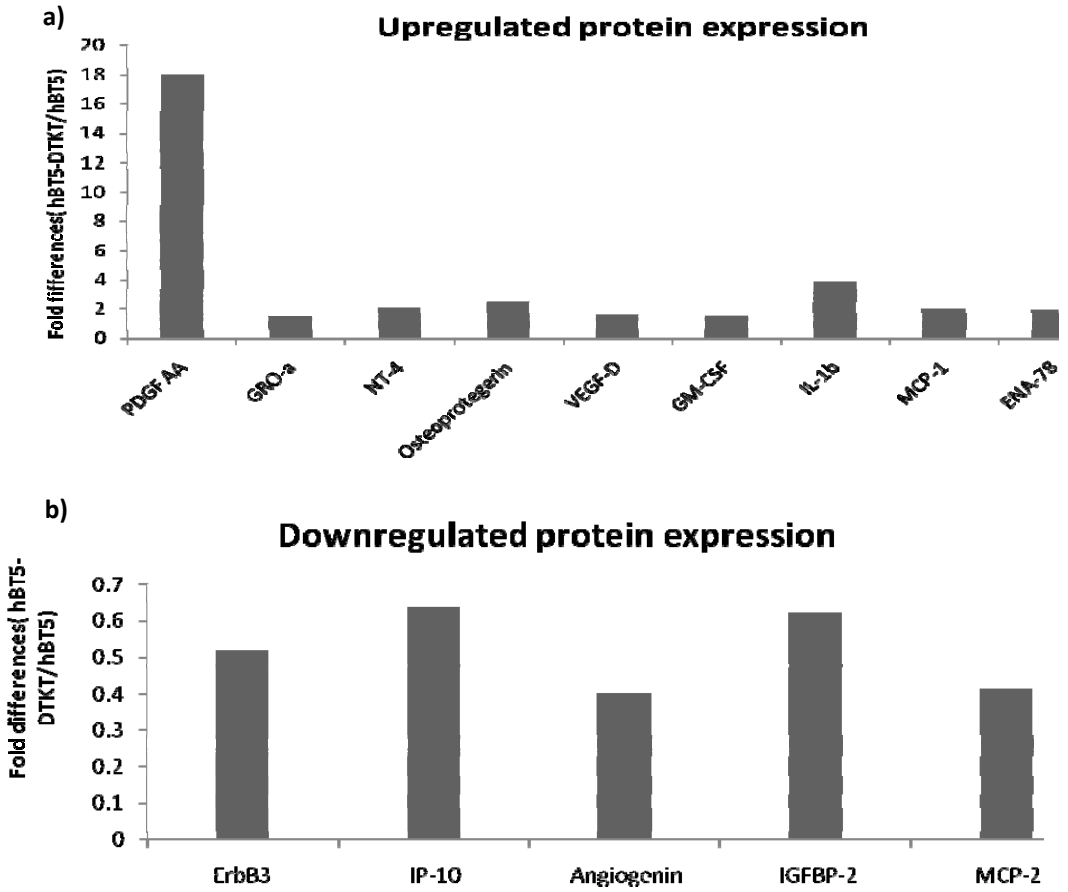


Figure 3.32: Differential regulatory protein expression of human PBMC to plasmid DNA at 5 hours

Human PBMCs were cocultured with 100 nM of plasmid DNA, pVAXhBlot5 (hBT5) and pVAXhBlot5-DTKT (hBT5-DTKT). Culture supernatants were collected at 5 hours and assayed for cytokines and chemokines via the protein array. Data was presented as fold differences of protein expression of hBT5-DTKT over hBT5.

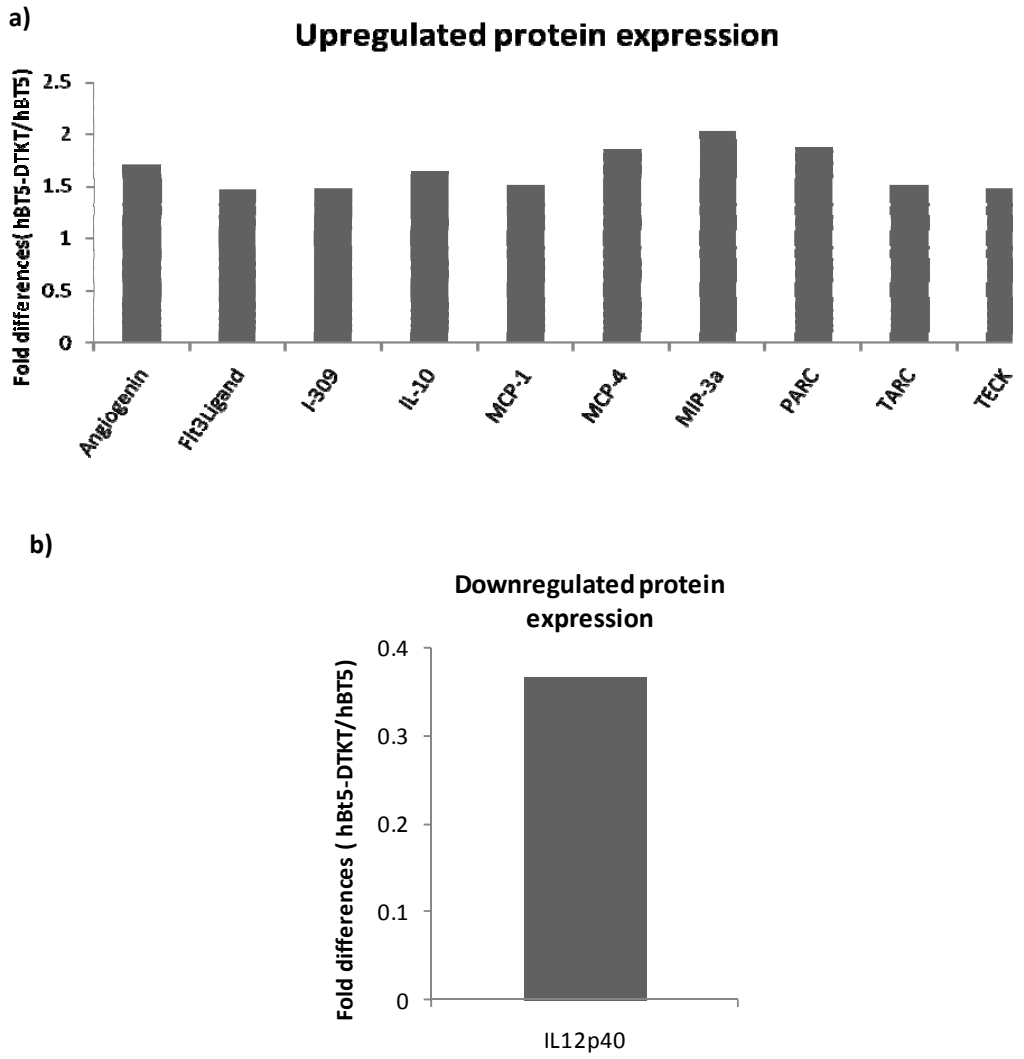


Figure 3.33: Differential regulatory protein expression of human PBMC to plasmid DNA at 24 hours

Human PBMCs were cocultured with 100 nM of pVAXhBlot5 (hBT5) and pVAXhBlot5-DTKT (hBT5-DTKT). Culture supernatant was collected at 24 hours and assayed for cytokines and chemokines via the protein array. Data was presented as fold differences of protein expression of hBT5-DTKT over hBT5.

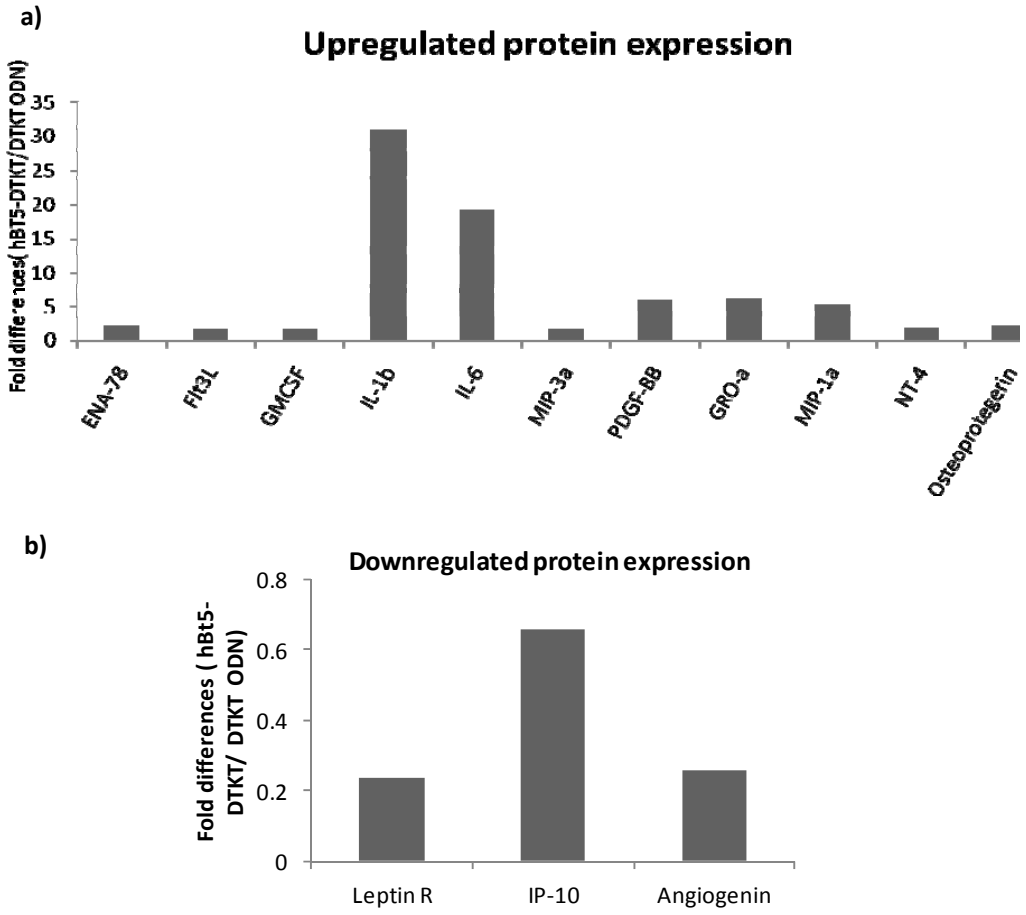


Figure 3.34: Differential regulatory protein expression of human PBMC to plasmid DNA or free CpG oligonucleotides at 5 hours

Human PBMCs were cocultured with 100 nM of pVAXhBlot5-DTKT (hBT5-DTKT) and free CpG ODN (DTKT ODN). Culture supernatants were collected at 5 hours and assayed for cytokines and chemokines via the protein array. Data was presented as fold differences of protein expression of hBT5-DTKT over DTKT ODN.

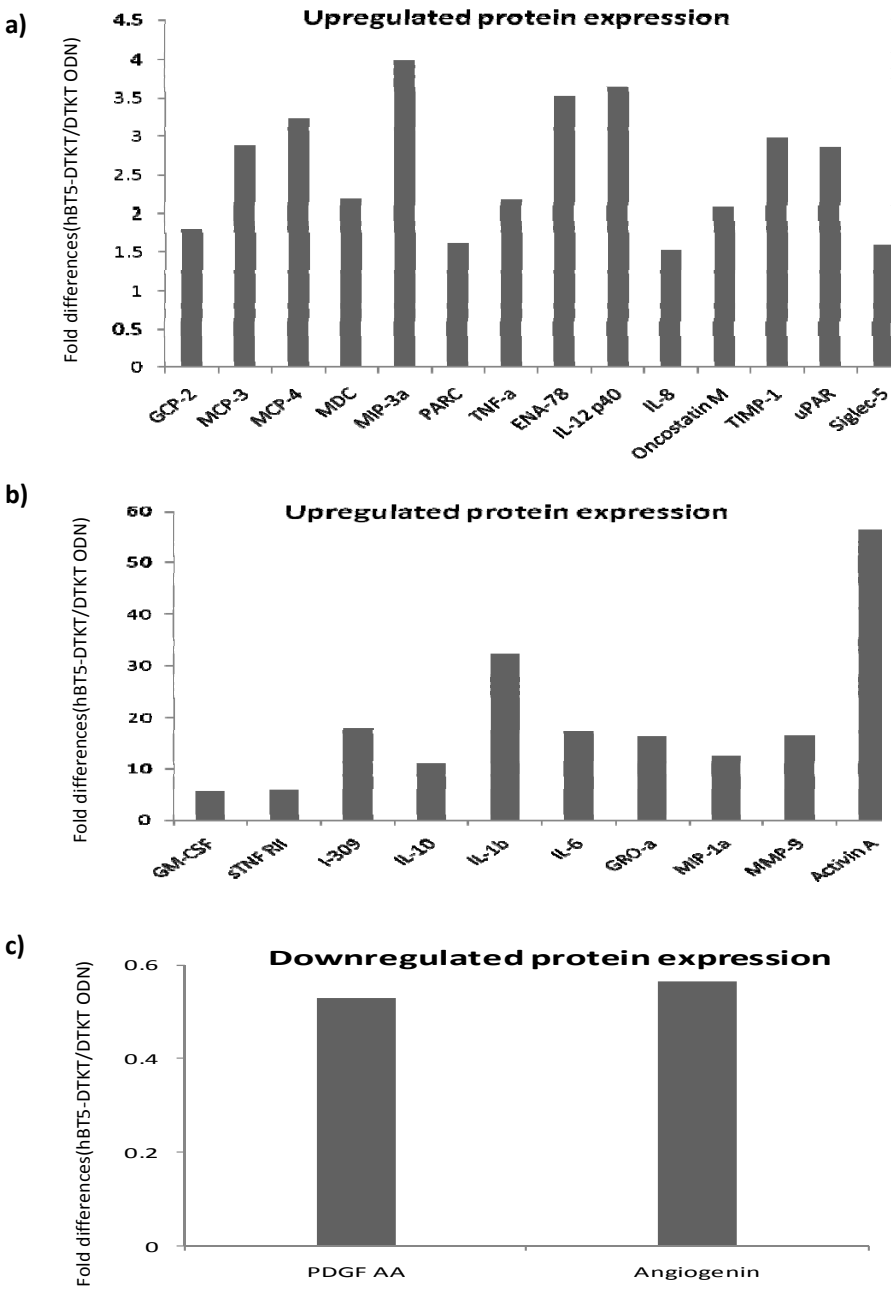


Figure 3.35: Differential regulatory protein expression of human PBMC to plasmid DNA or free CpG oligonucleotides at 24 hours

Human PBMCs were cocultured with 100 nM of pVAXhBlot5-DTKT (hBT5-DTKT) and free CpG ODN (DTKT ODN). Culture supernatant was collected at 24 hours and assayed for cytokines and chemokines via the protein array. Data was presented as fold differences of protein expression of hBT5-DTKT over DTKT ODN.

