UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

The Immune Response Of Pregnant Women And Neonates To Ovalbumin And β-Lactoglobulin In Relation To Maternal Dietary Intake Of Hen's Egg And Cow's Milk During Pregnancy And The Development Of Atopic Eczema In The Infant

by

Claire Ann Powell

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ABSTRACT

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THE IMMUNE RESPONSE OF PREGNANT WOMEN AND NEONATES TO OVALBUMIN AND β-LACTOGLOBULIN IN RELATION TO MATERNAL DIETARY INTAKE OF HEN'S EGG AND COW'S MILK DURING PREGNANCY AND THE DEVELOPMENT OF ATOPIC ECZEMA IN THE INFANT

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The increasing prevalence of allergic disease over the last 30 years has created a significant health/economic burden on sufferers, health services and the community. This necessitates continued research to understand the basic mechanisms and causes, and to identify targets for prevention or treatment. The present study investigates the maternal intake of egg and milk during pregnancy, its impact on the development, and the characteristics of the foetal immune response to those food allergens. Here we have focused on features of cord blood that could predict an allergic outcome, in particular characterisation of memory and homing markers. In carrying out these aims, the following hypotheses were addressed: (1) Atopy in early childhood (predominantly associated with food allergy) is related to maternal consumption of egg and milk allergens; (2) There is evidence of altered T cell function at birth in neonates who go on to develop atopic eczema and; (3) The clinical manifestations of food allergy in early childhood are determined by tissue specific homing molecules expressed on specifically sensitised cord blood mononuclear cells (CBMCs).

High risk pregnant women were recruited onto the study (n = 51) and had their dietary intake of egg and milk recorded. Mothers were divided into two groups dependent on their infant's development of atopic eczema (AE), or lack of development of eczema (NE), in the first 12 months of life.

Early development of AE in the infant had no association with maternal dietary intake of egg and milk. A tight correlation between maternal specific IgG, IgG_1 and IgG_4 and cord blood specific IgG, IgG_1 and IgG_4 were detected for both ovalbumin and β-lactoglobulin. Hence this confirms transplacental transfer of maternal specific IgG and IgG subclasses to the foetal circulation. Postnatally, higher ovalbumin IgG and IgG_1 were identified in AE infants. Expression of the skin specific homing molecule cutaneous lymphocyte antigen (CLA) was significantly increased at birth in infants who went on to develop eczema indicating a possible role for CLA+ T cells in cord blood in the future development of eczema. The cord blood from AE infants also showed an increased T cell memory (CD45RO+) response after culture with allergen. However, there was no specific T cell proliferation indicating the increased CD45RO expression was due to a phenotypic switch from naïve to memory, rather than a proliferative response. This hypothesis driven study was the first to establish a putative role for the expression of CLA in cord blood as a marker for future atopic eczema development.

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Declaration of authorship

I, **Claire Ann Powell** declare that the thesis entitled:

The Immune Response of Pregnant Women and Neonates to Ovalbumin and β-lactoglobulin in Relation to Maternal Diet during Pregnancy and Development of Atopic Eczema in the Infant

and the work presented in it are my own. I confirm that:

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Date: 27th January 2010

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List of Abbreviations

2-ME	2-mercaptoethanol
AE	Atopic eczema
APC	Allophycocyanin
AU	Arbitrary units
Bet v1	Betula verrucosa
BLG	β-lactoglobulin
BS	Blood sample
BSA	Bovine serum albumin
CBMC	Cord blood mononuclear cells
CCR	Chemokine receptor
CFSE	Carboxyfluorescein succininmidyl ester
CH	Constant heavy domain
CL	Constant light domain
CLA	Cutaneous lymphocyte antigen
CSR	Class switching recombination
DBPCFC	Double-blind placebo-controlled food challenge
DEPC	Diethylpyrocarbonate
Der p1	Dermatophagoides pteronyssinus
DPP10	Dipeptidylpeptidase
DTH	Delayed-type hypersensitivity
EDTA	Ethylene diamine tetra acetate
FFQ	Food frequency questionnaire
FITC	Fluorescein isothyocyante
FOXP3	Forkhead box P3
FucT VII	Fucosyltransferase VII
GPCR	G protein coupled receptor
HEP	Histamine equivalent in prick testing
HEV	High endothelial venules
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
LFA-1	Leukocyte function-associated antigen-1
LIF	Leukemia inhibitory factor

LPR	Late-phase reaction
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAFF	Ministry of Agriculture, Fisheries and Food
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK	Natural killer T cell
NE	No eczema
OD	Optical density
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP Cy5.5	Peridinin chlorophyll protein with a cyanine dye
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PRR	Pattern-recognition receptor
PSGL 1	P-selectin glycoprotein ligand 1
SC	Secretory componenet
SCORAD	Scoring atopic dermatitis severity index
SI	Stimulation index
SPT	Skin prick test
TBS	Tris buffered saline
Tc	Cytotoxic T cell
TCM	T cell central memory
TCR	T cell receptor
TEF	T cell effector function
TEM	T cell effector memory
T_H	T helper cell
TLR	Toll-like receptor
TMB	3,5,5-tetramethylbenzidene
Treg	Regulatory T cells
UHQ water	Ultra high quality water

VH	Variable heavy domain
VL	Variable light domain
WHO	World health organisation

Chapter 1: Introduction

1.1 The Immune System

The immune response consists of complex interactions involving highly specialized cells and humoral factors, which work together to clear infectious agents from the body. These interdependent components collectively guard against bacterial, fungal, parasites and viral infections, as well as inhibiting the growth of tumour cells. Many of the cell types within the immune system have specialized functions, such as release of pro-inflammatory cellular granules (e.g. mast cells), apoptosis of infected cells (e.g. T cells), and eradication of pathogenic bacteria (e.g. macrophages). However, not all immune responses protect from disease, both foreign allergens such as those found in house dustmite, grass pollen or food and autoantibodes created to self-antigen, (auto-immune disease) can cause disease as a consequence of inducing an inappropriate immune response.

The immune system has traditionally been divided into innate and adaptive components. Both are composed of various cell types and immunologically-active molecules. The innate immune response refers to non-specific defence mechanisms and consists of mechanical barriers including skin, mucosal membranes and low pH. Innate immunity within blood and tissue is delivered principally by phagocytic cells which are able to recognise a wide range of components present on bacteria (e.g. lipoproteins, lipopeptides) via their toll-like receptor; and the complement system which circulates in the blood and are activated by microbial membranes (Takeda & Akira, 2005). The adaptive immune response is triggered when a pathogen evades the innate immune system and generates a threshold level of antigen (Murphy et al., 2008) By definition an antigen is a protein molecule which often protrudes from the surface of a cell that can induce an immune response. In contrast to innate immunity, the adaptive response is highly specific for particular pathogens and is organized around two classes of specialized lymphocytes: T cells and B cells. Combined, these lymphocytes generate a huge repertoire of antigen specific receptors on their cell surface, and

provide memory and increased protection against subsequent re-infection with the same pathogen. Inappropriate activation of components of the adaptive response underlies both auto-immune and allergic disease.

1.1.1 B cells

B cells are able to interact with antigen via cell-surface immunoglobulin (Ig) receptors. When a naïve B cell is triggered upon encounter with a matching antigen it becomes activated and will engulf the antigen and digest it. The B cell will then display antigen fragments bound to its unique surface master histocompatibility complex molecule (MHC). The combination of antigen fragments and the MHC molecule is then presented to any T helper cells present. Cytokines secreted by the T helper cells (T_H) help the B cell to rapidly divide and differentiate into two separate lineages: effector cells (plasma cells that produce antigen-specific antibodies) and antigen-specific memory B cells (which are able on re-exposure to the antigen, to differentiate into antibody-producing plasma cells). Plasma cells do not express any surface Ig, but instead secrete large quantities of Ig as soluble antibodies (Benson et al., 2007)

1.1.2 Immunoglobulins

Igs represent a very large family of structurally and functionally different proteins and hence are divided into various groups (Woof & Burton, 2004). The different classes and subclasses of Igs are determined by the type of heavy chain the Ig possesses. The five main classes are: IgG, IgA, IgM, IgD and IgE (with $\gamma, \alpha, \mu, \delta, \varepsilon$ heavy chains, respectively) (Murphy et al., 2008) (Fig 1.1). Each class differs in size, charge, amino acid composition and carbohydrate content.

IgG is the most abundant Ig and is found both in vascular and extravascular spaces, as well as in secretions. The most important effector mechanisms of IgG are the activation of the complement system and promotion of phagocytosis, through Fcγ receptors present on the cell surface of phagocytic cells. Present in four forms (IgG_1, IgG_2, IgG_3, IgG_4),

it is the only antibody class able to cross the placenta and provide passive immunity to the foetus.

IgA is the second most prevalent and predominates in serum (90%), nasal secretions, saliva, tears and milk and also acts as a B cell receptor.

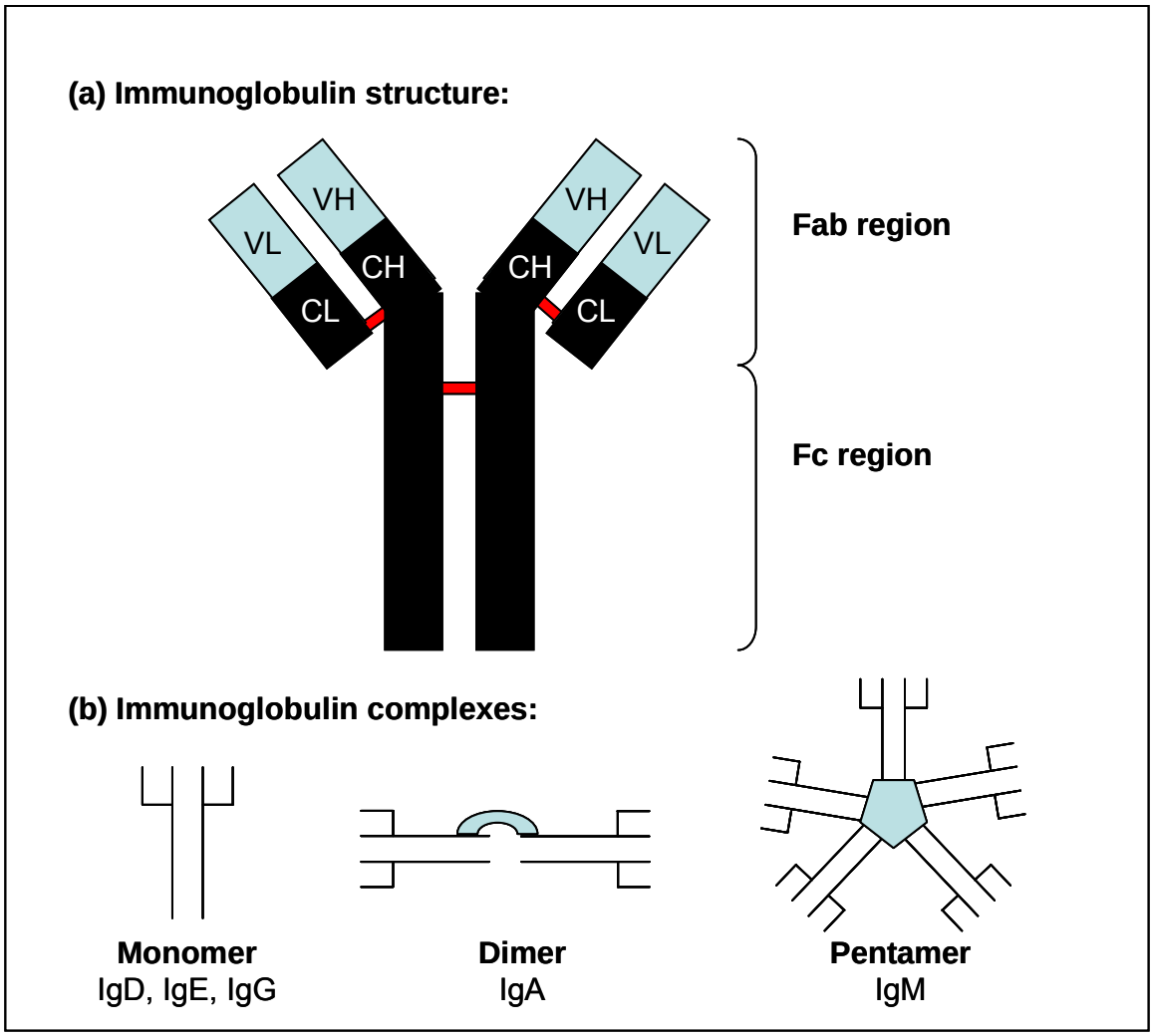

Figure 1:1 Basic Immunoglobulin Structure.

(a) The basic structural units of the immunoglobulin include two identical heavy chains (VH/CH), and two identical light chains (VL,CL). The heavy chains contain one variable domain (VH) followed by three constant domains (CH). The light chains contain one variable domain (VL) and one constant domain (CL). The immunologlobulin can also be divided into separate fragments. The Fab region (fragment antigen binding) is the area within the antibody which contains the antigen-binding site, and Fc region (fragment crystallisable) forms the lower part of the molecule and is so called because it readily crystallizes. (b) Immunoglobulin complexes are divided into: monomers (IgD, IgE and IgG); dimers (IgA); and pentamers (IgM).

Regarding other Igs, IgM acts as a monomeric cell surface receptor on B cells, but more commonly exists as a free-circulating pentamer of the typical four-chain Ig molecule and appears early in the immune response. IgM can also be found in breast milk, saliva, tears and in mucus, although secretory IgA (sIgA) is the predominant Ig in secretions. IgD is found solely as a monomeric antibody, and working in tandem with IgM, its primary function is as an antigen receptor on B cells. B cell antigen recognition by IgD and IgM is believed to play an important role in antigen-triggered lymphocyte differentiation, yet no biological function for the IgD class has been fully identified (Yuan et al., 2001).

Finally, IgE is also a monomeric Ig normally found in very low concentrations in human serum. There are two distinct cell surface receptors for IgE, the low-affinity FcεRII (CD23) receptor and the high-affinity FcεRI receptor. The former is found on leucocytes and lymphocytes, whereas the latter is found on the surface of basophils and mast cells, where it sensitizes cells on mucosal surfaces, such as the conjunctival, nasal and bronchial mucosa. Cross linking of IgE bound to its FcεRI receptor usually results in exocytosis (discussed later).

1.1.3 Origin of the T Lymphocyte

Production of new lymphocytes (lymphopoiesis), takes place in specialized lymphoid tissues, which includes the bone marrow for B cells and the thymus for T cells. T lymphocytes (T cells) arise from hematopoietic stem cells which are present in the bone marrow but migrate to the thymus gland to mature. During maturation in the thymus the T cell will express a specific membrane antigen receptor called a T cell receptor. Development and expression of this T cell receptor allows the T cell to detect and bind to its ligands and undergo selection based on its binding ability (Devereux et al., 2000). Stages of T cell development are identified by the expression of different surface markers including: the T cell receptor; CD3 (a signal transduction component of T cell receptor)

and co-receptors CD4 and CD8. Once matured, thymocytes can be divided into the subset populations of CD4 and CD8 positive.

T cells can be then be divided into the functional groups based upon their expression of surface antigen. This includes: (i) T helper cells (Th cells), which assist the function and differentiation of other immune cell populations, through their cytokine profile and are CD4+; (ii) cytotoxic T cells (Tc cells) that are able to directly destroy cells infected with foreign agents and are CD8+; and (iii) regulatory T cells (Treg) which can suppress or regulate the function of other immune cell types (Liu et al., 2006). Treg cells express high levels of the surface marker, CD25 (IL-2 receptor) and the transcription factor, forkhead box protein 3 (*Foxp3*) (Sakaguchi, 2005). They have a dedicated regulatory function, maintaining the crucial balance of an immune response and preventing autoimmune and allergic responses by effector T cells.

T_H cells can be divided into further subsets, T_H1, T_H2 and the more recent T_H9 and T_H17, based on their respective cytokine profiles, responses to chemokines and their interactions with other cells. Each T cell subset is able to promote different types of inflammatory responses. T_H1 cells are important in the control of intracellular bacterial infections, activating macrophages, and release of cytokines and chemokines to attract macrophages to the site of infection. In contrast, T_H2 cells are directed at killing extracellular pathogens by activating B cells, neutrophils and mast cells. Each cell subpopulation possesses specific homing potential based on the expression of adhesion and cytokine receptors which enable the cells to leave the lymphoid tissue and migrate, maturing into their functional groups (Colantonio et al., 2004).

1.1.4 The T-cell pathway

T cells that have never encountered an antigen before referred to as naïve T cells (Prescott & Jones, 2001). To maximise chances of an antigen encounter, naïve T cells continuously circulate from blood to

lymphoid organs and travel back to the blood, making contact with thousands of antigen presenting cells within the lymphoid tissues. When immature dendritic cells, a specific antigen presenting cell, in the peripheral tissues, comes into contact with antigen they become stimulated and migrate to draining lymph nodes via afferent lymphatics (Pulendran et al., 2001). During this migration, the morphology of the dendritic cell changes. They lose the ability to capture other antigen, and develop the capacity to process and display peptide antigens (fragments of protein initiallhy phagocytoses by the antigen presenting cell) in context with MHC class I and II on their cell surface (Banchereau & Steinman, 1998). An important difference between the two classes of MHC molecules is the source of peptides they present at the cell surface (Janeway, 2001). MHC-I class molecules, which are recognized by CD8+ T cells, present peptides derived from proteins synthesized in the cytosol. Peptides derived from proteins in intracellular vesicles are presented by the MHC-II class molecules and recognized by CD4+ T cells. Unlike T cells, B cells are able to recognise antigen alone.

1.1.5 The homing of naïve T cells to the lymph nodes

Immuno-surveillance by T cells is not achieved by random migration, but is highly specific and is tightly regulated by multi-step cascades of molecular interactions with microvascular endothelial cells. These channel the appropriate T cell subpopulations to a particular microenvironment; a process referred to as homing. Naïve T cells migrate to lymph nodes by forcing themselves through specialized high endothelial venules As explained in figure 1.2, the first step for T cell homing to lymph nodes or tissue involves at least three steps: (1) tethering and rolling (mediated by primary adhesion molecules); (2) exposure to a chemotactic stimulus provided by chemokines and G-protein coupled receptors (GPCR); and (3) arrest mediated by activated integrins. GPCR High endothelial venules express addressin molecules that bind to the L-selectins expressed on naïve T cells.

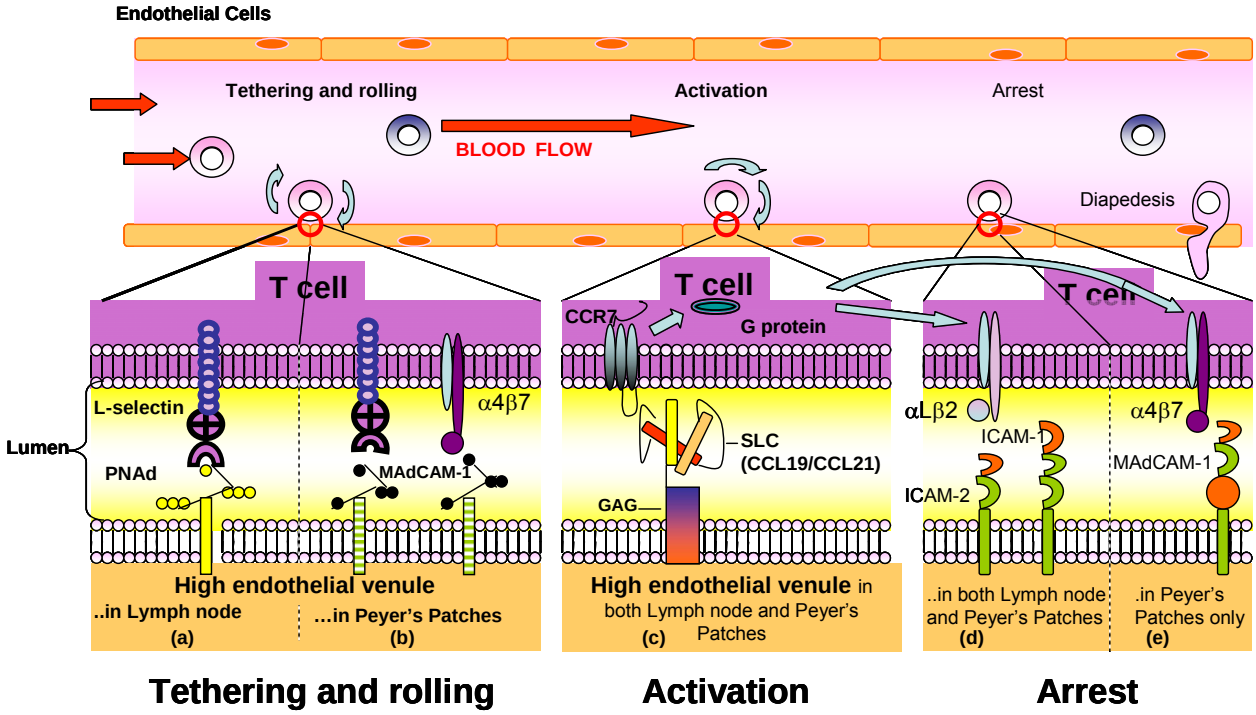

Figure 1:2 Migration of a lymphocyte out of the bloodstream into lymph nodes and Peyers Patches.

A circulating lymphocyte (pink) enters the high endothelial venule in the lymph node and Peyer's Patches (PP), it adheres weakly to the surface of the specialized endothelial cells by binding of L-selectin on the lymphocyte surface to peripheral-node addressin (PNAd) on the lymph node (a) (von Andrian & Mackay, 2000), and mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) on the endothelial cells of the PPs. Once the lymphocyte has tethered onto the endothelial in PPs $\alpha 4\beta 7$ integrin will interact with MAdCAM-1 to initiate rolling (b). The adhesion is sufficiently weak to enable the lymphocyte to roll along the surface of the endothelial cells (a & b). Chemokines are secreted by the stimulated endothelial cells (secondary lymphoid-tissue chemokine; SLC) and are presented on the luminal surface, they include CCL19 and CCL21 (Ober & Thompson, 2005). These expose themselves to the chemokine receptor 7 (CCR7) on the rolling lymphocyte which rapidly activates a signaling cascade through G protein for activation of a stronger adhesion system (c) (Hoffjan & Ober, 2002) $\alpha L\beta 2$ integrin binds to intercellular adhesion molecule 1 (ICAM-1) and intercellular adhesion molecule 2 (ICAM-2), this stops the cell from rolling and to start the process of diapedesis (d). In PPs the CCR7 activates $\alpha 4\beta 7$ integrin and $\alpha L\beta 2$ integrin. The $\alpha L\beta 2$ integrin must be activated for naïve T cells to home to PPs. The $\alpha 4\beta 7$ integrin is expressed on effector T cells, and homes to venules which express high levels of mucosal addressin-cell adhesion molecule 1 (MAdCAM-1).

1.1.6 Activation of T lymphocytes

For any newly encounted pathogen only a few naïve T cells express the T cell receptor with the correct specificity. Activation of the T cell receptor occurs via a MHC/peptide complex signal as well as a costimulatory signal between CD80/86 on the APC, and CD28 on T cells (Breinig et al., 2006). Several mechanisms are involved to ensure that once the naïve T cell encounters its specific antigen (with co-stimulatory molecules) on an APC, it is retained in the peripheral lymphoid organ, where it proliferates and differentiates into a variety of effector cells dependent on the stimulatory conditions and cytokine milieu (Banchereau & Steinman, 1998; Weninger et al., 2002). During the transition from naïve to the effector T cell phenotype, there is a dramatic sorting of adhesion molecules and chemokine receptors expressed by the T cell. This ensures that the specific T cells will preferentially home to those tissues in which their specific function is required (Kunkel & Butcher, 2002b).

1.1.7 Memory T lymphocytes

After the primary response peaks and the antigen has been cleared a large proportion of T cells that responded to the primary antigen encounter will die. The mechanisms which allow memory T cell precursors to survive when the majority of their brethren apoptose remains unclear. However, Madadmutil and colleagues did identify the homotypic form of CD8, CD8$\alpha\alpha$, was selectively expressed by CD8+ memory T cell precursors and was necessary for their survival (Madakamutil et al., 2004). Those cells destined to differentiate into memory cells offer immediate protection and a valuable robust anamnestic response. Two sub types of memory T cells, central memory and effector memory, have been identified (Table 1.1). They differ in their anatomical location, expression of cell surface markers and effector function. Recent evidence suggests the duration of antigenic stimulation, in addition to the type and amount of cytokines present during T cell

priming, influence the differentiation of effector T cells into the different memory T cell phenotypes (Geginat et al., 2001; Sallusto et al., 2004).

As explained in Figure 1.3, central memory T cells are CD45RO+ memory cells which also express CCR7 and $CD62L^{hi}$ (L-selectin) (Wherry et al., 2003). These two receptors are also present on naïve T cells, and are required for migration through high endothelial venules to secondary lymphoid tissue (Bingaman et al., 2005). Existence of central memory T cells provides the immune system with a strategic reserve of highly sensitive cells that remain within or close to the lymph node. Therefore, they can rapidly be recruited upon a second exposure to produce a faster immune response (Wherry et al., 2003) with effector T cells that are able to produce large amounts of IFNγ or IL-4 (Sallusto et al., 2004; Wherry et al., 2003). Both effector memory T cells and central memory T cells can be located in the blood and spleen. However effector memory T cells, which do not express CCR7, are located in non-lymphoid tissue, such as the skin and gut, dependent on cell surface expression of the specific homing marker. Both memory T cell subpopulations are able to acquire effector function (T_H1 or T_H2 cytokine production) upon restimulation with peptide *in vitro* (Masopust et al., 2001; Unsoeld et al., 2002; Wherry et al., 2003). However, in a model proposed by Wheery and colleagues, it was suggested that effector memory T cells are a transitory population that represent an “intermediate” cell type in the effector-to-memory transition. This was based upon the central memory T cell population showing better long-term persistence *in vivo* and improved ability to rapidly proliferate on re-encounter with the same pathogen when compared to the effector memory T cell subpopulation.

The different memory populations respond differently to a secondary antigen challenge. The effector memory T cell population, which contains T_H1, T_H2, and Tc cells, are capable of immediate recruitment to inflamed tissue. Cells that develop into T_H1 gain the ability to produce IFNγ and express the surface receptors CCR5 and CXCR3. Cells that mature into

T_H2 cells acquire the capacity of producing IL-4, and express surface chemokine receptors CCR4 and CCR3. In contrast central memory T cell, upon a secondary exposure will produce IL-12, help antigen-specific B cells (via cognate interaction) and eventually generate a new population of effector T cells (Sallusto et al., 1999b). In summary, there is now convincing evidence that antigen-specific CD4 and CD8 memory T cells exist as either central or effector memory T cell subpopulations which are heterogeneous in expression of chemokine receptors, adhesion, and costimulatory molecules.

Markers	Naïve T cell	Central Memory	Effector Memory
CD45RA	+	-	-
CD45RO	-	+	+
L-selectin	+	+	+/-
CCR7	+	+	-
CCR4	-	+	+
IFNγ	-	-	+/-
IL-4	-	-	+/-

Table 1:1 Identification markers for naïve, central and effector memory T cell subpopulations.

Characterisation of the surface markers expressed and the cytokines produced from naïve T cells, CD4+ central memory T cells and CD4+ effector memory T cell populations.

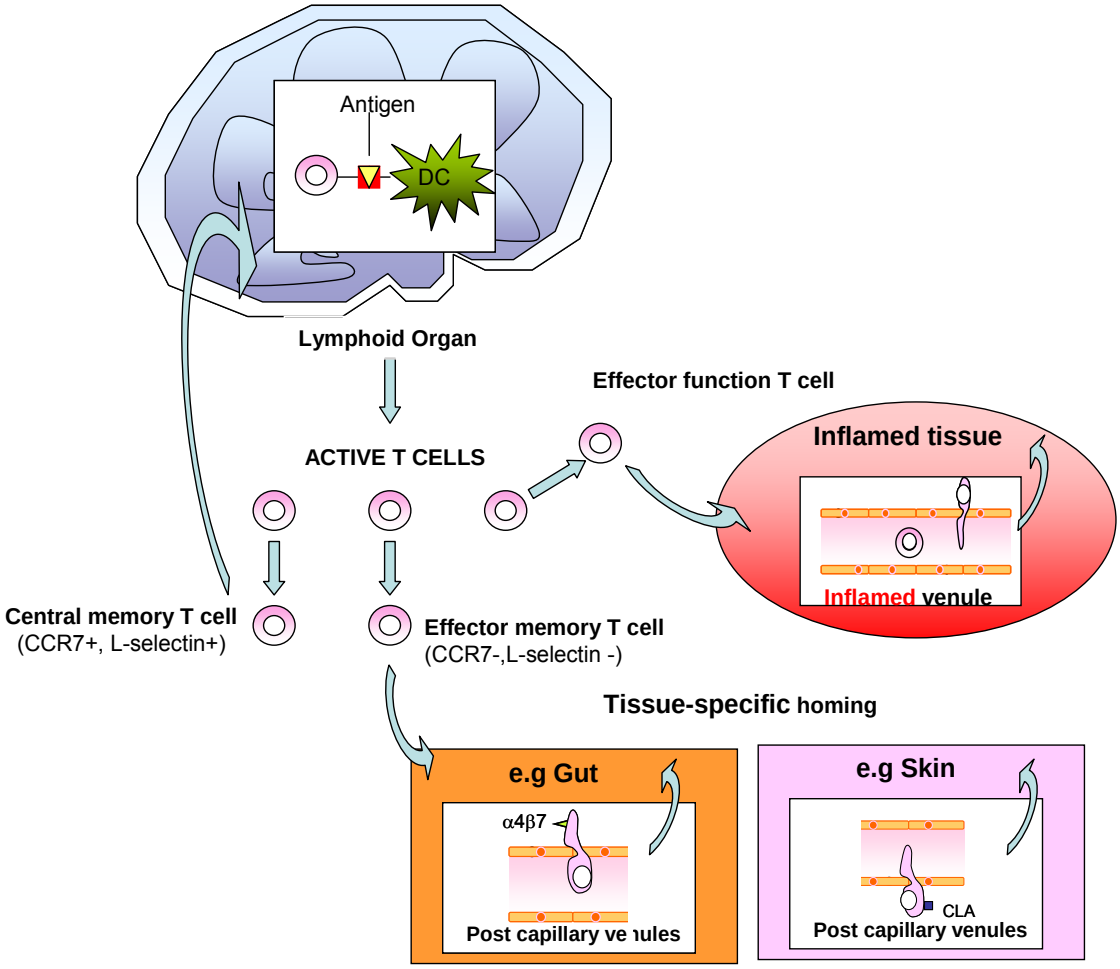

Figure 1:3 Migration pathways of T cells.

Specialized phagocytic cells of the innate immune system (not shown), including macrophages and dendritic cells ingest invading microbes or their products at the site of infection (as seen in inflamed tissue). These dendritic cells then mature and migrate via the lymphatic vessels to the nearest lymph node. Here they present the antigen to the naïve T cells present. After clonal expansion the activated T cells are divided into memory and effector T cells. The effector activated T cells migrate to the site of infection where they either help to activate macrophages or kill infected cells, thereby helping to eliminate the antigen

1.1.8 T lymphocyte tissue-specific homing

As mentioned previously, T cell subsets have a homing potential based on their surface expression of adhesion and chemokine receptors (1.1.5). These enable T cells to interact with organ specific micro-vascular endothelial cells, where the cognate ligands are selectively expressed,

allowing specific recruitment to the tissues (Colantonio et al., 2004). Both effector function T cells and effector memory T cells can home to different extralymphoid tissues, such as the skin and the gut (Mora & von Andrian, 2006).

1.1.8.1 Adhesion molecules for cutaneous and gut tissue-specific homing

The concept of specific tissue T cell homing arose from early studies which showed adoptively transferred lymphocytes were able to migrate preferentially to those tissues from which they had been originally isolated (Cahill et al., 1977).

The tropism for selective tissue is determined by the expression of specific tissue homing receptors following priming, and the expression of the corresponding ligands in the appropriate tissue venules (e.g. intestinal and cutaneous tissue venules). Several experimental observations have suggested the expression of specific homing molecules could be directed by the site of antigen presentation. Firstly, lymphocytes isolated from specific areas including the intestinal or cutaneous lymphoid organs, show preferential homing to their tissue of origin in adoptive recipients (Hall et al., 1977). Second, the presence of antigen specific T cells in human blood, following immunisation by oral or subcutaneous route, differentially express the gut homing marker $\alpha 4\beta 7$ integrin (Kantele et al., 1999). The unique microenvironment within cutaneous versus intestinal secondary lymphoid organ during the initial priming of the T cell is also thought to play a possible role (Picker et al., 1993).

After leukocyte tethering and rolling on the vessel wall (schematically depicted in Fig 1.2), transient adhesive interactions occur that are governed primarily by selectins. Two cell surface homing molecules that have been studied in depth include the $\alpha 4\beta 7$ integrin and the cutaneous leukocyte associated antigen (CLA). These two surface molecules identity T cell subsets which have preferential homing capacity for intestinal and skin tissues, respectively (Butcher et al., 1999).

The ligand for the integrin $\alpha4\beta7$, is the mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM-1) (Berlin et al., 1993; Kunkel & Butcher, 2002a), which is expressed by endothelial cells in the intestinal tract (Kunkel & Butcher, 2002a; Pabst et al., 2004).

T cells associated with homing to the skin are characterised by the expression of CLA, a carbohydrate determinant which is predominantly on P-selectin glycoprotein ligand 1 (PSGL1) (Fuhlbrigge et al., 1997), a mucin-like glycoprotein present on all leukocytes. The ligands for CLA include both E-selectin, a cell adhesion molecule expressed on endothelial cells, and P-selectin, a cell adhesion molecule on activated endothelial cells and platelets.

Chemokine receptors have also been indicated as having a possible role in T cell homing to the skin and gut (Kunkel & Butcher, 2002b). For example, the chemokine receptor, CCR4 has been implicated in the T cell tropism to sites of cutaneous inflammation. However, this in addition to CLA and $\alpha4\beta7$ are discussed in further detail in Chapter 5 (5.1).

It is thought that additional tissue-committed populations of T cell exist as T cells from other tissue compartments, including bronchoalveolar lavage fluid and liver, do not express CLA, $\alpha4\beta7$ or CCR4. This suggests that unknown combinations of adhesion molecules and chemokine receptors may mediate homing to these organs.

1.2 An Abnormal Immune Response - Allergy

The immune system normally responds to secondary exposure of a variety of microbial invaders with little or no damage to host tissues. However, inappropriate 'over-reactivity' is known as hypersensitivity and can lead to disease. Hypersensitivity is classified into four main categories, with a less understood fifth category being more recently described (Rajan, 2003). Types I, II, and III are reactions mediated by

antibody, or antibody and complement, whereas Type IV is mediated by cellular-mediated reactions (mainly by T cells and macrophages) and is referred to as delayed-type hypersensitivity (DTH) (Table 1.2). Allergy is often equated with type I hypersensitivity, which is characterized by overproduction of IgE antibodies against foreign proteins that are commonly present in the environment. However, the same immune response is normal and necessary for intestinal parasite elimination. Allergy may be defined as a state of hypersensitivity, induced by exposure to a specific antigen resulting in a harmful immune response on subsequent exposure. It is important to recognise that hypersensitivity responses are all (other than V) a component of a normal reponse to infection.

Type	Alternative Names	Mediators	e.g. Disorders
I	Allergy (immediate)	IgE	Atopy; anaphylaxis; asthma; hay fever; eczema
II	Cytotoxic, antibody-dependent	IgM or IgG (complement)	Autoimmune haemolytic anemia; Thromobocytopenia; Erythroblastosis fetalis;
III	Immune complex disease	IgG (complement)	Serum sickness; Arthus reaction; Systemic lupus erythematosus
IV	Delayed-type hypersensitivity (DTH)	T cells	Contact dermatitis; Graft rejection.
V	Autoimmune disease	T cells	Grave's disease; Myasthenia Gravis;

Table 1:2 Classifications of types of hypersensitivity

Coombs and Gell classification with the addition type (Type V), which is often used as a distinction from Type 2 (Rajan, 2003).

1.2.1 Epidemilogy

The most common form of allergy, IgE mediated hypersensitivity, has shown a large increase within the last few decades and now affects more than 25% of the population of the western society (Floistrup et al., 2006). The aetiology of the allergic immune response is complex and has shown to be influenced by many factors. Both environmental and genetic factors are believed to play an important role in the development of this multifactorial disorder.

1.2.2 Genetics of allergic responses

Numerous chromosomes and candidate genes have been associated with allergy. Chromosomes have been implicated in: asthma and the asthma-associated phenotypes (e.g. 1p, 2q, 4q, 5q, 6p, 12q, 13q, 14q, 19q, 20p and 21q); atopy (e.g. 3q, 4q, 6p, 11q, and 17q); and for elevated IgE levels (e.g. 2q, 3q, 5q, 6p, 7q, and 12q) (Hoffjan & Ober, 2002; Homey et al., 2002; Ober & Thompson, 2005). Zhang *et al* for example identified the gene PDH finger protein 11 *(PHF11*), a gene that influences IgE and asthma (Zhang et al., 2003). Other potential asthma susceptible genes or complexes that have been identified include ADAM metallopeptidase domain 33 (*ADAM33),* dipeptidylpeptidase 10 (*DPP10)*, SET domain, bifurcated 2 (*SETDB2),* neuropeptide S receptor 1 *(NPSR1)*, and serine peptidase inhibitor, Kazal type 5 (*SPINK5)* (Allen et al., 2003; Jang et al., 2005; Laitinen et al., 2004; Van Eerdewegh et al., 2002; Zhang et al., 2003). *ADAM33* was identified by Van Eerdewegh's group on chromosome 20p13 and was associated with the asthma phenotype and expressed in bronchial and other muscle tissue thought to be involved in the bronchial hyperresponse (Van Eerdewegh et al., 2002). As ADAM33 is not expressed by the cells mediating immune responses it is clear that genes associated with allergy are not necessarily the same as those affected the tissue specific manifestations. Similarly, the gene for DPP10, present on chromosome 2q14, has been suggested to be an asthma susceptibility gene (Allen et al., 2003). The precise functions of

these nominated genes remain poorly understood and the mechanisms which link these genes to atopy are still unclear.

There is consistent evidence from studies on twins, that genetic factors influence susceptibility to sensitization and atopy (Koppelman et al., 1999; Strachan et al., 2001). However, comparisons of monozygous twins have shown they often vary in their expression of atopy (Strachan et al., 2001). The division of Germany after World War II illustrated that despite a similar genetic predisposition for atopy, there was a marked divergence in the national prevalence rates for allergic disease (Lehmann et al., 2002). This suggests an introduction of environmental factors and genetic factors are important in determining the pattern of sensitization and atopy.

1.2.3 Environmental factors

The observed increase in allergic disease prevalence has occurred rapidly over a few decades, a time period that precludes genetic factors alone in the aetiology. There are clear differences in the prevalence of allergic diseases between rural and urban areas within one country. Several studies including the Swiss SCARPOL study have shown a strong association between growing up on a farm (with continuous exposure to a microbial rich environment) and a reduced risk to developing an allergic disease (Braun-Fahrlander et al., 1999). When Von Ehrenstein et al., compared children from two different rural Bavarian regions, the most pronounced allergo-protective effect of the farming environment was shown to be present at farming sites with livestock in the stables (Von Ehrenstein et al., 2000). These observations suggest that the farming environment may influence the developing immune system leading to a reduced risk of allergy.

Other environmental factors including increased exposure to indoor allergen, pollution or changes in diet and breast feeding have also been highlighted as having a possible role in the rise of atopic diseases. Both,

what time point and which environmental factors influence the development of atopy remains controversial, with little consistent evidence for some risk factors and both prenatal (Hagendorens et al., 2004) and early postnatal periods being proposed as the important time period for sensitisation (Garn & Renz, 2007).

The 'hygiene hypothesis', proposed the increased prevalence of atopic disease was due to lack of intense infections in the western world owing to improved hygiene, smaller family size, vaccinations and the widespread use of antibiotics, which may alter the human immune system in such a way that it responds inappropriately to a normally innocuous environmental antigen (Mattes & Karmaus, 1999; Strachan et al., 1989; Strachan et al., 2000). The immunological interpretation of the hygiene hypothesis involves the balance between T_H1 (associated with bacterial and viral infection, as well as autoimmune disease) and T_H2 (associated with helminth infection and allergic disease) cells (Matricardi & Bonini, 2000). It proposes that limited exposure of pathogenic allergens during early childhood leads to inadequate stimulation of T_H1 response, therefore favouring expansion of T_H2 cells which results in a predisposition to allergy.

In accordance with the hygiene hypothesis, exposure to foodborne and orofecal microbes, including *Toxoplasma gondii* and *Helicobacter pylori*, have also been recorded to reduce the risk of atopy development (Matricardi et al., 2000). Investigations into gut commensals have also shown differences in gut colonisation and type of gut bacteria, differ from those children at high and low risk of atopy (Sepp et al., 1997).

