Lymphocyte expression of costimulator molecules in early life

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SUMMARY

Infectious diseases are a major cause of morbidity and mortality in young children, particularly in developing countries. This has been attributed to the young child's relative lack of previous antigen exposure, and immaturity of the immune system.

In T-dependent antibody responses, costimulator molecules provide contact-mediated signals during interactions between T cells and B cells that regulate lymphocyte activation. This study investigated the hypothesis that costimulator molecules are differentially expressed on lymphocytes from neonates and young children compared with adults, contributing to limitations of T-dependent antibody responses in early life.

Flow cytometry was used to examine the expression of two groups of costimulator molecules (CD80, CD86, CD28, CD152 and CD40, CD154) on peripheral blood lymphocytes from adults and young children (2-20 months of age), and umbilical cord blood lymphocytes. The expression of these molecules was studied on adult and cord blood lymphocytes activated *in vitro* with PMA and ionomycin or plate-bound CD3 mAb. A method was also developed for removing contaminating erythroid cells from cord blood mononuclear cells required for functional studies.

The expression of CD80 and CD86 was similar on adult and cord blood B cells. Higher levels of CD28 expression and reduced surface expression of CD152 on cord blood T cells compared with adult T cells, suggested that neonatal T cells may be more responsive to activation than adult T cells. This difference in the relative expression of CD28 and CD152 may also influence cytokine secretion by neonatal T cells.

CD40 expression was equivalent or higher on B cells from cord blood and young children compared with adults. The kinetics of CD154 expression differed between adult and cord blood T cells activated with PMA and ionomycin or CD3 mAb, and were

affected by the presence of B cells. Cord blood T cells were capable of expressing adult levels of CD154 at certain time-points in both activation systems.

These results suggest that lymphocytes from young children should be able to deliver and respond to costimulatory signals. The differences in lymphocyte expression of these costimulator molecules in young children are unlikely to fully account for limitations in humoral immunity in early life, and may even represent a specialised adaptation for this stage of immune development.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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ABBREVIATIONS

Ag	antigen
ATCC	American Type Culture Collection
BSA	bovine serum albumin
CLL	chronic lymphocytic leukaemia
DMSO	dimethyl sulfoxide
FITC	fluorescein isothiocyanate
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
mAb	monoclonal antibody (ies)
MC	mononuclear cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
ND	not done
PBS	phosphate buffered saline
PE	phycoerythrin
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
RF10	RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine
SAC	Staphylococcus aureus Cowan I
TCR	T cell receptor
TNF	tumour necrosis factor
TRAF	tumour necrosis factor receptor associated factors
VCAM-1	vascular cell adhesion molecule-1
NK	natural killer

CHAPTER 1.

INTRODUCTION

1.1 The immune system in young children

1.1.1 Infectious diseases in young children

Children are highly susceptible to infectious diseases compared to adults. Infectious diseases are a major cause of morbidity and mortality in infants and young children, particularly in developing countries. The estimated child mortality rate (probability of death before 5 years of age) in 1998 was 8 per 1000 live births in high income, industrialised countries and 87 per 1000 live births in developing countries (World Health Organization, 1998). More than 65% of deaths in children under 5 years of age in developing countries are caused by infectious diseases, the most common being diarrhoeal diseases and acute lower respiratory tract infections (Fig. 1.1) (World Health Organization, 1998).

1.1.2 Immunisation of young children

One of the main strategies employed to protect young children from infection is immunisation. Immunisation induces protective immunity, which is conferred by the prolonged presence of circulating antibodies after the primary antibody response, and immunologic memory (Ahmed & Gray, 1996). The ultimate aim is to develop vaccines which have high efficacy, minimal side-effects, are easily administered (ie. single dose, combined vaccines), provide life-long immunity, and which can be given near the time of birth to ensure protection in early life and maximise compliance.

Unfortunately, the efficacy of many vaccines administered during the neonatal period or early infancy is sub-optimal (Christie & Peterson, 1951; Cowan *et al.*, 1978; Di

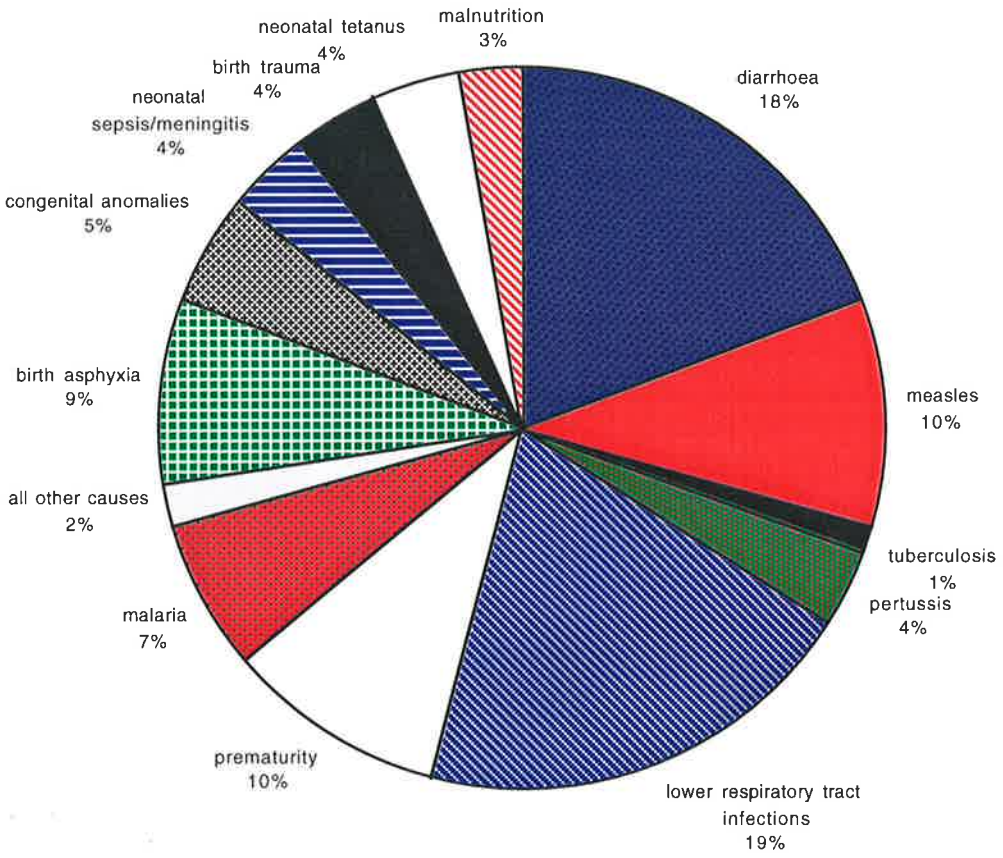


Fig. 1.1 Main causes of death in children under 5 years of age in developing countries, 1995 (World Health Organization, 1998). Total number of deaths was 10.4 million.