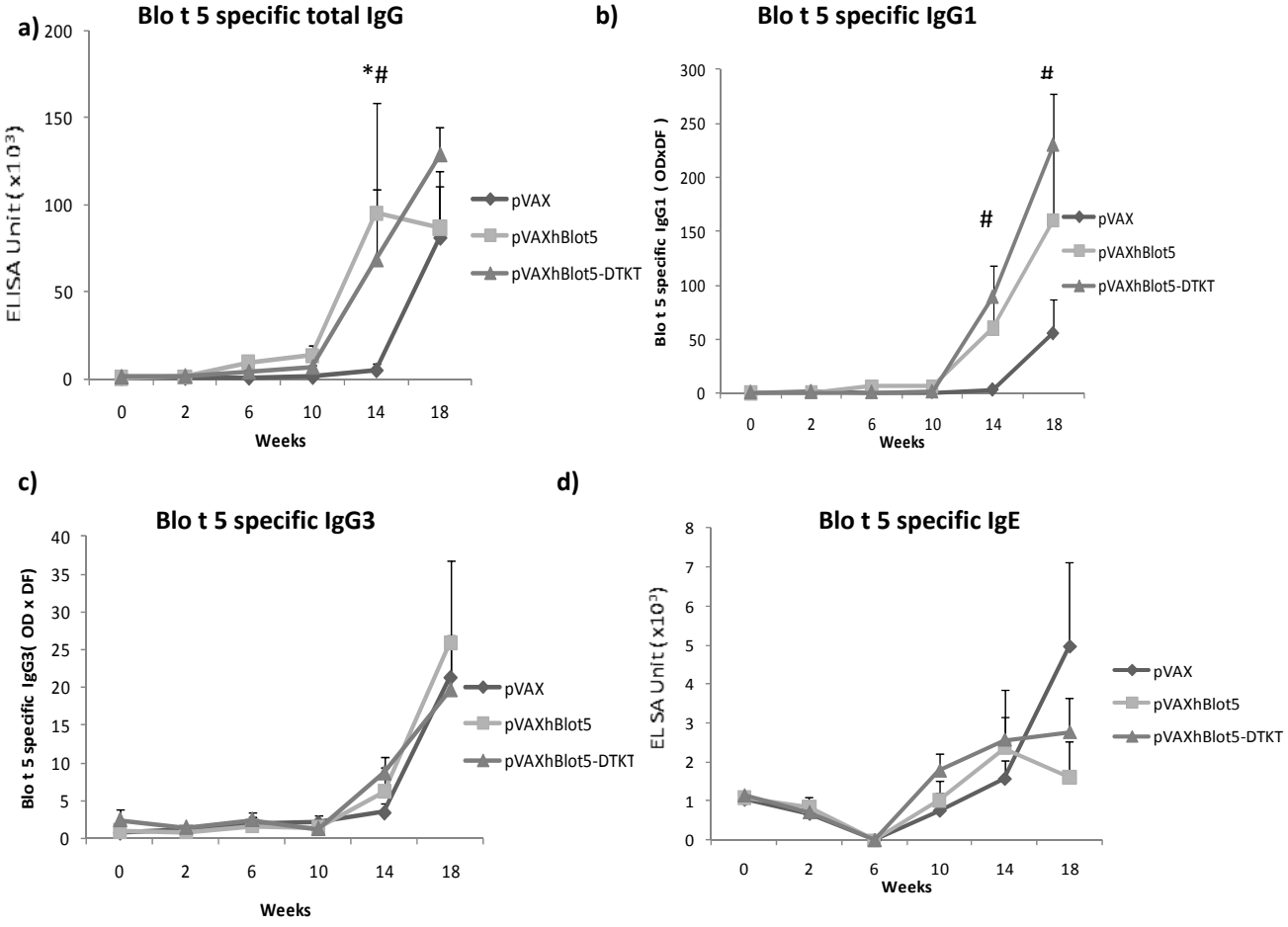


Figure 3.36: Allergen specific antibody responses in rhesus macaques intramuscularly immunized with plasmid DNA and subcutaneously immunized with Blo t 5 protein

Rhesus macaques were immunized with 2 mg of plasmid DNA thrice at 4 weeks interval. 4 and 8 weeks after the last immunization, the rhesus macaques were subcutaneously immunized with 500 µg rBlo t 5 protein adjuvanted with alum. Sera were collected from the rhesus macaques regularly. Titers of Blo t 5 -specific total IgG, IgG1, IgG3 and IgE were determined by ELISA. * stands for statistical significance between pVAX and pVAXhBlot5 groups, # stands for statistical difference between pVAX and pVAXhBlot5-DTKT groups, $p < 0.05$

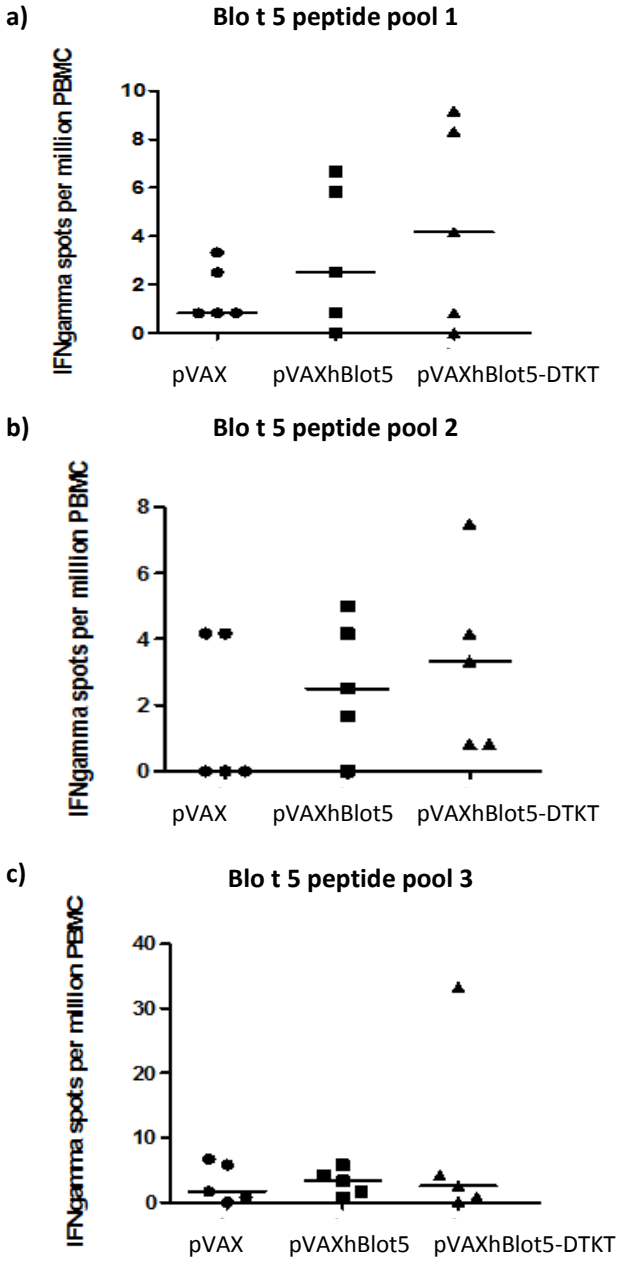


Figure 3.37: IFN-γ producing cells in rhesus macaques intramuscularly immunized with plasmid DNA and subcutaneously immunized with rBlo t 5 protein

Rhesus macaques were immunized with 2 mg of plasmid DNA thrice at 4 weeks interval. 4 and 8 weeks after the last immunization, the rhesus macaques were subcutaneously immunized with 500 µg rBlo t 5 protein adjuvanted with alum. Blood was collected from the rhesus macaques 2 weeks after the second subcutaneous immunization and IFN-γ forming spots of peripheral blood mononuclear cells in response to stimulation were determined by ELISPOT.

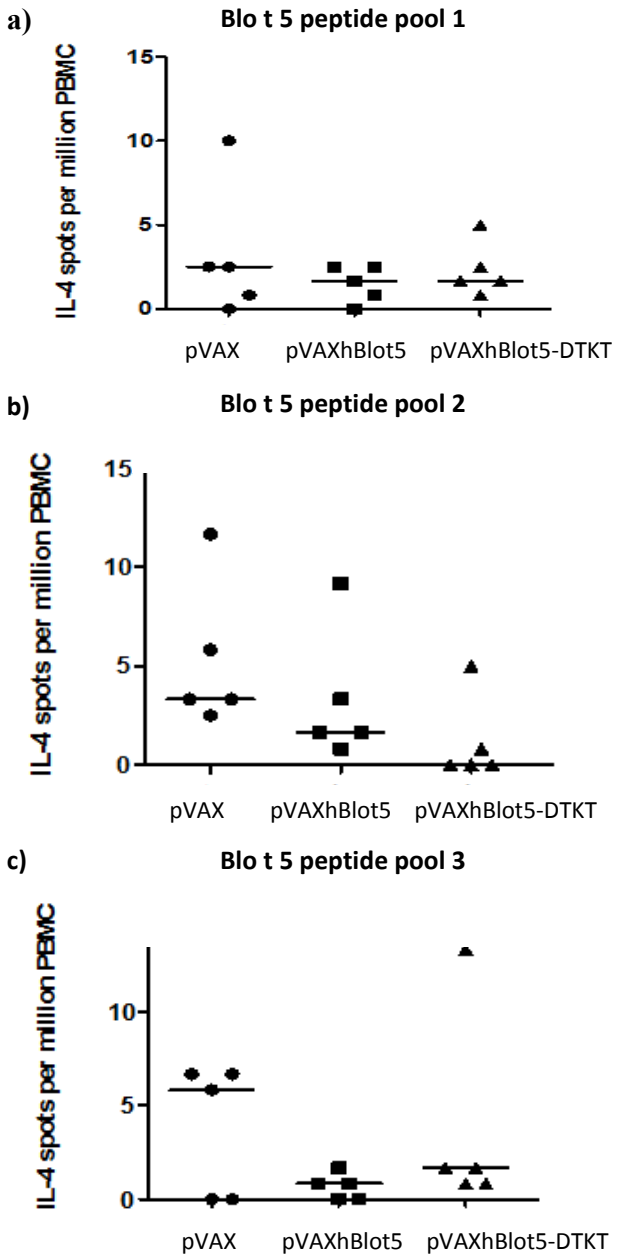


Figure 3.38: IL-4 producing cells in rhesus macaques intramuscularly immunized with plasmid DNA and subcutaneously immunized with rBlo t 5 protein

Rhesus macaques were immunized with 2 mg of plasmid DNA thrice at 4 weeks interval. 4 and 8 weeks after the last immunization, the rhesus macaques were subcutaneously immunized with 500 µg rBlo t 5 protein adjuvanted with alum. Blood was collected from the rhesus macaques 2 weeks after the second subcutaneous immunization and IL-4 producing spots of peripheral blood mononuclear cells in response to stimulation were determined by ELISPOT.

3.3 Discussion

DNA vaccine was an attractive modality for the treatment of allergic disease as it could be engineered to incorporate the gene of interest as well as molecular adjuvants. In addition, DNA vaccines were cheap, easy to produce and did not require cold storage. DNA vaccines could provide a long term source of antigen. However the main disadvantage of DNA vaccine was its low immunogenicity. Hence, in this study, multiple approaches were employed to increase the immunogenicity of the DNA vaccine. In this study, Blo t 5 allergen gene together with a signal peptide and a lysosome-targeting signal, LAMP-1 targeting sequence, was cloned into pVAX vector with the insertion of human CpG motifs into the backbone. Previous reports had shown that robust antigen specific Th1 responses could be induced by the use of LAMP-1 in HIV gag DNA vaccines (de Arruda et al., 2004; Marques et al., 2003). Electrophoration was also employed to increase the efficiency of plasmid DNA uptake (Babiuk et al., 2002; Mir et al., 1999; Uno-Furuta et al., 2001). This study had evaluated if the insertion of additional CpG motifs into the plasmid backbone provided an adjuvant effect by enhancing the immunogenicity.