1.2.4 Mechanisms involved in atopic allergic disease

Atopic allergic disease occurs when an individual produces allergen-specific IgE in response to an otherwise innocuous antigen. In the development of allergy, an individual will first become sensitized to a particular antigen and reach a state of hypersensitivity (Figure 1.4 (a)).

The antigen primed dendritic cells migrate to regional lymph nodes where they present the allergen via peptide-MHC complex to the specific naïve T cells. Dependent on the nature of the signals between the antigen presenting cell and T_H0 cells, T_H2 cells specific for the antigen are generated. These T_H2 cells secrete the cytokine IL-4, which is important for the growth and differentiation of B cells and stimulates the production of IgE. The secreted IgE circulates in the blood, sensitises basophils and tissue mast cells by binding tightly to the high affinity IgE surface receptor (FεRI) (Turner & Kinet, 1999). The additional binding of IgE to mast cells and basophils up-regulates FcεRI expression, permitting a lower threshold for cell activation, and increased secretion of inflammatory mediators.

These IgE-coated cells are now sensitized to the allergen and therefore, upon a second exposure to the specific allergen, cross linking of these IgE- FεRI complexes occurs (this is described as the effector phase). Cross-linking of IgE results in a cascade of signal transduction events causing the release of preformed mediators, including histamine, tryptase, and eotaxin (Figure 1.4 (b)), into the surrounding tissue. This can cause several systemic effects including vasodilation, nerve stimulation, mucous secretion and smooth muscle contraction and is referred to as the acute effector phase of the allergic reaction (Eigenmann, 2002). The typical clinical symptoms include; an immediate weal and flare reaction; sneezing and runny nose; or wheeze. Depending on the individual, the allergen and whether the mode of induction was at the skin, nose or airways, symptoms can be system wide (anaphylaxis) or localised to a particular body system, e.g eczema is localised to the dermis (Janeway, 2001). The response is also characterised by activation of the arachidonic acid pathway with the generation of newly formed mediators including prostaglandins and leukotrienes.

An acute response is often followed by a late-phase reaction (LPR), which is characterised in the skin by an oedematous, erythematous swelling; in the nose by sustained blockage; and in the lung by further

wheezing. At the start of LPR, leukocytes (including neutrophils, lymphocytes, eosinophils and macrophages) are attracted to the initial site by the mediators (leukotreines, postaglandins, eotaxins etc and cytokines) released earlier (Herzenberg et al., 2002). These cells are recruited and in turn release mediators which contribute to the progression of the inflammatory reaction. The LPR usually occurs 4 – 24 hours after the original reaction (Grimbaldeston et al., 2006)

T_H2 cytokines have been shown to play a major role in allergic pathology. Many allergic individuals develop debilitating and even life-threatening T_H2–driven IgE antibody responses. The T_H2 cells that produce cytokines IL-4 and IL-13, promote IgE production and can be inhibited by T_H1 cells that produce IFNγ (Homey et al., 2002; Umetsu & Dekruyff, 2006). Airway hyper-responsiveness is affected by IL-9 and IL-13, while IL-4, IL-9 and IL-13 have an effect on mucous hyper-secretion. In contrast, non-atopic individuals show mainly T_H1 immunity, characterised by IFNγ, that inhibits growth of T_H2 cells (Prescott et al., 1999).

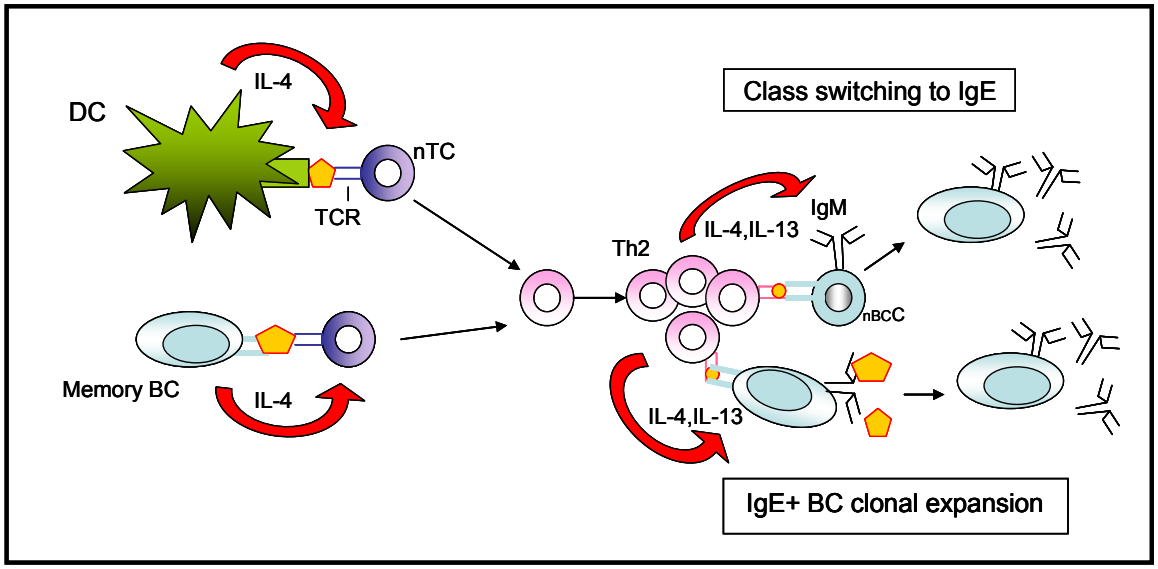

Figure 1:4 Sensitisation and memory induction.

Sensitisation to allergen and development of antigen specific B cell and T cell memory. Differentiation and clonal expansion of T_H2 cells leads to production of T_H2 cytokines including IL-4 and IL-13 which induce class switching to IgE. T cell activation in the presence of IL-4 leads to T_H2 differentiation. Adapted (Larche et al., 2006).

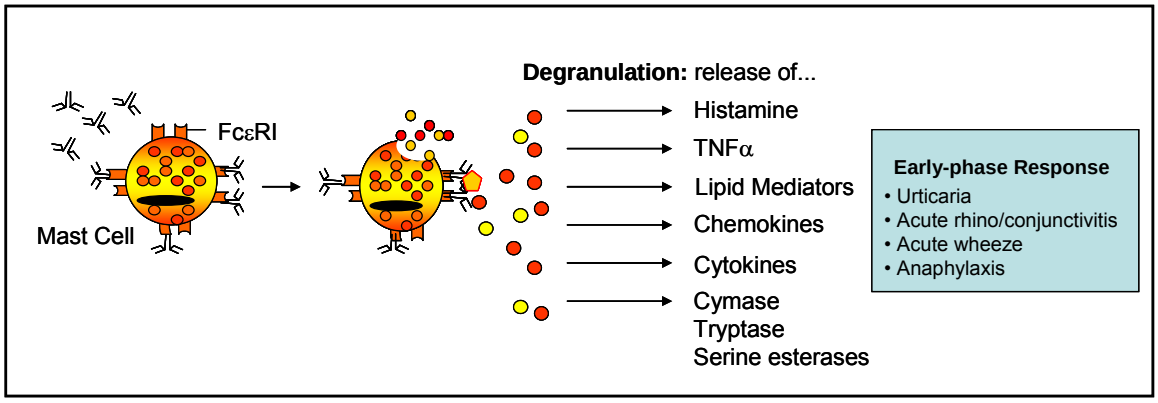

Figure 1:5 Early-phase reaction: Type 1 reaction

Early-phase reaction, where cross-linking of the mast cell (or basophil) FcεRI bound IgE by allergens results in the release of vasoactive amines, lipid mediators ,chemokines and cytokines which promote the immediate symptoms of disease.

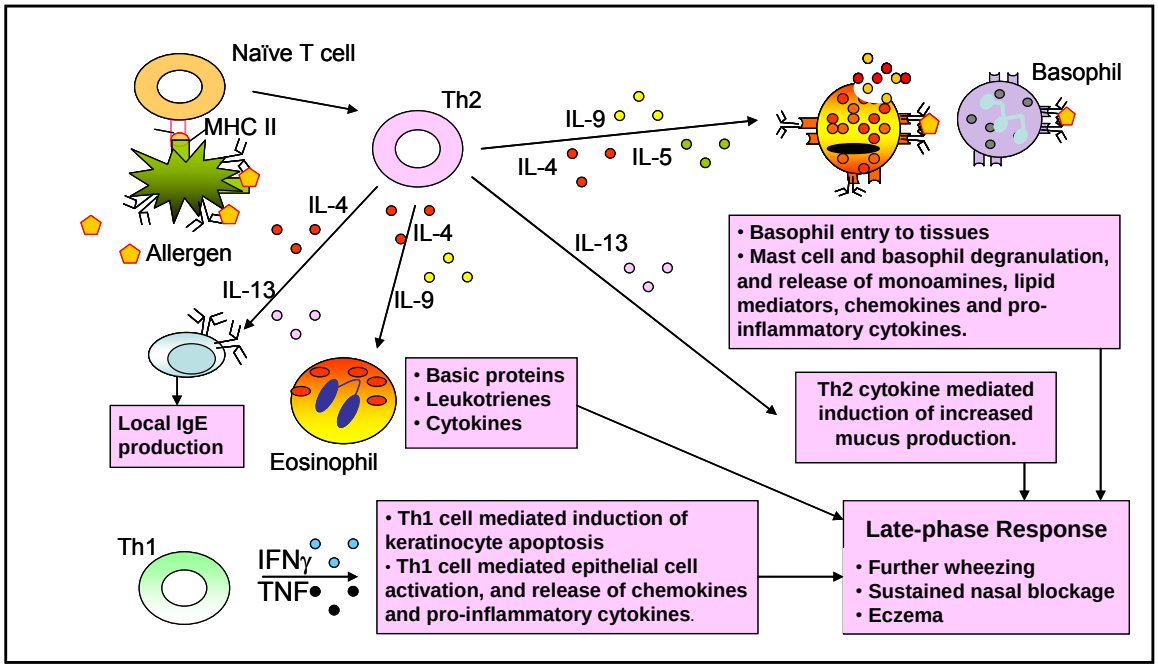

Figure 1:6 Late-phase response

Adapted (Larche, Akdis, & Valenta, 2006) Following migration to the sites of antigen exposure (influenced by cytokines and chemokines), activated antigen-specific T cells are reactivated. Local IgE production occurs in allergic rhinitis and asthma but is absent from allergic skin inflammation (atopic dermatitis). T_H1 cells, produce IFNγ and tumour necrosis factor (TNF) which induce apoptosis of skin keratinocytes, bronchial epithelial cells and pulmonary smooth muscle cells. Activated mast cells and basophils release histamines, chemokines and other cytokines, which all contribute to the late-phase allergic reaction.

1.2.5 Allergy development

Allergic disease has become a health/economic burden, with the disease severity varying from mild and intermittent, to continuous and intractable symptoms, depending on the individual. It causes loss of productivity, impairment of quality of life and a major source of suffering to those affected and also those who are closely related (Ben et al., 2004). Atopy is the production of specific IgE in an immune response following exposure to a common and normally harmless environment allergen (Coca and Cooke, 1923). There is a strong association between being atopic and the development of allergic disease such as asthma, hay fever or eczema (Berg et al., 1971). However, not everyone with atopy goes on to develop the clinical manifestations of allergic disease (Jarvis & Burney, 1998). This may be a consequence of either altered genetic predisposition to specific allergic disease as distinct from atopy or a failure to express specific homing molecules on sensitised cells.

Allergic disease often starts during early infancy with atopic eczema (inflammation of the skin) and food allergy (to common allergens including cows' milk and hens' egg) (Wahn et al., 1998), which appear to give way to aeroallergen sensitization and respiratory allergic disorders in later childhood (Croner et al., 1982; Tariq et al., 1998).

The atopic march usually refers to the association that infants who first develop atopic eczema and food allergy, often go on to develop rhinitis and asthma in later childhood. Eczema and sensitization to food allergens in infants and toddlers often improves by preschool age, when asthma and rhinitis may become more prevalent, along with sensitization to aeroallergens (Kulig et al., 2000; Nickel et al., 1997). Various epidemiological and birth cohort studies have investigated this atopic march pathway (Illi et al., 2004; Rhodes et al., 2001; Wahn et al., 1998). As discussed by Spergel and Paller, approximately half of the patients with atopic eczema will go on to develop asthma (Spergel & Paller, 2003). Rhodes *et al*, in a birth cohort study of one hundred babies of atopic

parents, investigated factors present in early life which may predict increased risk of asthma. They reported within their high risk subjects, skin sensitivity to hen's egg or cow's milk in the first year was predictive of adult asthma (Rhodes et al., 2001)

1.2.6 Eczema

Atopic eczema, bronchial asthma, and allergic rhinitis are classically considered to represent a common syndrome of atopic disease (Morar et al., 2006). Atopic eczema, also known as atopic dermatitis, is a common recurring inflammation of the skin and is reported to affect 15 – 20% of infants in the UK (Leung & Bieber, 2003) Atopic eczema is associated with cutaneous sensitivity to normally innocuous environmental or food allergens (e.g. house dust mite, egg, milk and peanut). It usually develops early in life, often becoming evident in the first year of life (Halkjaer et al., 2006). Other features of atopic eczema include: a disrupted skin barrier which often results in dry skin; pruritis; and increased specific IgE to environmental or food allergens. Genetic mutations independent of atopy have been implicated in atopic eczema. The main gene groups identified so far include those encoding for epidermal structural proteins (e.g. filaggrin) (Morar et al., 2007) and those encoding for essential components within the immune response (e.g. increased Th2 cytokine production) (He et al., 2003; Kawashima et al., 1998; Liu et al., 2000).

At least two forms of dermatitis have been identified, extrinsic and intrinsic. Both forms are associated with eosinophilia, however the extrinsic form, which affects 70 – 80% of individuals, is characterised by high levels of IgE specific to eniviromental and/or food allergens. While individuals with the intrinsic form (20 – 30%), do not demonstrate an IgE mediated sensitivity (Novak & Bieber, 2003). Within extrinsic atopic eczema, CLA+ memory T cells produce T_H2 cytokines, including IL-4, IL-13 (which induces isotype switching to IgE synthesis) and IL-5 (important in eosinophil development). (Hamid et al., 1994). However, while most studies have confirmed the presence of IL-5 and IL-13 in the skin of

extrinsic atopic eczema patients, some have failed to detect IL-4 (Akdis et al., 1999). This could be explained by a change in cytokines released. It has now become clear that IL-4 production is related to the type of skin lesion, and is present in acute dermatitis rather than in chronic dermatitis (Grewe et al., 1995). Skin biopsies from patients with atopic eczema that had been subjected to allergen-induced lesions, showed a biphasic cytokine profile (Eyerich et al., 2008). It is now known that in acute atopic eczema the Th2 profile predominates (e.g. increased IL-4, IL-5 and IL-13). However, within chronic atopic eczema, there is a predominant T_H1 cytokine pattern (e.g. increased IFNγ, IL-12, IL-5 and granulocyte-macrophage colony-stimulating factor)(Hamid, Boguniewicz, & Leung, 1994).

1.2.7 Food Allergy

As mentioned previously, food allergy is a common childhood manifestation of atopy and affects up to 8% of infants in early life (Sicherer & Leung, 2005). It is defined as any immunological mediated response to food that is reproducible under blinded conditions (Herzenberg et al., 2002). The foods most commonly responsible for allergic reactions in westernized societies include egg, peanuts, milk, tree nuts, fish, grains and soy. But there are many others, as potentially any food can provoke an IgE-mediated reaction (Eigenmann, 2003).

Food allergy symptoms are variable and may include: swelling of the lips, pharyngeal itching (oral allergy syndrome), laryngeal oedema (oral allergy syndrome), reflux, nausea, vomiting, abdominal pain, diarrhoea, asthma, rhinitis, angio-oedema, urticaria, eczema and anaphylaxis (Chung et al., 2007; Sigurs et al., 1994). The time and duration of the symptoms has also been shown to range from hours to days.

Food allergy can be divided into IgE- and non-IgE mediated responses, with non-IgE food allergy believed to result from immune mechanisms,

primarily involving lymphocytes and/or cytokines or other effector cells of the immune system (Eigenmann, 2002). In any type of manifestation of food hypersensitivity, T cells are present either as pivotal cells facilitating the production of food antigen-specific IgE antibodies, or as effector cells able to secrete autocrine and chemokine factors (Eigenmann, 2002). IgE-mediated reactions involve the release of mediators, including histamine (Fig 1.5), from mast cells which results in symptoms within 2 hours after exposure (Sicherer, 2002). A late-phase of other mediators (e.g. eotaxins, cysteinile leukotrienes), which attract local inflammatory cells may lead to symptoms 4 – 8 hours after exposure. Symptoms from non-IgE food reactions may develop 12 – 24 hours after exposure (Sicherer, 2002). Chronic food allergic disorders have been shown to typically affect the gut or skin (Sicherer & Sampson, 1999).

The onset age of food allergy depends on the food and at what stage it is introduced into the child's diet, with the first food allergy generally being to cows' milk, egg and wheat, which is introduced in the first year (Herzenberg et al., 2002). Results from the German MAS cohort study (Lau et al., 2002), a large birth cohort study which investigated the natural course of atopic disease from birth to 13 years of age, showed that children sensitized to hen's egg at 12 months showed increased risk of allergic sensitisation to aeroallergens at 3 years of age.

1.2.8 Tolerance

An important feature of the immune system is its ability to distinguish between self and non-self, as well as harmful and non-harmful antigens. The principal immune mechanisms that have been described in peripheral T cell tolerance include: cell death (with consequent clonal deletion); development of a non-responsive state in the T cell (anergy); and active suppression mediated by suppressor/regulatory cells. The latter mechanism was first described in a 1971 paper on infectious immunological tolerance by Gershon and Kondo, they proposed that

negative regulation could be an intervention with the activity of a positive T cell (Gershon & Kondo, 1971). This led to the notion of a specific subpopulation of T cells whose role was to limit immune responses (Okumura et al., 1977; Vadas et al., 1976). However, it has only been recently that this cell population, now called Treg cells, has become widely accepted and investigated by the scientific community.

Treg cells suppress T cell proliferation, inhibit IL-2 mRNA transcription and down-regulate pro-inflammatory cytokine production (IFNγ and TNF-α) (Sakaguchi, 2005; Shevach et al., 2006). It is generally agreed that Treg cell produce their suppressive effect either via cell-surface contact with the responder cells (as shown in *in vitro* experiments) (Levings et al., 2001; Thornton & Shevach, 1998) or via production of specific cytokines including IL-10 and TGF-β (as shown in *in vivo* experiments) (Tang et al., 2006).

Another primary factor involved with the development of peripheral tolerance after oral administration of allergen/antigen is the dose fed. Friedman and colleagues did suggest that high dosage of the oral antigen favours specific T cell unresponsiveness via anergy/deletion, while low oral antigen dosage has shown generation of Treg cell tolerance (Friedman & Weiner, 1994). However, later evidence has shown that these two forms of tolerance are not exclusive of each other and may actually overlap (Chen et al., 1998; Nakamura et al., 2001). Tregs have also been described in some studies to possess anergic properties (Taams et al., 2002; Tsuji et al., 2003). Consequently, both anergy/deletion and cell regulation may all form different aspects within the same process of tolerance.

1.3 Pregnancy immunology

Successful human pregnancy is an immunological paradox, ultimately representing the unique physiological phenomenon of a symbiosis of two semi-allogenic individuals, with the foetus developing in the potentially

hostile environment of the maternal immune system (Froebel et al., 1997). Despite the vast array of knowledge about the subject many questions still remain regarding this complex relationship and the possible sensitizing of the foetus in the womb.

1.3.1 Maternal immune responses during normal pregnancy

The immunological recognition of pregnancy is important for the maintenance of gestation, playing a crucial role in conceptus tolerance, as well as modulation of disorders of pregnancy (e.g. preeclampsia) (Agaard-Tillery et al., 2006; Sargent et al., 2006; Thellin & Heinen, 2003). The mechanisms of foetal tolerance are intricate and work at different periods of time in embryonic development.

Two distinct immunological interfaces have been described that occur during pregnancy (Sargent et al., 2006). The first interface occurs very early in pregnancy and is between maternal immune cells and the foetal trophoblast (the tissue that forms the wall of the blastocyst), present in the decidua (the lining of the uterus) (figure 1.7). The second immune interface, occurs later on in the pregnancy and involves interactions between the maternal immune cells (including macrophages, dendritic cells, T cells) and the syncytiotrophoblast (the outer layer of the trophoblast).

Trophoblast invasion of the uteroplacental blood flow is required to sustain foetal growth and development. When the blastocyst adheres to the uterus, foetal trophoblast cells differentiate and form a column of cells that invade the maternal decidua. This occurs during the first 18 weeks of gestation, forming an interface (interface 1) between foetal tissue and maternal immune system (Agaard-Tillery et al., 2006). The second interface, the placental villus, forms later in pregnancy and is composed of syncytiotrophoblast cells, which become surrounded by circulating maternal blood and have constant contact with the maternal immune system.

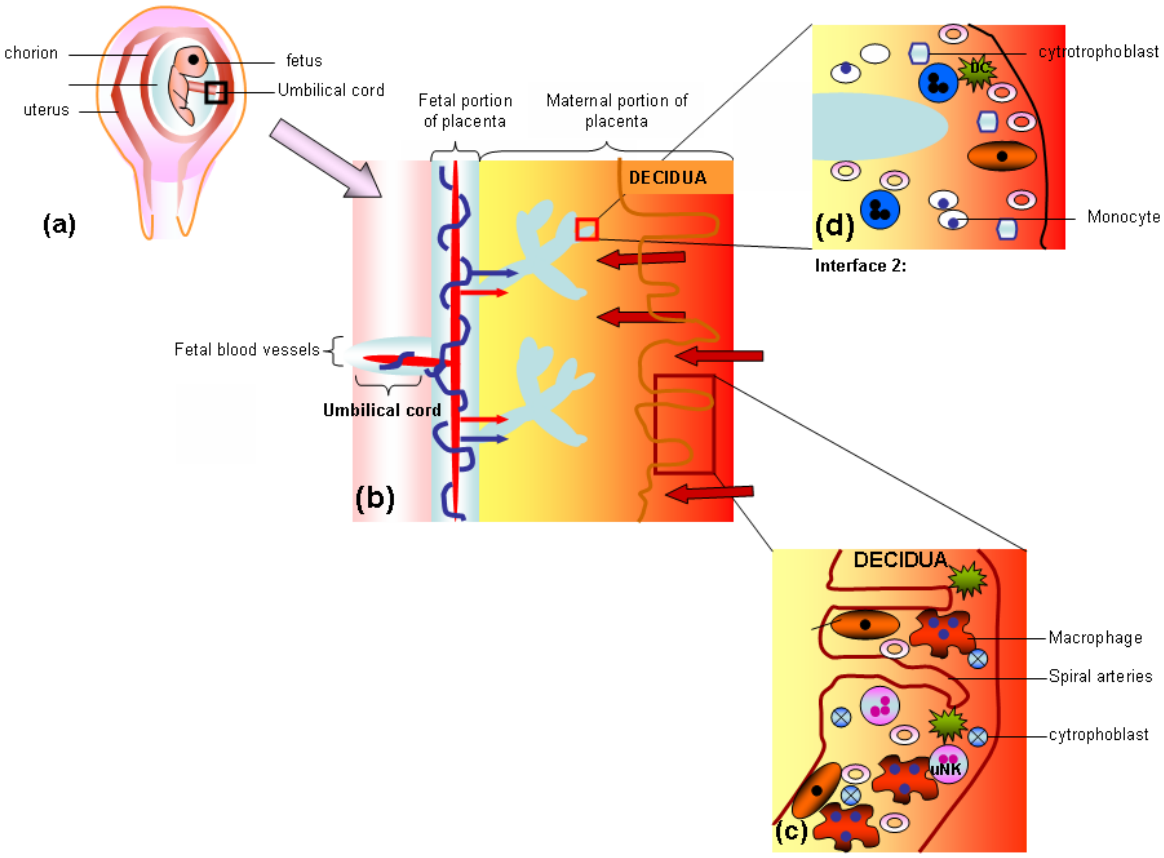

Figure 1:7 The two immunological interfaces of pregnancy.

The embryo is attached to the placenta by the umbilical cord (a). Embryonic blood vessels invade the placental tissue to form fingerlike villi. Maternal blood flows into the spaces surrounding the villi (d). In early pregnancy the first interface interactions occur in the decidua between maternal immune cells and the invasive extravillous trophoblast (c). This interface is dominant in early pregnancy but disappears during third trimester. Interface 2 occurs between maternal immune cells (including NK cells, T cells, mononocytes and DCs) and the syncytiotrophoblast that form the villous surface of the placenta. This interface occurs with the start of uteroplacental circulation at 8-9 weeks of pregnancy (d).

The maternal decidua, which has direct contact with the trophoblast, contains macrophages, dendritic cells and T cells (Piccinni, 2002). Decidual macrophages are alternately activated microphages that produce little oxidants and more immunosuppressive IL-10 and TGFβ2 (Haas et al., 1999). The down-regulation of T_H1 immunity (and therefore the apparent T_H2 skewing at the maternal-foetal interface) during gestation has been demonstrated consistently in humans and is regarded as more beneficial for the foetus. This is because it allows the foetus to

develop without the threat of harmful T_H1 cytotoxic responses being mounted against the trophoblast cell population. The T_H1/T_H2 hypothesis states that the foetus avoids maternal T cell rejection through T_H2 and Treg cytokine production of IL-4, IL-5, IL-6, IL-9, IL-10 and TGFβ. These reduce the production of pro-inflammatory molecules, such as IL-2 and IFNγ (Thellin & Heinen, 2003). Predominant T_H1 immunity during pregnancy is observed in miscarriages and preeclampsia (Dong et al., 2005), while at the foetal-maternal interface successful pregnancies are associated with T_H2 cytokine production. As high levels of progesterone are present at the foetal-maternal interface, it is believed to have a possible contribution in the T_H2 switch. Both IL-4 and IL-10 have been shown to have the ability to inhibit the development and function of T_H1 cells and macrophages (Piccinni, 2002). However, Sargent *et al* (2006) reported that normal pregnancy is a controlled state of inflammation, showing predominant immune interactions in the deciduas are between the placental trophoblast and maternal NK cells, instead of T cell

1.3.2 Transport across the Placenta

The placenta is a metabolically active organ and has been shown in animal models to to extract 40 – 60 % of the glucose and oxygen supplied by the uterine circulation (Harding, 2001). The remaining nutrients and metabolites are transferred across the placenta to the foetus by passive diffusion, facilitated diffusion, active transport, endocytosis or exocytosis. In brief, passive diffusion of oxygen, urea and carbon dioxide is limited to the placental exchange and blood-flow area. Glucose and lactate utilise facilitated diffusion involving transfer down a concentration gradient, aided by a transfer molecule. In contrast, active transport requires additional energy, in addition to a carrier protein, and is used for transport of amino acids.

Transfer across the human placenta can occur via a transplacental and a transamniotic pathway (Holloway et al., 2000). The exchange barrier is formed by the foetal endothelium and the syncytiotrophoblast layers. Maternal IgG crosses the placenta, transcellularly through the syncytium,

and is able to enter foetal circulation (Loibichler et al., 2002). Transfer begins around 16 weeks and increases during the last three months. The subclasses of IgG can cross the placenta in different concentrations, an order of: IgG1>IgG3>IgG4>IgG2 (Gasparoni et al., 1992) or IgG1>IgG3>IgG4>IgG2 (Drossou et al., 1995). Transplacental transfer of antigen (including β-lactoglobulin, ovalbumin and Bet v1) into the placenta has been observed (Loibichler et al., 2002) and is discussed in more detail (1.4.2) Transamniotic passage has also been observed, where allergens b-lactoglobulin and ovalbumin have been shown to travel from the decidua across the foetal membranes into the amniotic fluid (Edelbauer et al., 2004). This is discussed in more detail later (Chapter 3)

1.3.3 Antenatal Exposures

The intrauterine environment seems to play an important role in the pathogenesis of atopic disease. Other maternal characteristics and behaviours that have been associated with atopic outcome in the child include nutrition, smoking (Raherison et al., 2007), respiratory infections and the use of antibiotics during pregnancy (Hughes et al., 1999). Transplacental allergen exposure or maternal nutritional factors during late gestation could predispose the developing foetus to atopy.

1.3.4 Maternal diet during pregnancy

European guidelines recommend normal feeding during pregnancy and lactation as a low allergen content diet could potentially cause malnutrition in the expectant mother. The theoretical basis for suggesting dietary changes for mothers during pregnancy is the concept of intrauterine sensitisation, with evidence of allergen-specific T cell responses at birth. This suggests intrauterine exposure of antigens to the foetus and priming of the foetal immune system (Szepfalusi et al., 2000b), which may lead to sensitisation and theoretically the development of food allergy with antigen specific T cells. Thus, maternal diet during pregnancy

may influence foetal immune responses that could predispose the child to allergic disease. However, with conflicting results from cohort studies (Falth-Magnusson & Kjellman, 1992; Lilja et al., 1989a; Sausenthaler et al., 2007; Vance et al., 2005), the correct dietary advice for women during pregnancy remains unclear. It is proposed that allergen exposure occurs via transfer of allergens to the foetus by two possible routes: the dia-amniotic; and the diaplacental transport route (Holloway et al., 2000). It is not yet clear what transfer mechanism accounts for transplacental allergen passage, or at what stage during pregnancy it occurs (Loibichler et al., 2002). One study's observations suggested that foetal allergen exposure is a common phenomenon, implying the presence of allergens in foetal circulation may be part of the normal development of immunity (Loibichler et al., 2002).

1.3.5 Placental transfer of food antigen

Ovalbumin, along with house dustmite allergen (Der p1), has been detected in the amniotic fluid of pregnant mothers at 16 – 18wks gestation, and is detectable in foetal circulation at birth (Jones et al., 2001). Both ovalbumin and β-lactoglobulin, have been shown to stimulate proliferation and cytokine production from blood mononuclear cells collected at birth (Vance et al., 2004). With detection of ovalbumin and β-lactoglobulin in the amniotic fluid and the foetal circulation, this provides direct evidence of transamniotic allergen transfer as early as 26wks gestation (Loibichler et al., 2002). Cord blood mononuclear cells that are sensitised, also show a lower level of T_H1 production, which has been linked to the development of food-associated eczema in infants (Koch et al., 2006).

The maternal-to-foetal transport of IgG begins at 16wks gestation, increasing steadily from 22wks, with foetal IgG1 eventually surpassing maternal levels. Results from Vance et al., 2004 showed maternal serum OVA IgG concentration reflected egg consumption, indicating the

developing immune system may be exposed to varying dietary allergen doses as maternal IgG responses in pregnancy were closely related to infant IgG levels at birth. This suggests placental IgG should be considered as an early life factor in possible predetermination of infant atopy (Vance et al., 2004). Data from the Food Standards Agency (FSA) funded project TO7005, showed that either high or low levels of exposure to ovalbumin in dietary intake (as determined by maternal specific IgG) is related to a non-atopic phenotype of the infant. Hence, infants who had high exposure to ovalbumin may at a later date become actively tolerant. Whereas infants exposed to low levels of OVA may show no immune response to the allergen (figure 1.8).

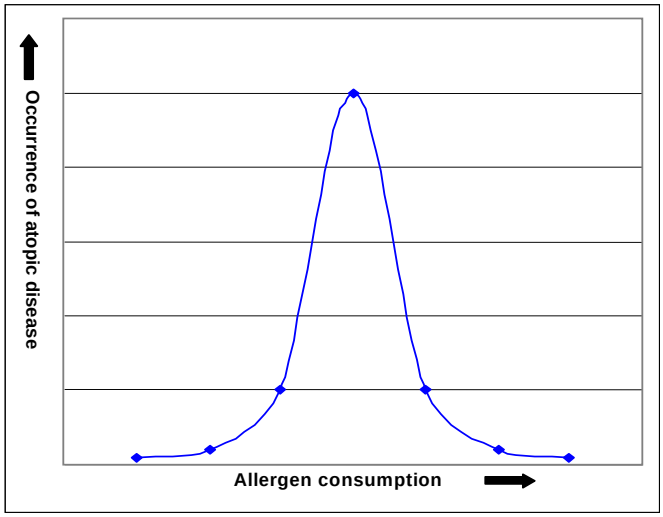

Figure 1:8 Graph signifying the relationship between maternal allergen consumption and occurance of atopic disease in the infant.

In summary, allergic disease has become a major public health issue, which results in large numbers of adults and children with often life-long medical conditions. Apart from the obvious detriment to quality of life, it also puts a large financial strain on the health care profession (Lapidus, Schwarz, & Honig, 1993). Hence increased understanding of the mechanisms underlying atopic disease and development of atopy should be considered an important area for detailed research.

1.4 Hypotheses and aims

As allergic disease often presents itself in early childhood has been proposed that perinatal or prenatal preventive strategies would be of benefit. Only a small number of studies have investigated the effect of maternal diet during pregnancy on the development of allergic disease in the offspring. There is even less data comparing maternal diet and the subpopulation of T cells present during pregnancy and in children with and without atopy. With such little information available within this area, the following hypotheses will be addressed in this thesis:

In those with a genetic predisposition:

- Atopy in early childhood is associated with maternal consumption of egg allergens, but this is modified by the nature of the maternal immune responses to the allergens.
- There is evidence of altered T cell function at birth in neonates who go on to develop atopic eczema.
- The clinical manifestations of allergy in early childhood are determined by tissue specific homing molecules expressed on specifically sensitised cord blood mononuclear cells (CBMCs).

In order to address these hypotheses the following aims will be fulfilled during this study:

1. To investigate the maternal immune response to ovalbumin allergens during pregnancy and how these change with dietary intake, gestational age and maternal atopic status.
2. To characterise the cord blood mononuclear cells at birth, and their responses to the allergens ovalbumin and β-lactoglobulin, in relationship to the development of eczema in the infant.
3. To relate organ specific allergic outcomes of the infants to the tissue specific homing molecules on sensitised cord blood mononuclear cells.

Chapter 2: Materials and Methods

2.1 Reagents

All the general laboratory reagents, materials and buffers are listed in appendix E.

2.2 Study population and questionnaire

2.2.1 Ethics

The investigations and samples collected were conducted following approval by Southampton and South West Hampshire Joint Research Ethics Committee (LREC04/Q1702/57). This study is registered with Southampton University NHS Hospitals Trust Research and Development.

2.2.2 Assessment visits

Parental atopic status was established upon recruitment by allergic history and a minimum of one positive skin prick test (SPT). Sibling atopic status was investigated on history alone. An initial recruitment questionnaire was completed with the Research Nurse on the first visit (Appendix B). Expectant mothers were given food frequency questionnaires (FFQ) to be completed between 20 and 24 weeks gestation, and again between 32 and 36 weeks gestation (Appendix A). The FFQs were reviewed with the Research Nurse at each assessment and blood samples (BS) were collected (figure 2.1). At birth cord blood was collected.

Infants were assessed at 6 and 12 months of age. Following a clinical assessment form and interviews with the parents, allergic symptoms were identified and recorded by a trained Research Nurse. The severity of eczema was assessed using SCORAD (**SCOR**ing **A**topic **D**ermatitis). SCORAD is a clinical tool used to assess the development and severity of atopic dermatitis (Rullo et al., 2008). General health and growth

parameters were recorded at each infant visit and a SPT performed (figure 2.1). All patients recruited onto this study were recruited by either myself, Research Nurse Norma Diaper or Dr Kathy Bodey (PhD).

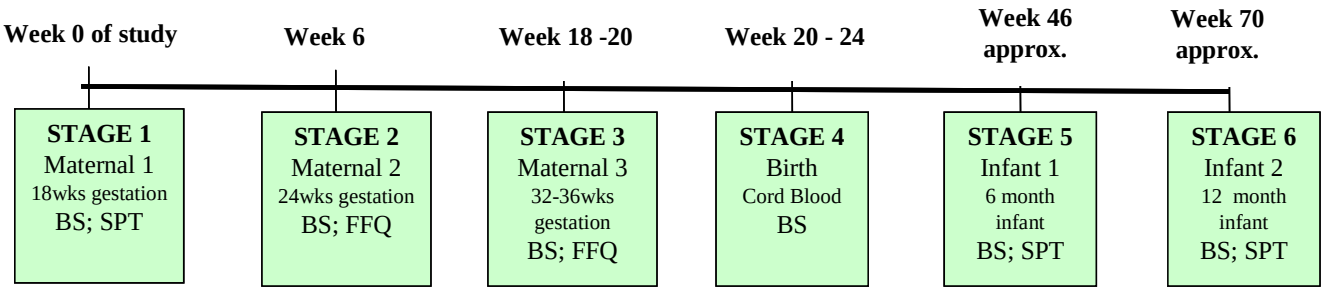

Figure 2:1 Study of timeline

Timeline of investigations: BS = Blood sample taken; SPT = Skin prick test; FFQ = Food frequency questionnaire.

2.2.3 Study Design and Subject recruitment

Within the original study design, 400 pregnant women, where the infant was at high genetic risk for atopy, were to be recruited to examine 'prenatal egg and milk allergen exposure in relation to tolerance or allergic sensitisation to food in infancy' (TO7044). Data from a previous FSA funded project (T07005) indicated that 40% of offspring born into families with genetic risk of atopy will present with atopic disease over the first 18 months of life. Thus it was anticipated that from the 400 recruited pregnant women, 160 infants in this cohort would develop atopic disease. Therefore recruitment of 400 mothers ensures that despite dropout, failure to collect all samples and inadequate or insufficient samples the necessary number of children with and without allergy with high medium or low dose exposure will be available.

However due to low recruitment within this study, it was appreciated the analysis would be underpowered and therefore the FSA removed their funding. Hence the study was stopped after 246 high risk pregnant women had been recruited. From those recruited a total of 51 samples

were selected for this smaller study, due to successful collection of samples and questionnaires including data from the infant follow up at 6 months. From these 51 subjects, 42 had infant's data from follow up at 12 months, and 25 of these infants had blood samples obtained.

As mentioned previously, infants in this study were divided into 2 groups, those who showed the presence of eczema before 12 months of life, and those who did not develop eczema within this study. In the original study design (n=400), infants were to be divided into those who showed senstitisation to egg and/or milk to those who were tolerant. However, due to the reduced number of infants recruited, very few showed egg or milk sensitisation (n = 2) by 12 months of age. However, a greater number of infants were diagnosed with atopic dermatitis during this study (n = 29). Thus, within this study we interpret the discrepancy between cellular and humoral responses to ovalbumin and b-lactoglobulin to demonstrate any distinct differences between those who developed eczema and those who did not. In addition, the original study design was to follow up the infant for the first 2 years of life. As the study was stopped early, data was collected up to the first 12 months. As atopic eczema usually develops within the first 12 months of life (Halkjaer et al., 2006) this was seen as an adequate main atopic end point for this study.

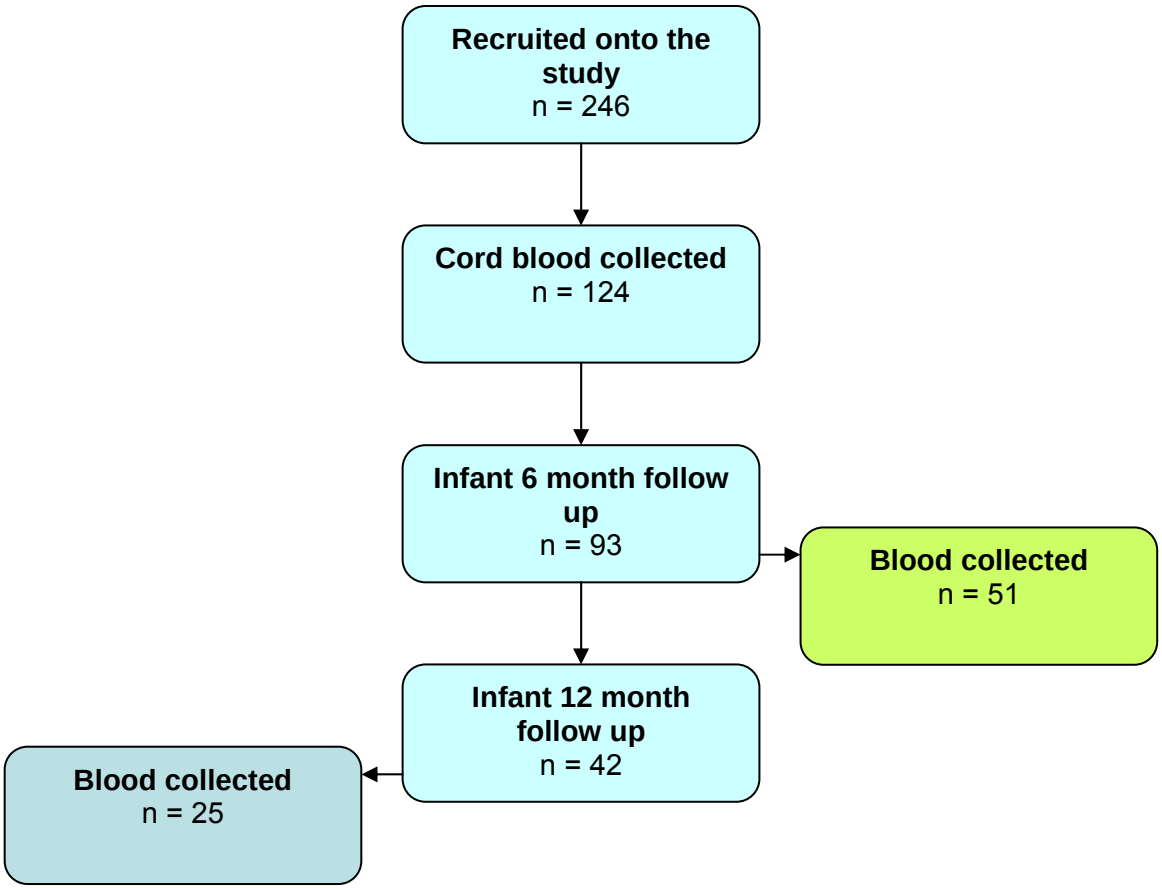

Figure 2:2 The study population

Flow chart showing the selection of mothers recruited onto this study. From the 246 atopic mothers originally recruited onto an original FSA funded study birth cohort (T07044), 124 cord bloods were collected, 93 infants were seen at 6 months and 42 infants at 12 months. Blood samples were obtained from 51/93 infants at 6 months and 25/42 at 12 months.