Sant'Agnese, 1950; Kayhty *et al.*, 1984; Kovarik & Siegrist, 1998; Kurikka *et al.*, 1995; Linnemann, 1973). Immunisation is therefore not usually commenced until 1-2 months of age and often multiple doses are required during the first year of life to induce protective antibody levels. A greater understanding of the neonatal immune system is required in order to overcome these difficulties. More knowledge about the functioning of the immune system in early life may also assist in the development of vaccines to some of the common childhood infections for which immunisation is currently unavailable.

1.1.3 Immaturity of the immune system in neonates and young children

A major contributor to the high susceptibility of neonates and young children to infection, and the poor response of neonates and infants to some vaccines, is the immaturity of their immune system (Wilson, 1986). Many of the components of innate immunity are limited in the human neonate (Kovarik & Siegrist, 1997; Miller, 1979; Wilson, 1986). Compared with adults, neonates have lower reserves of polymorphonuclear leucocytes and these cells have a decreased ability to migrate to the site of infection (Kovarik & Siegrist, 1997; Miller, 1979; Wilson, 1986). Under stress conditions, polymorphonuclear cells from neonates show deficiencies of phagocytosis and bactericidal activity (Kovarik & Siegrist, 1997; Miller, 1979; Wilson, 1986). Limitations also exist in the function of monocytes and macrophages, the complement system and natural killer (NK) cells in human neonates (Kovarik & Siegrist, 1997; Quie, 1990; Wilson, 1986).

Cell mediated and humoral adaptive immune responses are also limited in neonates. *In vitro* and *in vivo* studies have shown reduced cytotoxic T lymphocyte activity in human neonates (Adkins, 1999; Andersson *et al.*, 1981; Harris *et al.*, 1992; Wilson *et al.*, 1992). Cord blood lymphocytes proliferate in response to alloantigen but do not develop into functional cytotoxic lymphocytes (Adkins, 1999). Studies of young children with viral infections have demonstrated the presence of viral-specific cytotoxic T lymphocytes, but cytotoxic activity is reduced and these cells are rarely seen in children under 6 months

of age (Adkins, 1999).

Antibody responses to T-independent type 2 antigens, including the capsular polysaccharide antigens of encapsulated bacteria, are particularly poor in young children. Children under two years of age have a diminished antibody response to immunisation with polysaccharide antigens (Cowan *et al.*, 1978; Goldblatt, 1998; Kayhty *et al.*, 1984), and infections caused by encapsulated bacteria are a major cause of morbidity and mortality in this age group (Timens *et al.*, 1989). Neonatal B cells cultured with adult or neonatal T cells and polysaccharide antigens *in vitro* do not produce antibodies (Rijkers *et al.*, 1987), despite the presence of anti-polysaccharide precursors (Rijkers *et al.*, 1988). As a result of the poor immunogenicity of polysaccharide antigens in young children, conjugate vaccines have been developed against some of the common bacterial pathogens such as Haemophilus influenzae type b, Neisseria meningitidis and Streptococcus pneumoniae (Goldblatt, 1998). By coupling a polysaccharide antigen to a protein carrier, protective antibody responses can be elicited to polysaccharide antigens in children under 2 years of age (Stein, 1992).

This project focused particularly on T-dependent antibody responses in human neonates and young children. Antibodies confer protection from bacterial and viral infections by opsonisation, complement fixation, antibody mediated cellular cytotoxicity, neutralisation of bacterial toxins and neutralisation of viruses (Kovarik & Siegrist, 1998). During T-dependent antibody responses, activated CD4 positive T cells produce cytokines and deliver contact-mediated signals required for B cell differentiation into antibody secreting cells (Parker, 1993). Affinity maturation and memory B cell development are also features of T-dependent antibody responses (Ahmed & Gray, 1996; Berek, 1993; Stein, 1992). There are numerous *in vivo* and *in vitro* studies which show that T-dependent antibody responses in human neonates are functionally immature compared with adult responses.

1.1.4 Antibody responses in neonates and young children

Early studies of fetuses with congenital infections demonstrated that antibody responses occur as early as 16 weeks of gestation but are restricted to IgM (Gathings *et al.*, 1981). Umbilical cord blood lacks B cells expressing surface IgG or IgA (Wedgwood *et al.*, 1997) and peripheral blood from healthy term neonates generally contains only low numbers of antibody secreting cells, which only produce IgM antibodies (Lee *et al.*, 1991). In contrast, children between 1-48 months of age have circulating antibody secreting cells of all isotypes, often in higher numbers than adults (Lee *et al.*, 1991). Neonates with intrauterine or perinatal infections are more likely to have elevated numbers of antibody secreting cells, but IgM secreting cells are still more frequently increased than other isotypes (Stoll *et al.*, 1993). Serum levels of immunoglobulins do not reach adult levels for IgM, IgG and IgA until approximately 1 year, 3-6 years and 6-12 years of age, respectively (Allansmith *et al.*, 1968; Buckley *et al.*, 1968; Stiehm & Fudenberg, 1966). From these studies, it is evident that neonatal B cells have a limited capacity to switch to production of downstream immunoglobulin isotypes but this ability matures during early childhood.