To evaluate if CpG modified plasmid DNA enhanced the antigen presenting properties of antigen presenting cells, both pVAX-Blot5 and pVAXBlot5DTKT were used to stimulate either GMCSF or Flt3 ligand derived dendritic cells from mouse bone marrow *in vitro* as it was difficult to isolate dendritic cells in sufficient numbers *in vivo*. Antigen presenting properties of antigen presenting cells were assessed via upregulation of surface markers and cytokine production. This study had showed that modified and unmodified plasmid DNA elicited similar effects on bone marrow derived dendritic cells in terms of surface marker upregulation and cytokine

production. This could be due to the presence of alternate DNA sensor pathways such as the STING pathway that triggered type I interferon production, the AIM-2 inflammasome pathway/NOD-like receptor pathway that mediated proinflammatory cytokine production and the DNA mediated RNA polymerase III/RIG-1/IPS-1 pathway. Double stranded DNA structure was shown to be efficient in the activation of the STING pathway as single stranded DNA did not induce robust type I interferons. As both activation of the DNA sensor pathways and activation of TLR9 via CpG motifs converged at the downstream activation of NF-κB hence it was possible that the effect of additional CpG motifs was not observed due to the robust activation of the DNA sensor pathways. Besides that, TLR9 could interact with the phosphodiester 2' deoxyribose backbone of plasmid DNA and activate the intracellular signaling cascade (Haas et al., 2008). Furthermore, additional human CpG motifs inserted into the plasmid backbone may not be optimal for murine cells hence the adjuvant effect of CpG motifs was not profound.The enhanced immunogenic effect of human CpG motifs was reflected by using human cells as CpG modified plasmid DNA induced more robust production of proinflammatory cytokines from human peripheral blood mononuclear cells as compared to unmodified plasmid DNA. Plasmid DNA induced robust activation of the DNA sensor pathways may overwhelm the effect of activation of TLR9 by CpG motifs.

Dendritic cells were potent antigen presenting cells that could direct the differentiation of $CD4^+$ T cells. The combined effect of antigen dose, maturation status of dendritic cells and stimulation of dendritic cells with pathogenic-derived products affected the subsequent development of either a Th1 or Th2 response (Boonstra et al., 2003). High antigen doses had been suggested to promote Th1 cell

development while low antigen dose tended to favour Th2 cell development (Boonstra et al., 2003). Level of TLR9 expression varied in different dendritic cell subsets. In mice, bone marrow derived dendritic cells using GMCSF (GMCSF-DC) were myeloid dendritic cells that had low expression of TLR9 but high expression of TLR4 (Boonstra et al., 2003). In contrast, bone marrow derived dendritic cells using Flt3L (Flt3L-DC) were mainly plasmacytoid dendritic cells that have high expression of TLR9 and TLR7 but low expression of TLR2, TLR3 and TLR4 (Okada et al., 2003). Hence differential cytokine and chemokine production by bone marrow derived dendritic cells using GMCSF or Flt3 ligand may be due to the difference in TLR9 content. Flt3L-DC were known to be made up of 50% of $CD11c^{+}CD11b^{-}$ dendritic cells and another 50% of $CD11c^{+}CD11b^{+}$ dendritic cells while GMCSF-DC were reported to consist of a single population of $CD11c^{+}CD11b^{+}B220^{-}$ dendritic cells which were similar in phenotype to $CD11c^{+}CD11b^{+}B220^{-}$ dendritic cells induced by Flt3L (Gilliet et al., 2002). $CD11c^{+}CD11b^{-}$ subset from Flt3L-DC had been demonstrated to express the typical markers of mouse plasmacytoid DC precursors such as high levels of B220, CD45RB, Ly6c, GR-1, low levels of MHC class II and undetectable CD80 , CD86 levels (Asselin-Paturel et al., 2001; Nakano et al., 2001). In contrast, lower levels of CD45RB, Ly6c, GR-1, higher levels of CD80, CD86, MHC class II and undetectable levels of B220 were observed on $CD11c^{+}CD11b^{+}$ subset from Flt3L-DC and this phenotype was similar to that of splenic myeloid DC subsets (Moser and Murphy, 2000; Pulendran et al., 2001; Shortman, 2000). In this study, GMCSF-DC was found to produce higher levels of IL-6 and TNF-α as compared to Flt3L-DC upon stimulation with plasmid DNA. A possible reason could be that since Flt3L-DC consisted of 50% of $CD11c^{+}CD11b^{+}B220^{-}$ cells cells that resembled GMCSF-DC, only about 50% of DC were capable of producing IL-6 and

TNF-α in response to plasmid DNA stimulation while over 90% of GMCSF-DC could produce IL-6 and TNF-α. IL-6 was known to be the prototypic cytokine of conventional DC. (Yasuda et al., 2006) Besides that, $CD11c^{+}CD11b^{+}B220^{-}$ fraction of Flt3L-DC also showed a similar cytokine profile as GMCSF-DC with higher IL-6 and TNF-α than the $CD11c^{+}CD11b^{-}B220^{+}$ fraction which is in agreement with another published study (Gilliet et al., 2002). Hence $CD11c^{+}CD11b^{+}B220^{-}$ dendritic cells were more responsive to plasmid DNA stimulation and produced higher levels of cytokines and chemokines than $CD11c^{+}CD11b^{-}B220^{+}$ dendritic cells.

Development of immune responses could be affected by the CpG content in the plasmid backbone of DNA vaccines, hence a way to enhance the immune responses is to increase the number of CpG motifs (Coban et al., 2005; Kojima et al., 2002; Pontarollo et al., 2002). Synthetic CpG ODN had been used in many studies as they had proven to have potent adjuvant properties (Conforti et al., 2008; Sur et al., 1999; Weiner et al., 1997). CpG ODN had been demonstrated to induce robust upregulation of CD40, CD86 as well as IL-12p40, IL-12p70, IL-6 and TNF-α from GMCSF-DC (Dearman et al., 2009). In this study, CpG modified plasmid DNA was found to be more potent activators of dendritic cells in terms of upregulation of surface markers MHC class II, CD80, CD86, CD40 and production of proinflammatory cytokines such as IL-6, TNF-α when compared to free CpG ODN. Possible reason for the enhancement of antigen presenting functions of dendritic cells by CpG modified plasmid DNA could be that in addition to activating TLR9, the DNA plasmid backbone of plasmid DNA also activated the DNA sensor pathways.

This study had established an experimental allergy model in which an allergen Blo t 5 when adjuvanted with alum was capable of triggering mixed Th1, Th2 and Th17 immune responses. In addition, mixed humoral responses were also induced in such an experimental model with the production of IgG1, IgG2c (Th1 signature antibody) and IgE (Th2 signature antibody). This model provided a more accurate representation of the actual situation as allergic inflammation was now known to be not only a Th2 type mediated process, Th1 and Th17 type responses also played a role at enhancing Th2 immune responses (Randolph et al., 1999a; Sugimoto et al., 2004; Wakashin et al., 2008). This study showed that allergen gene vaccination could suppress production of Th1 cytokine IFN-γ, Th17 cytokine IL-17, Th2 cytokines IL-5, IL-13 and proinflammatory cytokine IL-6 from Blo t 5 specific T cells. Possible mechanisms could be via the induction of Treg cells with the subsequent production of immunosuppressive IL-10 or mediated by the effect of IFN-γ. The role of Treg in suppressing Th1, Th2, Th17 cells was highlighted in a study whereby Treg cells were ablated using human diphtheria toxin in 'knock in' mice with the insertion of the coding region of the toxin receptor at the 3' untranslated region of Foxp3 (Kim et al., 2007). Ablation of Treg cells resulted in inflammation at multiple organs and increased mortality which could be due to the increased activation of $Foxp3^{-}CD4^{+}$ T cells (Kim et al., 2007). Supporting evidence was apparent in another study which demonstrated that transfer of effector $Foxp3^{-}$ T cells into $Rag2^{-/-}$ recipient mice upregulated production of Th1, Th2, Th17 cytokines and inflammation in many organs (Chaudhry et al., 2011). In addition other studies reported that nTregs could suppress lung allergic responses through IL-10 and TGF-β production (Joetham et al., 2007; Presser et al., 2008). Allergen gene vaccination increased the production of immune suppressive cytokines such as IL-10 by splenocytes in response to rBlo t 5

protein when compared to pVAX vector group (Figure 3.27). Hence, it was possible that the suppression in Blo t 5 specific Th1, Th2 and Th17 cytokines was mediated through IL-10 producing Treg cells *in vivo*. Phospholipase A specific T cells from PBMC had been reported to produce IL-10 in response to phospholipase A with the subsequent induction of an anergic state in peripheral T cells (Akdis et al., 1998). Treg cells bear IL-10R and signalling via IL-10R was essential to suppress Th17 responses (Chaudhry et al., 2011). In addition STAT3, a transcription factor that drove the differentiation of Th17 had been found to bind to IL-10 locus in Treg cells and IL10R deficient Treg cells failed to produce IL-10 (Chaudhry et al., 2011). Given that Th1, Th2 and Th17 cells were also competent in producing IL-10, it would be reasonable to deduce that IL-10 produced by these cells could act in a feedback loop by acting on IL-10R on Treg cells and activating STAT3 with the subsequent production of more immunosuppressive IL-10 (Chaudhry et al., 2011). This IL-10 produced might control or suppress the effector cells. Role of IL-10 as a negative regulator of Th17 responses had been demonstrated in another study whereby IL-10R deficient Treg cells failed to suppress IL-17 and IL-22 by $CD4^{+}Foxp3^{-}$ T cells (Chaudhry et al., 2011). Besides that IL-10Rα had been found to be expressed on IL-17A producing $CD4^{+}$ T cells hence it may be probable that IL-10 could act directly and exert a suppressive effect on Th17 cells (Huber et al., 2011). IL-10 could be a possible mediator for the suppressive effect on antigen specific Th1, Th2, Th17 cytokines observed in allergen gene vaccinated mice.

Interestingly, splenocytes from allergen gene immunized groups produced significantly higher levels of IFN-γ than the pVAX vector group as well as the group that had been immunized intraperitoneally with rBlo t 5 protein adjuvanted with alum.

The immune regulatory role of IFN-γ had been previously reported and it had been shown that IFN-γ was capable of suppressing Th2 cytokines, IL-5, IL-13 as well as Th1 and Th17 type cytokines in the lung (Nakagome et al., 2009). It was hence possible that the suppression in the Th1, Th17 and Th2 cytokines seen in the allergen gene vaccinated group could be a result of the high levels of IFN-γ produced. Another possibility is that T_H1 T_R cells that develop in Th1 biased conditions could be induced. This antigen specific $CD4^+$ regulatory T cells were found to produce both IFN-γ and IL-10 and were capable of inhibiting effector T cell responses such as Th2 responses (Stock et al., 2004). TGF-β, also a regulatory cytokine produced by Treg cells, could also be a possible mediator but no difference was observed in this study. Hence it was speculated that IFN- γ could possibly play a role in the suppressive effect on antigen specific Th1, Th2, Th17 cytokines observed in allergen gene vaccinated mice.

pVAX vector vaccination group also showed suppression in IFN-γ and IL-17 production by Blo t 5 specific T cells. A probable reason was that the DNA plasmid backbone itself could activate different DNA sensor pathways as mentioned earlier. There were a number of DNA-sensing pattern recognition receptors such as the cytosolic sensor DAI (DNA dependent activator of IRF also known as ZBP1 and DLM-1), IFI16, absent in melanoma 2 (AIM-2) and TLR-9. Double stranded DNA was reported to bind to DAI with the subsequent phosphorylation of TANK-binding kinase-1 (TBK1) and activation of IRF3 (IFN regulatory factor 3), resulting in the expression of type 1 IFNs and proinflammatory cytokines (Takaoka et al., 2007; Wang et al., 2008). Importance of TBK1 was highlighted in another study where it was demonstrated that TBK1-dependent signalling was needed for DNA vaccine induced humoral and cellular immune responses *in vivo* (Ishii et al., 2008). The

binding of DNA to DAI could also activate another pathway, receptor interacting protein-1 kinase could also bind to DAI with subsequent activation of NFκB pathway (Kaiser et al., 2008). In addition, the phosphodiester 2' deoxyribose backbone of DNA also had been reported to activate TLR9 (Haas et al., 2008). It was observed in this study that Flt3L-DC produced type I interferons in response to plasmid DNA stimulation *in vitro*. Hence it was probable that type I interferon production stimulated by the DNA sensors pathways could be the suppressive factor for the attenuation of allergen specifc IL-17 and IFN-γ (Moschen et al., 2008).