2.2.4 Questionnaires

The initial recruitment questionnaire was based on the International Study of Asthma and Allergies in Childhood (ISAAC) protocol (Asher et al., 2001) (Appendix B). The questionnaire included questions on maternal past and present onset of atopic symptoms, specifically asthma, eczema, rhinitis and hayfever. Family history and seasonality of atopy was also investigated in the questionnaire.

The FFQ was developed and validated by Child Health dietician Mrs K Grimshaw and was based on the Ministry of Agriculture, Fisheries and

Food (MAFF) food portions questionnaire 'Food Portion Sizes' (Fish and Food Ministry of Agriculture, 2009). The main aim for the FFQ was to collect dietary intake and classify women according to the amount of egg and milk they eat. The food categories included in the FFQ were: bread; breakfast cereals/cereals bars; meat dishes; fish; savoury dishes; vegetable dishes; accompanying sauces, dressings, dips and sandwich fillers; biscuits; cakes; puddings/desserts; confectionary/snacks; jams/spread; and egg and milk products. For each food item, the mothers reported their average consumption over the past month according to 4 categories: "not eaten in the last month"; "once per month or less"; once per fortnight"; or "number of days per week". Quantitative intake of each food item was also gathered under the categories: "number and size of slices"; "number eaten on each occasion"; "number and size of spoonfuls" and "pint". The FFQ data was quantified using a mean gram protein value of the specific food item and was related to portion size. All dietary protein intake is reported in grams (g).

2.2.5 Skin testing

Subjects were tested on the volar surface of the forearm if adults, and on their back if an infant (figure 2.2). The skin was clean, dry and lotion-free. Each extract (Table 2.1) was applied in droplets on the skin at appropriate intervals (1.5cm) from each other and from the positive and negative controls. The skin was pierced with a 1mm lancet (ALK, Denmark) perpendicular to the skin, through the droplet. A new lancet was used for each solution. Excess solution was removed from the arm or back with clean tissue and reactions were measured after 15 minutes. A positive reaction to an allergen was confirmed by a weal with a diameter of ≥2mm for infants and ≥3mm adults; provided there was no reaction to the negative control, and an appropriate response to the histamine.

(a) **(b)** **(c)**

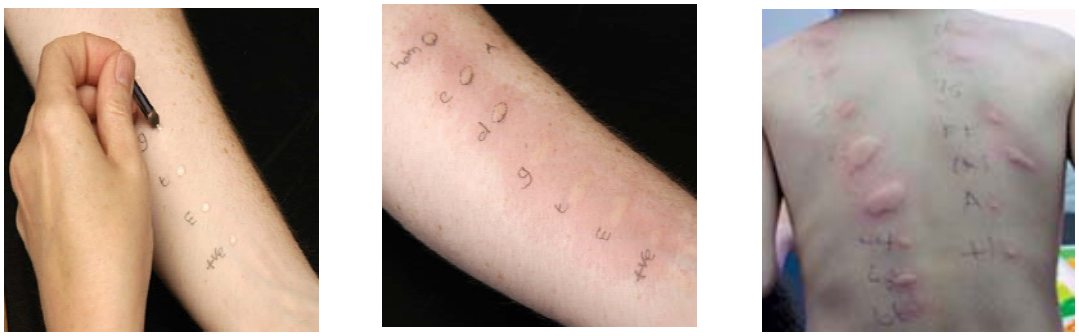

Figure 2:3 Skin prick testing.

(a) Skin prick tests are performed on the fore arm of adults. (b) Wheal diameter measurements are taken by drawing a dotted line around the circumference of each weal and then measuring the widest diameter. (c) Shows positive skin prick tests on an infant's back.

Skin prick test solution	**Concentration**
Histamine (positive) control	10mg/ml
Glycero-saline (negative) control	0.9%
House dust mite (*Dermatophagoides pteronyssinus*)	Soluprick standard quality (SQ), 10 HEP
Cat dander	Soluprick (SQ), 10 HEP
Grass mix pollen	Soluprick (SQ), 10 HEP
Mix tree pollen	Soluprick (SQ), 10 HEP
Dog dander	Soluprick (SQ), 10 HEP
Whole hen's egg	1:100 w/v
Cow's milk raw	1:20 w/v

Table 2:1 Solutions used for skin prick testing.

All solutions listed were used for both infant and adult skin prick testing and were supplied by ALK, Abello. When potency is expressed in HEP (Histamine Equivalent in Prick testing) it is related to the allergenic activity of the allergen product in the skin of the allergic patient. All skin prick test solutions were stored at 4°C.

2.3 Laboratory methods

All the general laboratory reagents, materials and buffers used in the methods of this thesis are listed in Appendix E.

2.3.1 Blood samples

At 18 and 36 weeks gestation, approximately 30ml of maternal blood was taken, and food frequency questionnaires for the previous 4 weeks were returned (Figure 2.1 stage 2 and 3). At birth, approximately 15ml of cord blood was collected by venepuncture of the umbilical cord into EDTA containing tubes (BD Vacutainer, UK) (Fig 2.1 stage 4). From the infant's visits, at the approximate age of 6 and 12 months (stage 5 and stage 6), venous blood samples were taken and a SPT performed.

2.3.2 Isolation and cryopreservation of mononuclear cells from blood

Peripheral venous blood was collected in lithium heparin vacuette tubes (BD, UK), placed on a roller (to prevent clotting) and processed within 2 hours of collection. Blood was layered onto an equal volume of Histopaque (Sigma, UK) and centrifuged for 30 minutes at 900 x *g* at room temperature (RT). The plasma was aliquoted into 2ml microtubes and frozen at -80^{o}C. The white mononuclear layer were re-suspended in ice cold phosphate buffered saline (PBS) and centrifuged for 10 minutes at 300 x *g*. The supernatant was discarded and the washing step repeated. The number of viable cells were calculated by exclusion dye, using a mixture of 10μl 0.04% Trypan Blue (Sigma, UK) to 10μl of sample on a haemocytometer. These cells were pelleted (400 x g, 4^{o}C, 10min) and resuspended in cell freezing medium (containing minimum essential medium, 8.7% dimtheyl sulfoxide and methyl cellulose) (Sigma, UK), at concentration of 5 x 10^{6} per ml, in Nunc cyro tubes (Nalge Nunc International). Cells were frozen at a rate of approximately 1^{o}C per minute using an isopropyl freezing chamber and placed in a -80^{o}C freezer

overnight. These were then transferred and stored in liquid nitrogen until required.

Blood serum samples were collected in the separator-serum (SST) blood-drawing gel tubes (Vacuatainer, BD), and were left to stand at RT for one hour before the serum was removed and stored in 2ml aliquots at -80^{o}C.

2.3.3 Thawing mononuclear cells from blood

The protocol used for thawing blood mononuclear cells was developed from work completed by Disi *et al,* which looked at maximizing the retention of antigen specific lymphocyte function after thawing (Disis et al., 2006). Their data showed that an increase in the media temperature to 37^{o}C was critical for viability and antigen specific function of T cells after cryopreservation.

Mononuclear cells were removed from liquid nitrogen storage, thawed quickly in a 37^{o}C waterbath with gentle agitation and transferred rapidly into 10 ml of pre-warmed (37^{o}C) medium (RPMI 1640 (Invitrogen, UK) with added 1% sodium pyruvate (Sigma, UK), 1% penicillin streptomycin (5000 units/ml) (Gibco, UK) and 5% Human AB serum (Sigma, UK). The cells were centrifuged at RT (300 x *g* for 7 min) and washed twice with warm media. Cells were resuspended in 5 ml of warm medium and cell number and viability was assessed using 0.04% Trypan blue (Sigma, UK) as previously described (2.2.2).

2.3.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.3.4.1 RNA isolation method

Cord blood mononuclear cells were pelleted at 300 x g for 5 minutes and the supernatant discarded. The pellet was resuspended in 1 ml TRIZOL Reagent (Sigma, UK) and left to stand for 5 minutes at RT. After this, 200μl of chloroform was added and the sample shaken vigorously for 15 seconds before being left to stand for 15 minutes at RT. After being

centrifuged at 12,000 x g for 15 minutes at 4 °C, the upper aqueous layer was removed and transferred into a fresh tube. The RNA was precipitated by adding 500 μl iso-propanol which was mixed gently and left to incubate for 10 minutes at RT. The sample was pelleted at 12,000 x g for 10 minutes at 4 °C and the supernatant discarded. After this the sample was washed with 1 ml 75% ethanol and pelleted again at 12,000 x g for 5 min at 4 °C. The supernatant was discarded and the pellet allowed to air dry. The sample was dissolved in 25 μl diethylpyrocarbonate (DEPC) water (Sigma, UK), and placed on a heating block for 10 minutes at 60°C to allow the RNA to fully dissolve in the water.

2.3.4.2 DNAse Treatment

22μl of RNA sample was taken and added to: 0.75 μl RNase Inhibitor (RNase In) (Promega, UK); 2.6 μl DNase I buffer10 X; and 1.5 μl DNAse I (Sigma, UK) and left at RT for 15 minutes. After this 2.6 μl stop solution was added and the samples left to incubate at 70°C for 10 minutes. Samples were then left to chill on ice for 5 minutes and stored at -80 °C overnight.

2.3.4.3 First strand synthesis

Samples were left to thaw on ice and the RNA concentration calculated using a spectrophotonycytometer, nanodrop ND1000 (Thermoscientficic, USA). From the sample, 1 μg of RNA was added to DEPC water to make up 11 μl total. To this 11μl sample, 1μl deoxynucleotide triphosphate (dNTPs) (10mM) and 1 μl oligo dT_{20} primer (Opuron, 50μM) were both added and the sample incubated at 65 °C for 5 minutes. The final end sample volume was 19 μl for the transcription reaction and additionally contained: 4 μl first strand buffer (5X); 1 μl Dithiothreitol (DTT) (0.1M); 1 μl reverse transcriptase superscript III (Invitrogen,UK); and 1 μl RNase inhibitor (Promega, UK). The sample was then placed on a therma cycler

which followed the programme of 50 °C for 40 minutes, 70 °C for 15 minutes and then held at 14 °C. cDNA was stored at -20 °C until required.

Oligonucleotide	Sequences
CLA-F	ACACAGCCATTGGGGGTTGC
CLA-R	CCTCGTGGCTGCTGGTTGA
AlphaE-F	CGGGGGCTCACGGTACAAACAT
AlphaE-R	CTGCGAGGGGCTGGCGGAGAG
Alpha4-F	TGGAGTGCCCCCTGATTTACGAAC
Alpha4-R	AAGCCATCTGCATTGAGGTCCACA
Beta7-F	GGACGGGAAGTTGGGCGGCATTTT
Beta7-R	GGGAGGCAGTGGGTGGCTTGGAGA

Table 2:2 PCR primers used for radiolabeling

2.3.4.4 RT-PCR

The master mix for each primer being used was made up in a sterile RNAse free 1.5 ml eppindorf and contained: 10 μl PCR buffer; 5 μl dNTPs; 5 μl forward primers; 5 μl reverse primers (Table 2.2), 2.5 μl DMSO and 0.3μl Taq DNA polymerase. This was made up to final volume of 49 μl with dH_2O and 1 μl cDNA was added. Amplification was carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems, UK) and the cycling programme illustrated in figure 2.4 was followed.

95 °C	12 minutes	
95 °C	1 minute	
65 °C	1 minute	**30 cycles**
72 °C	1 minute	
72 °C	5 minutes	
14 °C	10 minutes	

Figure 2:4 Settings used for the PCR reaction

2.3.4.5 Gel Electrophoresis

Agarose gels of final concentration 1.5% w/v were made by dissolving Ultra pure agarose (Seakem) in 1 X TAE buffer and a final concentration of 500 ng/μl ethidium bromide was added. Samples contained 3 μl green loading buffer and each gel was run with a DNA molecular weight marker (Promega, UK). Gels were eletrophoresed in 1 x Tris-acetate-EDTA (TAE) running buffer for 45 minutes at 80 V and then visualised under UV transillumination (M-20, UVP Inc, California USA).

2.3.5 In vitro stimulation of mononuclear cells

2.3.5.1 Development and optimisation of the stimulation method

Several papers have determined the optimal ovalbumin concentrations to use when stimulating peripheral blood mononuclear cells (PBMCs) (Kopp et al., 2000; Miles et al., 2003; Prescott et al., 1999). To optimise the concentration of the allergens used in this study, response curves for different concentrations of ovalbumin (Grade VII,Sigma, UK) were performed on both atopic and non-atopic bloods. Cultures of PBMCs (1.5 x 10^6 ml) from allergic and non-allergic donors were cultured with a number of different stimulants at different time points using different concentrations to determine the most effective time point and concentration. For antigen-specific stimulation, dose-response experiments were performed to determine optimal concentration required

for maximum antigen-specific T cell response. To determine the optimal stimulus of ovalbumin in PBMCs the experiment was performed on egg-allergic children with positive SPT results (n=3) and non-allergic children (n=2). Ovalbumin concentrations between 0 – 250μg were compared (Appendix E). In optimising this method each stimulant was also tested over a time period of 4 – 10 days with the maximal response being recorded each day. The control subjects showed no response to ovalbumin at any of the concentrations.

2.3.5.2 Method of stimulation

PBMCs were re-suspended at a concentration of 1.5 x 10^6 cells per ml in RPMI medium (Sigma, UK) and cultured in flat bottomed 24-well plates (Greiner, UK). The allergens ovalbumin and β-lactoglobulin were added at the optimal concentrations of 125μg/ml, while 1.25μg ml PHA (Sigma, UK) was added. Cells were incubated at 37°C in 5% CO_2 for 7 days. Proliferation was determined as described in section 2.2.6.2.

2.3.6 Proliferation Assays

2.3.6.1 Tritiated thymidine incorporation

In *vitro* measurements of mitogen- and antigen-induced blood lymphocyte proliferation was measured using [^{3}H]-thymidine incorporation. Thymidine is a specific precursor for DNA and is incorporated into new DNA in proliferating cells. Therefore, as ^{3}H-thymidine is incorporated into the DNA of proliferating cells, there will be an increase in the measured specific activity.

After 7 days of culture at 37°C in humidified 5% CO_2, [^{3}H]-thymidine was added at 0.5 μci/ml six hours before termination of the experimental period. The incorporated radioactivity was assessed using a Packard Top Count microplate scintillation counter (Packard). Proliferation was expressed as stimulation index (SI), which was calculated as the ratio between the mean counts per minute (c.p.m) values measured in the

stimulated cells and the mean c.p.m values measured for the unstimulated cells. An SI of 2 or more was considered as significant.

SI = (c.p.m in cultures stimulated with allergen) – (c.p.m. in unstimulated cultures) / (c.p.m. in unstimulated cultures)

2.3.6.2 Carboxyfluorescein Succinimidyl Ester – CFSE staining method

Studies have shown that different subsets of the lymphocyte population have different proliferation characteristics (Foulds & Shen, 2006). Proliferation of T cell subsets was measured using the carboxyfluorescein succinimidyl ester (CFSE, Sigma, UK). This fluorescent dye in its primary form is colourless and non-fluorescent, and consists of a fluorescein molecules containing 2 acetate moieties and a succininidyl ester functional group (Lyons, 2000). After passive diffusion into the intracellular environment, endogenous esterases remove the acetate groups to yield a highly fluorescent molecule. In addition, the succininmidyl ester reacts with intracellular amines, forming fluorescent conjugates that are retained and can be fixed with aldehyde fixatives. The cells were incubated with CFSE (5μM) for 20 min in the dark on ice and then washed with FACs buffer (% BSA, 0.1% sodium azide and PBS), and resuspended in medium (RPMI) with added serum. They were then incubated with conjugated antibodies for 30 min on ice, washed three times with FACs buffer and resuspended in 1% paraformaldehyde (PFA) (2.2.8.1). The cells were stored at 4° C for future analysis.

2.3.7 Preparation of lymphocytes from mononuclear cells for flow cytometry

Flow cytometry was used to phenotype the different subpopulations of lymphocytes present in infant peripheral blood and cord blood samples. All samples were processed blinded and later placed into one of two groups, dependent on infant showing either development of atopic eczema (AE) or no development of eczema (NE) by 12 months of age.

2.3.7.1 Development and optimisation

Many different types of fluorochromes are available for flow cytometry. When performing multicolour fluorescence studies there is a possibility of spectral overlap. To avoid this, preliminary experiments with different fluorochromes were performed. The fluorochromes used for this study were chosen as they showed least spectral overlap and were easily separated using fluorescence compensation. To determine the optimal concentration of the antibodies, each antibody was titrated to achieve maximal sensitivity at lowest possible concentration (table 2.3). Initial experiments were performed on a panel of 13 monoclonal antibodies (mAb) (table 2.3) recognizing constitutively expressed markers (CD3, CD4, CD8, CD28, CD30, CD45RA, CD45RO, CD69, CCR10, CCR4, β7 BD, UK. and CLA (MAC, UK.

2.3.7.2 Principles of flow cytometry

The principles of flow cytometry are shown in figure 2.5. In brief, single cells are passed through a flow chamber where they encounter a laser beam light source. The subsequent interaction results in a measurable scattering of the light source and a fluorescence emission from the fluorochromes conjugated to a monoclonal antibody, detected through a system of mirrors and optical filters. The first encountered mirror is a short pass mirror which allows light shorter than 560nm to pass and reflects light that is longer than that onto other optical filters. The light that is able to pass through this mirror reaches a beam splitter; this reflects 10% of the light to side scatter (SSC) detector and allows the remaining light to pass through. This light will continue to the FL1 detector (short wavelength, green fluorescence), CFSE and the fluorochrome fluorescein isothyocyanate (FITC) was used in this channel. There is a bandpass filter (BP) infront of the FL1 detector which will allow only light of wavelength 530 ± 30 nm to reach the detector. Light reflected by the 560 short pass (SP) mirror will reach a 610 long pass (LP) mirror, here wavelengths shorter than 610nm are reflected into the FL2 detector

(medium wavelength, yellow/orange fluorescence). Longer wavelengths will travel on and either be detected by the FL3 detector or reflected and detected in the FL4 channel. The fluorchromes phycoerythrin (PE) is detected in the FL2 channel, Peridinin chlorophyll protein with a cyanine dye (PerCP-Cy5.5) in the FL3 channel and allophycocyanin (APC) in the FL4 channel.

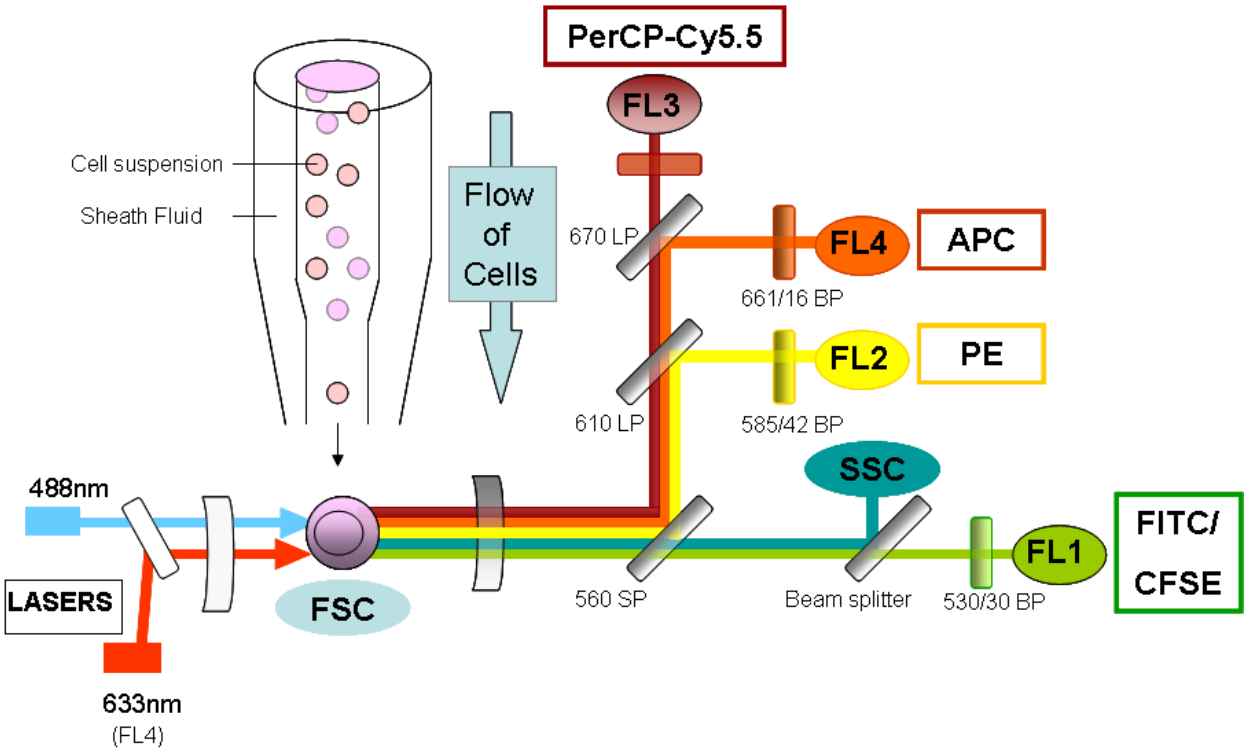

Figure 2:5 Flow cytometry

A schematic diagram of fluorescence detection by a flow cytometer. The fluorescence signals are directed to their respective detectors via a system of mirrors and optical filters. Showing the short pass (SP) and long pass mirrors. The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle.

Specificity	Clone	Isotype control	Manufacturer
Phycoerythrin (PE)-conjugated Antibody			
FoxP3	259D/C7	IgG1	BD Biosciences
CD3	HIT3a	IgG2a	BD Biosciences
CD4	IVT114	IgG1	BD Biosciences
CCR4	205410	IgG2b	R&D Systems
CCR7	3D12	IgG2a (rat)	BD Biosciences
CD28	CD28.2	IgG1	BD Biosciences
CD45RO	UCHL1	IgG2a	BD Biosciences
CD19	HIB19	IgG1	BD Biosciences
CLA	HECA-452	IgM (rat)	Mitenyi Biotec,
Beta-7	FIB504	IgG2a (rat)	BD Biosciences
Allophycocyanin (APC)- conjugated Antibody			
CD25	M-A251	IgG1	BD Biosciences
CD56	B159	IgG1	BD Biosciences
CD8	RPA-T8	IgG1	BD Biosciences
CD3	UCHT1	IgG1	BD Biosciences
CD127	40131	IgG1	R&D Systems
CCR9	248621	IgG2a	R&D Systems
Fluorescein isothyocyanate (FITC)- conjugated Antibody			
CD45RA	HI100	IgG2b	BD Biosciences
CD25	M-A251	IgG1	BD Biosciences
CD19	HIB19	IgG1	BD Biosciences
CD103	Ber-ACT8	IgG1	BD Biosciences
Peridinin-Chlorophyll-Protein Complex (PerCP)-conjugated Antibody			
CD3	SK7	IgG1	BD Biosciences
CD4	SK3	IgG1	BD Biosciences

Table 2:3 Monoclonal fluorochromes

Monoclonal antibodies against different human cell surface receptors used in flow cytometry analysis. All monoclonal antibodies were raised in mouse except where indicated and are categorised according to the flurochrome conjugate (fluorescein isothyocyanate (FITC); phycoerythrin (PE); allophycocyanin (APC); or Peridinin chlorophyll protein with a cyanine dye (PerCP-Cy5.5)). Isotype controls and manufacturer for each antibody are also listed.

2.3.7.3 Four colour flow cytometry method

Aliquots of 1 x 10^5 cells in 100μl PBS were incubated in a 96 V well plate (Greiner, UK) with blocking buffer at 4°C for 30min. Cells were washed with 150μl PBS and incubated with conjugated antibody at 4°C for 45min in the dark. All monoclonal antibodies were diluted in wash buffer to the optimal concentration and were conjugated to either: FITC, PE, APC or PerCP-Cy5.5 (Table 2.3). Control tubes were included in each test, these included FITC-, PE-, APC- and PerCP-Cy5.5 conjugated isotype control monoclonal antibodies.

Following incubation the plate was washed twice and the cells resuspended in 100μl FACs buffer. The cells were fixed in 1% PFA and stored at 4°C until ready to be analysed on the BD FACSAria^{TM} cell sorter (BD, UK).

2.3.7.4 Analysis of lymphocyte populations from PBMCs and CBMCs using flow cytometry

Flow cytometry analysis was carried out using BDFACSDiva software (BD, Biosciences). The first gate was drawn around the lymphocyte population identified on the forward scatter (FSC; indicating size) and side scatter (SSC; indicating granularity) dot plots (Fig 2.6 (a)). The different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and cellular contaminants.

A second gate was drawn round $CD3^+$ cells from a FSC and CD3 dot plot which was gated on the lymphocyte population, therefore identifying the T cell population (Fig 2.6 (b)). Subpopulations of T cells were classified with selective cell surface markers.

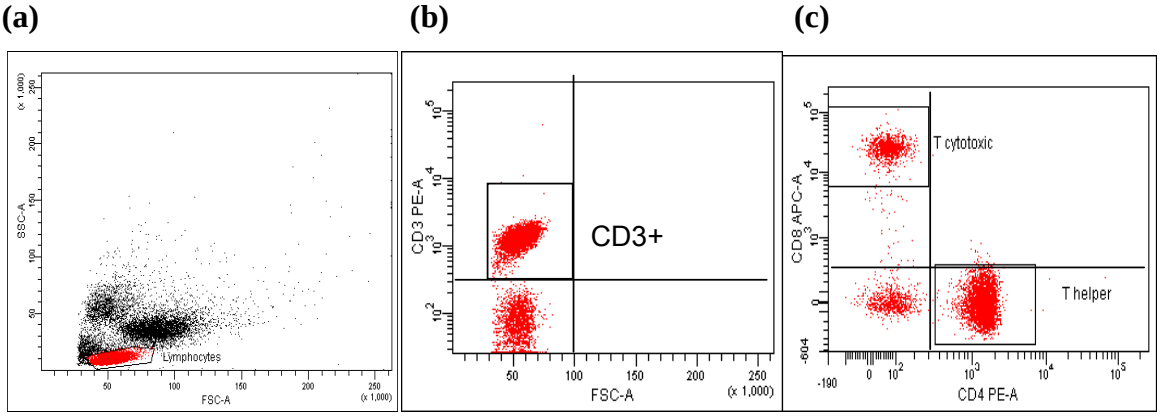

Figure 2:6 Analyses of flow cytometry data

Flow cytometry analysis dot plots gating. (a) A sample of human peripheral blood leukocytes. The intensity of the right-angle light scatter (side scatter) is plotted against the forward-angle light scatter (forward scatter) for each cell. The first gate identifies the lymphocyte population from the total PBMCs. (b) The FSC is plotted on the x-axis versus CD3 fluorescence on the y-axis. The gated cells are CD3+. (c) An example of dual-colour fluorescence staining histogram. Lymphocytes are stained with anti-CD4 in the PE channel (x-axis) and anti-CD8 in the APC channel (y-axis). The cytotoxic gate identifies the CD3+CD8+ T cells while the T helper gate identifies those cells that are CD3+CD4+.

2.3.8 Enzyme Linked Immunosorbant Assay (ELISA)

ELISA is a standard method for specific detection of immune mediators. Ovalbumin and β-lactoglobulin specific IgG, IgG1 and IgG4 concentrations were measured from maternal, cord and infant blood.

2.3.8.1 ELISA method for detection of specific IgG

Fifty μl per well of ovalbumin, β-lactoglobulin or bovine serum albumin (BSA) (100 μg/ml diluted in coating buffer) was plated onto 96 half well plates and incubated at 4 °C over night. The BSA coated plate was included in each experiment as a control for non-specific binding. The next day plates were washed with 100 μl per well assay buffer and blocked with 100 μl per well of blocking buffer for 2 hours at RT. Again, plates were washed and incubated with 50 μl of diluted samples and

standards for 1 hour at RT. Samples and standards were diluted in sample buffer (1:100 and 100 μg/ml – 1.56 μg/ml respectively). Plates were washed with wash buffer and 100 μl peroxidise conjugate rabbit anti-human IgG (DAKO, UK) diluted 1:6000 in sample buffer, was added to each well. After incubation of 2 hours the plates were washed 3 times with wash buffer before adding 100 μl of tetramethylbenzidene (TMB) substrate buffer and incubated in the dark for 30 minutes. The reaction was stopped with by adding 50 μl of 1M HCl and the absorbance read at A_{450nm}. Samples whose absorbance registered above the reference curve were repeated at a higher dilution to bring the optical density down to the linear part of the reference curve.

2.3.8.2 Calculation of allergen-specific IgG concentration

The optical density (OD) obtained from the standards and samples on the BSA-coated plate were subtracted from the OD obtained from the allergen-coated plates. The average was then taken of the duplicated results, unless there was a >10% difference between the results then sample was ignored. A standard curve from the standards OD and their known concentration was constructed. All sample ODs were compared to the standard curve and concentrations expressed as AU.

Isotype	**Detection**	**Manufacturer**	**Dilution**
HRP-conjugated rabbit anti-human IgG	IgG	Dako	1:6000
HRP-conjugated mouse anti-human IgG1	IgG1	Binding Site	1:3200
HRP-conjugated mouse anti-human IgG4	IgG4	Binding Site	1:1600
Avaidin HRP	Biotinylated	Sigma	1:2000

Table 2:4 Detection antibodies for ovalbumin and β-lactoglobulin specific immunoglobulin measurement

2.3.8.3 Measurement of allergen specific IgG1 and IgG4 – development and optimisation

An indirect ELISA to measure allergen-specific IgG sub-classes IgG1 and IgG4 was developed using commercially available enzyme-conjugated antibodies (Table 2.4). Concentrations to achieve maximum sensitivity for optimal detection of samples and standard curve were determined. Standard curves were developed using pooled allergic patients'serum.

2.3.8.4 ELISA method for detection of specific IgG1 and IgG4

A 96 half well plate was coated with either ovalbumin, β-lactoglobulin or BSA at 100 μg/ml in coating buffer, 50 μl per well and incubated at 4 °C overnight. Plates were then washed with 100 μl per well assay buffer three times and blocked with 100 μl per well of blocking buffer for 2 hours at RT. Plates were washed as above and incubated with 50 μl of diluted samples and standards for 2 hours at RT. Samples and pooled serum (used for standard curve) were diluted in sample buffer (1:50 and 1:10 – 1:640 respectively). Plates were washed with wash buffer and either 100 μl peroxidise conjugate mouse anti-human IgG1 or 100 μl peroxidise conjugate mouse anti-human IgG4 was added to each well (Table. 2.4). After an incubation of 2 hours at RT, the plates were washed a further 3 times with wash buffer before adding 100 μl of TMB substrate buffer and incubated in the dark for 30 minutes. The reaction was stopped with by adding 50 μl of 1M H_2SO_4 and the absorbance read at both 405 nm with reference set at 620 nm.

2.3.8.5 Calculation of allergen-specific IgG1 and IgG4 concentration

The optical density (OD) obtained from the standards and samples on the BSA-coated plate were subtracted from the OD obtained from the allergen-coated plates. The average was then taken of the duplicated

results, unless the difference between the results was greater than 10%, the samples were not included. A standard curve was constructed using the standard's OD against their known dilutions, whereby a 1:100 dilution of the pooled serum represented 0.01 AU. All samples OD were compared to the standard curve and concentration of allergen-specific IgG1 and IgG4 expressed in AU, with dilution taken into account.

2.3.9 Data entry and analysis

All data were analysed and double entered into SPSS (Version 15, Chicago, USA) for windows.

For data that did not show normal distribution (e.g. levels of immunoglobulin antibodies detected, proliferation) analysis was performed using non-parametric tests Mann-Whitney U test (comparing AE vs NE groups) and Wilcoxon tests (comparing those samples exposed to allergen to those that were cultured alone). Parametric tests including the t-test and paired t-test were used to analyse normally distributed data.

A probability level of <5% was considered statistically significant. Although numerous statistics were analysed in this study, multi-variable testing was not used as this is an exploratory study.

Chapter 3: Specific IgG responses to ovalbumin and β-lactoglobulin through pregnancy and first year of life in relation to diet and development of infantile eczema

3.1 Introduction

The aetiology of allergy is complex and involves multiple factors, including genetic predisposition (Cookson & Moffatt, 2000) and environmental influences. Several studies have considered the *in utero* environment and early life exposure to be an important time period in the development of atopy (Jenmalm & Bjorksten, 2000; Prescott et al., 2005; Warner et al., 1994). Early life factors that have previously been associated with the development of atopy include rapid foetal growth (Fergusson et al., 1997), the feeding of cows milk to the infant (Snijders et al., 2008), neonatal infections (Benn et al., 2004) and maternal smoking (Raherison et al., 2007) as well as other lifestyle habits. Environmental factors which may affect the foetus *in utero*, include the maternal intake of potential food allergens such as cow's milk and hen's egg as well as diet supplements such as vitamin D (Devereux et al., 2007). The maternal immune responses such as the transplacental transfer of allergen specific maternal IgG antibodies to the foetus during pregnancy may also play a role in the later development of allergy in the infant (Billington, 1992; Szepfalusi et al., 1997; Szepfalusi et al., 2000a). The relationship between foetal allergen exposure via the maternal diet, and development of atopic eczema in the infant remains to be elucidated.

The foods investigated in this study include cow's milk and hen's egg. Cow's milk is an important source of protein, especially for children. The proteins present in milk are divided into two different groups: the casein proteins and the whey proteins. The most abundant proteins are the whey proteins, α-lactoglobulin and β-lactoglobulin (Coultate T.P, 2007) with β-lactoglobulin (Bos d5) being the most abundant (approximately 3 – 4 g/L) (Blanc et al., 2008). β-lactoglobulin, which is the major allergen responsible for milk allergy, is absent in human milk (Wal, 2002). This present study investigated the immune response to this protein. With regards to egg allergy, most investigations have focused on ovalbumin (Gal d I) and ovomucoid (Gal d III). Only the predominant

glycophosphoprotein ovalbumin was investigated in this research project, this was primarily because previous studies have shown ovalbumin to induce a greater T cell specific immune response compared to ovomucoid in egg allergic children (Tay et al., 2007b). As both the T cell response (Chapter 5) and the development of egg allergy within children were investigated in this study, ovalbumin was thought to represent the more relevant egg protein for the context of this study.

Food-specific IgE antibodies are usually present in food allergy, with specific IgE titres or SPT weal diameters showing predictive values for clinical reactions (Roberts & Lack, 2000; Sampson, 2001). However, the clinical significance of food-specific IgG remains to be clarified. It remains unclear whether the levels of IgG are raised in allergy. Research groups have demonstrated IgG levels to be elevated, reduced and comparable to that of healthy controls (Hidvegi et al., 2002; Hill et al., 1993; Shek et al., 2005; Sletten et al., 2006; Vance et al., 2004b). These differences could be due to variation in the subject groups and the ELISA methods used. For most studies, the specific IgG titre was measured in arbitrary units meaning it is not possible to compare data to further investigate this conflict.

Increased cord blood IgE levels has been associated with mothers who have a positive history for atopy (Bergmann et al., 1995). Unlike IgE, IgG and its subpopulations are able to cross the placenta via active transport (Avrech et al., 1994; Englund, 2007). Both IgG and IgE levels in the cord blood have been investigated as possible predictive markers for infant atopic development (Bergmann et al., 1995; Casas et al., 2004; Chang et al., 2005; Edenharter et al., 1998; Iikura et al., 1989). Total IgE serum levels of 100 – 150 kU/1 are considered elevated in adults. In contrast, the cut-off levels for IgE in cord blood is not well defined (Allam et al., 2005). This has led to studies comparing cord blood IgE levels with future atopic development at different cut-off levels (Aichbhaumik et al., 2008; Hansen et al., 1993; Hide et al., 1991; Tariq et al., 1999; Yang et al., 2007). Dependent on these cut-off levels, positive predictive values for

atopy development has differed between studies (range 20 – 95%), with all showing low sensitivity but high specificity (Hansen et al., 1993; Hide et al., 1991). This implies that elevated cord blood IgE is a reliable predictive factor of future atopy development but normal or low levels are weak predictors for the absence of atopy.

High levels of food and inhalant allergen-specific IgG have been detected in cord blood from infants of atopic mothers compared to infants of non-atopic mothers (Jenmalm & Bjorksten, 2000). The significance of infant specific IgG, and the subsequent development of atopic disease, remains to be clarified. While some authors have reported high levels of food allergen specific IgG (Casimir et al., 1989; Vance et al., 2004a) and inhalant allergen specific IgG (Jenmalm & Bjorksten, 2000), in cord blood to be associated with decreased infant atopy, others have found no association (Zeiger et al., 1989) or even an increased association with allergy development (Iikura et al., 1989). Interestingly, data from Vance *et al*., showed infants with a mid-range concentration of ovalbumin and β-lactoglobulin specific cord blood IgG, were most likely to be atopic at 6 months compared to those with the higher or lower levels (Vance et al., 2004a).

Both IgG4 and IgE antibodies are markers of sensitisation and atopic disease (Kihlstrom et al., 2005). Elevated specific IgG4 concentrations have been reported in sera of patients with atopic eczema (Shakib, 1986). Increased levels of IgG1 antibodies to both ovalbumin and β-lactogloblin, have been detected in children with persistent sensitisation (Sletten et al., 2006; Vance et al., 2004b). However, other investigators have detected increased levels of IgG1 antibodies during early immunotherapy (Buchanan et al., 2007; Hedlin et al., 1991). Preliminary results from one study investigating egg oral immunotherapy in children with egg allergy, showed a significant increase in ovalbumin specific IgG levels in the infants after egg exposure (Buchanan et al., 2007). Interestingly, although it was only a small pilot study, all infants tolerated significantly more egg protein than before the start of the study, and two

subjects demonstrated oral tolerance (Buchanan et al., 2007). This suggests that exposure to the food is an important component for the generation of an IgG response.

3.1.1 Aim

The aim of this part of the study was to investigate ovalbumin and β–lactoglobulin specific immunoglobulins (IgG, IgG1 and IgG4) present in maternal, cord and infant blood samples, in relation to the maternal dietary intake of egg and milk protein and the subsequent development of eczema in the infant by the age of 12 months.

3.2 Subjects, samples and methods

3.2.1 Clinical Assessment

In brief (full details in Methods 2.1.2), detailed clinical information about atopic symptoms for asthma, eczema, hay fever, rhinitis and food allergy were taken and SPT responses to common food and inhalant allergens recorded (Appendix B). For infants, detailed clinical information about atopic symptoms for eczema and food allergy was taken by interview with the parents at both the 6 and 12 month visit (Appendix D). Expectant mothers completed FFQ (20 - 24 weeks and 32 - 36 weeks gestation) (Appendix C) and had blood samples were taken at each visit. For infants, SPTs were performed and blood samples were taken at both 6 and 12 month visits.

Of the 51 food frequency diaries handed out all 51(100%) FFQ1 were returned and 47 (92%) FFQ2 were returned. Diet was not monitored prior to 20 weeks because of the potential confounding effects of morning sickness. Although good validity and reliability of FFQ, specifically those looking at food allergens consumed during pregnancy, have been shown

(Venter et al., 2006), the weaknesses of FFQ do include problems with quantifying food intake and participant's recall (Cade et al., 2002). For this purpose the expectant mothers were also given food diaries in addition to the FFQs, which were filled out by themselves for the month duration at home and then the data transferred to the FFQ. This was done in the hope to eliminate the problems earlier suggested in addition to keeping the administration simple and, in principle, being able to measure the dietary intake and eczema development (Cade et al., 2002).