Vaccine studies indicate that antibody responses to T-dependent antigens mature during infancy. Generally, antibody responses to vaccines in the first weeks of life are of lower magnitude, slower induction and shorter duration than responses in older children and adults (Kovarik & Siegrist, 1998). Early studies showed that primary antibody responses against diphtheria and pertussis are lower when immunisation is initiated in children under 3 months of age compared with children 3-12 months of age (Christie & Peterson, 1951; Di Sant'Agnese, 1950). A number of subsequent studies have confirmed that antibody responses to tetanus, diphtheria and pertussis improve with increasing age of administration (Halsey & Galazka, 1985). During the first few weeks of life, neonates also have poor serological responses to immunisation with oral poliomyelitis vaccine (Halsey & Galazka, 1985). Despite this, administration of an additional dose of trivalent oral poliovirus vaccine at birth, followed by the routine 3-dose immunisation schedule,

can result in higher titres of neutralising antibodies during the first months of life (Halsey & Galazka, 1985; Weckx *et al.*, 1992). The seroconversion rate induced by measles vaccine is poor in children receiving the vaccine before 12 months of age (Linnemann, 1973; Preston, 1995). For this reason, measles vaccine is usually delayed until 12-15 months of age. Commencing immunisation with the Haemophilus influenzae type b conjugate vaccine at a later age also results in a higher specific antibody concentration being achieved (Kurikka *et al.*, 1996). In some studies it has been found that children receiving an initial dose of vaccine at birth can actually show poorer antibody responses to subsequent vaccine doses, compared with children in whom vaccination is commenced later (Baraff *et al.*, 1984; Isaacs, 1997). This is believed to occur as a result of tolerance induction.

Immaturity of the neonatal immune system is at least partly responsible for the poor efficacy of some vaccines in the first weeks of life (Halsey & Galazka, 1985), although interference by placentally-acquired maternal immunoglobulin also reduces antibody responses to some vaccines, as discussed below.

1.1.5 Maternal immunoglobulin

Maternal immunoglobulin to some extent compensates for the immature immune system of the young child in the first months of life. Maternal IgG is transferred across the placenta, especially during the last 4-6 weeks of pregnancy, so that the serum IgG levels of a full-term neonate are equivalent to or higher than those of the mother (Fischer *et al.*, 1997). Maternal immunisation is an alternative strategy for protecting very young infants from infection. Immunising mothers in the third trimester of pregnancy ensures that neonates acquire protective levels of maternally-derived IgG against infections such as neonatal tetanus or Haemophilus influenzae type b (Fischer *et al.*, 1997; Mulholland *et al.*, 1996).

The half-life of maternal IgG in the infant's circulation is 3-4 weeks (Fischer *et al.*,

1997). Ideally, vaccine schedules should be commenced early enough to induce protective levels of endogenous antibodies in children, before the loss of protective levels of maternal IgG (Fischer *et al.*, 1997; Kovarik & Siegrist, 1998). However, the presence of trans-placentally acquired maternal antibodies can suppress responses to immunisation against measles (Linnemann, 1973) tetanus (Booy *et al.*, 1992; Claesson *et al.*, 1989; Sarvas *et al.*, 1992), diphtheria (Bjorkholm *et al.*, 1995; Halsey & Galazka, 1985), pertussis (Booy *et al.*, 1992), Haemophilus influenzae type b conjugate vaccine (Claesson *et al.*, 1989; Kurikka *et al.*, 1995) and poliomyelitis (Halsey & Galazka, 1985; Perkins *et al.*, 1959). The timing of immunisation is therefore critical to ensure that vaccines are administered to children when a protective antibody response can be induced, but prior to the disappearance of protective levels of maternally-derived IgG. This is one of the reasons for administering repeated doses of vaccines during the first years of life.

1.1.6 Neonatal B cell antibody production *in vitro*

Neonatal B cells have a limited capacity to differentiate into antibody-secreting cells in response to *in vitro* stimulation. Activation of cord blood lymphocytes using T-dependent systems such as Staphylococcus aureus Cowan I (SAC), (Durandy *et al.*, 1990; Watson *et al.*, 1991) CD3 mAb (Splawski *et al.*, 1991) and pokeweed mitogen (Andersson *et al.*, 1981; Gathings *et al.*, 1981; Hayward & Lawton, 1977; Miyawaki *et al.*, 1981; Tosato *et al.*, 1980) generates plasma cells which express and secrete predominantly IgM, whereas adult lymphocytes also secrete IgG and IgA in these systems. In addition, cord blood lymphocyte IgM responses are lower compared with adult lymphocyte responses. The neonatal B cell response to pokeweed mitogen is particularly poor, with little or no generation of immunoglobulin-secreting cells. An assessment of B cell responsiveness to pokeweed mitogen, SAC and activated T-cell supernatants in IgD positive and IgD negative subsets of human adult peripheral blood B cells, has shown that most of the responsive cells are IgD negative (Jelinek *et al.*, 1986). Thus the absence of cells

secreting downstream isotypes in cord blood may reflect the absence of IgD negative post-switch memory B cells. In the presence of EL4 thymoma cells and adult or neonatal T cell supernatants, neonatal B cells are as capable as adult B cells of differentiating into IgM, IgG and IgA secreting cells (Tucci *et al.*, 1991). This suggests that neonatal B cells are not intrinsically deficient and can differentiate in response to some activation stimuli (Tucci *et al.*, 1991).

Co-culture experiments using adult and cord blood lymphocytes have indicated that the limited immunoglobulin secretion by neonatal lymphocytes in response to T-dependent stimuli is attributable to both B and T cells. Addition of adult T cells or activated adult T cell supernatants to neonatal B cells in several polyclonal activation systems enhances IgM, IgG and IgA secretion. However, the frequency of neonatal B cells secreting downstream isotypes and the level of production of these antibodies is still lower than that observed in adult lymphocyte cultures (Andersson *et al.*, 1981; Hayward & Lawton, 1977; Miyawaki *et al.*, 1981; Splawski *et al.*, 1991; Tosato *et al.*, 1980; Yachie *et al.*, 1995). Conversely, neonatal T cells or supernatants are deficient in providing help to adult B cells in polyclonal activation systems, and also exhibit suppressor activity which may be eliminated by irradiation or corticosteroids (Andersson *et al.*, 1981; Gathings *et al.*, 1981; Hayward & Lawton, 1977; Miyawaki *et al.*, 1981; Splawski *et al.*, 1991; Tosato *et al.*, 1980; Watson *et al.*, 1991).