Notably, pVAXBlot5-DTKT immunized group showed greater suppression in Th2 cytokines, IL-5 and IL-13, when compared to pVAXBlot5 immunized mice that had been immunized intraperitoneally with rBlo t 5 protein adjuvanted with alum (Figure 3.28). In addition, pVAXBlot5-DTKT activated dendritic cells also showed attenuated IL-17 and IFN-γ production in *in vitro* priming of Blo t 5 specific T cells as compared to pVAXBlot5 (Figure 3.21). A possible mediator of the suppressive effect could be GMCSF, as splenocytes from pVAXBlot5-DTKT immunized mice produced the highest levels of GMCSF (Figure 3.23). GMCSF has been implicated in the expansion of Treg cells, hence suppressive effect observed in pVAXBlot5-DTKT immunized mice could be attributed to GMCSF expansion of Treg cells (Sheng et al., 2008).

The importance and involvement of TLR9 had been a topic of interest after some studies revealed that TLR9 deficient mice elicited similar immune responses to DNA vaccination (Babiuk et al., 2004; Spies et al., 2003). However recently other studies had demonstrated that TLR9 was still involved in the activation by CpG DNA as it was shown that CpG DNA but not non CpG DNA bound to TLR9 hence inducing a

conformational change and activation of TLR9 via *in vivo* single molecule experiments (Chen et al., 2011). In addition, dendritic cells activation by plasmid DNA and subsequent priming of antigen specific $CD8^+$ T cell responses was found to be dependent on TLR9 *in vitro* and *in vivo* (Rottembourg et al., 2010). Expression levels of TLR9 in primary human cells were found to correlate with the responsiveness to CpG-DNA stimulation and the highest expression of TLR9 was found in pDCs and B cells (Bauer et al., 2001; Hornung et al., 2002). In mouse, TLR9 was expressed in pDCs, cDCs and B cells. Upon activation of TLR9, IL-15 was secreted by cDCs that in turn induced pDCs to express CD40L. In addition, CD40 expression on cDCs was also induced by TLR9 signalling. This CD40-CD40L ligation subsequently induced conventional dendritic cells to produce IL-12 that activate NK cells to produce IFN-γ (Kuwajima et al., 2006). Hence the increased levels of IFN-γ observed in the splenocyte cultures of pVAXBlot5-DTKT mice could also be due to the activation of NK cells brought about by the crosstalk between plasmacytoid dendritic cells and conventional dendritic cells upon TLR9 engagement by CpG motifs. In this study, the involvement of TLR9 was also verified via TLR9 inhibition experiments whereby inhibition of TLR9 attenuated sharply the production of IL-6, TNF-α, IL-12p40, IP-10, KC, MIP-1α, MIP-1β, MIP-2 and IFN-α from $CD11c^+CD11b^+B220^-$ DCs and IFN-α, IL-6, IP-10, IL-12p40 from $CD11c^+CD11b^-B220^+$ DCs. Hence TLR9 might be involved in the enhanced levels of IFN-γ observed in pVAXBlot5-DTKT immunized mice.

It was demonstrated in the allergy model that intraperitoneal immunization with Blo t 5 adjuvanted with alum induced distinct subsets of IL-17 and IFN-γ producing cells. The flexibility of Th17 phenotype had been demonstrated by the conversion of Th17

cells into Th1/Th17 cells in the presence of IFN-γ *in vivo* (Annunziato and Romagnani, 2010; Kurschus et al., 2010; Lexberg et al., 2010). Given that allergen gene vaccination had been shown to induce the production of considerable levels of IFN-γ by splenocytes, it is probable that Th17 cells can be converted to Th1 cells under the IFN-γ rich microenvironment. Another piece of supporting evidence came from a recent study in which a Th17 reporter mouse line had been used to show the plasticity of both *in vitro* and *in vivo* generated Th17 cells as these cells can change their phenotype into Th17/Th1 as well as Th1 cells (Kurschus et al., 2010). Allergen/alum immunization induced IL-17 producing cells could either be suppressed by IFN-γ or switched to other T helper phenotypes.

One of the major limitations of DNA vaccines was its limited efficacy in humans especially in their induction of humoral responses (Calarota et al., 1998; Coban et al., 2004; Epstein et al., 2002). In this study, by employing the use of molecular adjuvant, human CpG motifs, we proved by both *in vitro* and *in vivo* studies using murine model as well as human peripheral blood mononuclear cells and non-human primates of the enhanced immunogenic effect of CpG modified plasmid DNA. In this study we showed that pVAXhBlot5-DTKT induced human PBMC to secrete elevated levels of IL-6, MIP-1α and MIP-1β. This was in agreement with previous studies that reported that plasmid DNA with additional CpG motifs (either the D or K type) induced PBMC to produce IL-6 (Coban et al., 2005). Addition of human CpG motifs into the plasmid backbone showed enhanced stimulatory effect on human cells. Human CpG motifs were active in both mouse and humans while optimal CpG motifs for mice did not work in humans, as such human CpG motifs were employed so that its efficacy could be assessed by using both the murine and human system (Ishii et al., 2004). In

addition the use of humanized Blo t 5 gene sequence, humanized lamp targeting sequence and human CpG motifs made it more possible for this plasmid DNA to be applicable for human use. This study was one of the few studies that evaluated allergen gene vaccination in non-human primates. In this study, pVAXhBlot5-DTKT immunized macaques produced the highest level of Blo t 5-specific total IgG and IgG1, suggesting that incorporating CpG motifs into the plasmid backbone enhanced the immunogenicity of the DNA vaccine. Besides that, cellular responses were also improved in pVAXhBlot5-DTKT immunized non-human primates that elicited higher number of IFN-γ producing cells in response to stimulation with Blo t 5 peptide pools. Studies done by other groups had also reported that immune responses to hepatitis B vaccine and heat-killed leishmania vaccine had been strongly enhanced by the use of CpG ODN in non-human primates (Davis et al., 2000; Jones et al., 1999). However subsequent studies of DNA vaccines carried out in murine models should evaluate airway hyperresponsiveness and airway remodeling as these readouts serve as better indicators of the efficacy of DNA vaccines. However despite the success in using CpG ODN to improve the immunogenicity of vaccines, caution must be taken in the use of CpG ODN as it had been shown that coagulation serine proteases, plasminogen and thrombin production could be triggered by phosphorotioated oligonucleotides (Bokarewa et al., 2002). Hence the incorporation of CpG ODN into the plasmid backbone might be a safer way of boosting the immunogenicity of DNA vaccines by minimzing its side effects.

Chapter 4 Exploration on the Use of Cytokine Gene Delivery against Airway Inflammation and Allergic Responses

4.1 Introduction

Increased prevalence of allergic asthma had become a public health issue (Ozdoganoglu and Songu, 2011). Genetic vaccination was a promising alternative approach to treat allergic asthma compared to protein immunization as protein immunization was associated with potential anaphylactic side effects and synthesis of IgE. In addition, gene expression in the skeletal muscle had been reported to be detected for many months after injection due to the episomal existence of plasmids in muscle cells (Davis et al., 1996; Wolff et al., 1992). Besides allergen gene immunization, cytokine gene immunization was also extensively researched on as a modality to skew the underlying T helper type 2 immune responses to that of a T helper 1 response in allergy. As such, attention had been placed on pro Th1 cytokines such as IL-12, IL-18, IFN-γ and IL-10 in the past decade (Dow et al., 1999; Li et al., 2008; Maecker et al., 2001; Nakagome et al., 2005).

It had been demonstrated that vaccination with an OVA and IL-18 fusion plasmid DNA not only protected mice from airway hyperresponsiveness induction but also reduced allergen specific IgE production (Maecker et al., 2001). In addition, OVA and IL-18 fusion plasmid DNA could negate established airway hyperresponsiveness, decrease Th2 cytokine IL-4 and increase Th1 cytokine IFN-γ in vaccinated mice, this effect was not seen in groups vaccinated with OVA plasmid DNA only (Maecker et al., 2001). In another study, vaccination of mice with plasmids encoding IFN-γ and IL-12 induced strong Th1 responses hence protecting the mice against lung

inflammation, as well as IgE induction (Kumar et al., 2001). Besides IL-18 and IL-12, intravenous and intratracheal IFN-γ gene delivery also resulted in a significant attenuation of AHR and airway eosinophilia, in addition administration of IFN-γ via the intravenous route was also able to reduce serum IgE levels (Dow et al., 1999). However there were certain dangers with the use of these cytokine genes as they may result in overwhelming Th1 responses.

As such, we aimed to determine the role of regulatory cytokines in allergic diseases. Regulatory cytokines were known to modulate the immune responses instead of skewing it in a particular direction. The novel regulatory cytokine, IL-35 is a heterodimeric cytokine from the IL-12 family that was composed of Epstein-Barr-virus-induced gene 3 (Ebi3) and interleukin-12 alpha which had been found to be expressed at high levels in mouse $Foxp3^+$ Treg cells but not in resting or activated $CD4^+$ cells (Collison et al., 2007b). The immunosuppressive role of IL-35 had been demonstrated in murine models of collagen induced arthritis as well as inflammatory bowel disease (Collison et al., 2007a; Niedbala et al., 2007) but its role in Th2 mediated disease, for example, allergic asthma had not been determined. Allergic asthma is a chronic inflammatory respiratory disease affecting atopic individuals globally. Asthma is a heterogenous and chronic inflammatory disease, its' cellular inflammation profiles could either be characterized by eosinophilia or neutrophilia. The prevalence of allergy has been increasing steadily in recent years, even with the emergence of more potent drugs. Hence, more effective therapies are warranted. In this study, we aimed to evaluate the suppressive effect of IL-35 on the Th2 and Th1/Th17 mediated lung inflammation in two newly developed murine models. One

was induced by a Blo t 5-specific Th2 cell line and the other was induced by the direct intranasal sensitization of Blo t 5-specific TCR transgenic mice.

4.2 Results

4.2.1 Construction of pVAX-IL35

The Epstein-Barr virus induced gene 3 (EBI3) was amplified from the first strand cDNA prepared from lipopolysaccharide activated bone marrow derived dendritic cells. Amplified EBI3 had a *BamHI* site at the 5' site and a *HindIII* site at the 3' end. The p35 subunit of IL-12 (IL-12p35) was amplified from the first strand cDNA prepared from splenocytes. Amplified IL-12p35 had *EcoRI* site at the 5' end and a *NheI* site at the 3' end. Two complementary oligonucleotides encoded for 4 repeats of GGGS linker were designed and annealed with the *HindIII* and *EcoRI* sites at the sense and antisense strands respectively. Amplified EBI3, IL-12p35 and the double stranded linker were digested with respective restriction enzymes and ligated into *BamHI* and *NheI* sites of pVAX. The amino acid sequence of IL-35 constructed by the fusion of EBI3, 4 repeats of GGGS linker and IL-12p35 was shown (Fig 4.1).

4.2.2 Expression of EBI-3, IL-12p35 and IL-35 mRNA in lungs of mice treated with pVAX-IL-35

To evaluate the expression of single chain IL-35, mice were administered intratracheally with pVAX-IL-35 and total RNA was extracted from the lungs. EBI-3, IL12p35 and IL-35 mRNA expression was determined by real time PCR. EBI-3, IL-12p35 and single chain IL-35 mRNA expression was detected at cycle number 18, 23 and 31 respectively (Fig 4.2). Since the expression of the fusion single chain IL-35 transcript could be detected by PCR, that distinguished it from the endogenous EBI3 and IL-12p35 expression. Hence pVAX-IL-35 could be expressed in the lungs of mice.

4.2.3 IL-35 attenuated Blo t 5-specific Th2 cell line mediated allergic airway inflammation

To assess if local delivery of the new regulatory cytokine IL-35 can attenuate the Blo t 5-specific Th2 cell line induced inflammation by transfer of Blo t 5-specific Th2 cells and intranasal Blo t 5 protein challenge, a plasmid DNA encoding recombinant single-chain IL-35 was intratracheally delivered into the airway. Total cell infiltration in the BALF was reduced in mice that had been administered pVAX-IL-35 compared to those administered with pVAX (1.68×10^5 *vs* 3.35×10^5, $p < 0.05$) (Fig 4.3A). Intratracheal administration of pVAX-IL-35 also significantly reduced eosinophils, lymphocytes and neutrophils numbers in the BALF (Fig 4.3B). The reduction in eosinophils was most marked from 2.37×10^5 in pVAX treated mice to 7.0×10^4 in pVAX-IL35 treated mice (Fig 4.3B). In addition, Th2 effector cytokines, IL-5, IL-4 and IL-13 were also significantly attenuated in the BALF of experimental pVAX-IL-35 group compared to the control pVAX group (Fig 4.3C). Concomitantly with the suppressed Th2 cytokine concentrations in the BALF, CCL2/MCP-1, CXCL1/KC, CXCL5/LIX, CXCL9/MIG and CXCL10/IP-10 concentrations in the BALF were also attenuated in the BALF from mice of the experimental pVAX-IL-35 (Fig 4.3D). Hence, locally expressed IL-35 could suppress the Blo t 5-specific Th2 cell line mediated lung inflammation by reducing the Th2 effector cytokines and chemoattractant CC- and CXC- motif chemokines for the recruitment of polymorphic mononuclear cells and lymphocytes.