3.2.1.1 Patient Sera

Serum samples were collected at 16 weeks (n=45), 24 weeks (n=48) and 36 weeks gestation (n=43) from mothers at high risk of atopy. Foetal cord blood (n=37) was obtained through the technique of percutaneous umbilical blood sampling, and infant serum samples were collected at 6 (n=20) and 12 months (n=20) age.

3.2.1.2 Infant Outcomes

In their 6 and 12 months' postpartum visits, infant's allergic outcome were recorded with questionnaires, based on standard questions from the International Study of Asthma and Allergies in Childhood (ISAAC) and evaluated using the Scoring Atopic Dermatitis Severity Index (SCORAD). SCORAD, assesses the percentage of body surface area involved as well as the intensity of the "representative area," e.g. pruritus, insomnia.
If a SCORAD score was given to an infant at either visit or the parent had answered affirmatively to the eczema question ('Has your child ever had eczema?'), the infants were defined as having developed eczema in the first year of life and were placed into the atopic eczema group (AE). If no eczema was present at either visit and the response to the eczema question was negative the infants were allocated into the non-eczema (NE) group.

3.2.2 Laboratory methods

3.2.2.1 ELISA Techniques

Serum ovalbumin and β-lactoglobulin specific IgG, IgG1 and IgG4 concentrations were measured by in-house indirect ELISAs, developed and optimised as previously described (2.2.8). All concentrations were expressed in arbitrary units (AU), by comparison of the sample OD to the reference standard curve with the dilution of the sample taken into account. Samples with absorbance above the reference curve were repeated at higher dilution such that the absorbance fell on the linear part of the reference curve.

3.2.2.2 Statistics

As the levels of immunoglobulin antibodies to the allergens were not normally distributed the AE and NE groups were compared with Mann-Whitney U test. Correlations within groups were calculated using the Spearman's rank order correlation coefficient test. Measurements that appeared below the limit of detection by the ELISA were assigned "0" value for analysis. The Chi-square test was employed for categorical variables. A probability level of <5% was considered to be statistically significant. Data was analysed and double entered into SPSS (Version 15, Chicago, USA) for windows.

3.3 Results

3.3.1 Study population

Fifty one high risk atopic mothers (with personal or family history of allergy) were recruited for this study from an original birth cohort (n = 247). The maternal, paternal and infant characteristics included in the analyses are summarised in Table 3.1. Twenty eight offspring went on to develop eczema (AE) while 23 showed no development of eczema by 12 months.

3.3.2 SPT data

Upon recruitment, self-reported atopic disease within the maternal (AE = 69%, NE = 62%), paternal (AE = 31%, NE = 33%) and sibling (AE = 50%, NE = 60%) groups was similar in both the AE and NE populations (p = 0.199, p = 0.160 and p = 0.241, respectively). However, maternal SPT data showed maternal atopic sensitisation to be higher in the AE group (listed Table 2.1) in 22 out of the 28 (78.6%) mothers compared to 11 from the 22 (50%) mothers in the NE group (p=0.034) (Table 3.2).

All but one of the mothers had SPT performed. The most common allergens causing positive skin tests in the mothers was: house dust mite (HDM) (22/50); dog (21/50) and mixed grass pollen (18/50). SPT data was collected from 47 of the 51 infants at 6 months (AE n = 26, NE n = 21) and from 29 infants at 12 months (AE n = 19, NE n = 10) visit.

At both 6 and 12 months visit the AE infants showed a higher number of positive SPT results. However this was only significant at 12 months (p = 0.032) (Table 3.3). Of the allergens used in this study, the most common to cause a positive SPT result in the infants included the food allergens hen's egg, cow's milk, with the addition of the aero-allergen cat dander at 12 months (Table 3.3).

		Non-eczema infants (NE), **n = 22**	Atopic eczema infants (AE), **n = 29**	P
Maternal Characteristics				
Maternal age at birth of child (years)	mean (SD) min to max	32.8 (4.4) 24.5 – 40.2	31.3 (5.9) 20.0 – 40.0	0.826[2]
Maternal asthma	(%)	9 (40.9)	13 (46.4)	0.696[1]
Maternal eczema in childhood	(%)	11 (50)	17 (60.7)	0.613[1]
Maternal rhinitis/hayfever	(%)	10 (45.5)	17 (60.7)	0.461[1]
Positive SPT % to >1 allergens	(%)	11 (50)	22 (78.5)	0.034*[1]
Paternal Characteristics		**n = 19**	**n = 23**	
Paternal age at birth of child (years)	mean (SD) min to max	36.6 (7.1) 27.1 – 52.2	34.3 (8.3) 21.4 – 52.3	0.389[2]
Paternal asthma %	(%)	8 (42.1)	6 (26.1)	0.273[1]
Paternal eczema in childhood %	(%)	5 (26.3)	5 (21.7)	0.729[1]
Paternal rhinitis/hayfever	(%)	13 (68.4)	13 (56.5)	0.429[1]

Table 3:1 Parental age and history of atopic disease

Parental age and history of atopic disease in relation to the development of eczema (AE) or no development of eczema (NE) in the infant by 12 months of age. The mean and standard deviation (SD) are shown where appropriate along with the range (min to max). 2 T-test was applied, otherwise statistical analysis was performed using chi-square test.

		Non-eczema infants (NE)	Atopic eczema infants (AE)	p
Birth Characteristics		**n = 22**	**n = 29**	
Birth weight (g)	mean (SD) min to max	3507.5 (640.1) 2324 – 4725	3469.4 (575.7) 2680 – 4690	0.827[2]
Gestational age (wks)	mean (SD) min to max	39.8 (1.9) 35.0 – 42.0	39.9 (1.6) 36.1 – 42.0	0.783[2]
Sex - Male	(%)	13 (59)	18 (64)	
- Female	(%)	9 (41)	10 (36)	
Head circumference (cm)*	mean (SD) min to max	34.9 (1.3) 33.0 – 37.0	34.8 (1.8) 32.0 – 38.0	0.641[2]
Infant Characteristics at 6 months				
Weight (kg)	mean (SD) min to max	8.0 (0.8) 6.0 – 10.0	7.5 (0.9) 6.0 – 9.9	0.067[2]
Length (cm)	mean (SD) min to max	68.1 (2.6) 64.0 – 75.0	66.4 (2.2) 63.0 – 71.0	0.020*[2]
SCORAD score	mean (SD) min to max	Na	14.4 (12.5) 3.0 – 48.0	
Positive SPT to hen's egg	(%)	0	6 (23)	0.024*
Positive SPT to cow's milk	(%)	0	4 (15.3)	0.086
Infant Characteristics at 12 months				
Weight (kg)	mean (SD) min to max	10.2 (0.9) 9.0 – 12.0	10.1 (1.3) 8.0 – 14.0	0.800[2]
Length (cm)	mean (SD) min to max	76.5 (2.0) 74.0 – 80.0	73.3 (3.5) 70.0 – 84.0	0.780[2]
SCORAD score	mean (SD) min to max	Na	11.7 (7.9) 4 – 27	
Positive SPT to hen's egg	(%)	1 (10)	5 (25)	0.092
Positive SPT to cow's milk	(%)	1 (10)	2 (10)	0.177
Infant diet				
Age first fed formula (months)	mean (SD) min to max	2.5 (1.1) 1.0 – 4.0	3.5 (2.8) 1.0 – 10.0	0.257[2]
Age first fed solids (months)	mean (SD) min to max	4.5 (0.7) 3.0 – 6.0	4.7 (0.9) 2.5 – 6.0	0.434[2]
Age first fed egg (months)	mean (SD) min to max	8.4 (2.4) 4.0 – 12.0	8.5 (2.1) 6.0 – 12.0	0.949[2]

Table 3:2 Infant characteristics

Infant characteristics in relation to the development of eczema (AE), or no development of eczema (NE) by 12 months of age. The mean and standard deviation (SD) are shown where appropriate along with the range (min to max). 2 T-test was applied, otherwise statistical analysis was performed using chi-square test.

3.3.3 Infants clinical data

A logit regression was used to investigate whether the development of eczema in the infant could be predicted from birth weight, gestational age, sex, mode of delivery and head circumference measurements taken from this present study. All showed to be non-significant however, there is a continual trend for infants within the eczema group to show a lower mean for weight and length measurements at both 6 and 12 month visits.

SPT solution	SPT results at 6 months		SPT results at 12 months	
	AE Infants (n=26)	NE Infants (n=21)	AE Infants (n=19)	NE Infants (n=10)
Hen's egg	6*	0	5*	1
Cow's milk	4	0	2	1
House dust mite	1	0	1	0
Peanut	0	0	0	1
Cat dander	2	0	4	0
Dog dander	0	1	1	0
Mixed grass pollen	1	0	1	0
Mixed tree pollen	0	0	0	0

Table 3:3 The number of infants with positive SPT results to different food and aero-allergens at 6 and 12 months of age.

*From the 6 infants at 6 months who had a positive SPT for egg, 3 infants continued to have a positive reaction at 12 months while 3 infants no longer showed a positive reaction. However, 2 different infants had a positive reaction at 12 months when they didn't have one at 6 months. Positive wheal >2mm.

3.3.4 Maternal dietary consumption

Expectant mothers showed a significant correlation between mean dietary intake of milk protein at 20 – 24 weeks gestation (FFQ1) and 32 – 36 weeks gestation (FFQ2) ($p<0.001$). Mean egg protein intake recorded

in FFQ1 and FFQ2 also correlated ($p<0.001$). Mothers whose infants did not develop eczema, showed no significant differences in their dietary intake of egg or milk protein between the two time periods measured during pregnancy. However, mothers included in the AE group showed increased mean milk protein intake between FFQ1 (mean = 623.7 g; 95% confidence interval 529.9 to 717.5g) and FFQ2 (mean = 719.1g; 95% confidence interval 616.4 to 821.8g) ($p = 0.02$).

As expected both AE and NE mothers mean dietary intake of milk protein (measured in grams) was significantly higher than that of egg protein in both FFQs ($p>0.001$). The dietary mean monthly intake of milk protein in AE mothers was 623.7g during 20 - 24 weeks gestation (FFQ1) and 719.1g during 32 – 36 weeks gestation (FFQ2). The dietary mean monthly intake of egg protein was significantly lower at 110g during 20 - 24 weeks gestation (FFQ1) and 93.4g during 32 – 36 weeks (FFQ2). A similar pattern of egg and milk protein intake in the maternal diet was seen in the NE mothers (Milk: 721.4g; 710.7g: Egg: 93.4g; 101.5g, respectively). There was no significant difference in the mean dietary intake of hen's egg and cow's milk protein in the maternal diet of those in the AE group and the mothers in the NE group during the pregnancy (Figure 3.1). However, a power calculation has shown a sample size of 506 completed FFQs with atopic infant outcomes would be needed to detect a difference maternal dietary egg and milk protein intake and the development of eczema in the infant.

3.3.5 Maternal specific IgG to ovalbumin and β-lactoglobulin

Maternal ovalbumin and β-lactoglobulin specific IgG concentrations were determined at 12, 24 and 36 weeks gestation. No significant variation in specific IgG concentrations was detected between any of the time points. Furthermore, no significant difference for either ovalbumin or β-lactoglobulin specific IgG levels was detected between the mothers within the AE group or in the NE group, at any of the designated time points during pregnancy (Fig 3.2). There was a significant correlation between

maternal ovalbumin and maternal β-lactoglobulin specific IgG levels at both 24 and 36 weeks gestation ($p<0.001$ and $p<0.001$, respectively).

Maternal ovalbumin and β-lactoglobulin specific IgG1 and IgG4 were recorded at 36 weeks gestation, at birth and from the infant at both 6 and 12 month visits. No significant difference in either ovalbumin or β-lactoglobulin specific IgG1 or IgG4 was detected between AE or NE mothers. We detected no correlation between maternal dietary intake of egg protein and ovalbumin specific IgG ($p = 0.521$), IgG1 ($p = 0.464$) or IgG4 ($p = 0.531$) at 36 weeks gestation. Furthermore, no correlation was detected between maternal dietary intake of milk protein and β-lactoglobulin specific IgG and IgG subgroups (IgG $p = 0.509$; IgG1 $p = 0.053$; IgG4 $p = 0.084$) at 36 weeks gestation.

3.3.6 Relationship between maternal IgG subclasses and cord blood IgG subclasses

Cord blood plasma was available for 37 of the 51 (72.5%) babies. Ovalbumin specific IgG in the cord blood, directly correlated with the matching maternal specific IgG concentrations taken at 36 weeks gestation (Rho = 0.864, $p<0.001$). Ovalbumin specific IgG subclasses, IgG1 and IgG4 also correlated highly with the paired maternal levels (IgG1 Rho = 0.511, $p = 0.001$, IgG4 Rho = 0.865, $p = 0.001$) (Figure 3.3). Similarly, cord blood β-lactoglobulin specific IgG, directly correlated with paired maternal β-lactoglobulin specific IgG levels from 36 weeks gestation (Rho = 0.464, $p = 0.001$). Furthermore, maternal and cord blood β-lactoglobulin specific IgG subgroups, IgG1 and IgG4 also highly correlated (IgG1 Rho = 0.665, $p = 0.001$; IgG4 Rho = 0.591, $p = 0.001$) (Figure 3.3).

No correlation between maternal dietary intake of egg protein and cord blood ovalbumin specific IgG, IgG1 or IgG4 ($p = 0.643$; $p = 0.596$; $p = 0.834$) or maternal dietary intake of milk protein and cord blood β-lactoglobulin specific IgG, IgG1 or IgG4 ($p = 0.572$; $p = 0.226$; $p = 0.834$)

was detected. There was also no correlation between maternal dietary intake of egg protein and infant ovalbumin specific IgG or IgG1 at 6 or 12 months (6 months: $p = 0.991$; $p = 0.618$; 12 months $p = 0.542$; $p = 0.119$). However, a significant correlation between maternal dietary intake of egg protein and infant ovalbumin specific IgG4 was detected at 6 months ($p = 0.015$), but this was no longer apparent at the 12 months visit ($p = 0.511$). The only significant correlation detected with maternal dietary milk intake and infant β-lactoglobulin specific IgG and IgG subgroups was infant specific IgG1 at 6 months ($p = 0.001$).

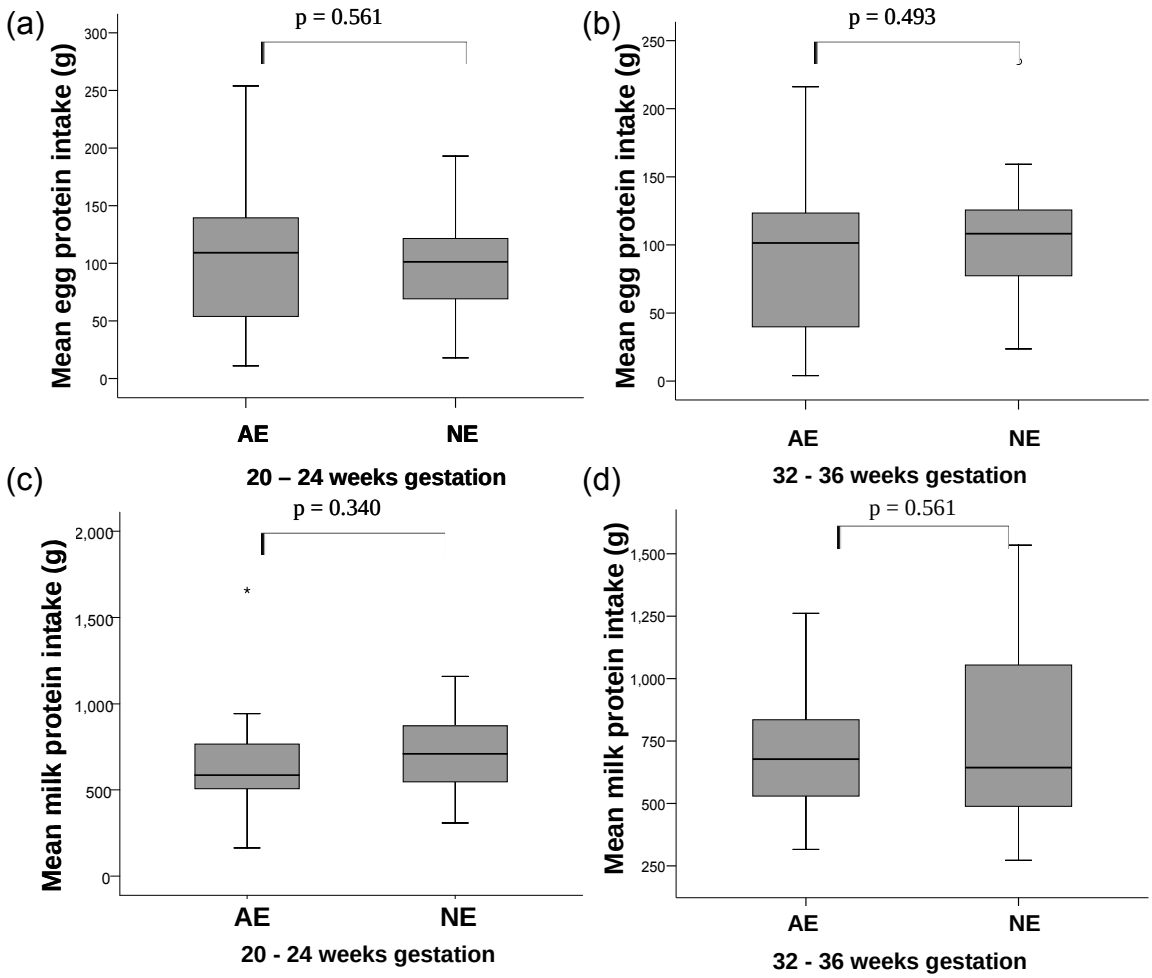

Figure 3:1 Maternal egg and milk dietary intake during pregnancy.

Mothers were divided into two groups according to the development (AE) or non-development (NE) of eczema in their infant. (a) The mean maternal dietary intake of egg protein during 20 – 24 weeks gestation (FFQ1) and (b) 32 – 36 weeks gestation (FFQ2) for both groups. (c) The mean maternal dietary intake of milk protein during 20 – 24 weeks gestation (FFQ1) and (b) 32 – 36 weeks gestation (FFQ2) for both groups. No significant difference of dietary egg or milk intake during pregnancy was seen between the two groups. The top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the line in the middle represents the 50th percentile. The whiskers (the lines that extend out the top and bottom of the box) represent the highest and lowest values that are not outliers or extreme values. Outliers (values that are between 1.5 and 3 times the interquartile range) and extreme values (values that are more than 3 times the interquartile range) are represented by circles beyond the whiskers.

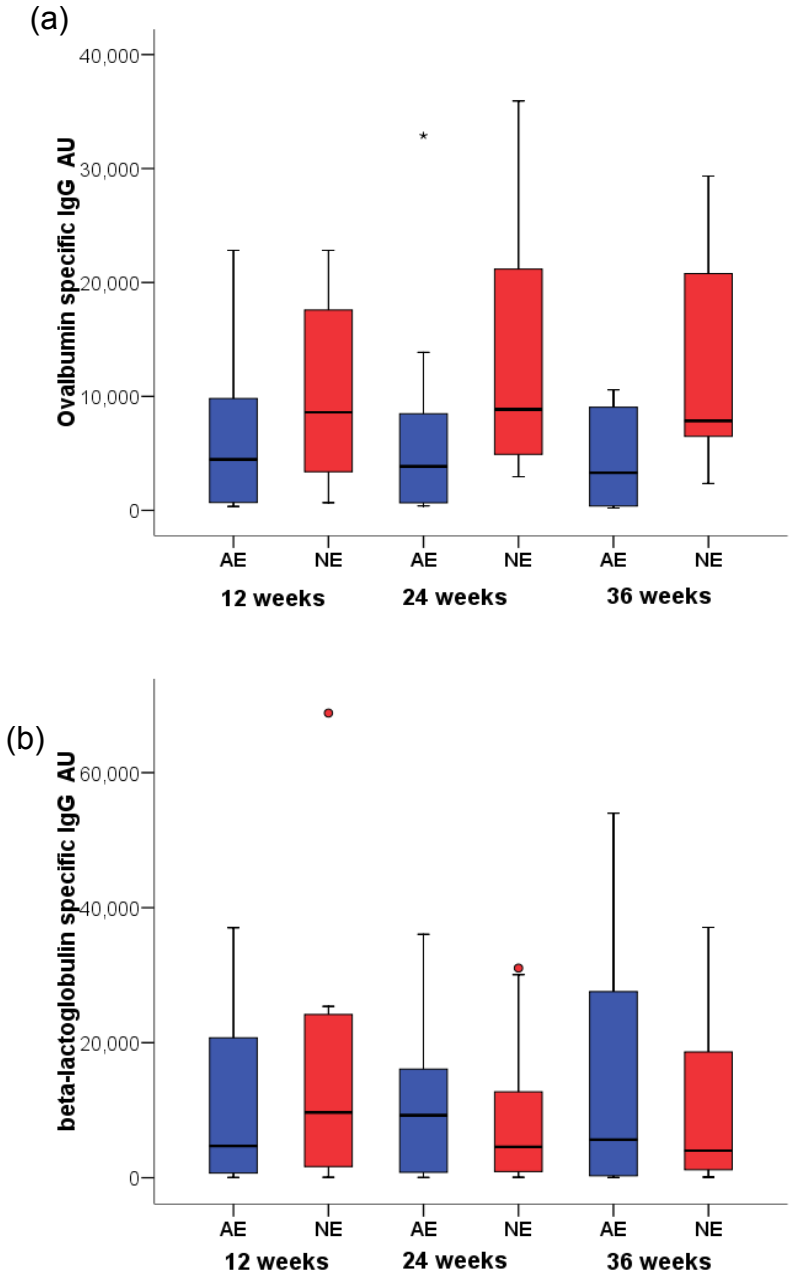

Figure 3:2 Maternal serum ovalbumin and β-lactoglobulin specific IgG concentrations at 12, 24 and 36 weeks gestation according to whether the infant developed eczema.

Ovalbumin (a) and β-lactoglobulin (b) specific IgG concentration (arbitrary units, AU) were measured by indirect ELISA. The box shows values in the interquartile range (25th and 75th percentiles). The central line shows the median value and the whiskers extend to the lowest and highest values, excluding outliers which are shown by the separate circles. Between the two groups (AE, NE) no significant difference for either ovalbumin or β-lactoglobulin specific IgG concentration was seen at any stage during pregnancy.

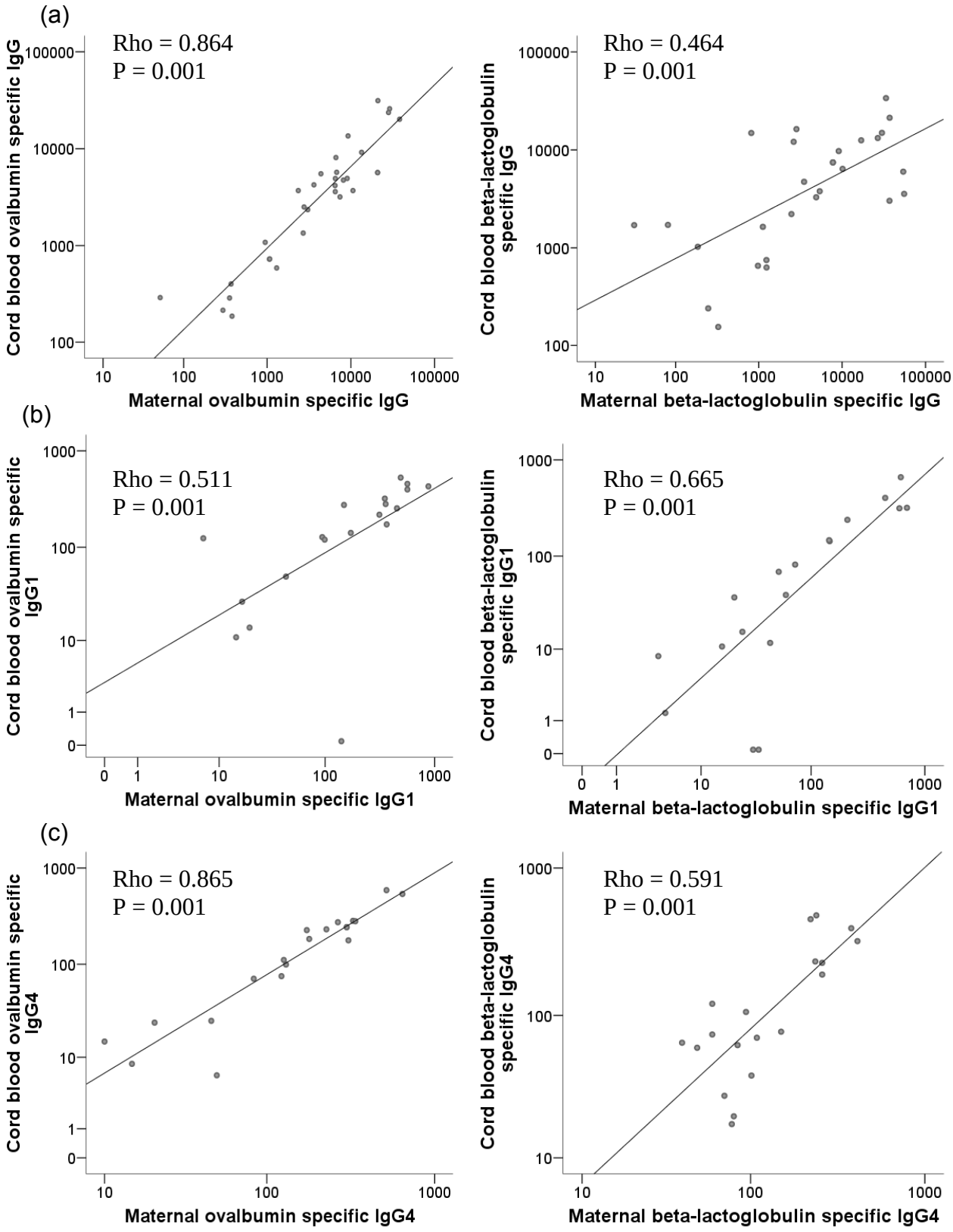

Figure 3:3 Specific concentrations in cord blood and matched maternal sera

Infant and maternal samples taken at 36 weeks gestation for ovalbumin and β-lactoglobulin specific: (a) IgG, (b) IgG1 and (c) IgG4

3.3.7 Correlation between maternal specific IgG and SPT results

No relationship between maternal dietary intake of egg and milk proteins during pregnancy and infant positive SPTs to one or more allergens was detected. However, we did observe a significant association between maternal positive SPT results and infant positive SPT results ($p = 0.041$); and maternal SPT and the future development of eczema in the infant ($p = 0.035$).

3.3.8 Infant diet

The response rate for the infant's diet questionnaire at the 6 months visit was high (n = 50 of 51 [98]). We investigated whether the introduction of egg into the infants diet was associated with sensitization to egg. None of the infants with a positive SPT reaction to egg at 6 months (n= 5) had had egg introduced into their diet. At the 6 months visit, no infants from the AE group and 7 infants from the NE group had been introduced to egg. No associations were found between the introduction of the other foods mentioned in the questionnaire (Baby cereal, wheat, cheese, baby rice and rusk).

3.3.9 Infant IgG overview

Ovalbumin-specific IgG was detected in all cord bloods while ovalbumin specific IgG1 was detected in all but two of the cord blood samples. The respective rate of detection of ovalbumin specific IgG and IgG1 in infants were: 21 from 26 samples for both IgG and IgG1 at the 6 months visit (81%) and 20 of the 24 (83%) and 19 of the 24 (79%) samples respectively, from the 12 months visit. Infant ovalbumin-specific IgG4 was less detectable with rate of detection: birth (28/37), 6 months (15/26) and 12 months (16/24).

The respective rates for the detection of β-lactoglobulin IgG and IgG1 were: birth (34/37:28/37), 6 months (19/26:14/26) and 12 months

(21:24/21:24). Specific β-lactoglobulin IgG4 was detected in less cord and infant blood samples (birth 24/37; 6 months 13/26; 12 months 10/24). Although not significant, there was a trend in the cord blood for both ovalbumin and β-lactoglobulin specific IgG mean to be higher in the NE infants.

3.3.10 Infant IgG and eczema

The study population as a whole, showed a decrease in ovalbumin specific IgG concentration between birth and 6 months of age ($p = 0.031$). This decrease in ovalbumin specific IgG continued to 12 months of age. However, children who developed eczema (AE) showed an increase in ovalbumin specific IgG between birth and 6 months (Figure 3.4). At the 12 months visit, children in the AE group showed significantly higher ovalbumin specific IgG concentrations than those children in the NE group ($p = 0.029$). This significant difference was also detected in ovalbumin specific IgG subclass, IgG1, at both the 6 months ($p = 0.029$) and 12 months visits ($p = 0.020$). In contrast, no increase was detected for ovalbumin-specific IgG4 levels between the two visits (Figure 3.4). Furthermore, both AE and NE infants showed a significant decrease of ovalbumin specific IgG4 from birth to 6 months ($p = 0.03$), which then remained constant at the 12 months visit. No significant difference in IgG4 levels was detected between the AE and NE infants at any of the time points in this study.

Unlike ovalbumin, there was no significant difference in β-lactoglobulin specific IgG levels from birth to the 12 months visit. Moreover, β-lactoglobulin specific IgG subclasses, IgG1 and IgG4 showed no significant difference between the two groups of infants at any of the time points during the study. Levels of infant ovalbumin specific IgG1 and IgG4 detected were always higher than those levels detected for β-lactoglobulin specific IgG subclasses.

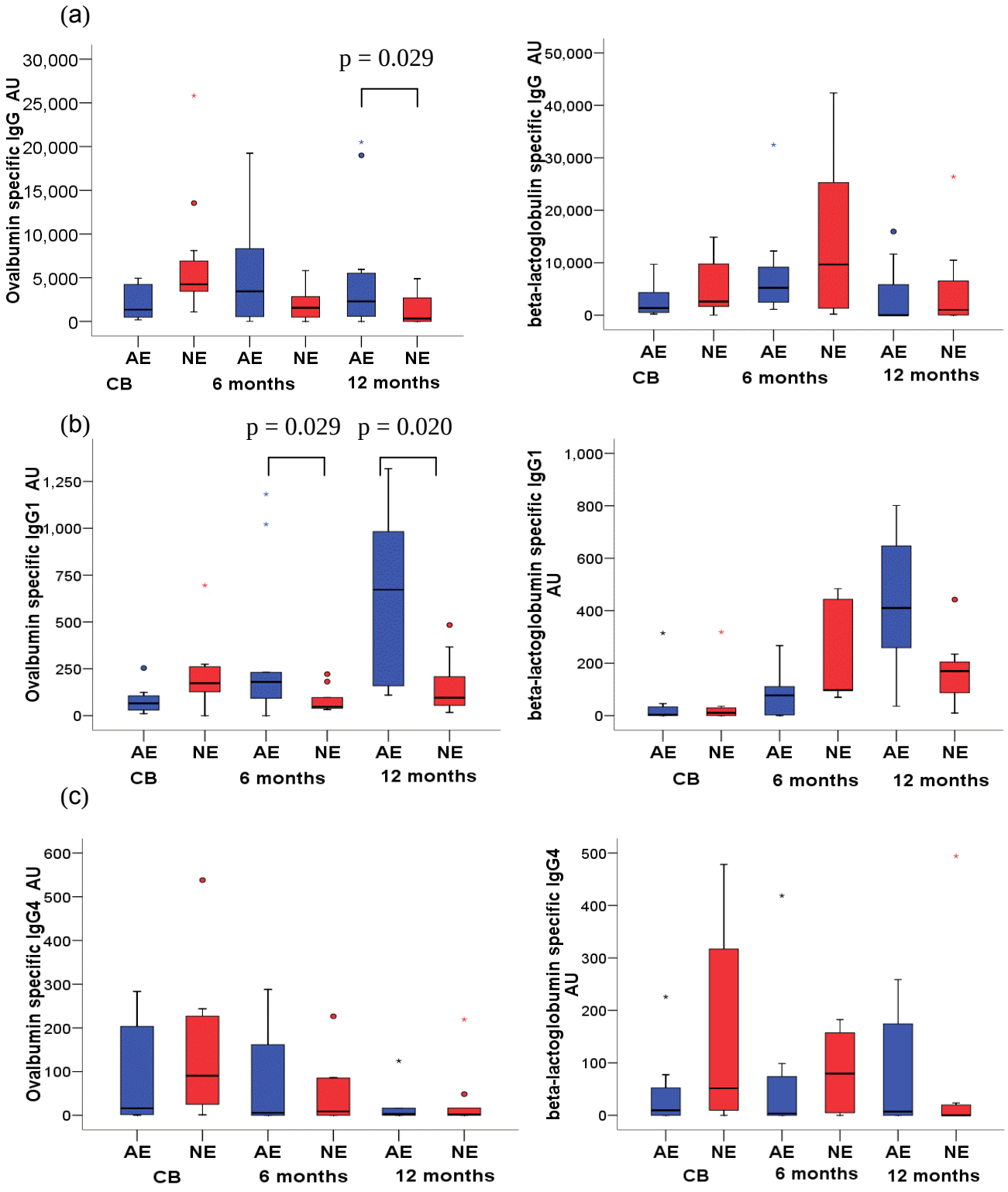

Figure 3:4 Ovalbumin and β-lactoglobulin specific IgG, IgG1 and IgG4 from birth to 12 months of age.

Serum ovalbumin and β-lactoglobulin specific (a) IgG, (b) IgG1 and (c) IgG4 detected in neonatal and postnatal samples from infants who went on to developed eczema (AE) or showed no development (NE) by 12 months. AE infants showed significantly higher ovalbumin IgG1 concentrations at 6 (p = 0.029) and 12 months (p = 0.020) gestation

3.3.11 Infant IgG and SPT

There was a significant association between the development of eczema in the infant and positive SPT results detected at 12 months ($p = 0.003$). However, no association was found between infant positive SPT results and infant specific IgG to either ovalbumin or β-lactoglobulin.

3.4 Discussion

The presence of eczema during infancy can cause considerable morbidity, in particular sleep and feeding disruption, economic cost, parental stress and the possibility of later development of other atopic diseases. A recent questionnaire based study into the prevalence of eczema in western Sweden, showed that eczema was present in one in five infants (Alm et al., 2009). With such a high prevalence and the possible development of other allergic diseases, infants that develop early atopic eczema represent a population of great interest.

The earliest potential nutritional influence on infant development of atopic disease is possibly the diet of the expectant mother. Evidence of placental transfer of both food and aero-antigens has been documented, with detection of the specific food allergens ovalbumin and β-lactoglobulin, in the amniotic fluid (Jones et al., 2001) and cord blood samples from both atopic and non-atopic mothers (Lovegrove et al., 1994). This has led to the question: 'does the maternal diet during pregnancy have the potential to influence the development of eczema in the infant?'

Using the quantified data on maternal egg and milk protein intake we were able to compare the maternal diet with detection of ovalbumin and β-lactoglobulin specific IgG, IgG1 and IgG4 in maternal, cord and infant blood samples. There was no correlation between the mean dietary egg

intake of the mothers and the maternal ovalbumin specific IgG detected. This is inconsistent with previous work within our group which demonstrated maternal ovalbumin specific IgG concentration to be reflective of maternal dietary egg consumption (Vance et al., 2004a). However, different conditions were used by Vance and colleagues who randomized pregnant women into either an egg exclusion diet or an unmodified healthy diet during pregnancy. Although many of the mothers in the egg exclusion diet group accidently consumed egg, the amount consumed remained significantly below the levels of those in the normal diet group (egg exclusion group: range 0.21 – 25.3 g; normal diet group: range 2.97 – 149.1 g). Vance *et al* reported egg specific IgG reflected dietary intake because of the significant fall in concentration in the sera of egg avoiding women. (Vance et al., 2005). However, this was different to our study where both AE and NE mothers were not asked to alter their diet and consumed similar levels of egg during pregnancy (AE: range 11.0 – 254.0 g; NE: range 17.97 – 193.14 g). This may indicate that a wider range in the amount of egg protein consumed between our two groups would be needed to find a correlation between maternal egg protein intake and ovalbumin specific IgG.

To analyse immunological materno-foetal transfer, we compared specific immunoglobulins in cord blood and maternal circulation. There was a tight correlation between maternal and cord blood levels of specific IgG and IgG subpopulations IgG1 and IgG4. Therefore, indicating the ability of ovalbumin and β-lactoglobulin specific IgG and IgG subclasses to travel across the placenta. Transport of IgG across the placenta has been previously documented and is an active, selective, intracellular process specifically mediated by the FcR_n receptor (Landor et al., 1998). Transfer of IgG across the placenta has been shown from about 16 weeks gestation (Thornton & Vance, 2002). Previous studies have reported levels of both ovalbumin and β-lactoglobulin specific maternal IgG and cord blood IgG to correlate (Casimir et al., 1989; Lovegrove et al., 1994; Vance et al., 2004a). However, the correlation between maternal and

cord blood β-lactoglobulin specific IgG1 and IgG4 has not been previously reported.

In the murine model, transfer of maternal allergen specific IgG to the developing foetus invoked tolerance in the offspring to ovalbumin and β-lactoglobulin (Polte et al., 2008). Studies in both mice (Seeger et al., 1998) and rats (Jarrett & Hall, 1983) have suggested a protective role for maternal IgG, showing elevated ovalbumin specific maternal IgG levels reduce expression of IgE antibodies in the offspring in an antigen specific fashion. However, in human studies, elevated cord blood IgG levels to food allergens have been associated with both increased (Iikura et al., 1989) and decreased (Casimir et al., 1989) atopic symptoms while other studies have reported elevated cord blood IgG levels to have no effect on atopic development (Lilja et al., 1991). In our study, although not significant, there was a trend for increased ovalbumin and β-lactoglobulin specific cord blood IgG levels in the NE group compared to the AE group. We were unable to confirm an association between low levels of cord blood specific IgG to ovalbumin and β-lactoglobulin and the development of eczema in the infant, although a trend was visible a larger study population would be needed to investigate the role of IgG and eczema development in the infant further.

As in previous studies (Hattevig et al., 1990; Vance et al., 2004a) ovalbumin specific IgG concentration of the whole study infant population fell significantly between birth and 6 months, thus, reflecting the natural loss of transplacentally acquired IgG. This decrease in concentration was not detected for β-lactoglobulin specific IgG, which remained stable. However, this could be explained as all infants had been exposed to cow's milk in their diet by their 6 months visit. It can be assumed this exposure has stimulated their own IgG antibody production to β-lactoglobulin. However, on analysis of the infant ovalbumin specific IgG levels at the 6 and 12 months visits, even though egg had been introduced into all infants' diets apart from two by 12 months, no significant increase in ovalbumin specific IgG was detected. This may be

reflective of quantity and frequency of the amount of egg protein consumed within their diet which was significantly lower than that of milk protein.

When we analysed the data further, the decrease of ovalbumin specific IgG between birth and 6 months was observed only in infants who did not develop eczema (NE infants). This observation could be accounted for endogenous production of ovalbumin specific IgG in the eczema infants. By the 6 months visit only seven infants had been introduced to egg and none were in the eczema group (AE). This would indicate that infants who went on to develop eczema showed heightened ovalbumin specific IgG antibody levels at 6 months without dietary egg exposure. As T_H2 responses drive a humeral rather than cellular response to antigens, it might be expected that both IgG and IgE antibodies would be increased in atopic sensitised subject. In the absence of digestion of egg, exposure could have still occurred through maternal sources or contact with the antigen through other means, such as food preparation.

The predominant IgG subclass, IgG1, showed the most significant difference when comparing ovalbumin specific concentrations with the development of eczema in the infants. The kinetics of ovalbumin specific IgG1 antibody responses at 6 and 12 months of age were similar to that of specific IgG, although the number of infants with detectable IgG1 levels was lower. Earlier studies have shown the half-life of IgG1 to be 48.4 days (Sarvas et al., 1993). From this it can be assumed that the levels of IgG1 detected in the infants samples at both the 6 and 12 months visits are reflective of the infants own IgG1.

Our data is limited to the first 12 months of life, therefore we were unable to ascertain if the levels of ovalbumin specific IgG1 in the AE group would continue to increase beyond this time. Infants who went on to develop eczema showed significantly higher ovalbumin IgG1 concentrations than the infants who never developed eczema. No significant difference

between the infants who developed eczema and those who showed no development of eczema with the β-lactoglobulin specific IgG1.

The observed pattern of an early IgG1 response with a later appearing IgG4 response to both allergens ovalbumin and β-lactoglobulin is the recorded response to subcutaneous antigens administered during immunotherapy (Hedlin et al., 1991; Lilja et al., 1989b). This all supports the concept that the specific IgG4 response is a later appearing response, which is part of a natural immune response to prolonged exposure to antigens as well as playing a possible role in tolerance.