1.1.7 Differences in immunoglobulin subclass production in neonates and young children

The ability of B cells to secrete certain immunoglobulin subclasses is also restricted in early childhood. Serum concentrations of IgG1 and IgG3 reach adult levels at a younger age than IgG2 and IgG4 (Miles & Riches, 1994). Low levels of IgG2 antibodies in the first 12-18 months of life probably contribute to the poor response of young children to infections with encapsulated bacteria, since this is a major component of the mature

antibody response to T-independent type 2 antigens. When naive adult B cells and neonatal B cells are stimulated with pokeweed mitogen and adult memory CD4 positive T cells, neonatal B cells do not produce IgG2, IgG4 or IgA2, whereas adult naive B cells are capable of secreting these antibodies (Yachie *et al.*, 1995). These results suggest that the inability of neonatal B cells to switch to production of certain isotypes is partially related to intrinsic immaturity and not just the result of a lack of antigen experience. Neonatal B cells can be induced to secrete IgG2 when stimulated *in vitro* with CD40 mAb, activated CD4 positive T cell supernatants and IL-10 but production is lower than that by naive adult B cells (Servet Delprat *et al.*, 1996). Neonatal CD4 positive T cells are less effective at inducing IgG2 secretion by adult and neonatal B cells (Servet Delprat *et al.*, 1996), indicating that the deficient secretion of IgG2 in neonates is related to immaturity of both B and T cells.

1.1.8 Cytokine secretion by neonatal T cells

The limited ability of neonatal CD4 positive T cells to provide help for T-dependent antibody responses may be due to altered cytokine secretion. Human neonatal T cells activated *in vitro* with superantigens, phorbol esters and ionomycin, Phytohaemagglutinin (PHA), Concanavalin A or CD3 mAb produce significantly less IL-4 compared with adult T cells (Hayward & Cosyns, 1994; Lewis *et al.*, 1991; Pastorelli *et al.*, 1990; Splawski & Lipsky, 1991). Secretion of IFN-γ by neonatal T cells is often lower than secretion by adult T cells, but can be induced at adult levels under certain activation conditions (Hayward & Cosyns, 1994; Lewis *et al.*, 1991; Miyawaki *et al.*, 1985; Pastorelli *et al.*, 1990; Splawski & Lipsky, 1991; Wakasugi & Virelizier, 1985; Wilson *et al.*, 1986). IL-2 secretion by cord blood T cells may be equivalent, higher or lower than secretion by adult T cells, and this depends on the method of activation used (Hayward & Cosyns, 1994; Lewis *et al.*, 1991; Pastorelli *et al.*, 1990; Pirenne Ansart *et al.*, 1995; Splawski & Lipsky, 1991). Reduced secretion of IL-2 by CD3-stimulated neonatal T cells may

contribute to poor differentiation of antibody secreting cells from neonatal lymphocytes in the anti-CD3 system, since supplemental IL-2 enhances IgM secretion and induces production of low levels of IgG and IgA (Splawski & Lipsky, 1991). Reduced secretion of TNF, IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) by activated neonatal T cells has also been reported (Pirenne Ansart *et al.*, 1995; Wilson *et al.*, 1992).

In vitro and *in vivo* studies of cytokine production by CD4 positive T cells from neonatal mice have suggested a preferential development of Th2 dominated responses (Adkins, 1999; Barrios *et al.*, 1996). Although human neonatal CD4 positive T cells do not produce high levels of IL-4 *in vitro*, they secrete IL-4 at priming which promotes the acquisition of a Th2 phenotype after repetitive *in vitro* stimulation (Demeure *et al.*, 1995). When stimulated with IL-12, human neonatal CD4 positive T cells secrete IL-4 as well as IFN-γ (Shu *et al.*, 1994), suggesting that Th2 deviation occurs in human neonates as well as in neonatal mice. Despite this, under certain conditions human infants are capable of developing mature Th1 responses *in vivo* (Adkins, 1999). As yet, the factors governing *in vivo* Th1 vs Th2 CD4 positive T cell responses in human neonates and infants have not fully been determined (Adkins, 1999).

A recent review of human and murine neonatal T cell function suggested that neonatal CD4 positive T cell responses to activation via the T cell receptor (TCR) complex are generally weak in the presence of antigen presenting cells with moderate costimulatory activity (Adkins, 1999). However, stronger TCR-independent stimulation can induce adult levels of cytokine secretion by neonatal CD4 positive T cells (Adkins, 1999). This indicates that neonatal CD4 positive T cells are not inherently deficient in their ability to secrete cytokines, but may require stronger costimulatory signals for activation (Adkins, 1999; Kovarik & Siegrist, 1998).

1.1.9 Unique phenotype of neonatal B and T cells

Neonatal B and T lymphocytes have a unique phenotype which suggests that they may be functionally different from mature adult lymphocytes. Neonatal B cells express higher levels of surface IgM than adult B cells (Macardle *et al.*, 1997). Cord blood B cells express a number of antigens that are associated with activation (Durandy *et al.*, 1990), but express lower levels of most cytokine receptors compared to adult B cells (Zola *et al.*, 1995). One of the most notable phenotypic features of neonatal B cells is that the majority are CD5 positive, whereas in adults this population represents only about 10-25% of peripheral blood B cells (Casali & Notkins, 1989; Youinou *et al.*, 1999). CD5 positive B cells from adults produce mainly IgM antibodies of low affinity, which react with a variety of self and foreign antigens (Casali & Notkins, 1989; Youinou *et al.*, 1999). It is possible that these polyreactive antibodies play a role in first-line defence against infection *in vivo*, and hence may fulfil an important function in the antigenically naive neonate (Casali & Notkins, 1989). CD5 may also deliver important signals via its ligands CD72, a pan-B cell marker (Cerutti *et al.*, 1996), and a second ligand that has been identified on activated murine splenic B cells and activated T cell clones (Biancone *et al.*, 1996).