4.2.4 IL-35 suppressed antigen-specific IgE, IgG1 and confered long term protection upon subsequent Blo t 5 challenge

To investigate whether systemic distribution of IL-35 can suppress Blo t 5-specific Th2 cells initiated Blo t 5-specific humoral responses, mice were intramuscularly injected with pVAX-IL-35 twice before the transfer of Blo t 5-specific Th2 cells. Mice were administered 2 courses of 3 consecutive days of intranasal Blo t 5 challenges and Blo t 5-specific IgE and IgG1 were determined. Mice that received intramuscular immunization of pVAX-IL-35 produced lower titers of Blo t 5-specific IgE on days 30 (755 ELISA unit *vs* 321 ELISA unit), 37 (372 ELISA unit *vs* 94 ELISA unit) and 84 (12865 ELISA unit *vs* 2838 ELISA unit) as compared to that of pVAX injected mice (Fig 4.4B). Suppression of Blo t 5-specific IgG1 production was persistent for the whole experimental period up to day 91 in the pVAX-IL-35 treated mice (Fig 4.4C). Delivery of plasmid DNA encoding IL-35 not only provided the short-term suppression of Blo t 5 specific IgE production after the first course (days 18-20) of intranasal Blo t 5 instillation but also the long term suppression of Blo t 5 instillation as evidenced from the significant suppression in Blo t 5-specific IgE on day 84 and IgG1 on both days 84 and 91.

4.2.5 IL-35 suppressed total IgE while levels of total IgM, IgA, IgG1 and IgG2c remained unchanged.

In order to determine if systemic distribution of IL-35 has any effect on the production of different immunoglobulin isotype, total serum IgE, IgM, IgG1, IgG2c and IgA were assayed and compared from both groups of mice that received either pVAX or pVAX-IL-35. There was a significant suppression in total serum IgE on days 84 (52 x 10^3 ELISA unit *vs* 210 x 10^3 ELISA unit) and 91 (13 x 10^3 ELISA unit *vs* 42 x 10^3 ELISA unit) from pVAX-IL-35 treated mice that subsequently received the Blo t 5 specific Th2 cell transfer and intranasal Blo t 5 challenges when compared

to pVAX treated mice (Fig 4.5A). On contrary, total serum IgM, IgG1, IgG2c and IgA levels are similar between pVAX-IL-35 and pVAX treated groups for 3 months (Fig 4.5B-E). Thus IL-35 exerted a long lasting suppressive effect on the production of total serum IgE at the time points after the second course of Blo t 5 intranasal challenges. These results indicate that systemic IL-35 did not exert a pan immunosuppressive effect on antibody production.

4.2.6 IL-35 suppressed IL-4 in the donor Th2 cells

To examine if IL-35 exert a direct suppression on the production of IL-4 by Th2 cells, mice were first intramuscularly injected with pVAX-IL-35 twice before the transfer of Blo t 5-specific Th2 cells and administered 3 consecutive days of intranasal Blo t 5 challenges (Fig 4.6A). Spleens were excised one week later. Splenocytes of both pVAX-IL-35 as well as pVAX treated mice that had been stimulated *in vitro* with PMA and ionomycin were stained intracellularly for IL-4. It was found that splenocytes from pVAX-IL-35 experimental group had a significant reduction in IL-4 expressing $CD4^+$ $TCR\beta^+$ cells (0.56 ± 0.14%) as compared to the pVAX-1 control group (0.73 ± 0.04%) (Fig 4.6B). In addition, IL-4 expressing $CD4^+$ cells were also significantly reduced in the pVAX-IL-35 experimental group (1.31 ± 0.3 %) as compared to the pVAX control group (1.93 ± 0.16 %) (Fig 4.6B). These results suggested that IL-35 may suppress the Th2 cytokine production of donor Th2 cells *in vivo*.

4.2.7 IL-35 reduced neutrophil infiltration, chemokines and proinflammatory cytokines in a neutrophilic airway inflammatory murine model

We next assessed if local delivery of pVAX-IL35 intratracheally can attenuate neutrophilia in Blo t 5 TCR transgenic mice. Intratracheal instillation of 400 μg of pVAX-IL-35 reduced the total cell infiltration in the BALF (1.17×10^6 *vs* 2.77×10^6) when compared to pVAX treated mice (Fig 4.7A). Concomitantly with the decreased cellular infiltration, lymphocytes (4.18×10^5 *vs* 1.23×10^6) and neutrophils (3.29×10^5 *vs* 9.92×10^5) infiltration were also significantly attenuated in the BALF of pVAX-IL35 treated mice (Fig 4.7B). Proinflammatory cytokines such as IL-1α, IL-6, IL-17, IFN-γ and other cytokines that play a role in promoting the survival, proliferation and activation of neutrophils and other cell types such as G-CSF and LIF also showed a significant reduction in the BALF of pVAX-IL35 treated group (Fig 4.7C). Chemokines that played a chemoattractant role for monocytes and activated T cells such as CCL3(MIP-1α), CCL4(MIP-1β), CCL5(RANTES), CXCL1(KC), CXCL-9(MIG) and CXCL-10(IP-10), were significantly attenuated in BALF of experimental pVAX-IL-35 group compared to the control pVAX group (Fig 4.7D). These results suggested that pVAX-IL-35 could suppress neutrophil chemoattractant and activation chemokines as well as proinflammatory cytokines, hence reducing neutrophilia.

Figures

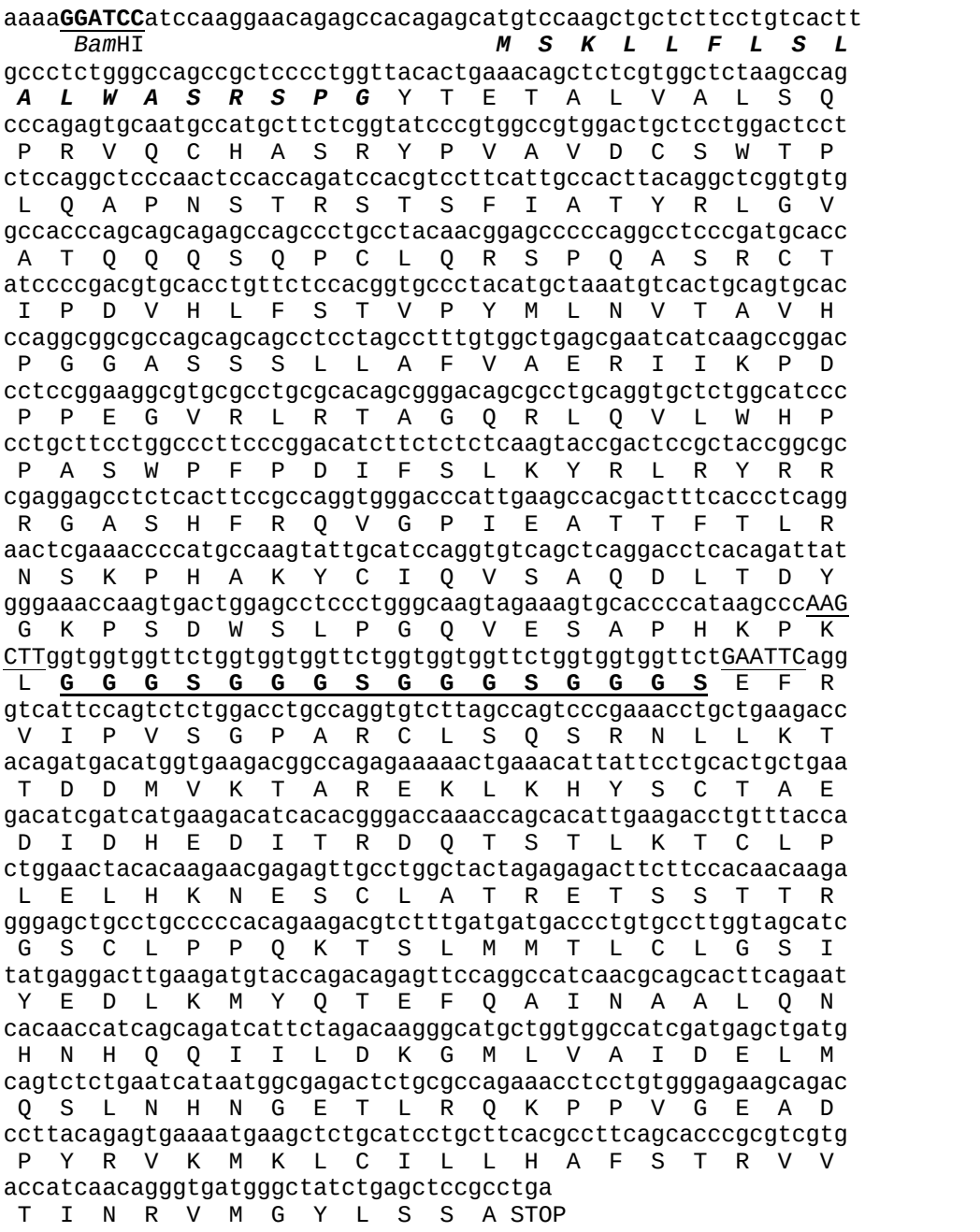


Figure 4.1: Nucleotide and amino acid sequences of constructed single chain IL-35

Single chain IL-35 was constructed by the fusion of EBI3, linker of 4 x GGGS repeats and IL-12p35. Leader sequence of EBI3 is in italics and highlighted, linker sequence is underlined and highlighted. Restriction enzymes sites are in captital letters and underlined. Single chain IL-35 was cloned into the multiple cloning sites of pVAX-1 at the *BamHI and NheI* sites.

Figure 4.2: **mRNA expression of EBI3, IL-12p35 and single chain IL-35 in the lungs**

Mice were given an intratracheal instillation of 400 µg of pVAX-IL-35 and they were sacrificed one day later. The lungs of mice were excised and total RNA extracted. RT-PCR was carried out to detect the single chain IL-35.

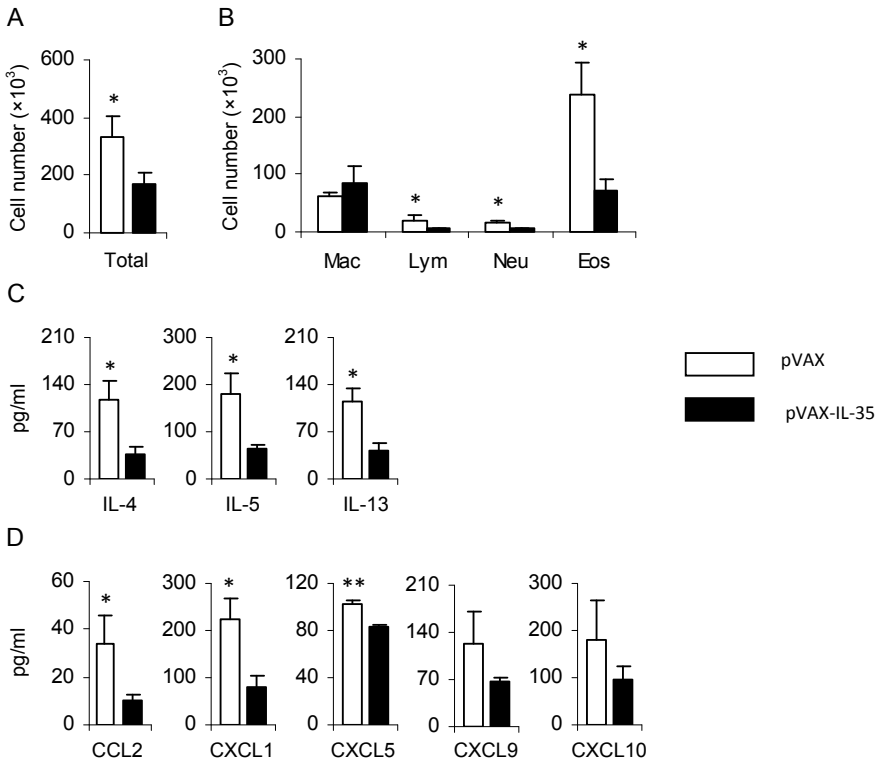


Figure 4.3: Effects of intratracheal instillation of pVAX-IL-35 on the suppression of Blo t 5-specific Th2 induced airway inflammation.

The C57BL/6 mice were given an intratracheal instillation of 400 µg of either pVAX control or pVAX-IL-35 (IL-35) on day 0, followed by intravenous injection of 2 x 10^6 Th2 cells on day 1 and intranasal instillation of 50 µg of Blo t 5 protein on days 2, 3, 4. The mice were sacrificed on day 5 and the BALF was obtained (A) Total cell counts (n = 6-7) and (B) differential cell counts for macrophages, lymphocytes, neutrophils and eosinophils in the BALF were performed. (C) Soluble mediators in the BALF was assessed by Milliplex multi-analyte profiling for cytokines IL-4, IL-5 , IL-13 and chemokines CCL2 (MCP-1), CXCL1 (KC), CXCL5 (LIX), CXCL9 (MIG) and CXCL10 (IP10).Results are presented as mean ±SEM. Data are the representative of two separate experiments,*: $p < 0.05$; **: $p < 0.005$.