The later onset of specific IgG4 may explain why little difference of specific IgG4 was detected between our infants who went onto develop eczema to those who did not, as our data was only collected for the first 12 months of life. This time period may not have been long enough to have seen the full development of the IgG4 response. Contradictory roles have been indicated for specific IgG4 antibodies, with elevated levels detected in tolerant atopic children and adults compared to non-atopic controls (Ruiter et al., 2007). It has been speculated that specific IgG4 could exert an immune modulating effect on the clinical manifestations of atopic disease (Kihlstrom et al., 2005).

However the protective effects of specific IgG4 are still debated. Evidence accumulating from the field of parasitology indicates, that IgG4, under certain conditions, effectively interfere with allergen-induced, IgE-mediated effector cell triggering, i.e. IgG4 acts as a blocking antibody (Hussain et al., 1992) With data indicating a striking similarity with respect to the type of antigen that triggers the IgG4 and IgE immune responses (Malbec et al., 1998). Since a marked difference in epitope specificity exists between the IgG4 and IgE antibodies, only a fraction of the allergen-specific IgG4 can interfere effectively with IgE binding. However, if no IgG4 antibody is induced by conventional immunotherapy, the therapy is likely to have been ineffective. One study reported

immunotherapy to only be considered as immunologically effective if a substantial increase of 10 to 100 fold in allergen specific IgG4 has been detected (Aalberse et al., 1993).

There have been conflicting results with the vigorous exclusion of egg and milk in atopic mothers diet during pregnancy. One study reported a trend towards a beneficial effect on infants born to atopic parents when mothers followed a milk-free diet during late pregnancy and lactation (Lovegrove et al., 1994). However, most studies have not generally supported a protective role in the maternal dietary exclusion of egg and milk. This includes a 5 year birth cohort by Willers and colleagues, which found no associations between maternal egg, milk or milk products consumption and longitudinal childhood outcomes (Willers et al., 2008). This present study was not an intervention study, and no dietary exclusions were placed upon the mothers recruited. As mentioned earlier, the dietary intake of both egg and milk in mothers whose infants went on to develop eczema were very similar to those who did not, at both stages of pregnancy that we examined. Hence, no correlation within our study population was seen between the incidence of infantile eczema and the maternal dietary intake of egg and milk during pregnancy.

No significant difference was observed in the time solids or egg was introduced into the infants' diet between those infants who developed eczema and those who did not. Although there was a trend for infants who developed eczema to be introduced to solids later on. This trend could again be due to the possible influence of reverse causation as early signs of eczema might delay the induction of solid food. However, recommendations regarding the introduction of foods to the developing infants' diet appear with very little epidemiological data and incomplete scientific evidence to support this belief of delayed introduction of certain foods to lower risk of atopy.

To summarise, most previous studies on ovalbumin and β-lactoglobulin specific IgG antibody responses from maternal, cord blood and infant

samples have not included analysis of the subclasses IgG1 and IgG4. Although this was a small study population, multiple samples were taken from mother, at birth and from the infants at the 6 and 12 months visits, to provide detailed longitudinal data. The aim of this study was to investigate the effect of the maternal diet on β-lactoglobulin and ovalbumin specific IgG/subclasses levels and the development of eczema in infants. The children within this study population who developed eczema showed significantly higher concentrations of specific IgG and IgG1 to ovalbumin. No significant difference was seen for ovalbumin IgG4 or any of the β–lactoglobulin specific IgG/subclasses.

The numbers in this study are limited and therefore caution is required in interpreting the data. However, these findings do suggest that further studies with larger sample sizes and of longer duration are necessary to substantiate our preliminary observation that alterations of ovalbumin and β-lactoglobulin specific IgG/IgG1 responses may be an indicator of infant eczema development. As children diagnosed with asthma have had significantly higher levels of ovalbumin IgG1 than non-asthmatics reported at birth (Warner et al., 2000), this expression of specific IgG1 may predate the expression of further atopic disease.

Chapter 4: The role of T cell memory subpopulations in cord blood in determining the development of infantile atopic eczema

4.1 Introduction

Children who develop atopic eczema are not identified until the disease has become established; thus, early interventions need to predict onset of disease. The identification of early markers within cord blood may help predict the development of atopic eczema, thus offering the chance to identify those children at most risk and target them for early intervention trials. We investigated immunological differences within specific T cell populations in cord blood mononuclear cell responses to ovalbumin or β-lactoglobulin and their relation to the development of atopic eczema within the infant.

The discovery of monoclonal antibodies by Kohler and Milstein over 30 years ago (Kohler and Milstein 1975), dramatically enhanced the use of immunofluorescence for identifying cell-surface antigens. In this chapter, highly specific monoclonal antibodies were used to identify specific cell populations that may influence the development of eczema, within cord and infant blood samples from high risk infants.

During the past decade studies have investigated different properties within cord, infant and adult blood, and their putative functions in the development of atopy. There has been a growing interest in the role of immune cells present in the cord blood, particularly in terms of their proliferative cytokine production capacity, with regard to their potential value as predictors for allergic outcome. The detection and proliferation capacity of food specific cord blood mononuclear cells has generated interest and supported the paradigm of *in utero* priming of the neonatal immune response (Edelbauer et al., 2004; Jones et al., 1996; Szepfalusi et al., 1997). Certain allergens, including ovalbumin and β-lactoglobulin, have been reported to cross the maternal-foetal barrier (Szepfalusi et al., 2000a) and have been detected in the amniotic fluid. Furthermore, raised proliferative responses to ovalbumin and β-lactoglobulin at birth have been associated with the development of eczema at 1 year of age in those infants who subsequently had a positive SPT to the same allergens

(Miles et al., 1996). However, the relationship between intrauterine allergen priming and the specific cellular immune responses detected within cord blood in relation to the development of allergy remains unclear.

As previously described (Introduction 1.1.6), after being presented with a specific antigen, a naïve T cell will become activated and differentiate into effector-memory T cells (Sallusto et al., 1999a). This is accompanied by changes in the characteristics of the cell including the release of chemokines and surface molecule expression.

In order to determine the presence of previously stimulated (memory T cell) and unstimulated (naïve T cell) populations with in the cord blood, sensitised to the food allergens ovalbumin and β-lactoglobulin, the isoform of the transmembrane glycoprotein CD45 was analysed.

The phosphates CD45, exists in different isoforms. The transition from a naïve T cell to an activated T cell, is accompanied by a shift from the expression of CD45RA+ to the CD45RO+ isoforms by the process of alternative splicing of the pre-mRNA (Clement, 1992; Ten Dam et al., 2000). Until recently it was unclear what regulated the shift from CD45RA to CD45RO upon T cell activation. However, work by Oberdoerffer and colleagues identified hererogeneous ribonucleoprotein L-like (hnRNPLL) as an important regulator of CD45 alternative splicing (Oberdoerffer et al., 2008). Subsequent experiments showed this protein increased after activation of the T cell, was able to bind to CD45mRNA, and was both necessary for the alternative splicing of CD45RA to CD45RO (Oberdoerffer et al., 2008).

There is contradictory data regarding expression of CD45RO in cord blood and the development of atopy. Studies have reported greater expression (van der Velden et al., 2001); lower expression (Miles et al., 1994) and no difference in the expression (Hagendorens et al., 2000) of CD45RO from the cord blood of atopic infants compared to non-atopic

infants. By including this cell surface marker we can detect any differences in memory expression within the cord blood of those infants who go on to develop atopic eczema to those infants who do not develop eczema.

As mentioned in chapter 1 (1.1.7), CCR7 is used to identify two different phenotypes of memory T cells: central memory (CCR7+); and effector memory (CCR7-), The former predominantly migrates to lymphoid tissue, and the latter to peripheral tissue (Gupta et al., 2006; Kaech & Ahmed, 2001; Moser & Loetscher, 2001; Sallusto et al., 1999b). At present there is very little published data regarding the expression of central and effector memory in cord blood and any possible role in the development of allergic disease.

The aim of this chapter was to determine whether the T cell phenotype in neonates could be used as a predictor of atopic eczema susceptibility. Thus, identification of T cell phenotypes in the cord blood, their proliferation capacity in response to allergen, and how this relates to atopic eczema outcome with hopefully broaden our understanding of atopic disease, specifically the development of atopic eczema.

4.2 Subjects, samples and methods

4.2.1 Subjects

As detailed in the methods (2.1.5), cord (n = 10) and infant (n = 10) blood samples were processed blinded and later placed into one of two groups, dependent on infant showing either development of atopic eczema (AE) or no development of eczema (NE) by 12 months of age.

4.2.2 Cell culture

Briefly, cells were incubated at a concentration of 1.25 x 10^6 per ml in RPMI medium (complimented with 5% human serum, 1% sodium pyruvate

and 1% penicillin streptomycin) in a flat bottomed 24-well plate. Both cord and infant blood mononuclear cells were stimulated with the final concentrations of mitogen and antigens: 1.25μg/ml PHA for 3 days, 125μg/ml β-lactoglobulin for 7 days and 125μg/ml ovalbumin for 7 days.

4.2.3 Cell staining

In brief (detailed account in 2.2.7) naïve T cells (CD45RA+ CCR7+), memory T cells (CD45RO+), central memory T cells (CD45RO+CCR7+) and effector memory T cells (CD45RO+CCR7-) were analysed using monoclonal antibodies. As described earlier (2.2.7.3) staining occurred in the dark, on ice for 35 minutes. Cells were then washed with FACs buffer and fixed with 1% PFA prior to analysis.

4.2.4 Flow cytometry analysis

As explained in detail previously (2.2.7) cells were analysed using 4-colour flow cytometry. Cultured cord and infant blood cells were first gated on the lymphocyte population via forward- and side-scatter properties and were analysed using a FACs Aria I (BD UK). Dead cells and monocytes were excluded via forward- and side-scatter characteristics. Data was analysed with BDFACSDiva 6.0 software (BD Biosciences).

4.2.5 Statistical Methods

Proliferation data of both cord and infant blood, which were not normally distributed, were analysed by non-parametric tests Mann-Whitney (comparing AE vs NE infants) and Wilcoxon tests (comparing proliferation to β-lactoglobulin and ovalbumin compared to unstimulated). Parametric tests were used for normally distributed data including surface expression (t-test, paired t-test). A probability level of <5% was considered statistically significant. Although numerous statistics were analysed within

this study, multi-variable testing was not used as this is an exploratory study.

4.3 Results

4.3.1 The study population

There was no significant difference between those who did or did not develop eczema with regard to birth weight and gestation age.

4.3.2 Cell viability

From the samples used within this study the cell recovery was between 72.1 – 93.4% with the median at 78.8%. The viability of the thawed cell population was between 89.2 – 94.6% with the median at 92.3%.

A number of samples collected for this study showed a very low cell count upon thawing. This may have been due to the amount of blood originally taken, the storing process or the actual thawing process itself. Due to their low cell count it was not possible to utilise all the samples collected. Therefore, those samples with satisfactory cell recovery and viability post thawing were chosen for this study.

4.3.3 Expression of CD3, CD4 and CD8 in cord blood from AE and NE infants

The percentage of CD3+, CD3+CD4+, and CD3+CD8+ lymphocytes present in the cord blood samples after different culture conditions is summarised in Figure 4.1. Firstly an evaluation of the CD3+ T cell populations present in cord blood was performed to examine whether there were any differences in expression between the two study groups. The data revealed no significant difference in CD3 expression in cord blood between the AE group (US, 48.72±2.21) and NE group (US, 54.10±5.98) (Fig.4.1). Furthermore, no significant change was detected in CD3 expression after culture with the food allergens β-lactoglobulin (p =

0.7268; p = 0.6428) or ovalbumin (p = 0.8415; p = 0.3537), in either AE or NE groups, respectively. However, a significant increase in CD3 expression was detected in both AE and NE cord blood after stimulation with the mitogen PHA (AE p = 0.0001, NE p = 0.0108).

The majority of cord blood CD3+ T cells co-expressed CD4 (AE 74.74±3.27; NE 75.96±2.13), with a smaller minority of CD3+ cells co-expressing CD8 (AE 19.20±3.79; NE 17.70±3.01). A small increase in CD4 expression was detected after stimulation with PHA, but upon further analysis this was not statisitically significant. Furthermore, no significant difference was detected in CD4 expression between the AE and NE cord blood samples. Expression of CD8 in AE cord blood remained constant after exposure to each stimulant, while a small increase was detected in the NE cord blood samples following culture with ovalbumin, β-lactoglobulin and PHA. However, this increase was only significant after culture with the antigen β-lactoglobulin (p = 0.023).

4.3.4 Expression of CD3, CD4 and CD8 in infant blood from AE and NE infants

The percentages of CD3+, CD3+CD4+, and CD3+CD8+ lymphocytes detected in infant blood are summarised in figure 4.2. The percentage of CD3+ T cells detected at 12 months was significantly higher than those detected at birth, both in the AE infants (p = 0.0064) and NE infants (p = 0.0079). The percentage of CD4+ T cells present within the CD3+ population in infant samples remained consistent with those detected at birth (AE cord blood 74.74±2.71, infant blood 72.52±3.92: NE cord blood 75.96±2.13, infant blood 72.5±4.28). Similarly the percentage of CD8+ T cells in infant blood showed no significant increase compared to the percentage detected in cord blood (AE cord blood 19.2±3.8, infant blood 23.7±2.94: NE cord blood 17.7±3.0, infant blood 25.36±4.73).

Similar to cord blood, the majority of CD3+ cells present in infant blood expressed CD4+, with a smaller minority expressing CD8 instead. Furthermore, the percentages of CD4+ and CD8+ T cells present were

similar for both AE and NE groups in the unstimulated infant blood samples (Fig. 4.2.). Upon stimulation with β-lactoglobulin, no significant difference in CD3+, CD4+ or CD8+ expression was detected in either AE or NE samples. On the contrary, following culture with ovalbumin there was a significant increase in CD3 expression in the AE group ($p = 0.0095$). This significant increase was not detected in the CD4+ or CD8+ subpopulations. Furthermore, both AE and NE infants showed significantly increased CD3 expression after stimulation with PHA ($p = 0.0001$, $p = 0.0009$, respectively). However, this increase in expression was not detected in the CD4+ or CD8+ subpopulations, in either AE or NE groups.

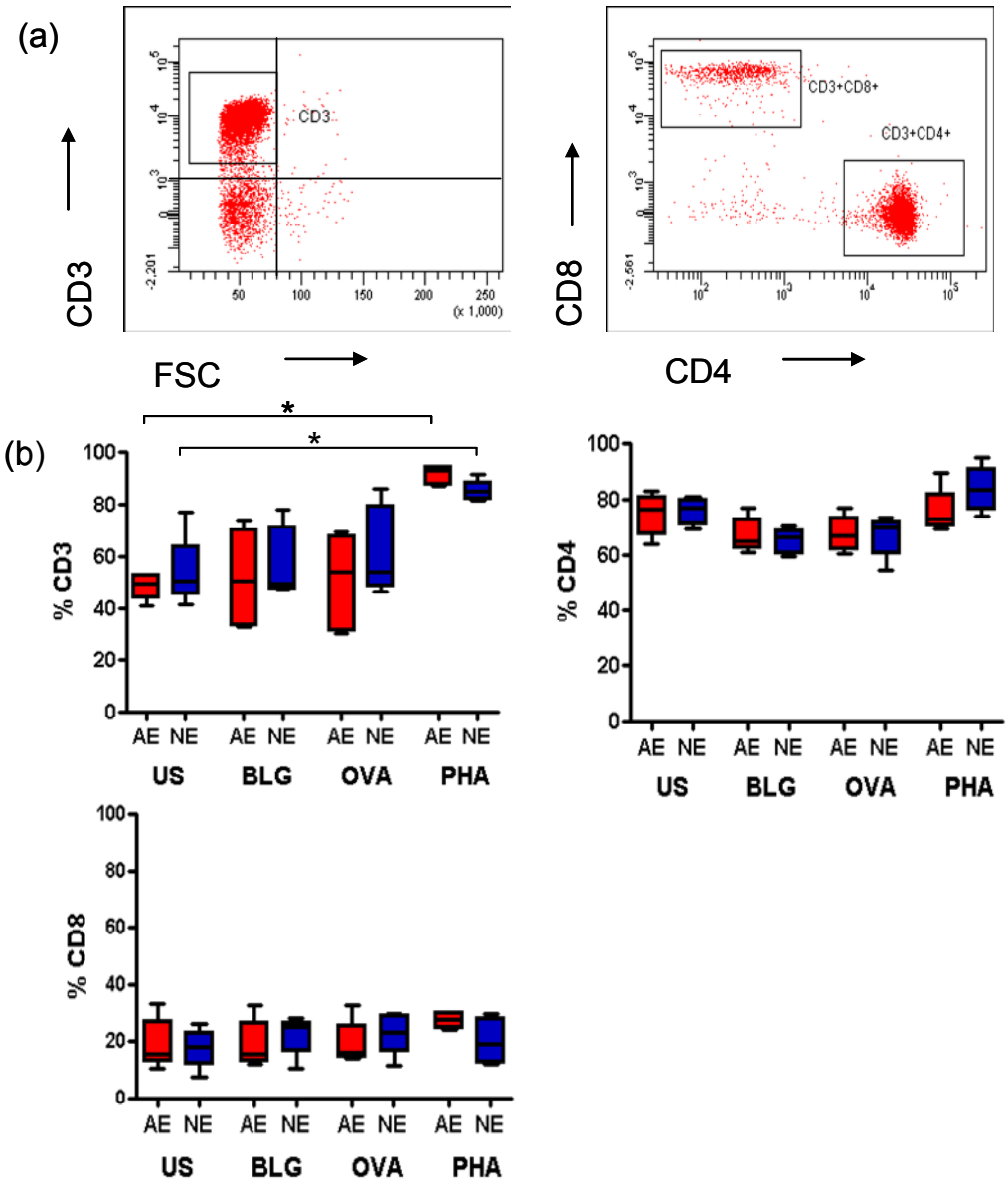

Figure 4:1 Percentage of CD3, CD4 and CD8 expressing cells in cord blood

Representative flow cytometry profiles from cord blood stained with CD3, CD4 and CD8. (a) From whole blood mononuclear cells the lymphocyte population was gated, followed by the CD3+ being gated. Within the CD3+ population the CD4+ and CD8+ population were identified. (b) The percentage of cell expressing CD3 was measure after being cultured or 7 days with no stimulant (US), β-lactoglobulin (BLG) and ovalbumin, and 3 days for PHA. There was an increase in CD3 expression after stimulation with PHA (*AE p = 0.0001, NE p = 0.0108). CD4 and CD8 expression are also shown. Cord blood was divided into two groups: those infants who developed eczema (AE)(red) and those who showed no development of eczema (NE)(blue) within the first 12 months of life.

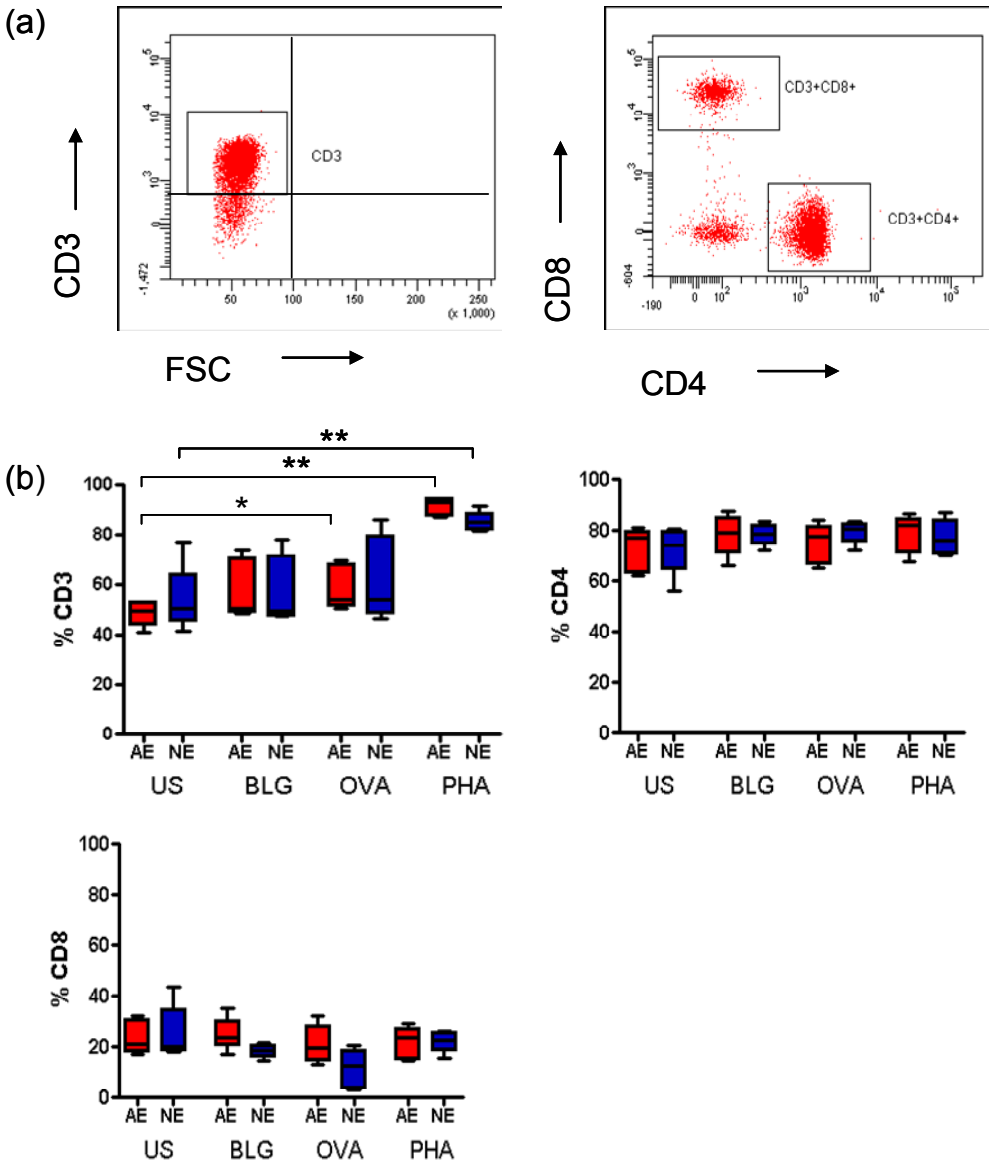

Figure 4:2 Percentage of CD3, CD4 and CD8 present in infant blood.

Representative flow cytometry profiles from cord blood stained with CD3, CD4 and CD8. (a) From whole blood mononuclear cells the lymphocyte population was gated, followed by the CD3+ being gated. Within the CD3+ population the CD4+ and CD8+ population were identified. (b) The percentage of cell expressing CD3 was measure after being cultured or 7 days with no stimulant (US), β-lactoglobulin (BLG) and ovalbumin, and 3 days for PHA. There was a significant increase in CD3 expression after stimulation with OVA in the AE group ($p = 0.0095$), and after stimulation with PHA in both AE and NE groups ($p = 0.002$, $p = 0.013$, respectively). Both. CD4 and CD8 expression are also shown. Infant blood was divided into two groups: those infants who developed eczema (AE)(red) and those who showed no development of eczema (NE)(blue) within the first 12 months of life.

4.3.5 Proliferative response to allergen in cord blood

In order to determine the specific cord blood T cell subsets that proliferated when cultured with ovalbumin, β-lactoglobulin, or PHA, cell division was analysed by CFSE-labelling. Proliferating CD3+, CD3+CD4+ and CD3+CD8+ T cell populations detected in cord blood samples are shown in figure 4.3. All cell populations investigated showed minimal proliferation to β-lactoglobulin or ovalbumin in both AE and NE cord blood. However, when challenged with PHA, significant proliferation was detected in all cord blood samples, clearly demonstrating the ability of the AE and NE cord bloods to proliferate ($p = 0.001$, $p = 0.0108$, respectively).

4.3.6 Proliferative response to allergen in infant blood

As shown in figure 4.4, following culture with β-lactoglobulin or ovalbumin, CD3+ cells from the AE infant samples showed significantly increased proliferation in comparison to the NE infant samples ($p = 0.0491$; $p = 0.0296$,, respectively). This difference was also detected in the CD3+CD4+ subpopulation, where once again the AE infants samples showed significantly higher proliferation compared to the NE infants after culture with β-lactoglobulin ($p = 0.0347$) and ovalbumin ($p = 0.0458$). Further investigation of the CD3+CD8+ population in the infant samples showed a significant difference between the AE and NE infants after stimulation with ovalbumin ($p = 0.001$) but not β-lactoglobulin. The highest proliferation seen within our study population came from those infants who had been diagnosed with either milk allergy (showed the highest proliferation after culture with β-lactoglobulin) or egg allergy (showed the highest proliferation after culture with ovalbumin). Both infants were in the AE group. Although those infants with the egg or milk allergy showed the highest proliferation, all infants within the AE group showed a higher proliferation than those in the NE group. All infants demonstrated significant proliferation to the control mitogen PHA (AE $p = 0.0002$; NE p

= 0.0009) and no significant difference between the two groups was detected (p = 0.9304).

4.3.7 Presence of naïve and memory T cells in cord blood

From the CD3+ T cell population, naïve and memory cells were identified by the expression of CD45RA or CD45RO isoforms. In contrast to adult peripheral blood, the majority of neonatal T cells in unstimulated cord blood expressed CD45RA (mean 89.01%, SD 6.54), with a much smaller population expressing CD45RO (mean 6.0%, SD 2.64) (Fig. 4.5). The percentage of CD45RA+ T cells was similar in AE and the NE cord blood samples. While all cord blood samples showed considerably lower expression of CD45RO+, there was no significant difference between the AE and NE unstimulated cord blood samples in the expression of CD45RA (p = 0.7330) or CD45RO (p = 0.8924). As illustrated in figure 4.5, there was a significant decrease in CD45RA expression after stimulation with β-lactoglobulin in the AE cord blood (p = 0.0047). Similarly, after stimulation with ovalbumin a significant decrease in CD45RA expression was also evident, this time in both the AE (p = 0.002) and NE (p = 0.035) cord blood samples. No significant difference in CD45RO expression was detected between the AE and NE samples in the absence of stimulant (p = 0.892) or after culture with either allergen (β-lactoglobulin, p = 0.144; ovalbumin, p = 0.243) or mitogen (PHA, p = 0.997). However, when comparing the expression of CD45RO in the AE group from unstimulated samples against those cells cultured with allergens, a significant increase was observed after culture with β-lactoglobulin (p = 0.015) and ovalbumin (p = 0.019). This significant increase was not detected in the NE group. Cord blood from both AE and NE infants showed a significant increase in CD45RO expression after culture with PHA (p = 0.0002; p = 0.0001, respectively).

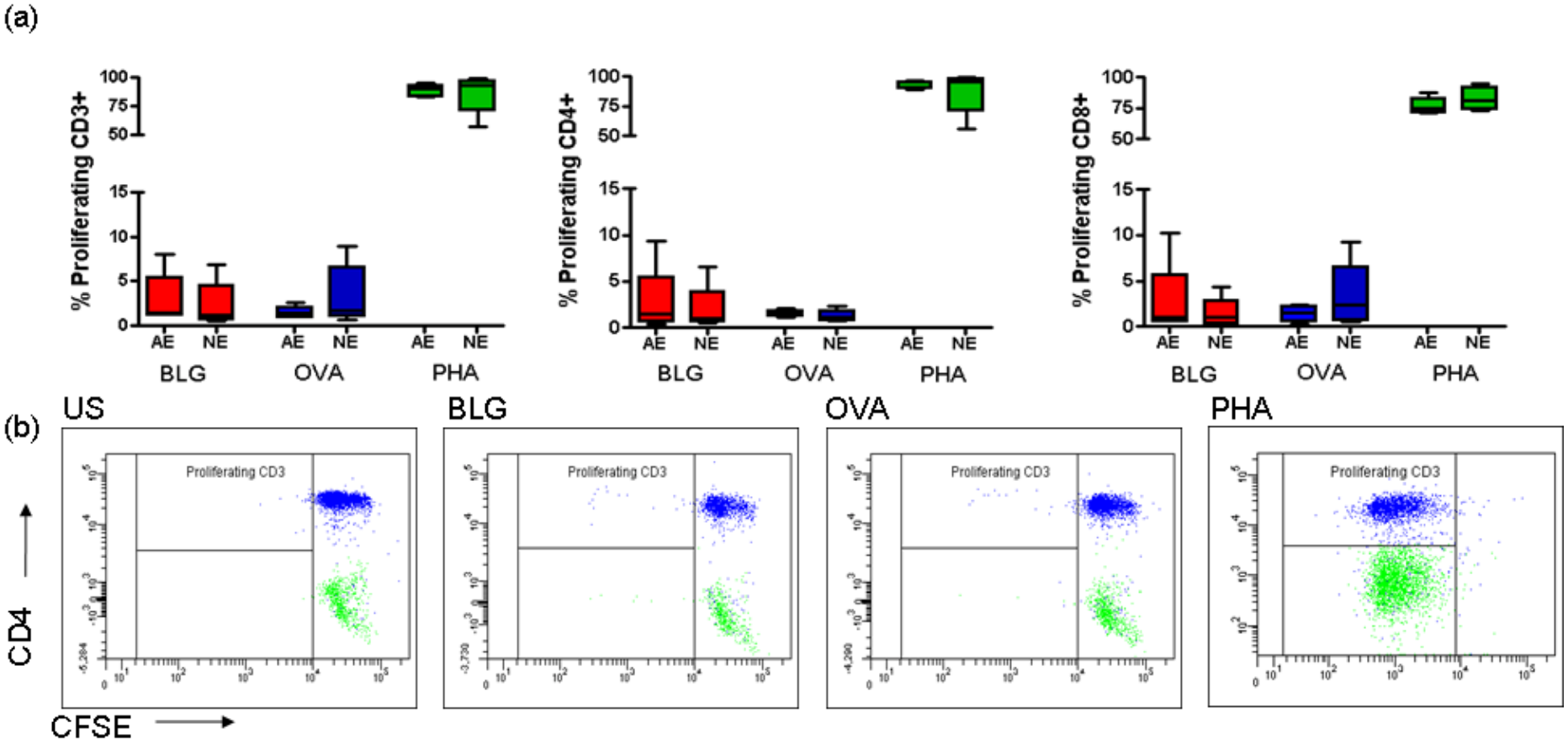

Figure 4:3 Detection of proliferating T cell subsets present in the cord blood.

(a) Comparison of the percentage of proliferating antigen specific T cell subsets after stimulation of cord blood mononuclear cells with β-lactoglobulin (BLG)(red), ovalbumin (OVA)(blue) and PHA (green) between AE infants and NE infants. Values represent the percentage of proliferating CD3+ T cells and the CD3+ T cells co-expressing CD4 or CD8. The horizontal lines represent the medians and the vertical lines extend to the maximum and minimum values. (b) Representative flow cytometry diagrams of infant blood mononuclear cells cultured with CFSE, when left in culture alone (US) or with an allergen (BLG, OVA) or mitogen (PHA). The CD3+CD4+ shown in blue and the CD3+CD8+ shown in green.

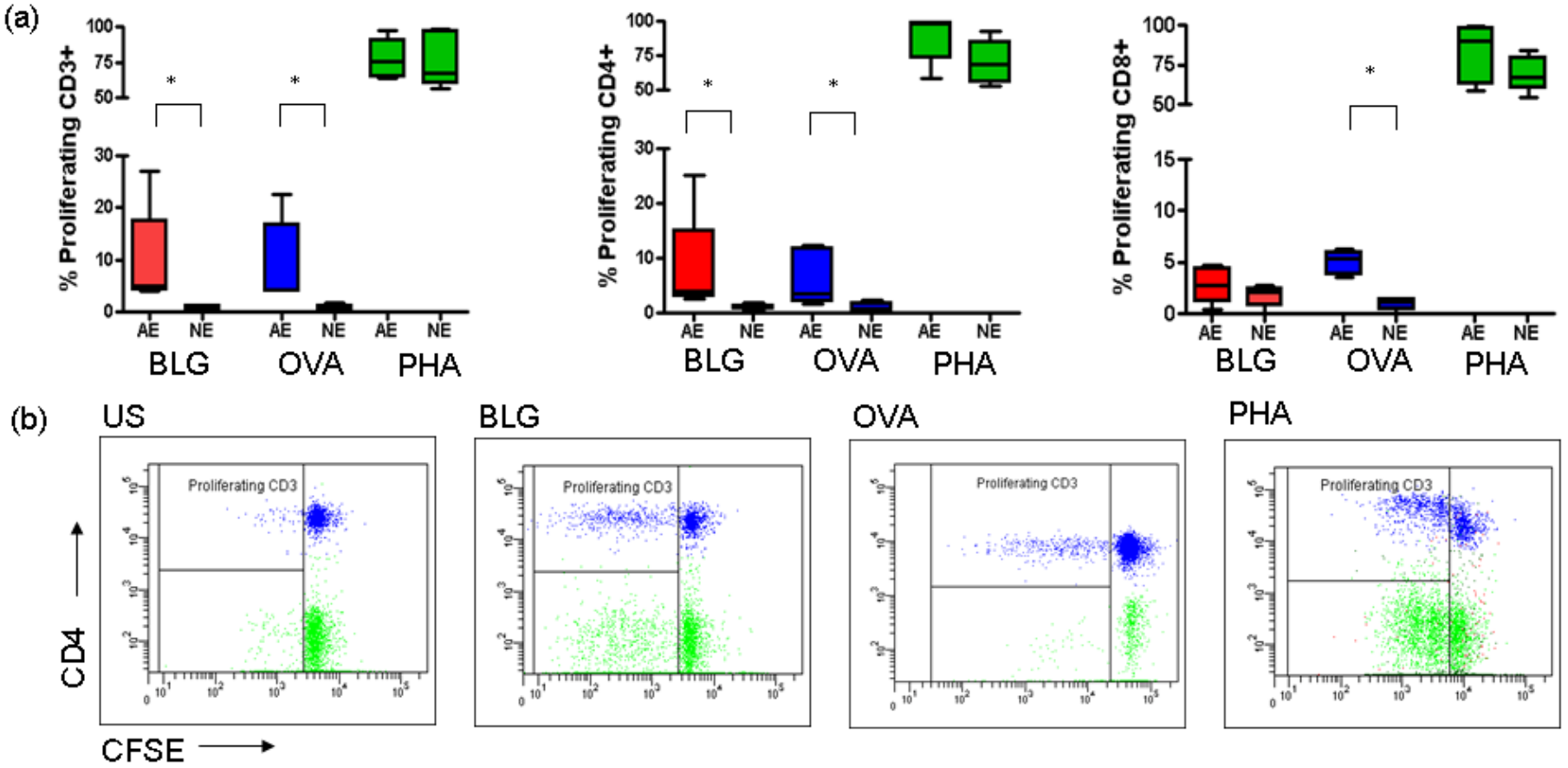

Figure 4:4 Detection of proliferating T cell subsets in infant samples.

(a) Comparison of the proliferating antigen specific T cell subsets after stimulation of infant blood mononuclear cells with β-lactoglobulin (BLG) (red), ovalbumin (OVA)(blue) and PHA(green) between AE infants and NE infants. Values represent the percentage of proliferating CD3+ , CD3+ CD4+ or CD3+CD8+ cells. The horizontal lines represent the medians and the vertical lines extend to the maximum and minimum values. (b) Representative flow cytometry diagrams of infant blood mononuclear cells cultured with CFSE, when left in culture alone (US) or with an allergen (BLG, OVA) or mitogen (PHA). The CD3+CD4+ shown in blue and the CD3+CD8+ shown in green.

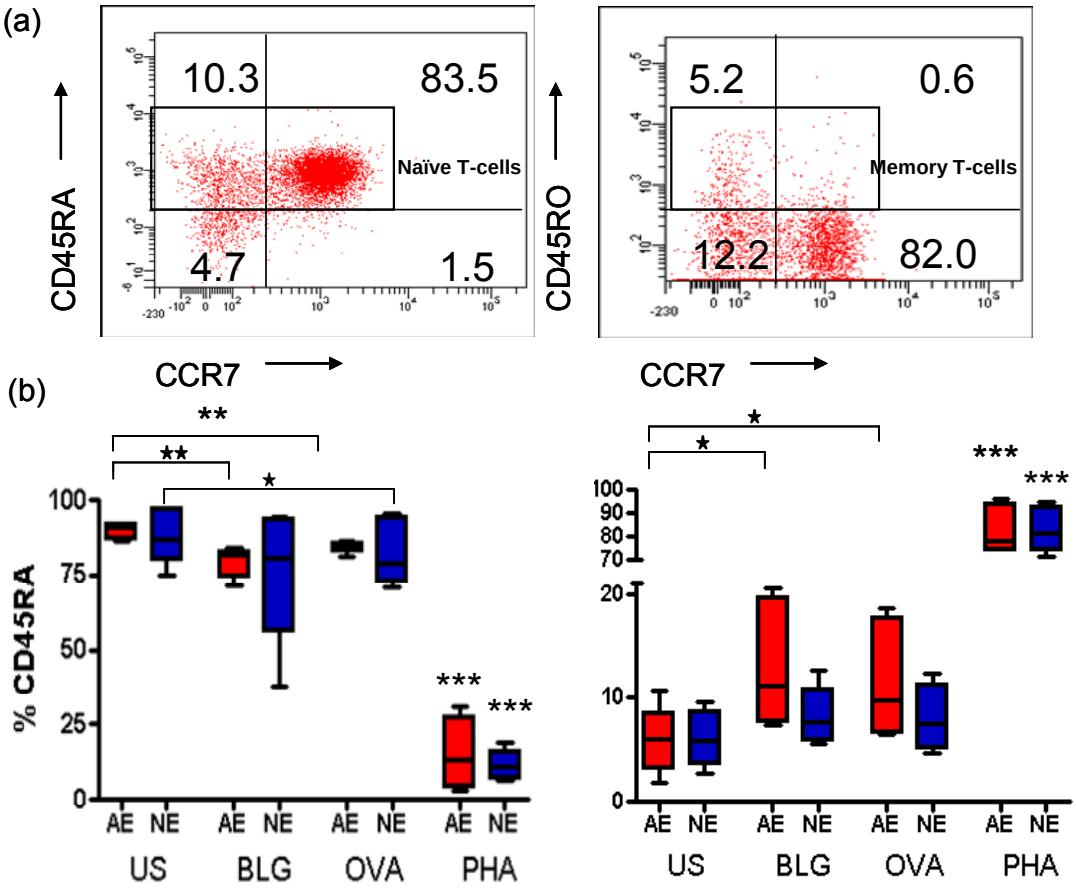

Figure 4:5 Naïve and memory T cells within cord blood.

(a) Representative flow cytometry profiles from cord blood stained with CD3, CD45RA or CD45RO and CCR7 monoclonal antibodies. The naïve and memory T cell populations are gated. (b) Graph representation of the percentage of CD45RA+ (naïve) and CD45RO+ (memory) T cells present in the cord blood of infants who developed eczema (AE) and those who showed no development of eczema (NE) at 12 months. Expression is showing unstimulated samples (US) and after culture with β-lactoglobulin (BLG), ovalbumin (OVA) and PHA.

4.3.8 CCR7 expression

Figure 4.6 shows the expression of CCR7 detected in cord blood after culture with no stimulant and after culture with the allergen β-lactoglobulin, ovalbumin and PHA. No significant difference in the expression of CCR7 between the AE or NE cord blood samples was observed when cultured in the absence of stimulant ($p = 0.8961$) or when cultured with either β-lactoglobulin ($p = 0.6797$), ovalbumin ($p = 0.4890$)

or PHA (p = 0.2100). However, both AE and NE infants displayed a significant decrease in CCR7 expression after culture with β-lactoglobulin (p = 0.0183; p = 0.0195) and ovalbumin (p = 0.0218; p = 0.0057). Furthermore, both AE and NE cord blood showed a significant decrease after culture with PHA (p = 0.0001; p = 0.0001).

4.3.9 Central and effector T cell memory expression in cord blood

Although CCR7 is strongly expressed on naïve T cells (CD45RA+), CCR7 expression on memory T cells (CD45RO+) is more varied. In cord blood both CCR7+ and CCR7- cells could be detected within the CD45RA+ naïve T cell and the CD45RO+ memory T cell populations (Fig. 4.7). Further analysis of the CD45RO+ T cell population revealed the majority of memory T cells to be of the effector memory cell phenotype (CD45RO+CCR7-) (US AE 79.54±4.043; US NE 74.78±6.824). A smaller percentage of memory cells within cord blood displayed the central memory phenotype (CD45RO+CCR7+) (US AE 18.80±4.104; US NE 17.60±5.627). No significant difference in the percentage of central or effector memory T cells in the cord blood, between the AE and NE infants was detected. Furthermore, no significant difference was detected between the two groups after culture with either allergen.