When compared to adult T cells, cord blood T cells differ in their expression of CD45RO and CD45RA, two isoforms of the leucocyte common antigen, CD45. CD45RA, expressed mainly by naive lymphocytes, is found on the majority of neonatal T cells, whereas CD45RO, considered a marker of previous antigen exposure, occurs only on a very small proportion of neonatal T cells (Aldhous *et al.*, 1994; Clement *et al.*, 1990; Wilson *et al.*, 1992). Using CD45RA and CD45RO as markers of naive and memory T cells, respectively, represents an over-simplification, since a proportion of T lymphocytes co-express both isoforms at low levels (Zola *et al.*, 1992) and CD45RO positive cells are capable of reverting to CD45RA positive cells in some circumstances (Rothstein *et al.*, 1991; Warren & Skipsey, 1991). Although few cord blood T cells express CD45RO at high levels, low intensity expression of CD45RO can be detected on approximately half of cord blood CD4 positive T cells (Zola *et al.*, 1992). It is suggested that these represent

cells in transition, either into CD45RO positive memory cells, or reverting from CD45RO positive to CD45RA positive cells (Zola *et al.*, 1992). It remains likely that the low number of neonatal CD4 positive T cells expressing CD45RO at high levels represents a lack of memory T cells, but this interpretation must be made with caution. Interestingly, cord blood CD4 positive CD45RA positive T cells stimulated with CD2 mAb and CD28 mAb show lower proliferation, CD25 expression, IL-2 secretion and IL-2 mRNA expression compared to adult CD4 positive CD45RA positive T cells (Hassan & Reen, 1997). This suggests that either the adult CD4 positive CD45RA positive T cell population contains memory T cells (possibly reverted from the CD45RO positive phenotype) or that cord blood CD4 positive T cells are intrinsically less responsive to stimulation than naive CD4 positive CD45RA positive adult T cells.

Neonatal T cells have a reduced density of surface CD3 molecules (Harris *et al.*, 1992) and decreased expression of adhesion molecules (Gerli *et al.*, 1993; Sanders *et al.*, 1988) which may limit the strength of activation signals delivered via the TCR (Adkins, 1999). This could explain why neonatal T cells have a higher requirement for costimulatory signals to achieve effective activation.

1.1.10 B cell memory development

Immunologic memory ensures a rapid, more effective secondary response on re-exposure to antigen (Gray, 1993). B cell memory is characterised by the ability, on second or subsequent exposure to a particular antigen, to produce antibodies of down-stream heavy chain isotype and of increased affinity (Silvy *et al.*, 1996). B cell memory is largely a feature of T-dependent antibody responses (Ahmed & Gray, 1996). Neonates and young infants do not develop an effective B cell memory response to some antigens. It has been shown that young children receiving their primary immunisation against diphtheria, tetanus and pertussis at 7 days of age have lower titres of pertussis agglutinins and diphtheria antitoxin in response to revaccination, compared with children whose

immunisation programme is started after 6 months of age (Di Sant'Agnese, 1950). This depends on the antigen, however, since tetanus antitoxin titres after revaccination are similar for both groups (Di Sant'Agnese, 1950). Another study has shown that neonates immunised at 1 day of age with a single injection of inactivated polio virus have strong memory responses when revaccinated at 6 months of age (Swartz *et al.*, 1989), which demonstrates that neonates can develop immunologic memory to some antigens. The ability to develop immunologic memory matures rapidly, as illustrated by children immunised in the first 6 months of life with Haemophilus influenzae type b conjugate vaccine, meningococcus conjugate vaccine or hepatitis B vaccine, who show strong memory responses when subsequently revaccinated (Goldblatt *et al.*, 1998; Leach *et al.*, 1997; Moyes *et al.*, 1990; Zepp *et al.*, 1997).

1.1.11 Germinal centres in young children

Differentiation of memory B cells occurs in germinal centres (Klaus & Humphrey, 1977). Primary follicles first appear around the 16th week of gestation and increase in size and number during fetal development, however, germinal centres are not usually evident until after birth, probably due to the lack of antigen exposure antenatally (Asano *et al.*, 1993; Luscieti *et al.*, 1980). Germinal centres are found at higher numbers in infants aged between 1-12 months than in any other age group (Luscieti *et al.*, 1980).

1.1.12 Affinity maturation

Memory B cells express antibody with high affinity for antigen, the outcome of a process that occurs in germinal centres known as affinity maturation. Affinity maturation results from accumulation of somatic mutations in the immunoglobulin variable gene regions, and subsequent selection and expansion of B cells expressing high affinity surface immunoglobulin (Berek, 1993; Wabl *et al.*, 1999). The ability of neonates and infants to

produce somatically mutated immunoglobulin genes has been examined by amplification of genomic VH6 sequences by polymerase chain reaction, detection of mutated clones using heteroduplex analysis and sequencing of mutated clones (Nicholson *et al.*, 1995; Ridings *et al.*, 1998; Ridings *et al.*, 1997). Mutated immunoglobulin genes occur in cord blood lymphocytes, but the number of mutated clones and the frequency of mutations in mutated clones is low (Ridings *et al.*, 1997). The frequency of mutations increases with age (Ridings *et al.*, 1998). In infants over 6 months of age there is also evidence of selection (Ridings *et al.*, 1998).

1.1.13 A role for costimulator molecules

During T-dependent antibody responses, protein antigen is taken up by antigen-presenting cells, processed and presented at the cell surface in association with major histocompatibility complex (MHC) class II molecules. The antigen-MHC II complex is recognised by the TCR complex on antigen-specific CD4 positive T cells. In addition to signalling through the TCR, effective activation of CD4 positive T cells requires contact-mediated signals delivered via the interaction of costimulator molecules on the T cell surface with their ligands on antigen presenting cells (Janeway & Bottomly, 1994; Parker, 1993). CD28 and CD152, on T cells, and their ligands CD80 and CD86, on antigen presenting cells, are widely considered to be the most important group of costimulator molecules regulating T cell activation (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994). In addition to providing cytokine-mediated help to B cells, CD4 positive T cells deliver contact-mediated signals to B cells during cognate interaction (Parker, 1993). The interaction between CD40, a pan-B cell marker, and CD154 (previously known as CD40 ligand), expressed by activated T cells, promotes B cell activation and differentiation during T-dependent antibody responses (van Kooten & Banchereau, 1996). CD40 signalling is believed to be critical for effective B cell activation.