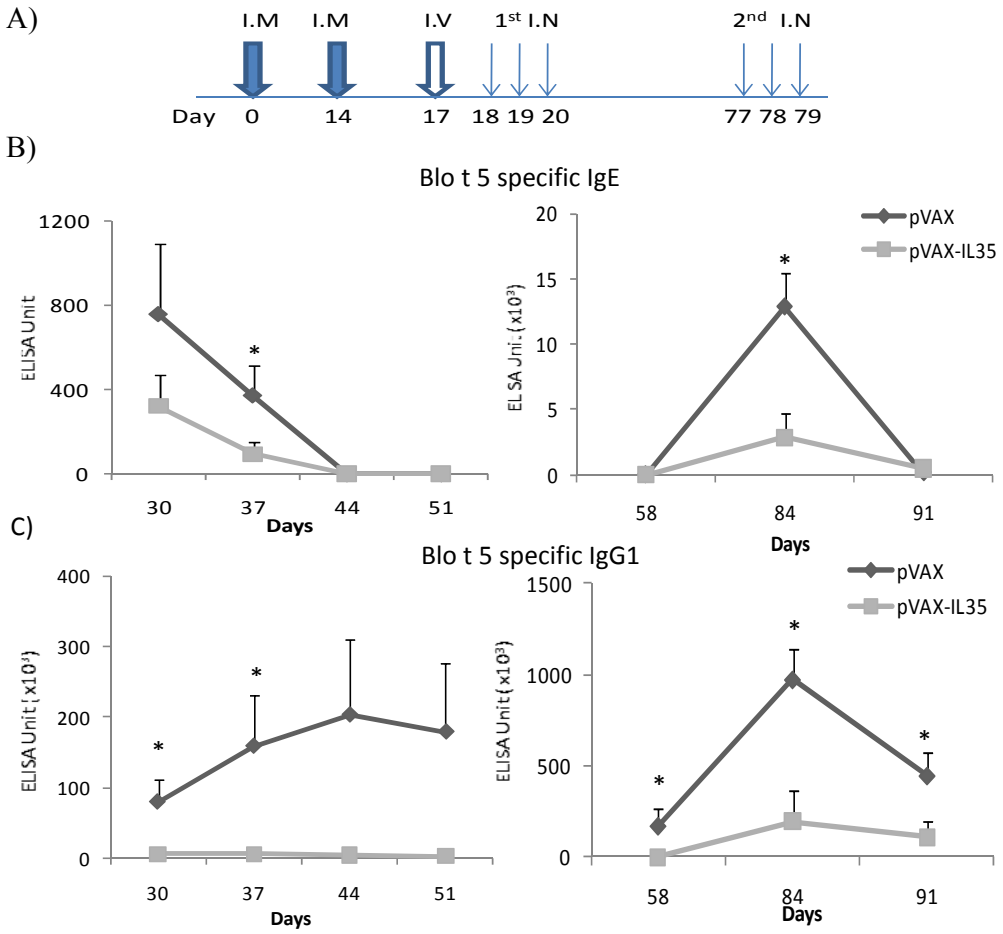


Figure 4.4: Effect of intramuscular injection of pVAX-IL-35 on humoral responses *in vivo*.

(A) The schematic diagram shows the protocol of the animal experiment. Mice (n=7) were given intramuscular (i.m.) injection of 50 µg of pVAX or pVAX-IL-35 on days 0 and 14. All mice were given the intravenous (i.v.) transfer of Blo t 5-specific Th2 cells on days 17 and followed by 2 courses (1st and 2nd) of 3 consecutive days of intranasal (i.n.) Blo t 5 challenges at indicated days. The levels of Blo t 5-specific IgE (B), IgG1 (C) were assayed by sandwich ELISA. Results are presented as mean ±SEM. Data are the representative of three separate experiments for results obtained within day 37 and two separate experiments for results obtained till day 91.*:$p < 0.05$.

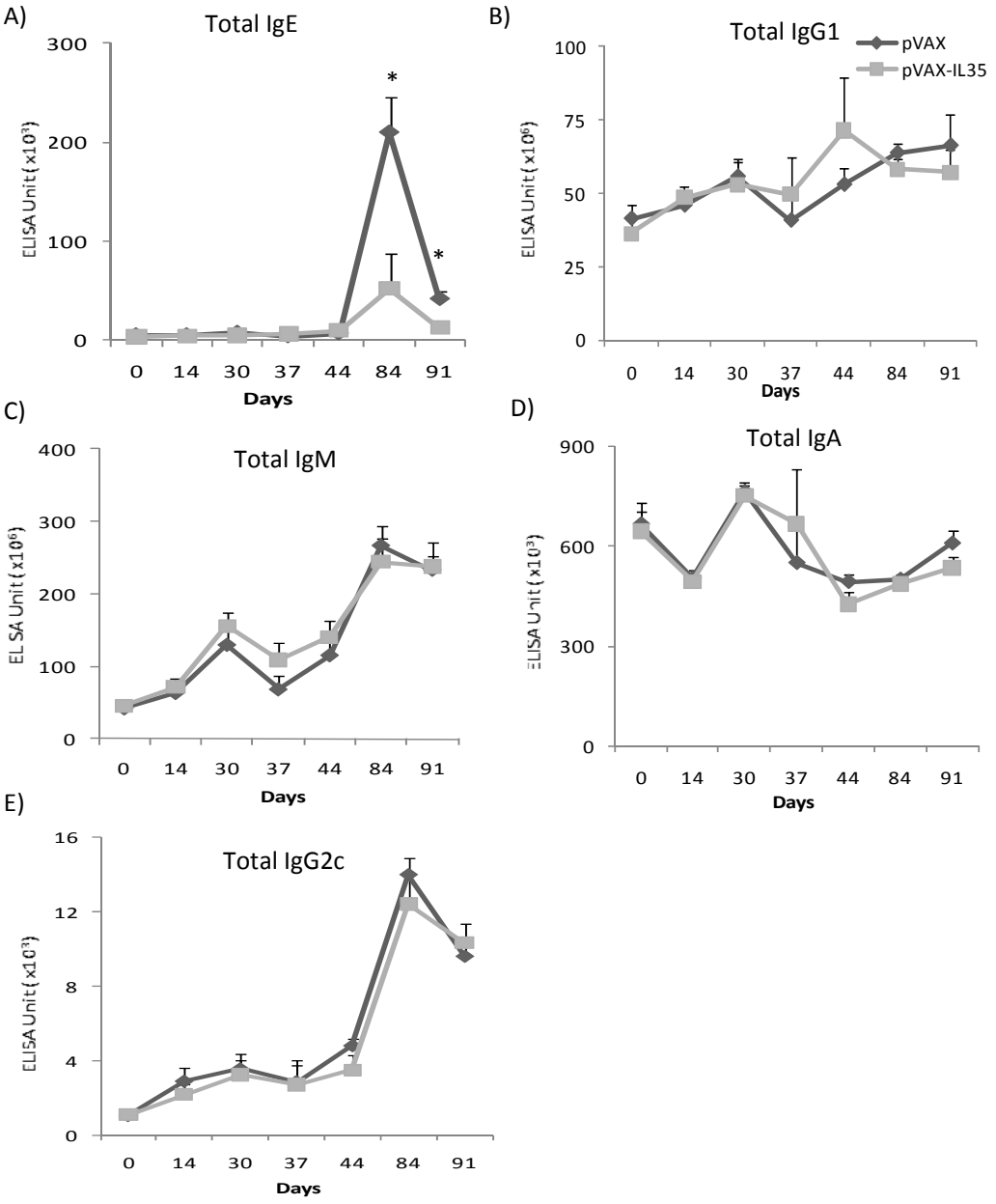
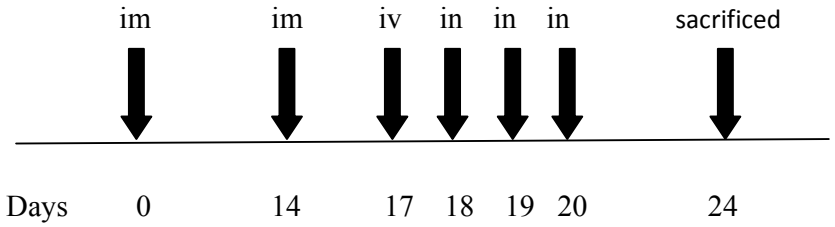


Figure 4.5: **Total serum immunoglobulin from mice treated with pVAX and pVAX-IL-35.**

The experimental protocol was the same as in Figure 4.4a. Sera were collected on indicated days and analyzed by sandwich ELISA. Results were presented as mean ± SEM (n = 7). Data were the representative of three separate experiments for results obtained within day 37 and two separate experiments for results obtained till day 91. *: $p < 0.05$.

A



B

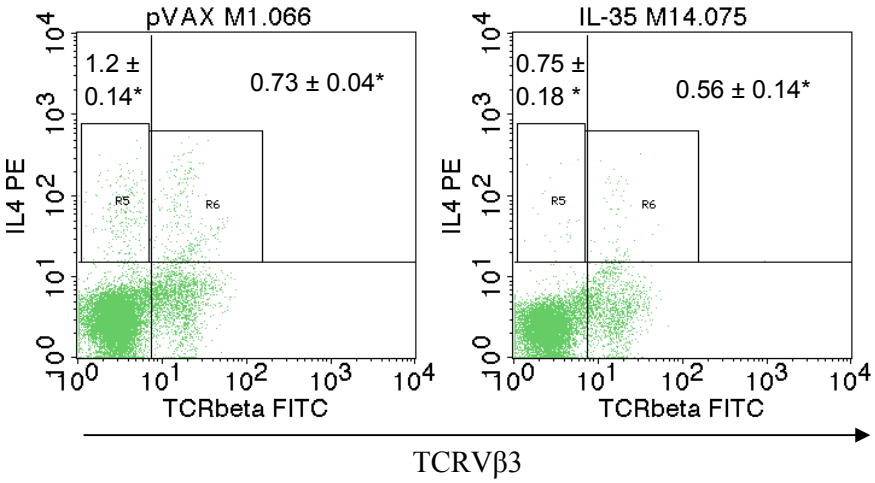


Figure 4.6: Effect of intramuscular injection of pVAX-IL35 on IL-4 in donor Th2 cells

A) The treatment timeline of mice. Mice (n = 7) were immunized with 50 µg of either pVAX empty vector or pVAX-IL35 twice at 2 weeks interval. The mice were then sensitized to Blot 5 by intravenous administration of 2 x 10^6 Blot 5 specific Th2 cells three days after the second immunization and then boosted with intranasal instillation of 50 µg of Blot 5 protein on 3 consecutive days. 1 week after the intranasal instillation, the mice were sacrificed and spleens were excised. B) Intracellular IL-4 staining of $CD4^+TCRV\beta3^+$ cells. Splenocytes of pVAX and pVAX-IL-35 treated mice were stimulated *in vitro* with PMA and ionomycin, in the presence of monesin for 8 hours. $CD4^+$ cells expressing both TCRVβ3 and IL-4 were analysed by flow cytometry. Data was presented as the mean ± SEM of the $CD4^+TCRV\beta3^+$ cells expressing IL-4 (n = 5-7). * statistical significance between pVAX-treated mice and pVAX-IL-35 treated mice, $p < 0.05$

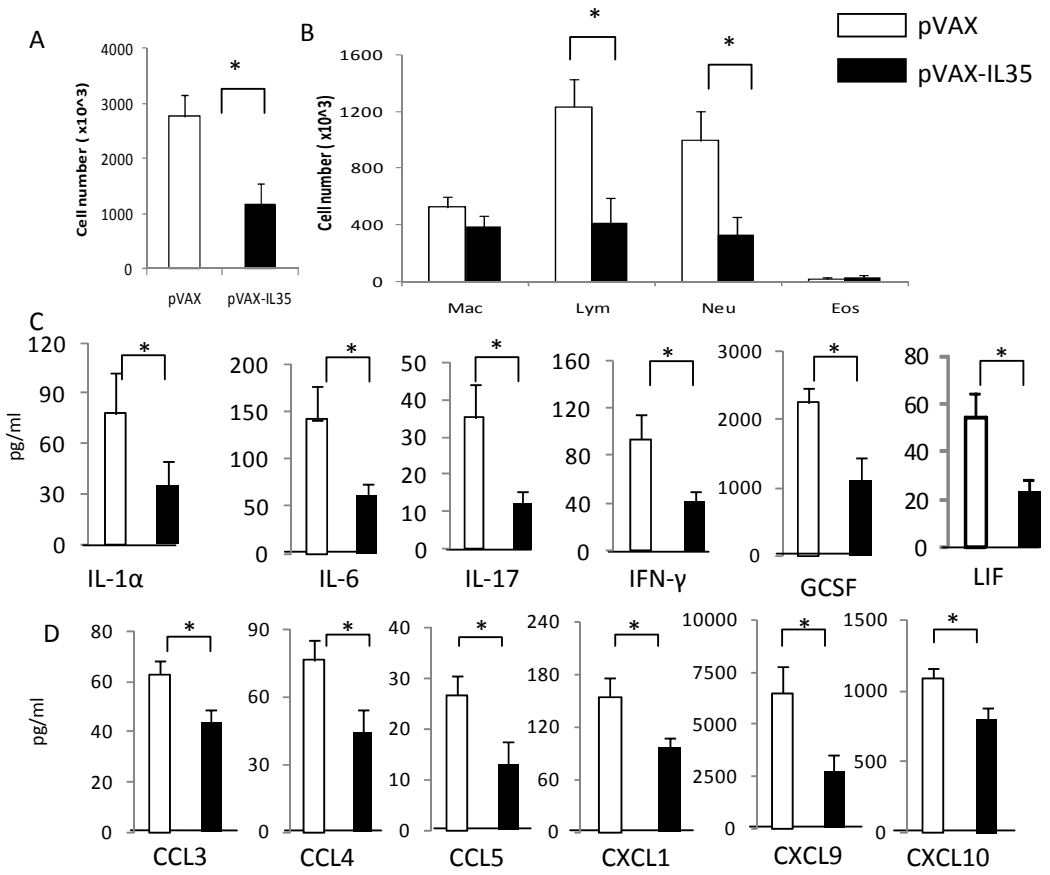


Figure 4.7: Effects of intratracheal instillation of pVAX-IL-35 on the suppression of airway inflammation in Blo t 5 TCR transgenic mice

The Blo t 5 TCR transgenic mice were given an intratracheal instillation of 400 µg of either pVAX control or pVAX-IL-35 (IL-35) on day 0, and intranasal instillation of 50 µg of Blo t 5 protein on days 1, 2, 3. The mice were sacrificed on day 4 and the BALF was obtained. (A) Total cell count (n = 8-9) and (B) differential cell counts for macrophages, lymphocytes, neutrophils and eosinophils in the BALF were performed. (C) Soluble mediators in the BALF was assessed by Milliplex multi-analyte profiling for cytokines IL-1α, IL-6, IL-17, IFN-y, GCSF, LIF and chemokines CCL3 (MIP-1α), CCL4(MIP-1β), CCL5 (RANTES), CXCL1 (KC), CXCL9(MIG) and CXCL10 (IP10).Results are presented as mean ± SEM. Data are the representative of two separate experiments,*: $p < 0.05$.