However, further investigation into each group revealed after culture with ovalbumin and β-lactoglobulin the AE group did show a significant decrease in central memory subpopulation compared to that recorded in the unstimulated sample (p = 0.0462, p = 0.0101 respectively). This decrease was also detected in the NE group, but was only significant after stimulation with ovalbumin (p = 0.0406), not β-lactoglobulin. Both AE and NE groups showed a significant decrease in central memory T cell populations in cord blood after culture with PHA (p = 0.0128; p = 0.0406, respectively).

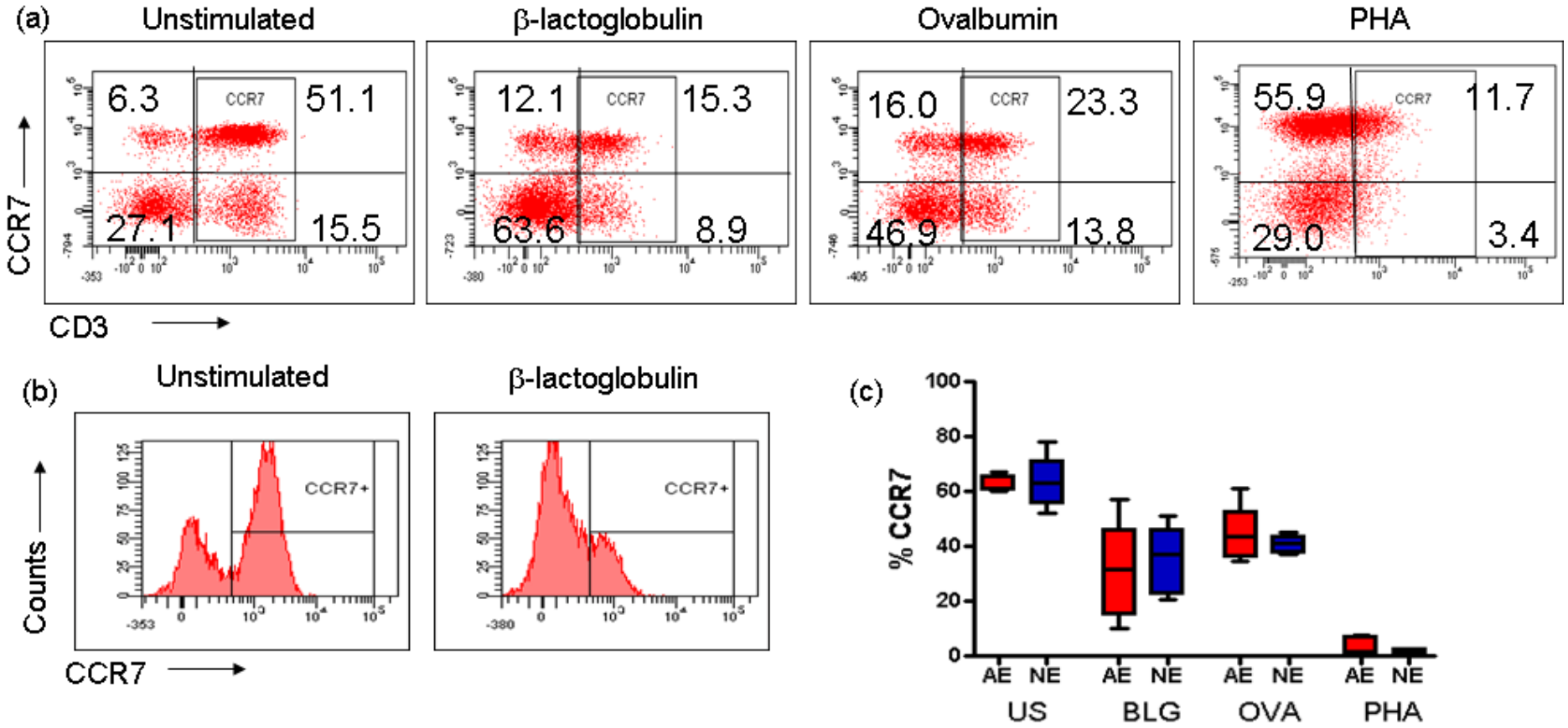

Figure 4:6. Expression of CCR7 in AE and NE cord blood.

(a) Flow cytometry analysis on a representative sample of cord blood mononuclear cells cultured: alone (unstimulated); with β-lactoglobulin; with ovalbumin; and PHA. Cells were gated on lymphocyte population via forward- and side-scatter properties and further gated on CCR7 population. (b) Histogram of representative cord blood CCR7 expression when cultured alone and when cultured with β-lactoglobulin. (c) Flow cytometry analysis comparing the expression of CCR7 in the AE (n = 5) and NE group (n = 5) after cultured alone (US), with β-lactoglobulin (BLG); with ovalbumin (OVA); and PHA

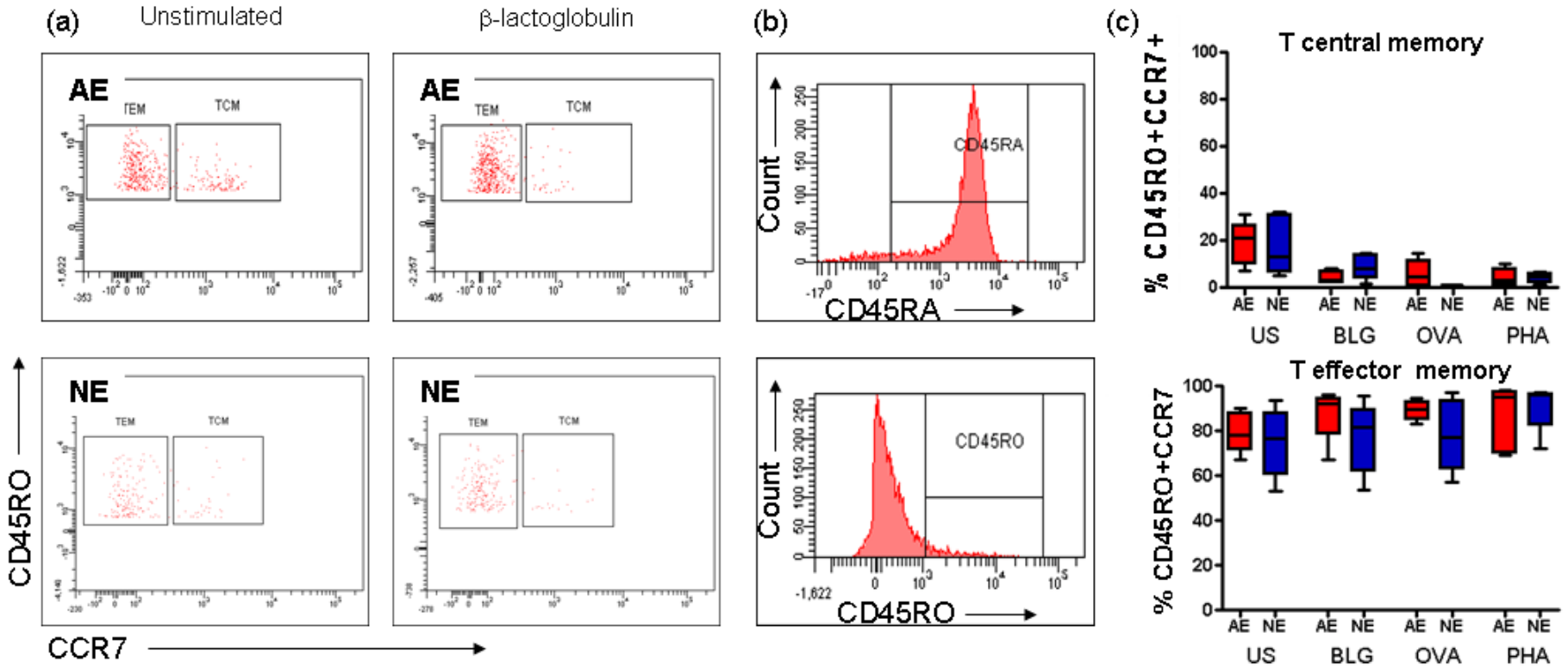

Figure 4:7 Expression of central and effector memory T cells in AE and NE cord blood.

(a) Flow cytometry analysis on a representative sample of cord blood mononuclear cells from AE and NE infants cultured alone (unstimulated) and with b-lactoglobulin. Cells were gated on lymphocyte population via forward- and side-scatter properties, CD3 and CD45RO, then further gated on CCR7. Showing the percentage of CD45RO as either central (CCR7+) or effector (CCR7-) memory phenotype. (b) Histogram of representative cord blood CD45RA and CD45RO expression when cultured alone. (c) Flow cytometry analysis comparing the expression of central and effector memory in the AE (n = 5) and NE group (n = 5) after cultured alone (US), with b-lactoglobulin (BLG); with ovalbumin (OVA); and PHA

4.4 Discussion

In this Chapter, flow cytometry was used for identification of different T cell subpopulations present in infant and cord blood samples. All samples were divided into two groups: infants who showed development of eczema by 12 months (AE group), and infants who showed no development of eczema by 12 months of age (NE group). To date, little is known about the immune profile of cord blood in relation to the future development of eczema. Therefore, one of the principle aims of this work was to establish if there was any significant difference in specific T cell subpopulations present in cord blood from the AE and NE infants. There was particular interest in naïve T cells and the different subclasses of the memory T cell populations (i.e. central and effector memory T cells).

To detect naïve and memory T cell populations present in cord blood, I analysed the expression of the surface marker CD45RA and CD45RO, respectively. Memory T cells respond to a specific antigen they have previously encountered. The presence of this cell phenotype within the neonate enables a more rapid immune response during the postnatal period to specific antigens. As expected, the data revealed strong expression of CD45RA and weak expression of CD45RO in the cord blood samples. Thus, reflecting the naïve status of the neonatal immune system. Furthermore, expression of CD45RO detected in cord blood was significantly lower than levels reported in adults (Devereux et al., 2001). Complementary to our data, an earlier study by Hagendoren and colleagues also reported similar CD45RA and CD45RO expression levels in cord blood (Hagendorens et al., 2000).

However, dissimilar expression of CD45RO was evident in the two groups after culture with β-lactoglobulin and ovalbumin. A significant increase in CD45RO expression was observed in the AE cord blood samples following culture with β-lactoglobulin and with ovalbumin. However, the NE group failed to show any significant change in CD45RO expression following culture with either allergen.

Possibly due to the small sample size within the two studied populations, the difference in CD45RO expression between the AE and NE infants following culture with either allergen was not statistically significant. Thus, our results may suggest, those infants who go on to develop eczema may show an increased memory phenotype immune response to specific allergen in their cord blood, but a larger study is needed to confirm and investigate these findings further.

In this study we reported no significant difference in the expression of CD45RO in unstimulated cord blood from AE or NE infants. In contrast to these data, Van der Velden and colleagues reported a correlation between greater expression of CD45RO in cord blood and increased risk of atopy development in the infant by the age of 12 months (van der Velden et al., 2001). However, contradictory to this data from Miles and colleagues showed infants at high risk of developing allergy had significantly decreased CD45RO expression in their cord blood, when compared to low risk babies (Miles et al., 1994).

Our results did show and confirm previous reports that cord blood CD45RA+ naïve T cells activated with PHA for a prolonged period in vitro, are converted to a CD45RO+ effector T cell phenotype (Clement et al., 1990; Early & Reen, 1999). Early and Reen, compared newborn and adult CD45RA+ T cells, detailing the kinetics of the phenotypic transformation of CD45RA to CD45RO. They reported the transformation occured significantly quicker in newborn naïve T cells than adult naïve T cells (Early & Reen, 1999). This rapid in vivo phenotypic conversion may be reflective of faster immune responses within the neonate; consequently providing an advantageous asset to an infant's immature, underdeveloped immune system. The fact that this process was more rapid after food allergen stimulation in those who developed eczema suggest some form of in utero priming or intrinsic difference (genetic or epigenetic).

The expression of CCR7 (a chemokine receptor that regulates homing to secondary lymphoid organs) was also investigated in the context of cord blood function in the present study. Results showed CCR7 to be expressed on the majority of the CD45RA+ T cell population, with more a variable expression detected on CD45RO+ T cells. These results are similar to those reported by both Jourdan *et al* and Sallusto *et al* (Jourdan et al., 2000; Sallusto et al., 1999b), who reported strong CCR7 expression on naïve T cells from cord and peripheral blood, respectively. Expression of CCR7 is vital in the mechanism by which T cells enter secondary lymphoid organs, without CCR7 expression cells do not adhere to the high endothelial venules after the initial tethering and rolling (Stein et al., 2000; Warnock et al., 2000). Hence, the cells are unable to enter important areas including Peyer's Patches or lymph nodes and would be unable to be primed by antigen presenting cells.

Dependent on the expression of CD45RO+CCR7+ or CD45RO+CCR7-, CCR7 has been shown to characterise central and effector memory T cells, respectively (Sallusto et al., 1999b). As stated previously, effector memory T cells are able to migrate to inflamed tissue and show immediate effector function. In contrast, central memory T cells express lymph node homing receptors and lack immediate effector function. However, central memory has been reported as possessing the ability to stimulate dendritic cells and differentiate into effector memory cells upon secondary stimulation (Sallusto et al., 1999b).

There has been no previous characterisation of central and effector memory T cells present in cord blood and their possible role in the development of atopy. Our results not only confirm the presence of both central and effector memory T cells within cord blood, but also show the majority of memory cells within cord blood are of the effector memory phenotype. This fits somewhat with a study that characterised the memory phenotype of CD8+ T cells in breast milk, where they showed the majority of T cells to be of effector memory phenotype (Sabbaj et al.,

2005). Although the role of increased effector memory T cells in the cord blood is unclear, it can be speculated that increased memory with an effector phenotype should have immunological benefits for the infant.

In our study, when cord blood was cultured with β-lactoglobulin, there was a significant decrease in the central memory T cell population in the AE infants, while culture with ovalbumin or PHA, resulted in a significant decrease of expression in both AE and NE infants. In line with the current data, it has been previously reported that the CCR7+ phenotype is rapidly lost upon antigenic stimulation (Sallusto et al., 2004). This loss of CCR7 expression also correlates with the differentiation of central memory T cells to effector memory T cells. Consistent with these observations two possibilities are likely. First, the neonatal immune response to allergen favours the effector memory phenotype. Second, the stimulatory conditions provided by these experiments favour effector memory T cell survival. Naïve and central memory T cells have been observed as more sensitive to apoptosis, whereas effector memory T cells have been shown to be more resistant to apoptosis (Gupta et al., 2005). This may also influence the expression of this phenotype after culture *in vitro* for 7 days.

In this study, subject numbers are too small to draw any firm conclusions regarding memory phenotype immune response in the neonate. However, given the importance of the neonatal immune response in both protecting progeny from infection and preventing inappropriate development of immune system, the presence of memory cells with an effector phenotype may be of great importance. It would be interesting to investigate on a larger study population the possible role of central and effector memory in the development of allergy, as the role of these effector memory T cells in the prevention of allergy remains to be elucidated.

As part of the search for markers to predict eczema development in children, investigation of CD3+ expression was performed in both cord

and infant blood. To this end, it was not possible to demonstrate a difference in CD3+, CD3+CD4+ and CD3+CD8+ expression on T cells within cord or infant blood between AE and NE subjects. This study did demonstrate a significantly increased percentage of CD3+ lymphocytes in peripheral blood from infants at 12 months of age compared to those detected at birth. This result correlates with other studies, such as de Vries and colleagues (de Vries et al., 2000) longitudinal analysis of the changes in lymphocyte subpopulations in a group of 11 infants who had their progress tracked from birth to 1 year of age. It was found that the number of T cells significantly increased at 1 week of age compared to the levels detected in the cord blood. This has caused some investigators to argue that neonatal lymphocyte subset values obtained from peripheral blood 3 - 7 days postnatal, are more appropriate at reflecting neonatal immune system than cord blood values, due to the impact of stress from child labour on the lymphocyte subpopulations (de Vries et al., 2000; Raes et al., 1993).

Strong suggestions that sensitisation occurs *in utero* have come from the study of neonates that exhibit symptoms of cow's milk allergy at first feeding (Host et al., 1988). A number of studies have found an association between elevated cord blood proliferative responses to allergens and foetal priming (Warner, 2004), and subsequently employed this as a possible early marker for the identification of neonates at high risk of developing allergy (Kobayashi et al., 1994;Miles et al., 1996;Warner et al., 1994). In contrast, some investigators have suggested that proliferation of cord blood to a range of antigens may not be a result of foetal priming as many of these proliferating cord bloods were functionally immature recent thymic emigrants (Thornton et al., 2004). None of the cord blood samples within our study population significantly responded to either ovalbumin or β-lactoglobulin. Immature allergen-induced cord blood proliferation has been identified by some studies as a key characteristic of neonates with an allergic predisposition and/or later manifestation of atopic disease (Haddeland et al., 2007; Kobayashi et al., 1994; Miles et al., 1996). However, within our study the

population of proliferating cord blood cells in the AE group was not different from that recorded in the NE group.

Due to previous reports of cord blood responses to ovalbumin and β-lactoglobulin, greater proliferation of cord blood mononuclear cells was expected. Lack of cord blood proliferation could be explained if the allergen did not cross the placenta. However, both ovalbumin and β-lactoglobulin have been shown to cross the placenta (Edelbauer et al., 2004; Szepfalusi et al., 2000b), but at present there is little data to indicate the amount of maternal dietary allergens necessary to expect foetal sensitisation. The sample size used in the present study could have been too small to detect specific proliferation. Alternatively, it could be because the cord blood samples had been cryogenically frozen and thawed prior to culture with the specific allergen. However, preliminary experiments with adult peripheral blood mononuclear cells showed the freezing process to have little effect on the proliferation of these cells. Data are quite limited regarding the effects of cryopreservation on allergen-induced specific proliferation. Despite the initial work performed herein, some investigators have detected dulled immune responses from cryopreserved cells, particularly when exposed to class II-restricted antigens (Shreffler et al., 2006; Weinberg et al., 1998), therefore leaving the issue of the effects of the freeze-thaw cycle open to further debate. Another possibility could be the nature of our study population, within our cord blood samples only one infant went onto develop egg allergy (n = 1), and one infant milk allergy (n = 1). Hence there may have been fewer samples that would have shown a proliferative response to these allergens.

Certain studies have raised doubt regarding the association between maternal allergen exposure and cord blood proliferative responses. Szepfalushi and colleagues indicated that cord blood proliferation was not increased during gestation and showed no correlation with maternal exposure to the allergens timonthy grass pollen and birch pollen (Szepfalusi et al., 2000b). Consistent with these data, Chen-Yeung et al,

also found no association between maternal house dust mite exposure and the specific proliferative cord blood response (Chan-Yeung et al., 1999). .

However, our study investigated proliferation in relation to development of eczema and here, the five infants who developed atopic eczema, failed to show any significant proliferation in their cord blood, suggesting that allergen-priming (towards ovalbumin and β-lactoglobulin) had not occurred prenatally within our AE and NE study population.

Contradictory to the cord blood data, significantly higher proliferation was detected in the AE infant samples compared to the NE infant samples after culture with β-lactoglobulin and ovalbumin. The highest proliferation to the allergen β-lactoglobulin was detected in the blood sample from the infant who had been diagnosed with milk allergy, while the highest proliferation to ovalbumin was seen in the infant who had had a positive diagnosis of egg allergy. These results indicate that events in the first year of life appear to be important in the development of eczema. On the contrary, a previous study by Tay and colleagues (Tay et al., 2007b), detected T cell proliferation and cytokine response to ovalbumin in children with and without egg allergy. Furthermore, these findings, in addition to those reported in earlier studies (Ng et al., 2002; Prescott et al., 1999), suggest that T cell populations specific for certain food protein may be part of the normal immune response rather than limited to food allergy. However, there has been no study whether food protein specific T cell proliferation is associated with eczema rather than food allergy.

There has been concern regarding the contamination of allergen preparations with endotoxins, and the affect on allergen-specific cellular preparations. High endotoxin contamination of commercially available products has been previously described (Brix et al., 2003). Eiwegger and colleagues (Eiwegger et al., 2008) investigated the endotoxin content of β-lactoglobulin, Ara h 1 (peanut allergen) and Bet v 1 (the major birch allergen), and found that endotoxin significantly increased proliferative

responses of mononuclear cells towards both β-lactoglobulin and Bet v 1. They further observed increased sensitivity in cord blood mononuclear cells compared to the peripheral mononuclear cells from adults, with increased proliferative responses to both purified allergens an allergen with endotoxins (Eiwegger et al., 2008). However, as none of the cord blood samples showed significant proliferation to either allergen, it can be speculated that the responses recorded for the infant samples were specific response to the allergen.

In summary, the data presented emphasises the complexity of the immune cell repertoire that resides within human cord blood, but also reveals some interesting divergence between T cell populations resulting in later atopic outcome. It has been shown that allergen stimulation is able to augment memory T cell expression in the cord blood of infants who developed atopic eczema, compared to those who did not develop eczema. This study also indicates the change in percentage of lymphocytes present at birth and early childhood.

Chapter 5: The presence and responses of homing T cells in cord and infant blood to the allergens ovalbumin and β-lactoglobulin

5.1 Introduction

The T cell population is divided into multiple subsets that possess different functional characteristics, including homing capacity. T cell migration and the ability to home to particular organs or tissues is a key aspect of T cell function. This phenomenon is a multi-step process mediated by complex interactions between adhesion molecules, chemokines and their respective receptors; the latter acting as markers of cell homing behaviour, which help delineate homing T cell subpopulations (Kunkel & Butcher, 2002b; von Andrian & Mackay, 2000). Differences in migratory behaviour between T cell subsets can be considerable. For example naïve T cells possess a very limited ability to migrate and can only home to secondary lymphoid organs by expression of the chemokine receptor CCR7. In contrast, effector T cells are capable of specific migration and home to peripheral tissues, such as the intestinal mucosa and skin (discussed in Chapter 1). As food allergy can present with various clinical outcomes (e.g. atopic dermatitis, allergic gastroenteritis etc), this could imply that the differential expression of homing markers found on T cells could relate to the different phenotypic outcomes of food allergy. The propensity of a specific individual developing atopic eczema as opposed to gut symptoms may depend on difference in the skin or gut seeking behaviour of their specific memory or effector T cells. In order to identify distinct homing T cell subpopulations in the present study, the expression of specific homing markers were examined. The cell surface markers of interest included the skin homing marker CLA (Ferenczi et al., 2002) and β7, which is involved in homing to the gut (Waldman et al., 2006).

The rat monoclonal antibody HECA-452 was first used to identify the cell surface receptor CLA in humans as having a putative role in skin homing nearly 20 years ago (Picker et al., 1990). They marked the expression of CLA on T cells at cutaneous sites, and the notable absence from T cells at non-cutaneous sites (Picker et al., 1990). CLA

binds to its vascular receptor E-selectin (CD62E), which is expressed on inflamed superficial dermal postcapillary venules and epithelial cells. Numerous studies have confirmed these findings and have also demonstrated CLA+ T cells to have a role in the pathogenesis of benign inflammatory skin conditions, including psoriasis (Schon & Boehncke, 2005), various forms of eczema (Incorvaia et al., 2008), vitiligo (van den et al., 2000) alopecia areata (Lacueva et al., 2005) and drug-induced eruptions (Torres et al., 1998). Circulating CLA+ T cells in skin allergy was originally described in atopic and contact dermatitis in a series of experiments performed in Davos, at the Swiss Institute of Allergy and Asthma Research (Santamaria Babi et al., 1995b). They also reported allergen-specific proliferation of CLA+ T cells in those patients with eczema (Santamaria Babi et al., 1995b). However it remains unclear what specific purpose CLA expression serves and its influence on the development of atopic eczema in children. Certain studies have recorded a significant increased percentage of CLA+ T cells in the blood (Mizukawa et al., 2008; Torres et al., 1998) and in skin lesions of patients with atopic eczema when compared with healthy groups (Mizukawa et al., 2008). Mizukawa and colleagues also reported that this increase in CLA+ T cells was related to the severity of the clinical symptoms. On the contrary, other studies have reported no difference in the proportion of CLA+ T cells in peripheral blood of atopic and non-atopic individuals (Campbell & Kemp, 1999; de Vries et al., 1997).

In adults, most CLA+ T cells express high levels of the chemokine receptor CCR4 (Campbell et al., 1999). However, the joint expression of CLA and CCR4 within cord blood and its possible role as a predictor for the future development of eczema has not been investigated.

Gut-homing T cells are defined by the expression of the integrin $\beta 7$. Integrins are heterodimers and are classified into groups dependent upon the shared β-chain. On leukocytes, the $\beta 7$ chain can dimerise with either alpha 4 ($\alpha 4$) or alpha E (αE). These chains are expressed only by leukocyte subsets and can mediate specific interactions between these

cells and extracellular matrix molecules, as well as with endothelial and epithelial cells (Cepek et al., 1994; Erle et al., 1994a).

The integrin $\alpha4\beta7$ is expressed by subsets of T cells and B cells, and activated myeloid cells (Bellon et al., 1994; Erle et al., 1994a). As mentioned previous in Chapter 1, the ligand for integrin $\alpha4\beta7$ is the glycoprotein mucosal addressin cell adhesion molecule (MAdCAM-1) and vascular cell adhesion molecule (VCAM-1, also known as CD106), which are expressed on venules present in the intestinal lamina propria (Briskin et al., 1997). The $\beta7$ subunit can also pair with αE to form $\alpha E\beta7$, which is expressed on intraepithelial lymphocytes located in the intestine and binds E-cadherin expressed on the basolateral surface of intestinal enterocytes.

In this part of the study, the aim was to investigate the presence of skin specific homing markers within cord or infant blood, and identify any relationship between CLA expression on the T cells detected and the development of atopic eczema in the infant. Furthermore we investigated any relationship between the specific proliferation of these homing markers to the common food allergens ovalbumin and β-lactoglobulin, and infantile eczema development. The longitudinal changes in the expression of homing markers from birth to 12 months of age were also investigated.

5.2 Subject, samples and methods

5.2.1 Subjects

As detailed in the methods (2.1.5), cord (n = 10) and infant blood samples (n = 10) were selected. All samples were placed into one of two groups; infants who developed atopic eczema (AE infants)(n = 5) and infants who did not develop atopic eczema (NE infants)(n = 5) in the first 12 months of life.

5.2.2 Cell staining

In brief (Methods 2.6), both cord blood and infant blood mononuclear cells were thawed and cultured with the fluorescent cell staining dye CFSE and the appropriate stimulant. After culture, cells were stained with combinations of specific monoclonal antibodies: anti-CLA PE; anti-CCR4 APC; anti-CD3 PerCP-Cy5.5; anti-β7 PE or the isotype controls. Staining occurred in the dark, on ice for 35 minutes. Cells were then washed with FACs buffer and fixed with 1% PFA.

5.2.3 Flow cytometry analysis

As explained in detail previously (Methods 2.7), cultured cord and infant cells were first gated on lymphocytes via forward- and side-scatter properties and were analyzed using a FACS Aria I (BD, UK). Dead cells and monocytes were excluded by forward- and side-scatter characteristics. Specific fluorescence for CLA, CCR4 and β7 expression was defined by the fluorescence of the isotype control for each sample (back-ground fluorescence).

5.2.4 RT-PCR

In brief, total RNA was isolated with TRI-reagent from cord blood mononuclear cells and processed with reverse transcriptase, as described in Methods 2.8. mRNA-specific oligonucleotide primer pairs were used and are summarised in Table2.2.

5.3 Results

5.3.1 CLA-expression on T cells in cord and infant blood

Flow cytometry analysis revealed expression of the skin homing molecule CLA to be present in cord blood. CLA expression was detected on both CD3+ and CD3- cells. We focussed on the expression of CLA on T cells and detected higher expression of CLA in cord blood from the AE infants (6.3 ± 2.3%) than the NE infants (3.0 ± 1.1%) ($p = 0.030$) (Fig. 5.1). Upon culture of cord blood mononuclear cells with the food allergens ovalbumin and β-lactoglobulin, no change in CLA expression was detected. There was also no significant difference in CLA expression after non-specific stimulation with the mitogen PHA.

In both infant groups, CLA expression at 12 months was significantly lower than detected at birth (AE infants $p = 0.043$, NE infants $p = 0.046$) (Fig. 5.2). Furthermore by 12 months of age no significant difference in CLA expression was observed between the AE (1.4 ± 0.2%) and NE infants (1.6 ± 0.2%) ($p = 0.53$). Similar to the cord blood samples, there was no increase or proliferation of CLA expression after culture with either ovalbumin or β-lactoglobulin.

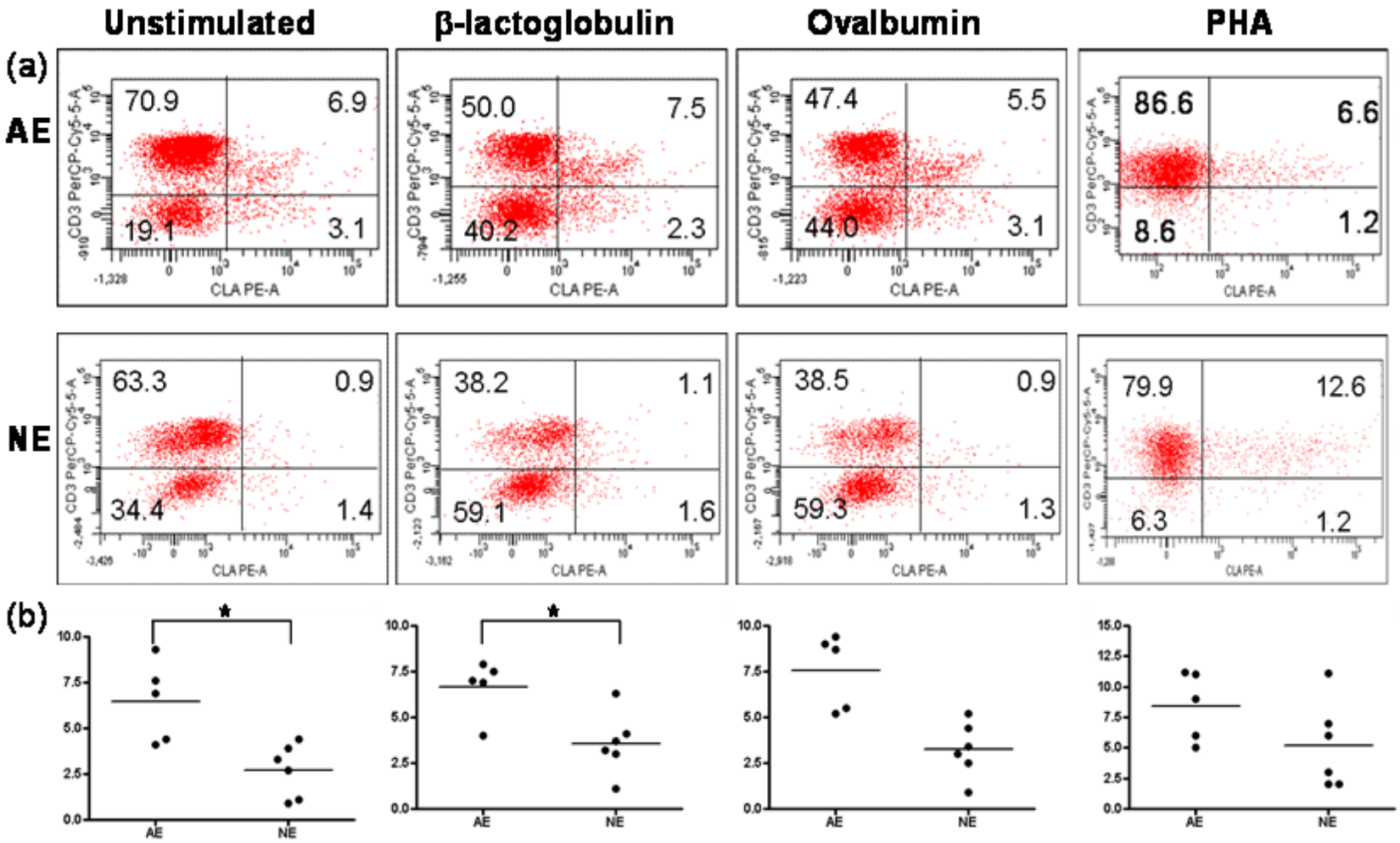

Figure 5:1 Percentage of T cells expressing CLA in cord blood samples.
(a) Representative FACS dot plots of cord blood cells stained with anti-CD3 and anti-CLA. Data were obtained from an AE infant who went on to develop eczema and a NE infant who showed no development of eczema in the first 12 months of life. Cord blood mononuclear cells were stimulated with β-lactoglobulin and ovalbumin. (b) Graphs showing % CLA expression detected in cord blood from infants grouped according to development (AE) or non-development (NE) of eczema.

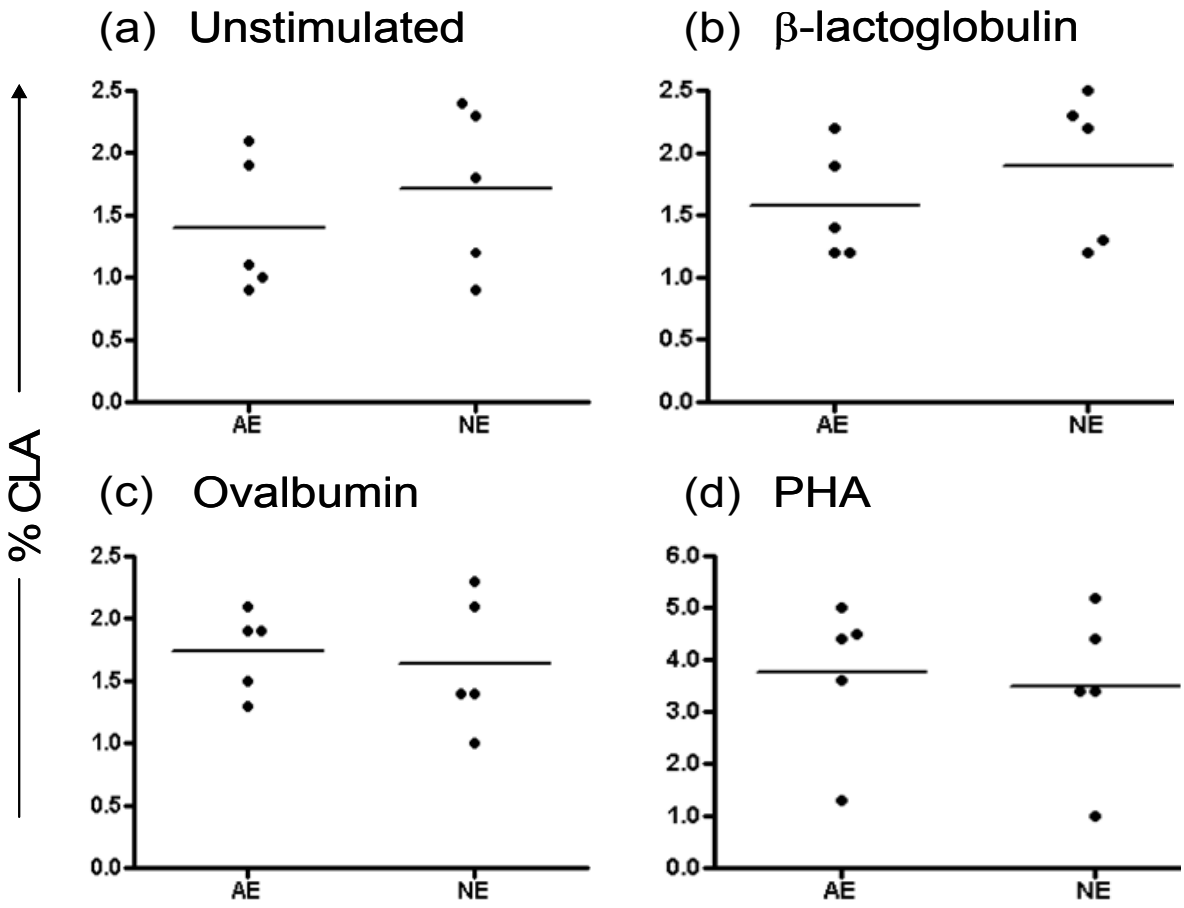

Figure 5:2 Percentages of T cells expressing CLA in infant blood samples.

The percentage of CD3+CLA+ cells detected in peripheral blood from infants who developed eczema (AE) and those who did not develop eczema (NE) at 12 months of age. There was no significant difference in expression of CLA between the two groups

5.3.2 No detection of CCR4+CLA+ T cells in cord or infant blood

As CCR4 has been associated with CLA expression in some adult skin homing T cells, we decided to investigate if the CLA+ T cells present in cord and infant blood also co-expressed CCR4.

There was significantly higher expression of CCR4 than CLA within both cord blood ($p = 0.04$) and infant blood samples ($p = 0.031$). However, the CLA+ T cells showed no co-expression of CCR4 in either the cord or infant blood samples (Fig. 5.3).

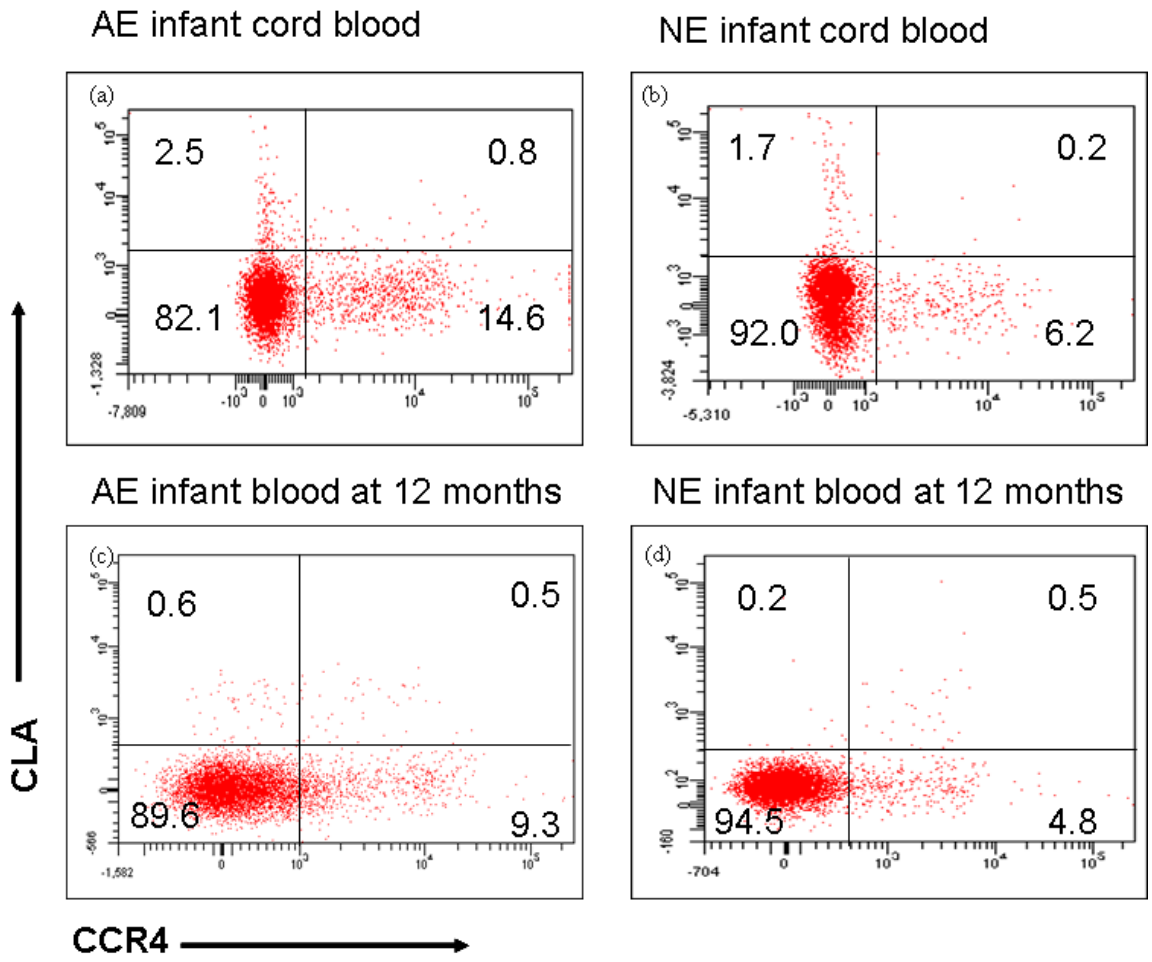

Figure 5:3 Presence of separate CLA+ T cells and CCR4+ T cells in cord and infant blood samples.

Representative FACS dot plots of cord blood cells (above) and infant peripheral blood cells (below) stained with anti-CCR4 and anti-CLA. The percentage present in the top right corner of each figure represents the percentage of cells that express both CCR4 and CLA collectively. As shown, there was no distinct CLA+CCR4+ expression detected in either the cord blood or infant blood.