This project examined the expression of the costimulator molecules CD80, CD86, CD28, CD152 and CD40, CD154 on cord blood lymphocytes from human neonates and peripheral blood lymphocytes from young children. It was postulated that the limitations of T-dependent antibody responses, isotype switching, affinity maturation and memory B cell differentiation in early life result from a relative deficiency of costimulator signals delivered to lymphocytes during cognate interaction. The basis for this hypothesis will be explained more fully after first describing these two families of costimulator molecules.

1.2 CD80, CD86, CD28 and CD152

CD80 (B7-1), CD86 (B7-2), CD28 and CD152 (CTLA-4) are all members of the immunoglobulin gene superfamily. CD80 and CD86 (expressed on B cells and other antigen presenting cells) are the ligands for CD28 and CD152 (expressed on T cells) (Fig. 1.2) (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994).

1.2.1 Structure of CD80 and CD86

CD80 is a 55 kDa glycoprotein and CD86 is a 70 kDa glycoprotein (Greenfield *et al.*, 1998). Both are expressed as monomers and have an extracellular region consisting of an IgV-like domain and an IgC2-like domain, a transmembrane domain and a short intracellular domain (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994) (Fig. 1.3). The cytoplasmic tail of CD86 is longer than that of CD80, with three potential sites of phosphorylation by protein kinase C (Hathcock & Hodes, 1996; June *et al.*, 1994). Overall amino acid conservation between CD80 and CD86 is approximately 25% (June *et al.*, 1994). CD80 has a slightly higher avidity for CD28 and CD152 than CD86 (Linsley *et al.*, 1994). CD80 dissociates more slowly from CD152 than CD86 (Linsley *et al.*, 1994).

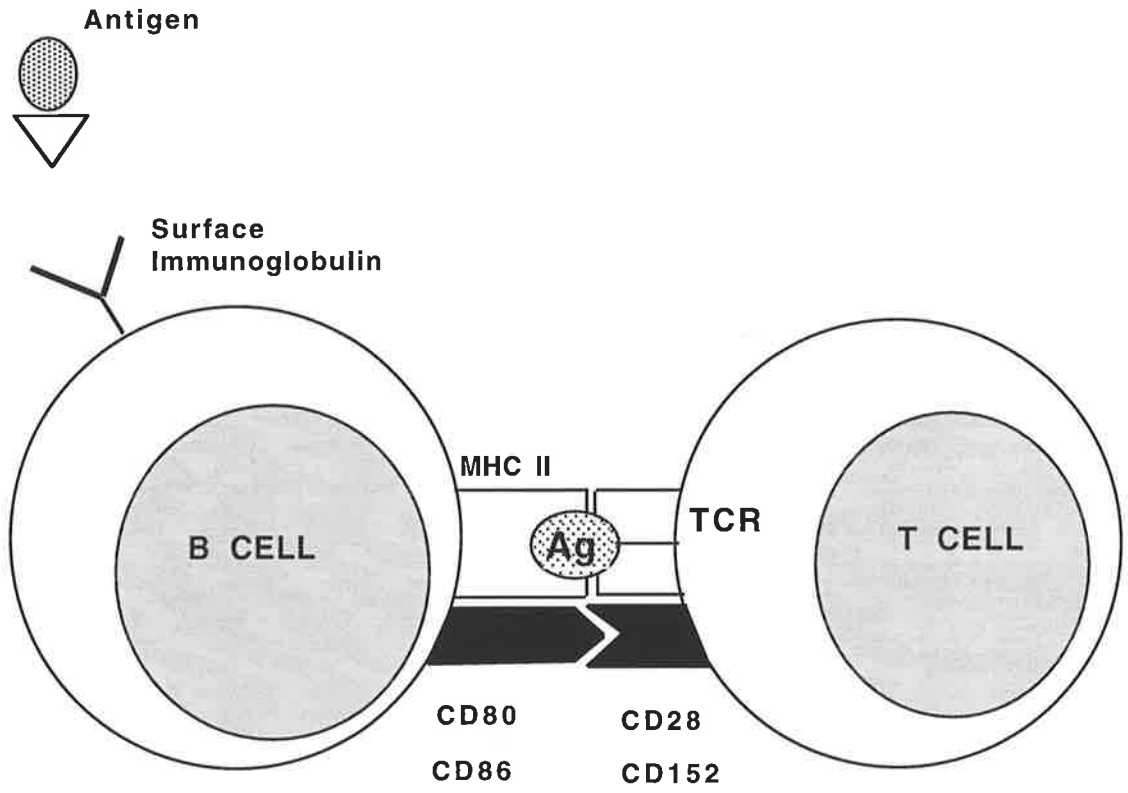


Fig. 1.2 CD80 and CD86 expressed on antigen presenting cells (including B cells), bind to CD28 and CD152 on the surface of CD4 positive helper T cells during cognate interaction and deliver signals that regulate T cell activation. (Ag = antigen; MHC II = major histocompatibility complex class II; TCR = T cell receptor).