4.3 Discussion

One of the most common chronic diseases in the world is asthma with around 300 million people of all ages and ethnic groups suffering from asthma (Masoli et al., 2004). The prevalence rate of asthma had been reported to increase with the increased urbanization of communities (Masoli et al., 2004). Asthma remains a major problem as main therapies to treat asthma currently such as glucocorticoids do not provide a long term solution to control asthma. As such, attention had been shifted to find new potent therapies for asthma and T regulatory cells as well as cytokines produced by T regulatory cells are gaining importance in the field. Cytokine gene immunization might be a promising approach. The potent inhibitory effects of IL-35, a cytokine produced by T regulatory cells, could be harnassed through the use of DNA vaccines.

Recent studies revealed that IL-35 was a novel cytokine that was constitutively expressed in murine $CD4^+CD25^+$ Treg cells and contributed to their suppressive function (Collison et al., 2007b). Other groups had shown in earlier studies that IL-35 was an inhibitor of Th17 cell differentiation *in vitro* and *in vivo* where IL-17 production was found to be suppressed with the simultaneous upregulation of IFN-γ synthesis in a mouse model of collagen induced arthritis (Niedbala et al., 2007). An immunosuppressive role of IL-35 in inflammatory bowel disease murine model also had been reported whereby wild type Treg cell recipients recovered from disease, had weight gain and decreased histopathology (Niedbala et al., 2007). IL-35 production by Treg cells had been found to be enhanced upon contact with conventional T cells, this suppression was dependent on IL-35 and IL-10, in addition maximal Treg cell suppression abilities was brought about by conventional T cell activation (Collison et al., 2009). However there had been no prior studies to determine the effect of IL-35

on allergic diseases. Allergic diseases conventionally had been thought to be only Th2 mediated with the characteristic infiltration of increased numbers of eosinophils, Th2 lymphocytes, activated mast cells and airway remodeling (Bousquet et al., 2000; Pascual and Peters, 2005). However, cellular infiltration profiles of asthma were not only characterized by eosinophilia, neutrophilia was also a characteristic feature of another subtype of asthma. Studies on neutrophilic asthma had shed light on the involvement of IL-17, the hallmark cytokine produced by Th17 cells (Hellings et al., 2003). Hence in our study, we employed both the eosinophilic and neutrophilic murine models of airway inflammation.

Studies on IL-35 had been hampered by the fact that production of recombinant IL-35 was challenging due to the low expression level and the poor stability of the recombinant IL-35 protein. To circumvent problems associated with the production of the recombinant IL-35 protein, we adopted both local and systemic plasmid DNA encoding IL-35 gene delivery approaches. Administration of pVAX-IL-35 had been found to exert immunosuppressive effects on effector Th2 cells when introduced both locally and systemically. Th2 cells were reported to express Th2 cytokines such as IL-5 and IL-13 which could augment the inflammation in allergic asthma (Hamid et al., 1991; Kay et al., 1991). Administration of IL-35 encoded plasmid intratracheally suppressed cellular infiltration into the lungs, in particular eosinophil and lymphocyte infiltration. Th2 effector cytokines as well as CC- and CXC- motif chemokines that were chemoattractants for polymorphic mononuclear cells and lymphocytes were also attenuated in BALF from mice that received pVAX-IL-35. Th2 cytokines, IL-5, IL-4 and IL-13 and chemokines such as CCL2 (MCP-1), CXCL1 (KC), CXCL5 (LIX), CXCL9(MIG) and CXCL10 (IP10) that played a chemoattractant role for neutrophils,

monocytes, macrophages and T cells were reduced in the BALF from mice administered with pVAX-IL-35 compared to mice that received pVAX.

IL-5 was the key cytokine that was involved in the recruitment, proliferation, differentiation and release of eosinophils from the bone marrow (Broide, 2002; Cho et al., 2004; Lopez et al., 1988; Rothenberg, 1998). Other Th2 cytokines like IL-4 and IL-13 facilitated the migration of eosinophils by increasing the expression of cell adhesion molecules in vascular endothelial cells (Meager, 1999). Another study had also demonstrated that IL-5 producing antigen-specific Th2 cells when adoptively transferred into unprimed mice can result in eosinophilic inflammation and this was IL-5 dependent as anti-IL-5 neutralizing antibody when administered abolished eosinophil infiltration (Kaminuma et al., 1997). In a separate study, it was demonstrated that the intranasal instillation of Th2 cell supernatant brought about eosinophilia and the production of specific chemokines, in addition IL-4 and IL-5 had been reported to stimulate higher production of MIP-2 and KC while IL-4 also induced higher MCP-1 production (Li et al., 1999). The association between IL-5 and eosinophilia had been demonstrated by the above mentioned studies. In our study, with the use of IL-35 encoding plasmid, the levels of IL-5, IL-13, IL-4, eosinophils and lymphocytes were significantly attenuated, which also implied at the alleviation of the inflammation seen in allergic asthma. Intracellular staining experiments also suggested that IL-35 suppressed IL-4 production by Th2 cells. Hence IL-35 might exert a suppressive effect *in vivo* by the attenuation of Th2 cytokines produced by the transferred Th2 cells, hence resulting in a decrease in the induction of production of chemoattractant chemokines, resulting in reduced eosinophilia and cellular infiltration. In addition, mice that received the pVAX-IL-35 treatment prior to the transfer of the

Blo t 5–specific Th2 memory cell line showed a long-term suppression of allergen-specific IgE, IgG1 and total serum IgE production, without affecting concentrations of other immunoglobulin isotypes in sera. This was the first study to demonstrate that DNA vaccine encoding IL-35 could be a potent immunosuppressive vaccine that was capable of long term protection against allergic asthma. This could be partly due to the long lasting *in vivo* expression of IL-35 by the intramuscular DNA delivery (Chua et al., 2009). That provided the constitutive source of IL-35 and maintained the long term protection effect on the Blo t 5-specific IgE and total IgE production 2 months later upon the second course of Blo t 5 intranasal challenges. An alternative possibility was that the initial IL-35 expression might also expand $CD4^+CD25^+$ Treg cells or induce a new regulatory population of cells iT_R35, that was responsible for conferring the long-term protection on the Blo t 5-specific IgE and total IgE production (Collison et al., 2010; Kochetkova et al., 2010; Niedbala et al., 2007).

All B cells initially expressed surface IgM, B cells in the germinal center could be stimulated to undergo class switch recombination of Ig heavy chains via the cytokine stimulation. Class switching from IgM to IgE was mainly regulated by two combined signaling from Th2 cells. Cytokines like IL-4 and IL-13 together with CD40 could induce IgE isotype switch (de Vries et al., 1993; McKenzie et al., 1999; Poulsen and Hummelshoj, 2007). IL-4 also activated class switch recombination to IgG1 in mouse and IgG4 in humans (Hellman, 2007; Snapper et al., 1988). Total serum IgE was partly controlled by IL-13 as a strong association of single nucleotide polymorphisms in the IL-13 gene with the total serum IgE is well recognized in humans (Graves et al., 2000; Maier et al., 2006). In addition,a functional single nucleotide polymorphism in the distal IL-13 promoter among variants linked to total serum IgE had been verified

that indicated the association of IL-13 level with the total serum IgE (Kiesler et al., 2009). Long-lived IgE producing plasma cells were not easily exterminated by therapeutics as they did not undergo cell division and were often located in localized areas like the bone marrow (Shapiro-Shelef and Calame, 2005). IgE though present in much lower levels than IgG, played a vital role in the induction of inflammation as it was able to bind to and cross-link high affinity receptors on mast cells and basophils. Immense efforts had been spent on clinical studies on antibodies against the receptor-binding region of human IgE (Nowak, 2006). However, this might not be the optimal solution due to its high cost as huge quantities of recombinant protein had to be administered to the patients (Buhl, 2005). Thus DNA vaccines encoding IL-35 might be a promising therapeutic tool as it had been shown to attenuate allergen specific and total IgE. The suppressive effects of IL-35 on Blo t 5-specific IgE and total serum IgE could be through the inhibition of Blo t 5-specific Th2 effector cytokines IL-4 and IL-13 by IL-35 as these cytokine levels in the BALF were also reduced in pVAX-IL-35 treated mice.

Besides suppressing Th2 mediated eosinophilia, IL-35 also had a suppressive effect on neutrophilia. There was significant reduction in Blo t 5 challenges induced neutrophils as well as lymphocytes in the airway of Blo t 5 specific TCR transgenic mice. In addition, neutrophil chemoattractant and activation chemokines, IL-17, IFN-γ as well as proinflammatory cytokines in the BALF were also significantly attenuated in the pVAX-IL-35 treated group. Allergen inhalation had been reported to increase IL-17 expression in airways of murine models (Hellings et al., 2003). The involvement of IL-17 in the bronchial neutrophilic influx had been confirmed via the use of anti-IL17 monoclonal antibody before allergen inhalation that attenuated

neutrophilia (Hellings et al., 2003). Neutrophil count had been reported to have a positive correlation with IL-17 mRNA expression in sputum of asthmatic patients (Bullens et al., 2006). In addition, severe asthmatics had also been found to have increased neutrophils in their airways (Jatakanon et al., 1999). Besides targeting at cytokine genes, IL-17 target genes also included neutrophil attracting chemokines as well as CC chemokines such as CCL2/MCP-1 (Dragon et al., 2007; Hata et al., 2002; Henness et al., 2004; Jones and Chan, 2002; Khader et al., 2007; Ruddy et al., 2004; Shen and Gaffen, 2008; Takaya et al., 2002; Witowski et al., 2000). All this evidence hinted that IL-17 played a major role in the recruitment and activation of neutrophils during an inflammatory response via the induction of chemokines and cytokines. In a recent study, inducible costimulator positive positive Treg cells produced IL-35 and this resulted in the suppression of IL-17 as well as the reversal of established IL-17 dependent AHR in murine models (Whitehead et al., 2011).The suppressive effect of IL-35 on neutrophilia could be through the inhibition of IL-17.

Another study had reported that the adoptive cell transfer of a TH17 polarized population into mice could convert to IFN-γ producers that can produce either both IFN-γ and IL-17 or IFN-γ only in the lung (Ashino et al.). This IFN-γ was found to be vital for the induction of airway hyperresponsiveness (Ashino et al.). IFN-γ could also enhance Th2 mediated immune responses (Bocek et al., 2004). In our study, we too had seen IFN-γ and IL-17 in the BALF of the mice that had neutrophilic inflammation in the lungs, importantly, IL-35 treated mice showed reduction in both IFN-γ and IL-17 in the BALF compared to controls. This suggested that both cytokines IFN-γ and IL-17, responsible for neutrophilia and airway hyperesponsiveness had been suppressed by IL-35. Hence reduction of IL-17 thus seemed to be a promising

strategy to alleviate neutrophilic asthma compared to anti-inflammatory agents that may have non-specific mode of action. However, it had also been pointed out that the use of anti-IL-17 monoclonal antibody increased circulating IL-5 as well as IL-5 levels in the BALF in anti-IL-17 antibody treated mice compared with controls (Hellings et al., 2003). In addition, anti-IL17 monoclonal antibody also elevated eosinophilia (Hellings et al., 2003). Thus anti-IL-17 antibodies might not be the best therapeutic tool. IL-35 may prove to be a better candidate given its robust suppressive effect on eosinophilia and neutrophilia, besides eosinophilia was not significantly upregulated with the use of DNA vaccine encoding IL-35.