5.3.3 CCR4 expression on T cells in cord and infant blood

Further analysis of the CCR4+ cells present in cord blood, showed the majority to be CD3+ (92.3 ± 4.01). Increased CCR4 expression was detected in unstimulated cord blood from the AE infant group (12.8 ± 1.7%) compared to the NE infant group (7.1 ± 1.7%) ($p = 0.044$) (Fig.

5.4). However, this significant difference in CCR4 expression between the two groups was no longer detected after culture with β-lactoglobulin, ovalbumin or PHA, although the AE group did show a continual trend for higher expression of CCR4 than the NE group.

Further analysis of AE cord blood, showed CCR4 expression significantly increased after culture with β-lactoglobulin ($p = 0.0054$) and ovalbumin ($p = 0.0021$), when compared to unstimulated. Significantly higher expression of CCR4 was also detected in the NE group after stimulation with ovalbumin ($p = 0.0233$) but not β-lactoglobulin (Fig 5.4(b)).

Although there was a larger population of CCR4+ cells detected at birth than at 12 months of age this was not significant ($p = 0.0540$). By 12 months of age, there was no longer a greater expression of CCR4 in the AE infants compared to the NE infants. Furthermore, there was no significant difference in CCR4 expression between the AE and NE infants following culture with ovalbumin, β-lactoglobulin or PHA.

Next we investigated any difference of CCR4 expression after culture with allergen or mitogen compared to when cultured alone (Fig. 5.4 (b)). At 12 months of age, infants in the AE group showed significantly increased CCR4 expression after stimulation with β-lactoglobulin ($p = 0.0159$) and ovalbumin ($p = 0.0154$). Significantly higher expression of CCR4 was also detected in the NE group after culture with β-lactoglobulin ($p = 0.0159$) and ovalubumin ($p = 0.0317$).

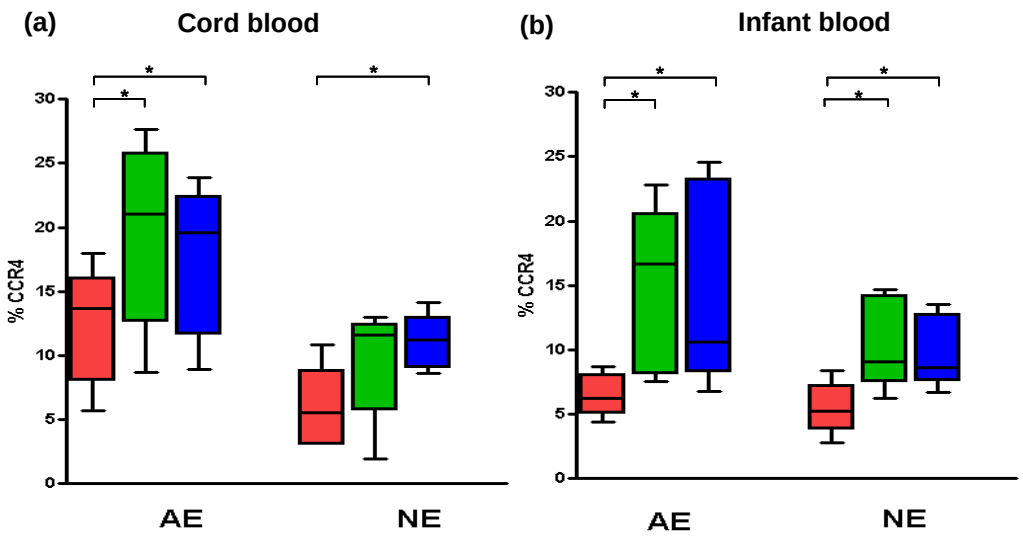

Figure 5:4 Percentage of T cells expressing CCR4 in cord and infant blood.

Measured by flow cytometry after being in culture for 6 days with no stimulant (red), with β–lactoglobulin (green) or ovalbumin (blue). The two groups of high risk atopic infants are shown: those with eczema at 12 months (AE)(n = 5) and those with no eczema development at 12 months (NE)(n = 5). (a) CCR4 expression in the cord blood was significantly higher in the AE group after culture with β-lactoglobulin (p = 0.0054) and ovalbumin (p = 0.0021) compared with unstimulated. Cord bloods from the NE groups showed increased CCR4 expression after culture with ovalbumin (p = 0.0233). (b) Levels of CCR4 expression at 12 months. Both AE and NE infants showed increased expression of CCR4 after culture with β-lactoglobulin (AE p = 0.0159; NE p = 0.0159) and ovalbumin (AE p = 0.0154; NE p = 0.0317)

5.3.4 The expression of β7 on T cells in the cord and infant blood

The anti-β7 specific antibody was used in the present study to identify gut homing cells. The percentage of lymphocytes that expressed β7 in unstimulated cord blood obtained from AE infants did not differ significantly from that of AE infants (AE 32.4 ± 6.6; NE 31.9 ± 6.9; p = 0.31) (Fig. 5.5). Both infant groups demonstrated higher expression of β7 than the CLA. Similar to CLA expression, no significant difference in the expression of β7 was detected after stimulation with ovalbumin or β-lactoglobulin in either the AE or NE infant group (BLG p = 0.686, p =

0.89; OVA p = 0.50, p = 0.686 respectively). However, after non-specific stimulation with PHA there was increased expression of β7 from both infant groups (AE p = 0.04, NE p = 0.04). The expression of β7 was also studied in lymphocytes isolated from infant peripheral blood. At 12 month, there was a significant reduction in the percentage of β7 expressing cells compared to levels detected at birth (p = 0.043). Similar to the cord blood samples, there was no significant proliferation detected after stimulation with ovalbumin or β-lactoglobulin. However, after stimulation with the mitogen PHA there was significant proliferation of β7 expression.

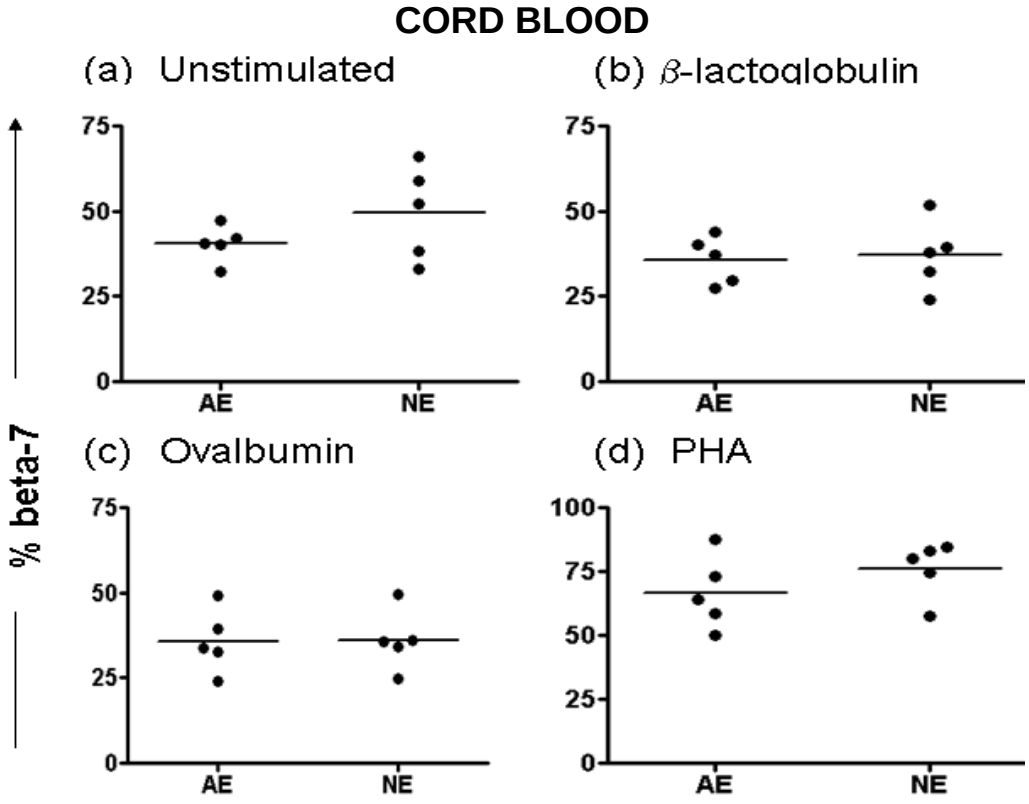

Figure 5:5 Percentage of CD3+β7+ cells detected in the cord blood after different culture conditions.

The percentage CD3+β7+ cells from cord blood taken at birth was measured by flow cytometry after being in culture for 6 days with: (a) no stimulant (unstimulated); (b) with β-lactoglobulin; (c) ovalbumin and (d) mitogen PHA. The two groups of high risk atopic infants are shown: as those who developed eczema by 12 months (AE) (n = 5) or those who did not develop eczema by 12 months (NE) (n = 5). There was no significant difference in β7 expression between the two groups.

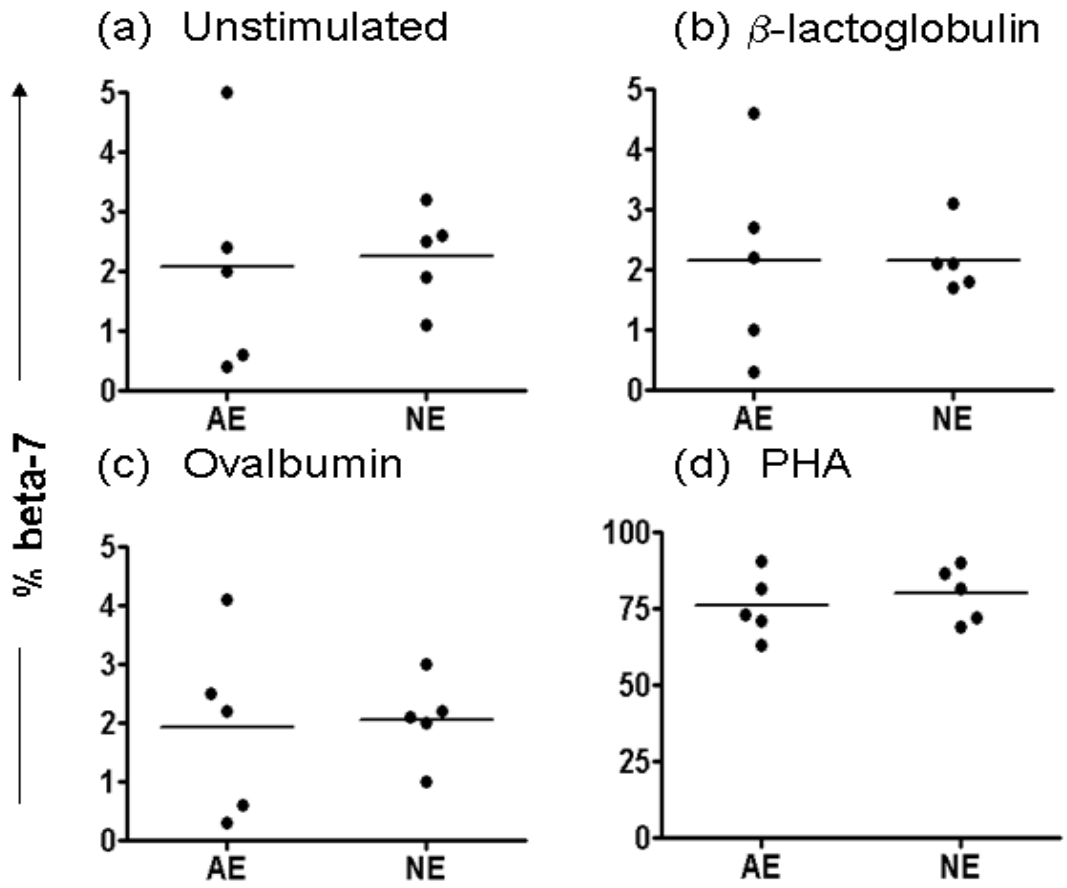

Figure 5:6 The percentage CD3+β7+ cells from infant blood taken at 12 months of age.

Expression of β7 T cells was measured by flow cytometry after being in culture for 6 days with: (a) no stimulant (unstimulated); (b) β-lactoglobulin; (c) ovalbumin and (d) mitogen PHA. The two groups of high risk atopic infants are shown: those who developed eczema by 12 months (AE)(n = 5) and those who did not develop eczema by 12 months (NE)(n = 5). There was no significant difference in β7 expression between the two groups.

5.3.5 RT-PCR analysis of homing markers present in cord blood

To confirm the expression of the homing markers by an alternative method, we used RT-PCR to investigate expression of CLA, αE, α4 and β7 in two cord blood samples. As shown in figure 5.12, the RT-PCR data

demonstrated that a DNA fragment of the expected size was found with all the markers tested (CLA = 549 bp, α4 = 463 bp, αE = 461bp and β7 = 462 bp). The presence of alpha chains (αE and α4), which are able to form hetrodimers with the β7 subunit, were also investigated. Data from the RT-PCR showed the β7 subunit to be the strongest expressed but both alpha chain subunits were also detected in the cord blood. These data confirmed the flow cytometry findings.

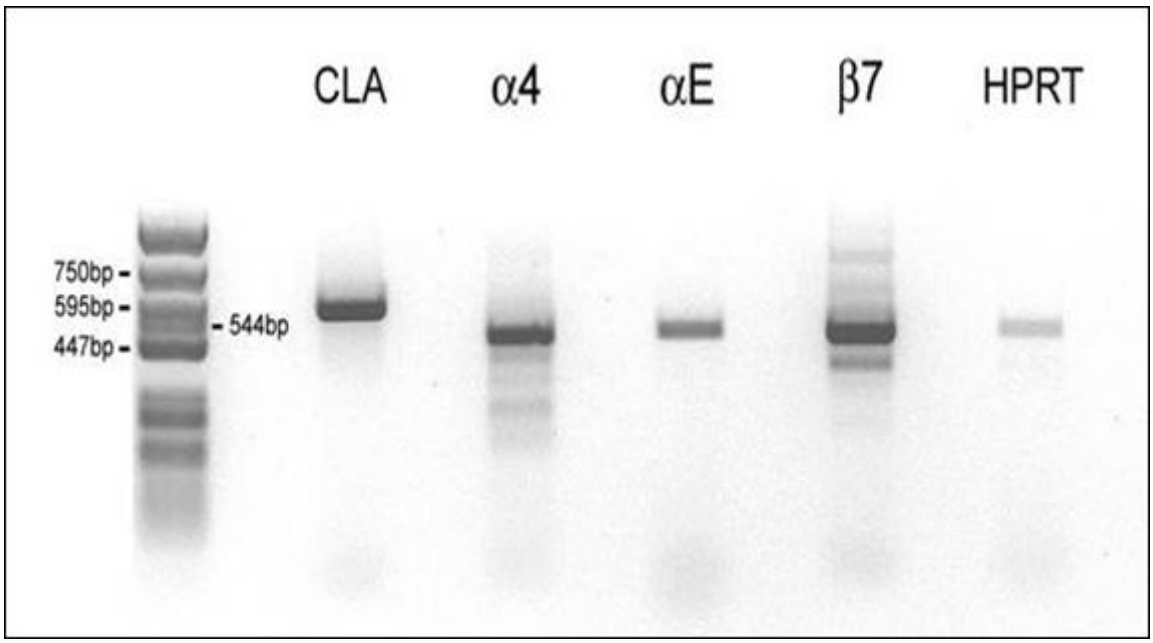

Figure 5:7 CLA, α4, αE and β7 mRNAs isolated from cord blood.

Total RNA was isolated, reverse transcribed, and amplified by PCR using specific primers as described in methods and materials (2.12).

5.4 Discussion

In humans the first lymphocytes appear in the gut and peripheral lymph nodes at around 14 - 15 weeks gestation (Spencer et al., 1986). These lymphocytes offer the developing foetus a basic functional immune system capable of mounting an adequate immune response and possibly directing lymphocyte homing. The the principle aim of this work was to compare the presence of homing markers on lymphocytes at birth (i.e. in cord blood) and at 12 months of age, then examine these temporal expression patterns in the context of atopic eczema development. Hence, homing marker expression could be compared in infants who later developed eczema and those that did not, as well as enabling comparisons between their immune responses to the food allergens ovalbumin and β-lactoglobulin.

Expression of CLA was detected in all cord and infant blood samples by flow cytometry. Although CLA has been detected in cord blood previously (Arrighi et al., 2001; Campbell & Kemp, 1999d;Kohno et al., 2001) this is the first study to illustrate increased CLA expression in cord blood from infants who went on to develop eczema. This observation has led to the hypothesis that increased CLA expression within the cord blood could predict future development of atopic eczema.

There has been contradictory data regarding CLA expression in the development of atopic eczema. An early study by de Vries and colleagues, found no difference between the percentage of CLA+CD3+ cells in peripheral blood of atopic and non-atopic adults (de Vries et al., 1998). These findings correlated with a later study by Campbell and Kemp who also reported no difference in the proportion of CLA+ T cells in atopic and non-atopic children (Campbell & Kemp, 1999b). In contrast, other studies have reported increased expression of CLA in those with atopic eczema (Seneviratne et al., 2007). Our data both confirms and elaborates on previous findings demonstrating CLA

expression to be present in cord blood and increased in individuals who go on to develop atopic eczema. However, the percentage of CLA expression within the infant blood samples was significantly lower than that detected within the cord blood samples. Furthermore, there was no longer a significant difference in CLA expression between infants who developed atopic eczema and those who did not. The decreased CLA expression observed in infant peripheral blood, relative to cord blood, could be due to the movement of these CLA expressing T cells to the skin. A recent study by Clark and colleagues, which focused on T cells isolated from normal human skin, reported ~ 1 x 10^6 T cells/cm^2 to be present in the skin surface (Clark et al., 2006). These findings suggest there is a large pool of T cells within normal (non-inflamed) skin, which are able to respond immediately to an antigenic challenge. Clark and colleagues estimated that the majority (95%) of CLA+ effector memory T cells were present in the skin. This would indicate that only a minority of CLA+ T cells are present in the circulation and could explain the decreased CLA expression detected in the infant samples.

Other studies have reported CLA expression to be induced after culture with food-allergens in food allergic patients with atopic eczema (Abernathy-Carver et al., 1995; Reekers et al., 1999). Therefore, after observing the presence of a small population of CLA+ T cells in cord blood we were keen to investigate if this specific subpopulation proliferated to either ovalbumin or β-lactoglobulin. We detected no proliferation of CLA+ T cells after culture with either of our food-allergens or mitogen in the cord or infant blood samples. Hence, there was no significant difference in CLA proliferation between those who developed atopic eczema and those who did not develop eczema in either the cord or infant blood samples. Contradictory to our results, Abernathy-Carver and colleagues reported an expansion of CLA+ T cells from children with milk-induced eczema after culture with the milk protein casein (Abernathy-Carver et al., 1995). Reekers and colleagues also reported increased CLA expression on allergen-specific T cells in birch pollen-related food induced atopic eczema (Reekers et al., 1999).

However, contrary to our study, the children recruited by Abernathy-Carver *et al* had been diagnosed with milk-induced eczema following a specific criteria which included: a diagnosis of atopic eczema according to the guidelines of Hanifin and Rajka (Hanifin & Rajka, 1980); a positive SPT to milk; the presence of milk-specific IgE in the sera; and a positive double-blind placebo-controlled food challenge to milk. In the present study, although the AE infants were diagnosed with atopic eczema, it was never diagnosed as specific to egg and/or milk. Only two of the ten infants samples analysed by flow cytometry showed a positive SPT to either egg (n=1) or milk (n=1) (summarised in Chapter 4). As no infants were subjected to egg or milk double-blind placebo-controlled food challenges, or had specific IgE detected in their serum, it is not possible to ascertain whether these subjects had either egg- or milk-induced eczema. However, although elevated specific IgE in response to a variety of different food and inhalant allergens have been implicated in atopic eczema (Sampson & Albergo, 1984). Results of SPT in patients with atopic eczema frequently fail to correlate with double-blind placebo-controlled food challenge (DBPCFC) (May, 1976). Furthermore, it should also be noted that not all allergy are IgE-mediated, about 40 -50% of milk allergy has been reported as non-IgE mediated (Host & Halken, 1990). All this suggests the importance of investigating other immune responses such as the tissue-specific T cells, in the development of atopic eczema.

The cells were cultured with β-lactoglobulin and ovalbumin because egg and milk allergy are the most common food allergies affecting young children (Eggesbo et al., 2001;Host & Halken, 1990). Furthermore, we focused on these allergens as children with sensitization to egg or milk have been reported to be at higher risk of persistent atopic eczema and demonstrate higher objective SCORAD scores (Wolkerstorfer et al., 2002). From our data we can speculate that the infants within the AE groups did not develop allergen-specific eczema to ovalbumin or β-lactoglobulin, although this is the most prominent type for the age group

we investigated (Wolkerstorfer et al., 2002). As both specific (ovalbumin and β-lactoglobulin) and non-specific (mitogen PHA) stimulation of cord blood T cells did not increase CLA expression, it is likely that specific conditions not employed in this study are necessary for the induction of this marker. However, a study by Kojima and colleagues which looked at patients with a previous history of eczema when exposed to hen's egg, observed increased CLA expression after using the same culture conditions and allergen as we have for this present study, but on older children (10 – 94 months, mean = 40 months) (Kojima et al., 2003). Interestingly, Kojima *et al* observed that those patients without eczema exacerbations following oral challenge had significantly lower percentage of CLA expression on T cells than subjects that showed late onset exanthematous reactions (Kojima et al., 2003). Hence, these data implied that those with atopic eczema who had outgrown their egg allergy had a reduced CLA+ T cell population after stimulation with ovalbumin.

The percentage of CLA+ lymphocytes we detected in both cord and infant blood was much lower than the 10-15% recorded previously in adults by our group and in other studies (Bordignon et al., 2005; Clark et al., 2006). Campbell and Kemp, examined the association between age and CLA expression, and showed CLA expression increased from <1% in cord blood to >10-15% in healthy adults (Campbell & Kemp, 1999a). Campbell *et al* therefore detected lower CLA expression than in our cohort, and found this percentage increased with age. Our data demonstrates a slightly different pattern, with higher levels in the cord blood than those described by Campbell and Kemp, that actually decreased by 12 months of age. However this discrepancy could be because the data from Campbell and Kemp was generated from a small study, where only four cord blood samples were examined, all of which were from non-atopic children.

As discussed in Chapter 1, when the model of central and effector memory was first described, all T cells expressing homing receptors

specific for peripheral tissue were classed as effector memory and unable to enter lymph nodes (Sallusto et al., 1999b). However, this division is less apparent for CLA+ T cells, where a subpopulation are known to express CCR7 and L-selectin, therefore possessing the ability to enter the skin, blood and/or lymph nodes. This specific subpopulation has been termed central memory skin-homing T cells and has been reported to represent the majority (80%) of CLA+ T cell present in the circulation (Campbell et al., 2001). The marker CCR7 was used in this study, but not in conjunction with CLA, so it remains to be clarified whether the specific phenotype of the CLA+ T cells detected in cord blood are the central memory skin-homing subpopulation. Nevertheless we can speculate that the CLA+ T cell population detected would be of the CLA+CCR7+ phenotype on basis of a number of key data. Firstly, the majority of CLA+ cells within circulation have previously been reported as CLA+CCR7+ (Clark et al., 2006). In addition, CCR7 expression is stronger on cord blood lymphocytes than in adults (Loria et al., 2005). Further investigation using CLA along with the CCR7 marker would allow us to distinguish the specific phenotype of these T cells expressing CLA.

Several distinct T cell subsets have been identified to possess different chemokine receptors (Sallusto et al., 2000). Like CCR7, the expression pattern of CCR4 on T cells is highly contentious and much conflicting data exists in the literature. Generally, in adults most CLA+ T cells express high levels of CCR4. Specifically >95% expression has been demonstrated in peripheral blood CLA+ T cells.(Campbell et al., 1999) and high levels have also been shown to be common in CLA+ T cells isolated from normal skin (Clark et al., 2006). In contrast however, only 60% of CLA+ T cells in the circulation expressed CCR4 (Clark et al., 2006). In comparison, our data showed the CLA+ subpopulation detected in cord blood did not express CCR4, and furthermore the inversely correlated expression of CLA and CCR4 actually appeared to distinguish two separate T cell subpopulations in both infant and cord blood samples. Adding to the apparent dichotomy that exists between

CCR4+ and CLA+ T cell subpopulations, CCR4+ T cells unlike CLA can be expressed on both cutaneous and non-cutaneous T cell subpopulations. However, increased expression of CCR4 and CLA in combination on T cells is thought to enable preferential migration towards mature dendritic cells, which promotes secretion of a large array of chemokines (Gerard & Rollins, 2001), including ligands for the receptors CCR4, CCL17 and CCL22 (Iellem et al., 2001).

Intestinal lymphocyte homing is principally mediated by β7 chains, which are able to form a heterodimer complexes with either alpha-E (αE) or alpha-4 chains (α4). The latter combination forms the integrin α4β7, which is the cell surface receptor for the MAdCAM-1 ligand, a glycoprotein that is expressed by gut endothelial cells. Lymphocytes expressing this receptor are able to enter the Peyer's Patches. MAdCAM-1 is within the immunoglobulin-like adhesion receptor superfamily. Another example of cells specific to gut homing are the αEβ7-expressing intraepithelial lymphocytes, which bind E-cadherin, a calcium dependant adhesion molecule expressed by epithelial cells (Colantonio et al., 2004; Hamann et al., 1994). Interestingly, >95% of intraepithelial lymphocytes located in the intestines express αEβ7 (Erle et al., 1994b), while only <2% of peripheral blood T cells express this marker (de Vries et al., 1997a).

In the current study, we did not evaluate gut symptoms in the infants. Thus, we were unable to compare expression of the gut homing marker and clinical relevance. For the purpose of this study the gut homing marker was used as a negative control. We hypothesised that CLA expression would be increased in infants with eczema not β7, as eczema is a disease of the skin.

Cells expressing β7 were detected in cord blood samples from all high risk infants. However, there was a large difference in the percentage of β7 expressing cells between individual subjects and no significant

difference was detected between the AE and NE cord blood samples. Moreover, the β7+ subpopulation showed overall a lack of significant proliferation to the food allergens studied (β-lactoglobulin and ovalbumin).

Similar to our data, expression of β7 integrin has been previously recorded at birth (Haddeland et al., 2007; Kohno et al., 2001). As the majority of $\beta7^+$ cells in peripheral blood have been shown to express the gut homing receptor integrin α4β7 (Rodriguez et al., 2004), it was originally expected that this would be the phenotype detected in cord blood. However, Kohno and colleagues identified the αEβ7 phenotype present in the cord bloods of both allergic and non-allergic controls (Kohno et al., 2001). Interestingly, they reported proliferation of αEβ7+ cord blood cells to the milk protein casein in cord blood from both milk-allergic and non-allergic infants with the infants who went on to develop dermatitis (milk-allergic) showing much higher percentage of αEβ7-positive T cells. αEβ7 has been identified as both a homing marker for the gut and an activation marker (Brew et al., 1995). In our study, we stimulated with the milk protein β-lactoglobulin, which failed to cause any proliferation in the cord blood samples. This could suggest that while casein-reactive T cells are present in cord blood of atopic and non-atopic individuals, within our subjects no β-lactoglobulin (or ovalbumin) reactive β7 expressing cord blood T cells were present. This is despite proliferation to both these allergens in our subjects in our CD3 experiments (see Chapter 4). In addition, Kohno and colleagues found integrin αEβ7 was no longer induced when they investigated T cells from older patients with food allergy induced atopic dermatitis (Kohno et al., 2001). Consistent with these results our data also showed much lower detection of β7 expressing cells in infants and again, no proliferation upon stimulation with either ovalbumin or β-lactoglobulin.

It has been demonstrated that the integrin αE is expressed on T cells infiltrating the skin in certain cutaneous inflammatory diseases including

atopic dermatitis (de Vries et al., 1997a; Simonitsch et al., 1994). This phenomenon lends credence to the widely-held theory that luminal antigens could participate in the onset of eczema. The expression of the skin homing marker CLA requires a glycosylation process, where an additional single fucose molecule is added to the P-selectin glycoprotein-1 expressed by fucosyltransferase VII (FucTVII) (Fuhlbrigge et al., 1997). Hence, skin tropism (through expression of CLA) is reliant on factors that up-regulate lymphocyte gene expression of FucTVII.

It has been reported that some gut bacteria, in an attempt to establish a niche, have the ability to regulate host fucosyltransferase gene expression, therefore altering the composition of the glycocalyx (Backhed et al., 2005). It remains unclear if FucTVII is activated via this route, but it is clear that FucTVII is essential for expression of sialyl Lewis-x , which has also been detected on *Helicobacter pylori* and other similar gut flora (Bergman et al., 2004). The possible up-regulation of FucTVII by the gut bacteria could block T_H1 responses, and therefore favour the T_H2 response (Bergman et al., 2004), leading to a possible accumulation of the T_H2 skin-tropic phenotype within the intestinal activated lymphocytes. Interestingly, as reviewed by Murch, it has been shown that probiotics that are unable to regulate host FucTVII could possibly reduce mucosal conversion of lymphocytes to this specific phenotype, therefore preventing eczema development (Murch, 2005).

In addition to its role in intestinal homing, $\alpha4\beta7$ is also known to function as a vital factor in the homing of lymphocytes to mucosal tissues. It has been reported that anti-$\alpha4$ monoclonal antibodies are able to prevent the adhesion of murine lymphocytes to peyer's patch high endothelial venules (Holzmann, McIntyre, & Weissman, 1989). The majority of CD4+ and CD8+ T cells express moderately high levels of $\alpha4\beta7$ and low levels of $\beta1$. Like $\alpha E\beta7$, $\alpha4\beta7$ has been detected in cord blood (Erle

et al., 1994a). An earlier study also indicated high levels of the α4 subunit in neonates (Pilarski et al., 1991).

It is clear from our data that there is substantial expression of β7 by parturition, hence suggesting that the immune system of newborns has the capacity for lymphocyte recruitment to mucosal lymphoid organs. Utilising flow cytometry we were able to distinguish cells expressing β7, but were unable to identify the specific heterodimer phenotype of these cells. However, data from RT-PCR analysis showed that both the α4 and αE subunits were being expressed in cord blood, suggesting that the β7 population detected by flow cytometry could be a mixture of both α4β7+ and αEβ7+ cells.

The study by Erle and colleagues also compared the level of α4β7 between neonatal and adult blood, reporting markedly lower detection of α4β7 in the latter (Erle et al., 1994a). In the present study we showed a loss of β7 expression between birth and 12 months. It was previously thought that this change in expression was likely to occur around the time of initial activation of the nTC. However, the infant samples at 12 months showed a dramatic decrease in β7 expression with a smaller decline in nTC also detected.

This chapter addresses key issues concerning the expression of tissue-specific homing T cell phenotypes that are present at birth and early childhood, and the putative role of these in the development of eczema. The data shows that expression of the surface homing markers β7 and CLA on lymphocytes changes during early postnatal life. This is similar to the expression of other homing markers such as L-selectin, which has been reported to alter its expression pattern during the *in vivo* transition to the memory T cell phenotype (Hamann et al., 1997). This type of temporal expression pattern may be exhibited by β7 and CLA, and hence explain the current findings. Although both markers show similar temporal expression patterns, it was demonstrated that β7

expression was higher than that of CLA. However, only the latter marker provided a significant difference between the two infant groups, which demands further investigation in a larger cohort to fully ascertain its role in the development of eczema.

Chapter 6: General discussion and plans for future work

6.1 Overview

The prevalence of atopic eczema has been increasing since World War II (Williams et al., 1999). A UK based survey from 2003 reported eczema affects approximately 23.1% of infants at some time during childhood (Burr et al., 2006). In infants, atopic eczema is often the first sign of the allergic state and most commonly presents itself in the first year of life associated with food allergy (Halkjaer et al., 2006). Current treatments for allergic diseases are primarily aimed at relief of symptoms already established. Hence there is a requirement for better fundamental understanding of the risk factors that lead to allergy development, to hopefully enable improved preventative therapeutic strategies, rather than relying on a palliative approach to care.

Previous studies have identified that both prenatal and postnatal events play a role in allergy development. However, the consequences of altered immune responses, within the mother and infant at birth, and the later development of infant atopic eczema, remain poorly understood. The fundamental aim of the work presented in this thesis was to investigate the relation between maternal dietary intake of hen's eggs and cow's milk during gestation, as well as the T cell phenotypes present in the cord blood, in relation to the development of atopic eczema in the infant. With its ability to cross the placental barrier, ovalbumin and β-lactoglobulin specific IgG and IgG subclasses (IgG1 and IgG3) from mother and infant were investigated in relation to development of eczema in the infant. The primary aims of this study (1.5) were: to describe the characteristics of the maternal and infant humoral immune response to the food allergens ovalbumin and β-lactoglobulin, and study how these alter with maternal dietary intake of hen's eggs and cow's milk during pregnancy (Chapter 3); to characterise cord blood mononuclear cells responses to ovalbumin and β-lactoglobulin in relation to development of atopic eczema in the infant (Chapter 4); and finally, to relate organ specific allergic outcomes of the

infants to the tissue-specific homing molecules on sensitised cord blood mononuclear cells (Chapter 5). In this final chapter the main findings from previous chapters have been drawn together, highlighting the conclusions and indicating future lines of investigation.

6.2 Hypothesis 1: Atopy in early childhood is associated with maternal consumption of egg and milk allergens, but this is modified by the nature of the maternal immune responses to these allergens.

The first objective was to investigate the levels of egg and milk protein consumed in the maternal diet in relation to the development of eczema in the infant. Dietary intake of egg and milk allergens during pregnancy has previously been a concern due to the concept of intrauterine sensitisation. The detection of ovalbumin and β-lactoglobulin in the amniotic fluid and the foetal circulation provides direct evidence of transamniotic allergen transfer as early as 26 wks gestation (Loibichler et al., 2002). There has previously been uncertainty and mixed opinions regarding maternal diet and atopic outcome of the infant Therefore, this hypothesis was proposed to investigate if those mothers who had a higher, lower or medium intake of egg and milk allergens in their diet, had infants at higher risk of developing eczema.

This study showed no correlation between maternal dietary intake of egg or milk protein and the development of eczema in infants by 12 months of age. Other investigations into the maternal diet and the development of allergic disease have included maternal peanut consumption (Hourihane et al., 1996; Lack et al., 2003), food avoidance (Kramer & Kakuma, 2003) and fruit and vegetable intake (Stazi et al., 2002), all of which found no conclusive relationship between the food being investigated and the development of allergic disease in the infant.

At present the lack of evidence showing any benefit from allergen restriction in the maternal diet and atopy development has lead the government to suggest no restriction on dietary intake of allergenic foods (www.dh.gov.uk/en/Healthcare/Children/Maternity/Maternalandinfantnutrition). While epidemiological and immunological studies have indicated that modification or supplementation of the maternal diet during pregnancy may reduce development of allergic disease, under-nutrition of the developing foetus may affect the development of the foetus lungs and immune system (Devereux & Seaton, 2005; Langley-Evans, 1997). A recent longitudinal study by Willers *et al* demonstrated intake of apple and fish within the maternal diet during pregnancy to have protective role against the development of asthma and eczema (Willers et al., 2007). This correlates with an earlier study by Romieu *et al*, which also confirmed maternal fish intake during pregnancy to have a protective role in the development of eczema at 12 months of age, and against atopic wheeze at 6.5 years of age (Romieu et al., 2007). The conflicting data and advice within the literature regarding the maternal diet during pregnancy, leaves expectant mothers unclear as to what they should be eating during pregnancy, and breast-feeding. The best example of this confusion relates to the peanut. In June 1998, the UK government published a report on peanut allergy suggesting that "pregnant women who are atopic or for whom the father or any sibling of the unborn child has an atopic disease, may wish to avoid peanuts and peanut products during pregnancy and breast feeding" (http://cot.food.gov.uk/cotreports/cotwgreports/cotpeanutallergy). However, it has become apparent that mothers with family history of atopy and those without, have been avoiding peanuts during pregnancy (Dean et al., 2007). Avoidance of peanut through pregnancy and early infancy was first suggested because it was thought that peanut consumption during pregnancy would influence the development of peanut allergy in the infant. However, the government has now withdrawn the advice about avoiding peanuts during pregnancy and early life (http://cot.food.gov.uk/pdfs/cotstatement200807), after it was shown there was no substantial evidence to support this advice and that the prevalence of peanut sensitisation in the UK had

continued to rise (Hourihane et al., 2007). A recent study that investigated the prevalence of peanut allergy amongst Jewish children within the UK and Israeli population, found the prevalence of peanut allergy was significantly lower in the Israeli children (1.85% vs 0.17%; $p < 0.001$). This difference in prevalence may have been due to the earlier introduction of peanut into the diet of the infants from Israel (Du et al., 2008). These issues highlight the need for larger studies into the effect of both the maternal and weaning diet in relation to the development of atopic disease in the child.

Serum IgG plays an essential role in host defence, which becomes apparent with the recurrent infections seen in those with IgG deficiency. IgG has the unique ability of being the only immunoglobulin class able to cross the placental barrier, and both ovalbumin and β-lactoglobulin specific IgG complexes have been detected in cord blood (Vance et al., 2004a). It is therefore reasonable to speculate that maternal dietary intake of egg and milk protein as well as levels of specific IgG which will be actively transported across the placenta to the foetus, may play an important role in the development of allergic disease.

For the allergen specific IgG subclasses studied, there was a predictable strong correlation between maternal and cord blood specific IgG levels. This finding confirms that both β-lactoglobulin and ovalbumin specific IgG complexes possess the capacity to cross the placenta. To my knowledge, the present study is the first to report a correlation in the β-lactoglobulin specific-IgG subclasses IgG1 and IgG4.

Thus, the data does not support the hypothesis that the development of eczema in the first 12 months of life is associated with maternal dietary intake of egg or milk protein, or the maternal IgG immune responses to the allergens. However, this was a small exploratory study that only looked at 51 maternal samples. The small sample size of the study and narrow range of differences in the dietary intake between the two groups,

makes it difficult to draw firm conclusions. Hence further research is required with a larger study number.

Given the difference in mean values and mean standard deviation a power calculation using our study data indicated that a sample size of 655 individuals per group would be required in order to show a significant difference in the maternal dietary intake of egg. A smaller sample size of 85 individuals per group would be required to show a significant difference in the dietary intake of milk between those mothers in the AE group and those in the NE group. When comparing ovalbumin and β-lactoglobulin specific IgG between the two groups, a power calculation showed that 2018 individuals per group would be required to show any significant difference in the β-lactoglobulin specific IgG between the two groups. However, analysis of the ovalbumin specific IgG in the statistical software nQuery Version 7, showed the difference between the AE and NE mothers was insufficient to be able to perform a power calculation to predict the sample size required.

6.3 Hypothesis 2: There is evidence of altered T cell function at birth in neonates who subsequently develop atopic eczema.

It has been reported that most patients who suffer with atopic eczema possess an altered cellular immune profile (Bos et al., 1993). The above hypothesis was proposed to investigate the immunological profile, specifically the memory T cell population present in the cord in relation to the development of atopic eczema in the infant.

Using flow cytometry, strong expression of CD45RA and weak expression of CD45RO was detected in cord blood samples, thus reflecting the naïve status of the foetal immune system at birth. Increased memory T cell expression was detected within the cord blood of AE infants after culture with β-lactoglobulin and ovalbumin (4.3.6). In correlation with these data, a previous study has also recorded

significantly increased CD45RO+ cord blood T cells in those infants who went on to develop atopy by 12 months, compared to those who did not (van, V et al., 2001).

Further investigation of the increased CD45RO expression after culture with the food allergens, showed this was not a proliferative response but instead indicated a phenotypic switch of the CD45 isotype from CD45RA to CD45RO. This rapid phenotypic conversion may be reflective of faster immune responses within the neonate; consequently providing an advantageous asset to an infant's immature, underdeveloped immune system at first exposure to pathogens. However, it appears to also be the first sign of an altered immune response which favours the development of atopy.

The lack of cord blood proliferation within our study could be explained by the low number of neonates who went on to develop egg (n = 1) or milk (n = 1) allergy. However, these data are important as they highlight those who go on to develop atopic eczema with an increased memory response at birth, compared to those who did not develop eczema. This strongly indicates to either, an innate difference in the T cell repertoire which may be a consequence of genetic or epigenetic influences or to *in utero* priming of the foetal immune response.

The proliferation of cord blood mononuclear cells to inhalant and food allergens reported in other studies, has suggested initial T cell priming may indeed occur *in utero* (Devereux, Seaton, & Barker, 2001; Prescott et al., 1998; Szepfalusi et al., 1997). However, within our study population there was no evidence of T cell proliferation to the food allergens ovalbumin or β-lactoglobulin, even though it has been documented that the foetus is exposed to ovalbumin (Vance et al., 2005). Although antigen-induced T cell proliferation may be a marker of antigen exposure, it may not be sensitive enough to detect the very low concentrations of exposure that may occur *in utero*. However, the higher rate of in-vitro inducibility of memory T cells on exposure to food allergen suggests that

some form of priming has occurred in those susceptible to developing atopic disease.