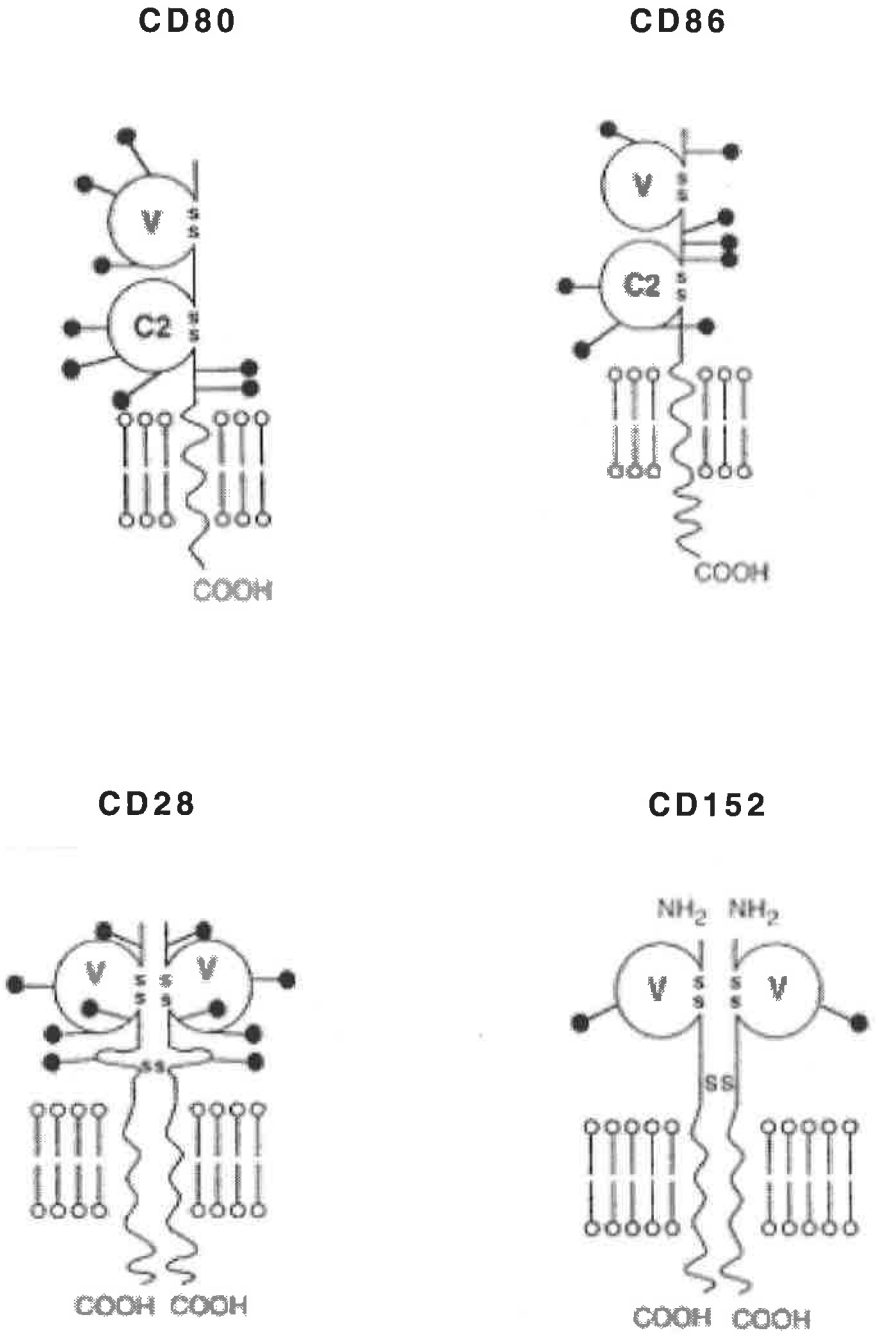


Fig. 1.3 Structure of CD80, CD86, CD28 and CD152 (Barclay *et al.*, 1997). CD80 and CD86 are expressed as monomers and CD28 and CD152 are expressed as homodimers. (V = IgV-like domain; C = IgC2-like domain; SS = disulphide bonds; sites of N-glycosylation are indicated by ●—).

1.2.2 Expression of CD80 and CD86

CD86, but not CD80, can be detected at low levels on resting B cells. Both molecules are also expressed by a number of antigen presenting cells including monocytes (Azuma *et al.*, 1993a), macrophages (Vyth Dreese *et al.*, 1995), dendritic cells (Azuma *et al.*, 1993a; Vyth Dreese *et al.*, 1995) and Langerhans cells (Hathcock & Hodes, 1996; Lenschow *et al.*, 1996). CD80 and CD86 are both expressed by activated T cells but are not detectable on resting T cells (Azuma *et al.*, 1993a; Azuma *et al.*, 1993c). In human tonsils or lymph nodes, CD80 is expressed on centroblasts and centrocytes but not mantle zone cells, with strongest staining occurring in the dark zone (Vyth Dreese *et al.*, 1995). CD86 is preferentially expressed on centrocytes in the light zone, particularly the apical light zone, and on clusters of interfollicular T cells (Vyth Dreese *et al.*, 1995).

Surface expression of CD80 and CD86 by human and murine B cells can be upregulated by stimulation with mitogens including Epstein Barr virus, bacterial lipopolysaccharide, pokeweed mitogen and SAC (Azuma *et al.*, 1993a; Freedman *et al.*, 1987; Hathcock *et al.*, 1994; Lenschow *et al.*, 1993; Valle *et al.*, 1991). Cross-linking MHC class II (Boussiotis *et al.*, 1993; Koulova *et al.*, 1991; Nabavi *et al.*, 1992) or surface immunoglobulin (Boussiotis *et al.*, 1993; Freedman *et al.*, 1987; Freeman *et al.*, 1989; Hathcock *et al.*, 1994; Lenschow *et al.*, 1994; Liu *et al.*, 1995; Valle *et al.*, 1991) also upregulates B cell expression of CD80 and CD86. Finally, CD80 and CD86 expression on human or murine B cells can be increased by ligation of CD40, using CD40 mAb presented by CD32 transfected L cells (Azuma *et al.*, 1993a; Ranheim & Kipps, 1993), soluble CD154 mAb (Roy *et al.*, 1995) or CD154 transfected cells (Liu *et al.*, 1995).

CD86 expression on murine B cells stimulated with bacterial lipopolysaccharide (Lenschow *et al.*, 1993), IL-4 (Stack *et al.*, 1994) or cross-linking surface immunoglobulin (Hathcock *et al.*, 1994; Lenschow *et al.*, 1994) increases within 6 hours of stimulation and reaches maximal levels by 24 hours, whereas CD80 expression peaks between 48 and 72 hours. In addition, CD86 is expressed at higher levels on the majority

of activated B cells while CD80 expression is lower and restricted to a subset of activated B cells. Similar patterns of expression and kinetics have been noted in human B cells (Boussiotis *et al.*, 1993; Freedman *et al.*, 1987; Valle *et al.*, 1991). CD80 mRNA can be detected in human splenic B cells 4 hours after stimulation by cross-linking surface immunoglobulin, with peak levels occurring between 4-12 hours and a gradual decline thereafter (Freeman *et al.*, 1989).

Expression of CD80 and CD86 is induced on human (Azuma *et al.*, 1993c) or murine (Hathcock *et al.*, 1994) T cells activated with CD3 mAb or Concanavalin A. CD80 and CD86 are constitutively expressed by human monocytes, and can be upregulated by treatment with IFN-γ (Azuma *et al.*, 1993a). Interestingly, treatment of murine peritoneal macrophages with IFN-γ upregulates CD86 expression but downregulates CD80 expression (Hathcock *et al.*, 1994).