Besides the secretion of IL-35, T regulatory cells also secreted IL-10 and TGF-β. The pleiotropic cytokine, TGF-β, was mainly secreted by eosinophils while other cells such as mast cells, lymphocytes, macrophages, fibroblasts and epithelial cells also produced TGF-β (Leivonen et al., 2005; Samarakoon et al., 2005; Xu et al., 2003). Although TGF-β had been reported to suppress allergen-induced inflammation when delivered intratracheally (Joetham et al., 2007), it had also been demonstrated to be involved in the stimulation of mucus hypersecretion, globet cell proliferation as well as tissue remodeling (Makinde et al., 2007; McMillan et al., 2005). Airway remodeling, peribronchiolar extracellular matrix deposition, proliferation of airway smooth muscle cell and mucus production in the lung was attenuated upon neutralizing anti-TGF-β antibody administration to mice with established airway inflammation in a treatment model (McMillan et al., 2005). Another study reported that intratracheal instillation of recombinant TGF-β protein in mice brought about an increase in collagen deposition in the airways as well as increased mRNA expression of collagen I and III (Kenyon et al., 2003). In addition, in asthmatic patients, serum

TGF-β was significantly elevated compared to control patients (Manuyakorn et al., 2008). In bronchoalveolar lavage fluid of asthmatic patients, TGF-β1 was also elevated and further increased when exposed to allergen (Redington et al., 1997). Due to the detrimental effects of TGF-β in asthma, TGF-β, though also a cytokine produced by T regulatory cells, was not considered as a candidate for DNA vaccine in our study. Mortality was significantly increased in mice with overexpression of IL-10 in the lung when they were challenged intratracheally with *P.aeruginosa* bacteria compared to control mice (Sun et al., 2009a). Bacterial clearance was also hindered in mice that had overexpression of IL-10 in the lungs (Sun et al., 2009a). As such, IL-10 was also not an ideal candidate for DNA vaccine. Hence with the possible detrimental effects of TGF-β and IL-10, they were not considered as therapeutic tools in our study although they were also produced by Treg cells.

Initially, there had been some controversy to the presence of IL-35 in humans. In contrast to murine models, it had been demonstrated that human $CD4^+CD25^+Foxp3^+$ T reg did not show a constitutive expression of IL-35, in addition neither EBI3 nor p35 mRNA was upregulated upon the induction of Foxp3 (Bardel et al., 2008). However recently, IL-35 was found to be expressed and needed by human T regulatory cells to exert the maximal suppressive effect (Chaturvedi et al., 2011). In addition, IL-35 from human T regulatory cells also converted suppressed human T conventional cells into new regulatory population of cells iT_R35 that also exerted suppressive effect via IL-35 (Chaturvedi et al., 2011). In another study, it had been demonstrated that human rhinovirus activated dendritic cell (R-DC) could induce $CD4^+$ and $CD8^+$ peripheral blood T cells to produce and release IL-35 through the inhibitory signals delivered from R-DC to T cells via B7-H1 and sialoadhesin. This

showed that human IL-35 producing T reg could be induced (Seyerl et al.). IL-35 had also been detected in the $CD4^+$ T cells obtained from peripheral blood of chronic Hepatitis B patients but not from healthy subjects (Liu et al., 2011). All this suggested the presence of immunosuppressive effect of IL-35 in human immune system, hence the IL-35 gene delivery might be feasible for application in humans.

IL-35 fusion protein was relatively unstable and may be technically difficult to produce and store. With the use of IL-35 encoding DNA vaccines, this problem is circumvented as DNA vaccines make use of the host machinery to produce the recombinant protein. In addition, the relative ease of production of DNA vaccines, low cost, stability and safety as well as no requirement for cold chain were more advantages of DNA vaccines. In conclusion, in this study we showed that IL-35 was a very promising therapeutic tool against effector T cells mediated airway inflammation as it exerted a potent suppressive effect on cellular infiltrates and cytokines/chemokines in the BALF, allergen specific IgE and IgG1 production as well as total IgE production.

Chapter 5 Conclusion and Perspectives

5.1 Conclusions

The first part of the study demonstrated an allergy model whereby intraperitoneal immunization of mice with rBlo t 5 protein adjuvanted with alum elicited a mixed T helper phenotype consisting of Th1, Th2 and Th17 immune responses. This mixed Th1, Th2 and Th17 immune responses could be suppressed by allergen gene vaccination. pVAXBlot5-DTKT immunized mice showed a trend of greater suppression in Blo t 5 specific IL-5, IL-6, IL-13 and IL-17 when compared to pVAXBlot5 immunized mice. In addition to the enhanced suppressive effect on T helper cytokine production, pVAXBlot5-DTKT immunized mice also elicited stronger humoral responses as evident from the higher levels of Blo t 5 specific IgG1 and IgG2c when compared to pVAXBlot5 immunized mice. Mice immunized with Blo t 5 constructs effectively suppressed Blo t 5 specific IgE production induced by intraperitoneal immunization with rBlo t 5 protein adjuvanted with alum. CpG modified plasmid DNA immunized mice showed a greater suppression of Blo t 5 specific T effector cytokines as well as enhanced Blo t 5 specific IgG2c production as compared to unmodified plasmid DNA immunized group.

In vitro studies carried out using dendritic cells derived from bone marrow with either GMCSF or Flt3L demonstrated the differences of cytokine and chemokine profiles in response to pVAXBlot5 or pVAXBlot5-DTKT stimulation. Flt3L-DCs were found to be more responsive to pVAXBlot5-DTKT stimulation as it produced higher levels of G-CSF, IL-1α, KC, MCP-5, VCAM-1, VEGF, MCP-1, PF-4, TCA-3 while GMCSF-DCs produced elevated levels of MIP-1α . In addition, subsets of Flt3L-DCs also responded differently to plasmid DNA stimulation. $CD11c^{+}CD11b^{+}B220^{-}$ DCs

produced elevated amounts of IL-6, IL-12p40 and IP-10 when compared to $CD11c^{+}CD11b^{-}B220^{+}$ DCs in response to pVAXBlot5-DTKT stimulation. Both pVAXBlot5 as well as pVAXBlot5-DTKT elicited similar levels of cytokine production and upregulation of surface markers on dendritic cells. TLR9 was involved in the activation of dendritic cells by plasmid DNA as inhibition of TLR9 sharply attenuated the production of cytokines and chemokines.

pVAXBlot5-DTKT elicited significantly higher production of IL-6, MIP-1α and MIP-1β from human peripheral blood mononuclear cells than pVAXBlot5. This enhanced immunogenic effect of pVAXBlot5-DTKT was not observed in mouse cells. The protein array was used to explore the differences between pVAXBlot5-DTKT and pVAXBlot5 stimulated human peripheral blood mononuclear cells and a dynamic change in the upregulation profile of cytokines and chemokines at early (5 hours) and later (24 hours) timepoints was observed. At an early timepoint of 5 hours, pVAXhBlot5-DTKT induced significant upregulation of PDGF-AA, GRO-α, NT-4, IL-1β, osteoprotegerin, VEGF-D, GMCSF, MCP-1 and ENA-78 and downregulation of ErbB3, IP-10, angiogenin, IGFBP-2 and MCP-2 compared to pVAXhBlot5. At a later timepoint of 24 hours, pVAXhBlot5-DTKT induced significant upregulation of angiogenin, Flt3 ligand, I-309, IL-10, MCP-1, MCP-4, MIP-3α, PARC, TARC and TECK with downregulation of IL-12p40 as compared to pVAXhBlot5. *In vivo* analysis was carried out by the immunization of non-human primates with pVAX-1, pVAXhBlot5 and pVAXhBlot5-DTKT. These monkeys were subsequently received subcutaneous immunizations with rBlo t 5 protein adjuvanted with alum. Immunogenicity of allergen gene constructs was demonstrated by faster kinetics and levels of Blo t 5 specific IgG productions. In pVAXhBlot5-DTKT immunized group,

there was a trend of higher production of Blo t 5 specific total IgG and IgG1 as compared to pVAXhBlot5 group. Allergen gene immunized groups showed attenuation of Blo t 5 specific IgE as compared to pVAX-1 immunized group. Notably, pVAXhBlot5-DTKT immunized group yielded the highest number of IFN-γ producing cells and lowest number of IL-4 producing cells in response to stimulation with three Blo t 5 peptide pools.

The second part of the study demonstrated the use of the immunoregulatory cytokine, IL-35, in the suppression of two airway inflammation models. The allergic airway inflammation model was induced by adoptive transfer of Blo t 5 specific Th2 cells followed with Blo t 5 challenges and the neutrophilic airway inflammation model was induced by intranasal instillations of Blo t 5 in Blo t 5 specific TCR transgenic mice. In the allergic airway inflammatory model, intratracheal administration of pVAX-IL-35 significantly reduced total cellular infiltration, eosinophils, lymphocytes and neutrophils numbers in the BALF. In addition, CCL2/MCP-1, CXCL1/KC, CXCL5/LIX, CXCL9/MIG and CXCL10/IP-10 concentrations in the BALF were also attenuated in mice of the experimental pVAX-IL-35. Intramuscular immunization of mice with plasmid DNA encoding IL-35 prior to the transfer of Blo t 5-specific Th2 cells provided both short term as well as long term suppression of Blo t 5 specific IgE production after intranasal Blo t 5 instillation. In addition to the suppression in Blo t 5 specific IgE, IL-35 also exerted a long lasting suppressive effect on the production of total serum IgE and IL-35 did not exert a pan immunosuppressive effect on antibody production. A possible mechanism behind the suppressive effect of IL-35 might be the via suppressive effect on the Th2 cytokine production of donor Th2 cells as

pVAX-IL-35 treated mice was found to have reduced IL-4 expressing $CD4^{+}TCRV\beta3^{+}$ cells.

In the neutrophilic airway inflammatory model, intratracheal delivery of pVAX-IL35 could also attenuate neutrophilia in Blo t 5 specific TCR transgenic mice. Total cellular infiltration and lymphocytes were also significantly reduced in pVAX-IL35 treated mice. Proinflammatory cytokines and chemokines such as IL-1α, IL-6, IL-17, IFN-γ, G-CSF, LIF, CCL3(MIP-1α), CCL4(MIP-1β), CCL5(RANTES), CXCL1(KC), CXCL-9(MIG) and CXCL-10(IP-10) also showed a significant reduction in the BALF of pVAX-IL35 treated mice. pVAX-IL-35 treatment could suppress neutrophil chemoattractant and activation chemokines as well as proinflammatory cytokines and hence reduce neutrophilia.

In summary, the results from the first part of the study had shed light on the probable regulatory effects of allergen gene vaccination. Allergen gene vaccinated groups produced higher levels of IFN-γ and IL-10, this may suggest the possible induction of T regulatory cells, T_H1 T_R cells, by allergen gene vaccination with the subsequent suppressive effect on the mixed T cell responses induced by intraperitoneal immunization with Blo t 5 protein adjuvanted with alum. In addition, CpG motif modified plasmid DNA was also found to have enhanced efficacy on human cells and in non-human primates. This will contribute valuable information to help in the development of more potent vaccines in prevention of allergic asthma. The use of IL-35 as a cytokine gene in DNA vaccines had revealed the potent suppressive effect of IL-35 on eosinophilia as well as on neutrophilia. Hence IL-35 had great potential in

the control of airway inflammation and this may be useful in the development of therapeutic tools to control airway inflammation.

5.2 Future Studies and Perspectives

Future Studies:

There are several aspects in this study that require further investigation.This study had shown that immunization with pVAXBlot5-DTKT resulted in a suppressive effect on the mixed Th1, Th2 and Th17 immune responses elicited by intraperitoneal immunization with rBlo t 5 protein adjuvanted with alum. To evaluate if the proposed mediators such as IFN-γ, IL-10 or GMCSF are involved, depletion studies using neutralizing antibodies can be carried out. An alternative way for evaluation of the possible mediators is to use knock out mice. This will enable us to evaluate the mechanism behind the mode of action of DNA vaccines.

Protein array studies on plasmid DNA stimulated human peripheral mononuclear cells had revealed that pVAXBlot5-DTKT stimulated human PBMC had higher production of cytokines and chemokines as compared to pVAXBlot5 stimulation. These mediators may shed light on possible mechanisms or signalling cascades due to the effect of additional CpG motifs.

pVAX-IL-35 had been demonstrated to play a suppressive role in this study, however it has yet to be evaluated if IL-35 could also control established airway inflammation. Hence to evaluate if pVAX-IL-35 could suppress established airway inflammation, mice could be first induced to develop airway inflammation via the transfer of Blo t 5 specific Th2 cells and intranasal Blo t 5 challenges before the subsequent delivery of pVAX-IL-35. This will contribute knowledge to the efficacy of IL-35 as a therapeutic tool to treat airway inflammation.

The suppressive effect of IL-35 had been shown to be mediated by IL-35 induced Treg cells, IL-35 producing inducible costimulator positive Treg cells or IL-10 producing $CD39^{+}CD4^{+}$ T cells. However it has yet to be evaluated if IL-35 can directly exert the suppressive effect on effector T cells like Th2 cells and that deserves further attentions and studies.

Perspectives

1) As CpG modified plasmid DNA showed enhanced immunogenicity on human cells and in non-human primates, this suggests that backbone modification is still a useful approach to improve the immunogenicity of DNA vaccines.

2) Inhibition of TLR9 attenuated the cytokines and chemokines production from plasmid DNA stimulated dendritic cells hence it implied that TLR9 though non essential but it is still involved, hence strategies to target at TLR9 is still useful.

3) DNA vaccines are a promising tool against allergic diseases as they have been shown to suppress a mixed T helper phenotype response.

4) IL-35 had been shown to elicit suppressive effect in neutrophilic and eosinophilic airway inflammation murine models, hence the potent immunosuppressive effect of IL-35 can be harnessed for use in allergic diseases.

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