This is the first study to further characterise the memory phenotype within cord blood into the subclasses, central and effector memory. The majority of cord blood memory T cells were identified as effector memory. These data are important into leading the way to further knowledge about the development of the neonatal immune response.

There was no significant difference in CD3, CD4 or CD8 expression in the cord blood of AE or NE infants (Chapter 4). Analysis with CFSE showed no significant cord blood T cell proliferation after culture with ovalbumin or β-lactoglobulin. However, at 12 months significantly higher proliferation was detected in the AE infants. As expected, those infants who had developed milk and egg allergy showed the highest proliferation. However, proliferation of infant peripheral blood mononuclear cells was higher for all those in the AE group compared to those in the NE group.

6.4 Hypothesis 3: The clinical manifestations of allergy in early childhood are determined by tissue specific homing molecules expressed on specifically sensitised cord blood mononuclear cells

Distinct populations of T cells are recognised by expression of trafficking molecules that allow cells to home to a specific anatomical site within the body. The homing molecules on T cells specific for the skin and gut have previously been documented (Ferenczi et al., 2002; Waldman et al., 2006). It has been proposed that organ-specific trafficking T cells, specifically those that home to the skin, may play a role in atopic eczema development. Therefore the above hypothesis was proposed to investigate the homing markers present, specifically the skin homing molecule CLA, in cord blood and infant in relation to the development of atopic eczema in the infant.

As reported in Chapter 5, this is the first study to indicate a relationship between increased expression of the skin homing molecule CLA in the cord blood and the development of atopic eczema in the infant. As very low levels of CLA expression have been detected in cord blood previously (Arrighi et al., 2001; Campbell & Kemp, 1999; Kohno et al., 2001) and the reported increased expression of CLA with age (Campbell & Kemp, 1999a) and in those with atopic eczema (Seneviratne et al., 2007).

Although it was hypothesised that the clinical manifestation of atopic eczema in early childhood would be more determined by CLA specific skin homing molecules present in cord blood. It was originally thought that there would be a more significant difference in the CLA expression between the AE and NE infants at 12 months of age (after they had established eczema) rather than in the cord blood. However, when CLA expression was investigated at 12 months there was a decrease compared to those levels detected at birth. Furthermore, there was no longer a significant difference between those infants who had atopic eczema and those who did not. A possible explanation of this could be that the CLA positive cells in the cord blood had preferentially homed to the skin and did not remain in circulation.

Abernathy-Carver and colleagues previously reported CLA expression on casein stimulated CD3+ T cells, was critical for the development of milk allergic eczema in milk allergic children (Abernathy-Carver et al., 1995). However, within our study population no neonates responded to ovalbumin or β-lactoglobulin. This may be explained by the low number of neonates within this study who went on to develop egg (n = 1) and milk (n = 1) allergy.

As this was a hypothesis driven, despite the small number of samples analysed, the significant difference detected in CLA expression is the cord blood is less likely to be a chance finding due to carrying out multiple analysis. However, it would be nice to see this data replicated in a larger

study. A pilot study that investigates the core blood from infants who go on to develop eczema in addition to development of either egg or milk allergy should also be performed. In addition to informing sample-size calculations for a larger trial in the future, a pilot study would also indicate if those infants who go on to develop egg or milk allergy have increased levels of ovalbumin or β-lactoglobulin specific CLA expressing T cells.

6.5 Summary of relevant findings not addressed by the original hypotheses of this study

In addition to the data collected to address the hypotheses raised in this study, there were additional data collected in addition to that directed at addressing the original hypotheses.

6.5.1 Infant IgG

By 12 months of age all infants had been frequently exposed to cow's milk but not hen's egg. The higher β-lactoglobulin specific IgG detected in the infants blood was thought to be reflective of the higher quantity and frequency of the protein being consumed in the diet. Although FFQs were not collected for the infants diets, parents were asked on the infant clinical questionnaire at both 6 and 12 month visits what foods had been introduced and at what age. The food types that were covered in the questionnaire included: Baby cereal; cheese; wheat; egg; fish; fruit; meat; baby rice; baby rusk; vegetables and yoghurt.

Infants who went on to develop atopic eczema showed heightened ovalbumin specific IgG antibodies at 6 months although no egg had been introduced into their diet. This observation could be explained by inadvertent egg ingestion through either maternal sources transferred in breast milk, or contact with allergen through other means including food preparation leading to transcutaneous or inhaled transfer.

One of the major differences between AE and NE infants was ovalbumin specific IgG1. At 6 months of age AE infants had a significantly higher ovalbumin IgG1 concentration than the NE infant group. The same trend was also identified at 12 months of age. Taken together, these data have shown that infants who develop eczema have a distinctive pattern of ovalbumin specific IgG1, compared with those who do not develop eczema. This enhanced humoural response could be caused by increased T_H2 cells (Del Prete et al., 1991). As mentioned in Chapter 5, CCR4 is expressed preferentially on T_H2 cells (Bonecchi et al., 1998; Pappas et al., 2006) and there was significantly increased CCR4 expression in the AE cord blood, indicating the possibility that those who develop eczema have increased T_H2 expression. However, Morimoto *et al* (2005) detected in the murine model that the functionality of CCR4 does not always correlate with the expression of CCR4, but may emerge in the later stages of T_H2 differentiation, indicating that T_H2 cells may express the receptor but fail to produce the T_H2 chemokines (Morimoto et al., 2005).

To summarise this point, children who developed atopic eczema had significantly higher ovalbumin specific IgG1 at 6 and 12 months of age. However, the numbers within this study were limited and therefore clinical extrapolation must be guarded. A larger study would be needed to investigate this finding further. These results concur with similar studies, including a Swedish study which reported atopic symptoms in the first 8 years of life was associated with increased concentration of ovalbumin IgG1, as well as IgG4 and IgG3, at 6 months age (Jenmalm & Bjorksten, 1998)

6.5.2 CLA and CCR4 expression

The carbohydrate molecule CLA is not the only skin homing molecule to be identified. Two chemokine/chemokine receptor pairs have also been

implicated as involved in T cell migration to the skin. Circulating CLA+ T cells also express CCR4 (>75%) and CCR10 (~30%) (Campbell et al., 1999;Homey et al., 2002;Hudak et al., 2002). Within this present study we compared CLA and CCR4 expression on cord blood T cells. Although the exact role of CCR4 receptor in T cell skin homing is not clear, our data showed that although both CLA and CCR4 expression was detected in the cord blood, they did not co-express and were two distinct T cell populations. CCR4 is also expressed by some T_H cells that lack CLA expression (Campbell et al., 1999) and has been identified as a marker of T_H2 cell (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Sallusto et al., 1998a). Data from the present study would suggest that CLA+CCR4+ T cells are not present at birth and must develop later in life. It can also be speculated that the CCR4+ population detected in cord blood was reflective of the increased T_H2 environment within pregnancy. However, further study is required, particularly investigating coordination between CCR4 and T_H2 cytokine expression in the cord blood.

6.5.3 Is pregnancy an important time to prevent/promote allergy?

Previous studies have described the atopic mother as having an important role in the development of allergy, as infants born to atopic mothers have an increased risk of developing atopic eczema than those born to an allergic father (Ruiz et al., 1992).

Within this present study we identified the presence of specific T cell surface molecules at birth, which may be able to predict the development of atopic eczema in the infant, indicating immune abnormalities are already present at birth in those who go on to develop atopic eczema. As there has often been a marked progression from atopic eczema with food allergy to asthma (the atopic march), it becomes crucial to understand the early development of atopic eczema and identify possible markers/targets for early preventative and interventional strategies.

Many studies have indicated that *in utero* environmental influences can have an effect on atopy development. Work by von Mutius' group has shown in several cross-sectional studies that maternal exposure to farming has the benefit of decreased risk of allergic disease in the off-spring (Schaub et al., 2009; von Mutius et al., 2000). There is an emerging consensus that environmental factors influencing the developing immune system *in utero* substantially influence the subsequent risk of childhood atopic disease (Miles et al., 1996; Prescott et al., 1998; Prescott et al., 1999; Warner et al., 1994).

The main environmental factor investigated in this study was maternal dietary intake of egg and milk protein. Although no relationship was detected between maternal dietary egg and milk intake and the development of atopic eczema, a number of other dietary factors have been previously indicated to influence atopy. A previous study reported maternal intake of fish to be beneficially associated with eczema, and maternal intake of apple beneficially associated with asthma (Willers et al., 2008). Another study suggested that vitamin D levels within the maternal diet may be protective against asthma and wheezing (Erkkola et al., 2009). However, a greater understanding of the role of maternal nutrition and the maternally derived allergen exposure to the developing foetus, in relation to the immunopathogenesis of allergic disease is crucial to develop an effective intervention approach.

6.6 Limitations of the study

Some of the limitations of this study have been previously outlined within this thesis. However some that should be remarked upon include the dividing of examined cord and infant blood samples into two groups, despite heterogenous phenotypes within these groups (e.g. those who had eczema and went on to develop allergy and those who had eczema but no allergy). However, further distinction of these groups was not feasible within this study due to small numbers.

All infants recruited onto the present study were high risk infants (i.e. one or both parents or sibling having being diagnosed with either hay fever, food allergy, eczema or rhinitis). All cord and infant blood samples analysed by flow cytometry (Chapter 4 and Chapter 5) had atopic mothers. It would have been preferable to have included another control group, where the infant was not at high risk of developing atopy. If this non-atopic group had been included there may have been a more significant difference in some of the T cell responses and phenotypes investigated within the present study. However, with the lower rate of eczema in infants of non-atopic parents, a much larger cohort would have been required.

Samples had to be cryogenically frozen prior to cell culture and this may have had consequences in regard to the cells sensitivity to the allergens. Although preliminary results showed no significant difference in the expression of CD3, CD4 and CD8 in adult blood pre and post cryogenic preservation. There was no investigation into other markers investigated in this study.

The infants' last review was at 12 months of age. Although the highest atopic eczema incidence rate has been found to be during the second half year of life, with a decline in incidence rate thereafter (Halkjaer et al., 2006), some of the infants included in the NE group may have gone on to develop atopic eczema later. Therefore, a follow-up time period of 12 months for diagnosing atopic eczema may be too short, although most studies do choose this time frame.

Therefore this was an exploratory study and we cannot draw firm conclusions between the immunological markers and the presence of atopic eczema without a larger confirmatory study.

However, the strengths of this study are detailed hypotheisis driven exploratory investigations that examine the immunological markers in both cord and infant blood in relation to the development of eczema. They

provide indications for markers that would be of great interest to further investigation within a larger study population.

6.6.1 Future Work

The present study has identified a number of areas where future research is required. Work that needs to be performed includes:

1. Continuation of preliminary work on identifying CLA expression in cord blood as a predictor of atopic eczema development. A larger study, should include a population of low risk neonates to distinguish any difference in CLA expression between infants at high and low risk of atopy, and a comparison of those born to atopic and non-atopic mothers, as well as those who go on to develop eczema.

2. Continued investigation into T cell memory expression within cord and infant blood samples. Holt and colleagues suggested post-natal maturational delay of T cells in some high risk infants (Holt et al., 1995). We identified increased memory expression in the AE cord blood samples after culture with β-lactoglobulin and ovalbumin. However, we did not investigate memory expression at 12 months. Therefore, further investigation into the evolution of infant memory expression is needed to: (1) Investigate the relation between memory expression in cord blood and in the infant at 12 months of age; and (2) to identify any altered immune memory in specific allergen responsive T cell sub-sets within those infants who go on to develop eczema compared to those that do not and those at low risk.

3. Fundamental investigation into other associated skin homing markers (e.g.CCR10) on the CLA+ population identified in cord and infant blood. A greater understanding of the mechanisms of T

cell homing to the skin is important to understand the pathophysiology and potential therapies for eczema and eczema development. Both CCR4 and CCR10 have qualities which suggest a role in T cell homing to the skin. Data from Soler and colleagues, who compared CCR4 expression with CCR10 in human cutaneous T_H trafficking, found within the skin homing T_H population CCR10 was associated with the effector phenotype (Soler et al., 2003). Homey et al, showed most skin-infiltrating T cells (>90%) found in patients with atopic eczema, psoriatic and allergic-contact dermatitis expressed CCR10. (Homey et al., 2002). Further study will be required to investigate if CCR10 is expressed on the CLA+ population detected in the cord blood and if CCR10 expression plays any role in atopic eczema. This could easily be addressed by analyzing the CCR10 expression by adding a biotinylated anti-CCR10 fluorochrome with the other fluorochromes (e.g. antiCLA, antiCCR4, antiCD3 and antiCD4).

Although the cord blood CLA+ T cell subset did not show proliferation to ovalbumin or β-lactoglobulin, it would be interesting to investigate if this cell population had been previously activated (*in utero*). A comparison of the activation marker HLA-DR expression would identify if these cells were activated would identify if these cells had been primed *in utero*.

4. Investigation into the APC present in cord blood. APC play a major role in the initiation of the immune response, it would be interesting to investigate this cell population in the development of atopic eczema. Although Hagendorens and colleagues showed no difference in circulating dendritic cells between newborns at risk and those not at risk of developing atopy, a third population of dendritic cells (CD11c-, CD123dim+ cells) was identified (Hagendorens et al., 2000). It can be speculated that these cell may be involved in a protective or generative role in the

development of allergy in newborns, as these cells would be the first line of defence *in utero* as allergens (including ovalbumin) are known to be able to cross the placenta. Further investigation into this cell population, along side the T cell population within cord blood would help clarify the neonatal mechanisms possibly involved with the development of atopy.

5. Follow up review of the infants. Atopic individuals may manifest hypersensitivity response to a particular allergen in the skin alone, as in atopic eczema, or in extracutaneous tissue alone (e.g. the lung), or in multiple tissues (e.g. either simultaneously in the skin and lungs or more often presenting at different times (Santamaria Babi et al., 1995a)). As our infants were last seen at 12 months, no development of asthma was detected. However atopic eczema is usually the first atopic disease to appear and asthma generally presents later in life (Burgess et al., 2008). The physiological mechanisms involved with determining the specific tissue of disease expression are unclear. It is important to follow up these children and investigate the regulation of the homing receptor expression on the T cells later in infancy.

There is a high prevalence of atopic eczema in infants (15 – 20%) in the UK (Kay et al., 1994). When Emerson and colleagues investigated the estimated costs associated with atopic eczema, they were £47 million for the U.K. with £30 million spent by the NHS and £17 million spent by the families of those with affected children (Emerson, Williams, & Allen, 2001). At present, there is poor knowledge and support regarding eczema preventative advice. Further research into the skin homing marker could help in understanding the cellular components involved in atopic eczema. It is important to continue the work started in this thesis. An appropriately powered study with a larger number of subjects and more control groups could offer the chance for a clearer understanding behind the development of eczema and the markers involved. At present there are no clear

preventative steps in preventing the development of eczema. However, with the detection of CLA as an early marker for the future development of eczema, further study could focus treatment targeted at this homing molecule for preventing eczema on high risk groups.

6.7 Summary

This study is the first to compare the relationship between maternal dietary intake of egg and milk protein during pregnancy, with development of atopic eczema in the infant. Prenatal approaches to preventing allergic disease are an exciting area of study with the potential to lead to significant public health interventions. The results presented in this thesis are encouraging, particularly the identification of CLA as a potential marker for the future development of atopic eczema. Hence this exploratory study has proposed some very interesting markers, such as the central and effector memory phenotype and the presence of skin-homing T cells within cord blood, which perhaps in a much larger study could be shown to have a role in the development of eczema.

The identification and knowledge of a marker that could predict atopic eczema development would identify those to target for preventative strategies. However, fully powered long-term follow up studies are necessary to develop concrete approaches and more detail in understanding the onset of atopic eczema and its role within later atopy development.

Appendices

Appendix A: Materials

A.1 General reagents

Reagent	Supplier
Bovine serum albumin (fraction V)	Sigma, UK
Heat-inactivated human AB serum	Sigma, UK (stored at -20°C)
RPMI 1640 with 2 mM L-glutamine	Invitrogen, UK
Sodium peroxide	Sigma, UK (stored at -20°C),
Penicillin streptomycin with 5000 units/ml Penicillin and 5000μg/ml Streptomycin.	Gibco, UK
Carboxyfluorescein diacetate succinimidyl ester, final concentration 2μg/ml	Sigma, UK (stored at -80°C)
Sodium azide	Sigma, UK
Cell freezing medium containing minimum essential medium, dimethyl sulfoxide (8.7%) and methyl cellulose	Sigma, UK

A.2 Buffers

All buffers (shown below) were prepared with ultra high quality reverse osmosis water purified through a Ropure ST (Barnstead) system. All reagents were stored at room temperature unless otherwise stated.

General buffers

Buffer	Composition
PBS	NaCl 8% w/vol, KCl 0.2% w/vol, KH_2PO_4 0.25% w/vol, Na_2HPO_4 1.4% w/vol, ddH2O, pH 7.4
Wash buffer	1% Bovine Serum Albumin (BSA) (stored at 4°C), 0.01% NaN_3, PBS
Blocking buffer	Wash buffer, 10% Human serum (stored at -20°C)

ELISA buffers

Buffer	Composition
Coating buffer	0.05M carbonate-bicarbonate buffer (Sigma, UK)
Wash buffer	UHQ, 10% Tris buffered saline (TBS), 0.5% Tween 20
Blocking buffer	Wash buffer, 3% BSA (stored at 4°C)
Sample buffer	Wash buffer, 1% BSA (stored at 4°C)
TBS 10x	200mM Tris, 9% NaCl and pH adjusted to 7.4 with HCl

In-house stock solutions for flow Cytometry

Material	Composition
Serum free medium	500 ml AIM V medium and 500µl 2ME
FACs buffer	1% BSA, 0.1% sodium azide, pH 7.4, (store at 4°C) made up in PBS
FACs blocking buffer	FACs buffer + 5% human serum (store at 4°C)
FACs fixing buffer	PBS, 4% Paraformaldehyde (PFA), add PFA to PBS and warm to 56 °C until PFA dissolves (~60min), pH 7.4 (store at 4 °C)
FACs saponin buffer (Permeabilization buffer)	PBS, 1% BSA, 0.1% sodium azide, 0.1% saponin

A.3 Apparatus

General laboratory equipment

Application	Apparatus	Supplier
FACS	FACSAria	Becton Dickinson, UK
	PS-Tube, 4.5ml	Greinerbio-one
Cell Culture	Pipettes, 3ml	Fisher Scientific
	Pastetes	Greinerbio-one
	Cryogenic tubes	Greinerbio-one
	15/50ml Centrifuge tubes	Greinerbio-one
Cytokine Bead Array	Microcentrifuge	
	FACSCalibur	Becton Dickinson, UK
	CaliBRITE™ 3 beads	Becton Dickinson, UK
	CaliBRITE™ APC beads	Becton Dickinson, UK

Appendix B: Recruitment Questionnaire

PREDICTING FOOD ALLERGIC CHILDREN

RECRUITMENT QUESTIONNAIRE

STUDY NUMBER: ☐☐☐☐ ☐☐

Moth = 01 Fath = 02 (Last two digits)

Name: . . . . . .

Address: . . . . . .

. . . . . .

Sex: Male ☐ Female ☐

Date of Interview: ☐☐ ☐☐ ☐☐☐☐

I am going to ask you some questions about yourself

1. Date of Birth : ☐☐ ☐☐ ☐☐☐☐

2. Have you ever had wheezing or whistling in the chest <u>at any time in the past?</u> Yes$_1$ ☐ No$_2$ ☐

If you have answered 'no' please skip to question 7a

3. Have you had wheezing or whistling in the chest <u>in the last 12 months?</u> Yes$_1$ ☐ No$_2$ ☐

If you have answered 'no' please skip to question 7a

4. How many attacks of wheezing have you had <u>in the last 12 months</u>? (Number of episodes separated by 7 days)
None$_1$
< 3$_2$
3 or more$_3$

5. <u>In the last 12 months</u>, how often, on average, has your sleep been disturbed due to wheezing?
Never woken with wheezing$_1$
Less than one night per week$_2$
One or more nights per week$_3$

6. In the last 12 months, has wheezing ever been severe enough to limit your speech to only one or two words at a time between breaths?

Yes$_1$ ☐ No$_2$ ☐

7a. Have you ever had asthma?

Yes$_1$ ☐ No$_2$ ☐

If 'no' go to question 8

7b. Was this confirmed by a Doctor?

Yes$_1$ ☐ No$_2$ ☐

7c. How old were you when you had your first attack of asthma?

. . . . . years

7d. Have you ever spent a night in hospital because of asthma.

Yes$_1$ ☐ No$_2$ ☐

If yes,

7e. How many admissions in the last 12 months?

. . . . . .

8. In the last 12 months, has your chest sounded wheezy during or after exercise?

Yes$_1$ ☐ No$_2$ ☐

9. In the last 12 months, have you had a dry cough at night, apart from a cough associated with a cold or chest infection?

Yes$_1$ ☐ No$_2$ ☐

10. Have you used any medicines to treat asthma, or any other breathing problems, at any time in the last 12 months? (including inhalers, puffers, pills capsules, tablets, syrups, nebulisers)

Yes$_1$ ☐ No$_2$ ☐

Please list:

. . . . . .
. . . . . .
. . . . . .

11a. Do you usually cough first thing in the morning in the winter?

Yes$_1$ ☐ No$_2$ ☐

11b **If yes**, do you cough like this on most days for as much as 3 months each year?

Yes$_1$ ☐ No$_2$ ☐

12a. Do you usually bring up any phlegm from your chest during the day, or at night, in the winter?

Yes$_1$ ☐ No$_2$ ☐

12b **If yes**, do you bring up phlegm like this on most days for as much as

Yes$_1$ ☐ No$_2$ ☐

3 months each year?

I now want to ask you some questions about skin problems

13. Have you <u>ever</u> had an itchy rash which was coming and going for at least 6 months? Yes$_1$ ☐ No$_2$ ☐

If you have answered 'no', please skip to question 18a

14. Have you had this itchy rash at any time <u>in the last 12 months?</u> Yes$_1$ ☐ No$_2$ ☐

15. Has this itchy rash <u>at any time</u> affected any of the following places:

	Yes$_1$	No$_2$
the folds of the elbows	☐	☐
behind the knees	☐	☐
in front of the ankles	☐	☐
under the buttocks	☐	☐
or around the neck, ears or eyes?	☐	☐
body	☐	☐
arms	☐	☐
legs	☐	☐

16. Has this rash cleared completely at any time <u>during the last 12 months?</u> Yes$_1$ ☐ No$_2$ ☐

17. <u>In the last 12 months,</u> how often on average have you been kept awake at night by this itchy rash. (please tick which applies)

Never in the last 12 months$_1$
Less than one night per week$_2$
One or more nights per week$_3$

18a. Have you <u>ever</u> had eczema? Yes$_1$ ☐ No$_2$ ☐

18b. **If yes**, at what age did it first occur? . . . . . years

19. Have you used any medicines to treat eczema, dermatitis or any other skin problems at any time in the <u>last 12 months</u> (including creams, lotions, powders, bath oils, pills, capsules, tablets) Yes$_1$ ☐ No$_2$ ☐

Please list:. . . . . .
. . . . . .
. . . . . .
. . . . . .

I now want to ask you some questions about nasal problems
<u>All</u> questions are about problems which occur when you <u>do not</u> have a cold or the flu

20. Have you <u>ever</u> had a problem Yes$_1$ ☐ No$_2$ ☐

with sneezing, or a runny, or blocked nose when you DID NOT have a cold or flu?

If you answered 'no' please skip to question 25

21. In the past 12 months have you had a problem with sneezing, or a running or blocked nose when you DID NOT have a cold or the flu?

Yes$_1$ ☐ No$_2$ ☐

If you answered 'no' please skip to question 25

22. In the past 12 months has this nose problem been accompanied by itchy-watery eyes?

Yes$_1$ ☐ No$_2$ ☐

23. In which of the past 12 months did this nose problem occur? (Please tick any which apply)

January	☐	May	☐	September	☐	1 = Spring
February	☐	June	☐	October	☐	2 = Summer
March	☐	July	☐	November	☐	3 = Autumn
April	☐	August	☐	December	☐	4 = Winter
						5 = > one season

24. In the past 12 months how much did your nose symptoms interfere with your daily activities? Please tick which applies

Not at all$_1$
A little$_2$
A moderate amount$_3$
A lot$_4$

25. Have you ever had hayfever or rhinitis?

Yes$_1$ ☐ No$_2$ ☐

26. Have you used any medicines to treat hayfever, rhinitis or any other nasal problems, at any time in the last 12 months (including sprays, solutions, pills, capsules or tablets)

Yes$_1$ ☐ No$_2$ ☐

Please list:
. . . . . .
. . . . . .
. . . . . .
. . . . . .

I have already asked you about medicines for asthma or other skin problems, hayfever or other nasal problems. I now want to ask you about medicines for other problems

27. Have you used any other medicines at any time in the last 12 months?

Yes$_1$ ☐ No$_2$ ☐

Please list:
. . . . . .

. . . . . .
. . . . . .
. . . . . .

28. Have you ever had a course of injections for allergies at any time in your life? Yes$_1$ ☐ No$_2$ ☐

29. Have you ever had a course of injections for allergies in the last 12 months? Yes$_1$ ☐ No$_2$ ☐

I now want to ask you some questions about you and your family

30. How many children have you had with your present husband/partner? ☐☐

31. How many of these children have:
- Asthma ☐
- Eczema or skin allergy ☐
- Hay fever or nasal allergy ☐

32. What is the name of your husband/wife/partner? ……..

33a. How many brothers have you? ☐

33b How many of your brothers have ever had:
- Asthma ☐
- Eczema or skin allergy ☐
- Hay fever or nasal allergy ☐

34a. How many sisters have you? ☐

34b How many of your sisters have ever had:
- Asthma ☐
- Eczema or skin allergy ☐
- Hay fever or nasal allergy ☐

35. Did your father ever have:
- Asthma Yes ☐ No ☐
- Eczema Yes ☐ No ☐
- Hay Fever Yes ☐ No ☐

36. Did your mother ever have:
- Asthma Yes ☐ No ☐

	Eczema	Yes ☐	No ☐
	Hay Fever	Yes ☐	No ☐

		Size mm
37. SPT results	Neg	☐☐
	HDM	☐☐
	Cat	☐☐
	Dog	☐☐
	Grass Pollen	☐☐
	Tree Pollen	☐☐
	Egg	☐☐
	Cow's Milk	☐☐
	Peanut	☐☐
	Alternaria	☐☐
	Aspergillus	☐☐
	Positive	☐☐

Bloods Taken? Yes / No

Name of Interviewer……

Signature / Designation……

Appendix C: Food Frequency Questionnaire

NAME……..DATE OF BIRTH……

NO. OF WEEKS PREGNANT……STUDY NO……

Please describe your eating habits OVER THE LAST MONTH by answering the following questions. Please tick the relevant box. Thank you.

Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
BREADS											
Standard bread (white, brown or wholemeal)											Number and size of slices
Milk bread											Number and size of slices
Weight Watchers bread											Number and size of slices
Brioche											Number and size of slices
Bagels											Number eaten on each occasion
Croissant, Pain au chocolat											Number eaten on each occasion
Danish pastries											Number eaten on each occasion
BREAKFAST CEREALS/CEREAL BARS											
Breakfast Cereal											

											Type of Cereal Number and size of spoonfuls*
Muesli											Type of Cereal Number and size of spoonfuls*
Porridge/Ready Brek											Type of Cereal Number and size of spoonfuls*
Breakfast cereal bar											Type of Cereal Bar Number eaten on each occasion
Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
MEAT DISHES											
Casserole – tomato based (including those made with a ready made sauce)											Number and size of spoonfuls*
Casserole – tomato based (including those made with a ready made sauce)											Number and size of spoonfuls*
Curry– tomato based (including those made											

with a ready made sauce)											Number and size of spoonfuls*
Curry– milk/cream based (including those made with a ready made sauce)											Number and size of spoonfuls*
Lasagne/ Mousaka											Number and size of spoonfuls*
Meat pie/ Pastie											Type and number and size of spoonfuls*
Corned Beef											Amount eaten
Chicken Kiev/ Chicken Cordon Bleu											Type and number eaten
FISH											
Fried/Boiled – All varieties											Type of Fish Number and size of piece
Fish in Breadcrumbs											Type of Fish Number and size of piece
Fish in Batter											Type of Fish Number and size of piece
Fish in sauce (including cook in a bag)											Type of Fish Number and size of piece

Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
SAVOURY DISHES											
Quiche/ Savoury Flan											Type of Flan Number and size of slices
Pizza											Type of Pizza Number and size of slices
Cheeseburger											Type and number eaten
Crispy Pancakes (frozen)											Type and number eaten
Savoury pancakes											Type and number eaten
Scotch Egg											Number eaten
Macaroni Cheese											Number and size of spoonfuls*
Pasta Carbonara											Number and size of spoonfuls*
Toad in the Hole											Number and size of

											spoonfuls*
Yorkshire Pudding											Number and size of spoonfuls*
VEGETABLE DISHES											
Fresh Pasta Egg Noodles											Number and size of spoonfuls*
Egg Fried Rice											Number and size of spoonfuls*
Mashed Potato											Number and size of spoonfuls*
Creamed potato											Number and size of spoonfuls*
Vegetable in cheese sauce											Number and size of spoonfuls*
Prepared salad (eg coleslaw, potato salad)											Number and size of spoonfuls*

Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
ACCOMPANYING SAUCES, DRESSINGS, DIPS AND SANDWICH FILLINGS											
White sauce, Cheese sauce,											

Onion sauce, Béchamel sauce,											Type of sauce Number and size of spoonfuls*
Hollandaise sauce, Béarnaise sauce,											Type of sauce Number and size of spoonfuls*
Tartare sauce, Mayonnaise, Salad Cream, Seafood dressing, Blue Cheese dressing											Type of sauce Number and size of spoonfuls*
Soured Cream based dips											Type of dip Number and size of spoonfuls*
Sandwich fillings (eg Coronation chicken, Chicken tikka etc.)											Type of dip Number and size of spoonfuls*
Sandwich spread											Number and size of spoonfuls*
BISCUITS											
Semi-sweet biscuits (eg digestive, rich tea etc.)											Type and number eaten

Other sweet biscuits											Type and number eaten
Half covered chocolate biscuits (eg chocolate hobnob, jaffa cake etc)											Type and number eaten
Full covered chocolate biscuit (eg Rocky, classic, kitkat)											Type and number eaten

Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
CAKES											
Sponge cakes (eg madiera, fruit, Victoria etc)											Type of cake Number and size of spoonfuls*
Sponge cake bar (eg mini rolls, jamaca ginger cake)											Type of cake Number and size of spoonfuls*
Doughnut, yum-yums											Type of cake Number and size of spoonfuls*
Custard tart											

											Number and size of spoonfuls*
PUDDINGS/DESSERTS											
Sponge Puddings (eg treacle, chocolate etc)											Type of pudding Number and size of spoonfuls*
Bread puddings (eg bread and butter, queen of puddings)											Type of pudding Number and size of spoonfuls*
Cheesecake type dessert											Type of dessert Number and size of slices
Lemon meringue pie											Number and size of slices
Crème caramel (homemade)/Luxury mousse (ie made with eggs)											Type of dessert Number and size of spoonfuls*
Ice cream (including choc bars)											Type, number and size of spoonfuls*
Traditional trifle (ie made with sponge)											Number and size of spoonfuls*
Waffles/pancakes											Type of food Number and size of portion

Yoghurt/ yoghurt type dessert											Type of dessert Size of portion

Food eaten	FREQUENCY OF CONSUMPTION										Amount per day on days eaten
	Not eaten in last month	Once per month or less	Once per fortnight	Number of days per week							* Spoonfuls: Specify teaspoon (tsp), dessertspoon (dsp), tablespoon (tbs) or large serving spoon (ss)
				1	2	3	4	5	6	7	
CONFECTIONERY/SNACKS											
Milk chocolate											Type and amount eaten
Milk chocolate covered bars (e.g. mars, bounty, lion bar etc)											Type and amount eaten
Other chocolate confectionery (e.g. smarties, minstrels, maltesers)											Type and amount eaten
Crisp/savoury snacks											Type and amount eaten
Nuts											Type and amount eaten
JAMS/SPREADS											
Jam/Marmalade											Number and size of spoonfuls*
Lemon curd											Number and size of spoonfuls*
Chocolate spreads											Number and size of spoonfuls*
Chocolate nut spreads											Number and size of spoonfuls*
EGG AND MILK PRODUCTS											
Milk (any type)											Amount (g ½ pint ; 200 mls etc)
Cream (any type)											Number and size of spoonfuls*
Cheese (any type)											Type and amount eaten

Appendix D: Infant questionnaire

PREDICTING FOOD ALLERGIC CHILDREN

CLINICAL ASSESSMENT FORM

NAME OF CHILD…….

DATE OF BIRTH……..

ADDRESS…….

TELEPHONE NUMBER……..

GP / HV DETAILS…….

SURGERY / HV TEL NO…….

DATE OF VISIT…….

VISIT NUMBER……..

STUDY NUMBER……..

SKIN PRICK TESTING

Has your child had any anti-histamines in the past week? YES / NO

If YES, which one……..

(size in millimeters)

Negative Control		Cow's milk, raw	
House Dust Mite		Hen's egg, whole	
Cat Dander		Peanut	
Dog		Alternaria	
Mixed grass Pollen		Asp. fumigatus	
Mixed Tree Pollen		Positive Control (Histamine)	

Environmental Details:

Mother Smoker? Yes 1 / No 2 ☐

Number per day? _____ ☐☐☐

Has there been any change in smoking habits since last visit? Yes 1 / No 2 ☐

Did you smoke during Pregnancy? Yes1 / No 2 ☐

Post Natally? Yes1 / No 2 ☐

Father smoker? Yes1 / No 2 ☐

Number per day? _____ ☐☐☐

Has there been any change in smoking habits since last visit? Yes1 / No 2 ☐

Another individual smoking at home? Yes 1 / No 2 ☐

Number per day? _____ ☐☐☐

Pets

Pets at home? Yes 1 / No 2 ☐

What is it? Cat Yes 1 / No 2 ☐

Dog Yes 1 / No 2 ☐

Other rodent Yes 1 / No 2 ☐

Feathered Yes 1 / No 2 ☐

Has there been any change in pets at home? Yes 1 / No 2 ☐

Cooking

How do you cook? Gas? Yes 1 / No 2 ☐

Electric? Yes 1 / No 2 ☐

Has there been any change in cooking appliance? Yes 1 / No 2 ☐

Skin Problems

Has your child had an itchy rash at any time in the last 12 months?

Yes 1 / No 2 ☐

Has this itchy rash at any time affected any of the following places:

The folds of the elbows Yes 1 / No 2 ☐

behind the knees Yes 1 / No 2 ☐

in front of the ankles Yes 1 / No 2 ☐

under the buttocks Yes 1 / No 2 ☐

around the neck, ears, eyes Yes 1 / No 2 ☐

body Yes 1 / No 2 ☐

arms Yes 1 / No 2 ☐

legs Yes 1 / No 2 ☐

Has this rash cleared completely at any time during the last 12 months?

Yes 1 / No 2 ☐

In the last 12 months how often on average has your child been kept awake at night by this itchy rash. (Please tick which applies)

Never in the last 12 months Yes 1 / No 2 ☐

less than one night per week Yes 1 / No 2 ☐

one or more nights per week Yes 1 / No ☐

5. Has your child been diagnosed with eczema? Yes 1 / No 2 ☐

If yes at what age did it first occur?_____

Have you used any medicines to treat eczema or any other skin problems at any time in the last 12 months (indcluding creams, lotions, bath oils)

Yes 1 / No 2 ☐

_____

_____

_____

8. Has your child suffered from any other skin allergy, such as urticaria/hives? ☐

Yes1 / No 2

Respiratory problems

1. Has your child ever had wheezing or whistling in the chest in the past. (Eg. On running around). Yes 1 / No ☐

2. Has your child had wheezing or whistling in the chest in the last 12 months? ☐

Yes 1 / No 2

3. How many attacks of wheezing has your child had in the last 12 months? (Number of wheezy episodes separated by 7 days) ☐

0 Yes 1 / No 2 ☐

<3 Yes 1 / No 2 ☐

3 or more Yes 1 / No 2 ☐

4. In the last 12 months, how often, on average, has your child had a dry cough at night,
apart from a cough associated with a cold or chest infection?

0	Yes 1 / No 2	☐
<3	Yes 1 / No 2	☐
3 or more	Yes 1 / No 2	☐

5. Has your child ever had asthma confirmed by a doctor? Yes 1 / No 2 ☐

6. Current Medication (e.g. inhalers) Yes 1 / No 2 ☐

…….

…….

…….

…….

Seasonal Allergic Rhinitis:

1. Has your child suffered from any of the following problems when they do not have a cold or flu?

Sneezing	Yes1 / No 2	☐
Runny nose	Yes1 / No 2	☐
Itchy Nose	Yes1 / No 2	☐
Blocked Nose	Yes1 / No 2	☐

2. In the past 12 months has this nose problem been accompanied by itchy watery eyes? Yes1 / No 2 ☐

3. In the past 12 months when did this nose problem

occur? (1= Spring, 2 = Summer, 3 = Autumn, 4 = Winter, 5 = > 1 season)

January	☐	May	☐	September	☐
February	☐	June	☐	October	☐
March	☐	July	☐	November	☐
April	☐	August	☐	December	☐

4. Has your child complained of a sore itchy throat or palate? Yes1 / No 2 ☐

5. How much did this interfere with their daily life and activities?

Not at all Yes1 / No 2 ☐

A little Yes1 / No 2 ☐

A moderate amount Yes1 / No 2 ☐

A lot Yes1 / No 2 ☐

6. Have you used any medicines or nasal sprays? Yes1 / No 2 ☐

……

……

7. Has you child been diagnosed with hay fever or rhinitis? Yes 1 / No 2 ☐

Abdominal Symptoms:

1. Has your child avoided any particular foods in the last 12 months? Yes1 / No 2 ☐

_____

_____

2. Has you child had any of the following reactions to food?

Diarrhoea Yes1 / No 2 ☐

Vomiting Yes1 / No 2 ☐

Stomach Pains Yes1 / No 2 ☐

Swelling of the lips/face Yes1 / No 2 ☐

Breathing difficulties Yes1 / No 2 ☐

Skin Rash/Flare up of eczema Yes1 / No 2 ☐

Behaviour change Yes1 / No 2 ☐

Loss of consciousness Yes1 / No 2 ☐

3. Has your child been seen by a hospital consultant for feeding problems or weight loss in the past? Yes1 / No 2 ☐

If Yes, give details……..

4. Has your child

a) been admitted to hospital Yes1 / No 2 ☐

If Yes, give details……..

b) been seen as an outpatient in the hospital Yes1 / No 2 ☐

If Yes, give details……..

Health

Any ongoing medical problems? Yes 1 / No 2 ☐

_____

_____

Current Medication? Yes 1 / No 2 ☐

_____

_____

Vaccinations to date? Yes 1 / No 2 ☐

_____

Infant Feeding

1.Did you breast feed your baby? Yes 1 / No 2 ☐

If Yes,

2. For how long did you breast feed? ☐☐

Answer in baby's age (weeks / months)

3. Are you still breast feeding? Yes 1 / No 2 ☐

4. When did you first give infant formula? ☐☐

Answer in baby's age (weeks / months)

Which one?.....

Solid Food Introduction

1. At what age did you first give solid foods to your baby? ☐☐

Answer in baby's age (weeks / months)

2. What did you first give?.....

3. Have you introduced any of the following:

FOOD TYPE	**Yes [1] / No [2]**	**AGE (WEEKS / MONTHS)**
Baby Cereal		
Wheat		
Cheese		
Eggs		
Fish		
Fruit		
Meat		
Meat with vegetables		

Baby Rice		
Baby Rusk		
Vegetables		
Yoghurt, and other desserts		

1.1. Growth

Weight: ☐☐●☐☐

Weight centile: (please circle)

<0.4th [1]	0.4th [2]	0.4-2 [3]	2 [4]	2-9 [5]
9 [6]	9-25 [7]	25 [8]	25-50 [9]	50 [10]
50-75 [11]	75 [12]	75-91 [13]	91 [14]	91-98 [15]
98 [16]	>98 [17]			

Length (cms) _____ ☐☐☐●☐

Length centile: (please circle)

<0.4th [1]	0.4th [2]	0.4-2 [3]	2 [4]	2-9 [5]
9 [6]	9-25 [7]	25 [8]	25-50 [9]	50 [10]
50-75 [11]	75 [12]	75-91 [13]	91 [14]	91-98 [15]
98 [16]	>98 [17]			

Examination

Clubbing: YES_1/NO_2 ☐

Chest deformity: YES_1/NO_2 ☐

Wheeze: YES_1/NO_2 ☐

Crackles: YES_1/NO_2 ☐

Eczema: YES_1/NO_2 ☐

Scorad ……..

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