1.2.3 Signalling via CD80 and CD86

No signalling role has been demonstrated for CD80.

A recent study has demonstrated that ligation of CD86 by CD28 delivers a direct signal to the B cell (Jeannin *et al.*, 1997). Secretion of IgE and IgG4 by human tonsillar B cells stimulated with CD40 mAb and IL-4 or IL-13 is enhanced in the presence of the CD86 mAb, IT2.2 (Jeannin *et al.*, 1997). In contrast, no enhancement of immunoglobulin secretion is seen with other CD86 mAb or with CD80 mAb. IT2.2 also enhances the expression of the ε mRNA transcripts, B cell proliferation and CD23 expression. The signal transduction pathway of CD86 has not yet been elucidated.

1.2.4 Structure of CD28 and CD152

Human CD28 and CD152 show 31% amino acid homology and consist of a single IgV-like domain, a transmembrane region and a short intracellular domain (Fig. 1.3) (Hathcock & Hodes, 1996; June *et al.*, 1994). Both are 44 kDa glycoproteins and are expressed as homodimers (Greenfield *et al.*, 1998). Conservation of the CD152 amino acid sequence between animal species is high, with 74% overall homology between human and murine CD152, and complete identity between the cytoplasmic domains, suggesting an important signalling function (June *et al.*, 1994). CD152 binds to CD80 and CD86 with higher avidity than CD28 (Linsley *et al.*, 1994).

1.2.5 Expression of CD28 and CD152

CD28 is constitutively expressed by 80% of human peripheral blood T cells (95% of CD4 positive T cells and 50% of CD8 positive T cells) (Hathcock & Hodes, 1996) and a proportion of thymocytes (Turka *et al.*, 1990). Surface CD28 expression has also been demonstrated on plasma cells and NK cells (Hathcock & Hodes, 1996). CD152 is not expressed on resting T cells, but is induced with T cell activation (Lenschow *et al.*, 1996). CD152 is expressed at similar levels on a small percentage of activated CD4 positive and CD8 positive T cells (Linsley *et al.*, 1992). The maximum level of CD152 expression on activated T cells is approximately 2-3% that of CD28 expression (Linsley *et al.*, 1992). CD152 mRNA and surface expression are largely confined to CD28 positive T cells (Lindsten *et al.*, 1993; Linsley *et al.*, 1992). However, a rare subset of CD28 negative T cells also expresses CD152 (Damle *et al.*, 1994), and in CD28-deficient mice, normal levels of CD152 expression can be induced (Green *et al.*, 1994). Interestingly, human peripheral blood B cells activated with membranes from activated T cells also transiently express CD152 (Kuiper *et al.*, 1995).

Surface expression of CD28 on human peripheral blood T cells or CD3 positive thymocytes stimulated with plate-bound CD3 mAb is increased approximately ten-fold after 24 to 48 hours (Turka *et al.*, 1990). There is a concurrent increase in CD28 mRNA

levels (Turka *et al.*, 1990). CD28 mRNA is also induced by culturing T cells with phorbol 12-myristate 13-acetate (PMA) (Lindsten *et al.*, 1993; Turka *et al.*, 1990). Surprisingly, treatment of CD28 positive T cells with a combination of PMA and ionomycin causes downregulation of CD28 mRNA expression (Lindsten *et al.*, 1993). CD28 expression can be upregulated by stimulation through the TCR and the integrin ligands, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Damle *et al.*, 1994). Interestingly, ligation of CD28 on activated CD4 positive T cells induces transient down-regulation of surface CD28 expression and CD28 mRNA levels (Linsley *et al.*, 1993).

T cell surface expression of CD152 is induced by activation of human peripheral blood lymphocytes with CD3 mAb or anti-TCR mAb plus ICAM-1 or VCAM-1 (Damle *et al.*, 1994; Linsley *et al.*, 1992). CD152 mRNA expression by human T cells may be induced by activation with PMA alone or PMA plus PHA (Lindsten *et al.*, 1993; Linsley *et al.*, 1992). CD152 mRNA levels in T cells activated with PMA are increased by addition of ionomycin (Lindsten *et al.*, 1993). Surface expression on human peripheral blood lymphocytes can be detected 2-4 days after activation (Linsley *et al.*, 1992). In murine splenocytes stimulated with CD3 mAb, surface CD152 expression is first detected after 24 hours of activation and reaches peak levels by 48 hours, returning to resting levels by 72 hours (Krummel & Allison, 1995; Walunas *et al.*, 1994). Ligation of CD28 stimulates CD152 mRNA expression and can enhance CD152 mRNA expression induced by immobilised CD3 mAb (Lindsten *et al.*, 1993).

Much of the cell-associated CD152 is intracellular, with a relatively small proportion being expressed on the cell surface (Leung *et al.*, 1995; Vyth Dreese *et al.*, 1995). Human peripheral blood T cells activated for 3 days with CD3 mAb or PHA show intracellular staining for CD152 that is approximately 5-fold higher than levels of surface staining (Leung *et al.*, 1995). The cytoplasmic domain of the CD152 molecule has a tyrosine-containing motif which localises the protein to a perinuclear Golgi or post-Golgi compartment (Leung *et al.*, 1995). CD152 appears to cycle between the cell surface and

intracellular stores (Linsley *et al.*, 1996). Incubation of activated T cells with labelled CD152 mAb at 37°C shows uptake of the mAb into endosomes, whereas cells incubated with the mAb at 4°C do not accumulate the mAb intracellularly (Linsley *et al.*, 1996). Both surface and intracellular CD152 expression are polarised towards the site of TCR engagement (Linsley *et al.*, 1996).

1.2.6 Role of signalling via CD28

Activation of naive CD4 positive T cells depends on T cell recognition of the antigen/MHC II complex on the surface of the antigen presenting cell. Binding of the TCR to the antigen/MHC II complex delivers 'signal 1' for T cell activation, but this alone is insufficient to drive proliferation and differentiation of naive CD4 positive T cells and a second signal is required. 'Signal 2' is delivered by the binding of costimulator molecules on the T cell surface to their ligands on antigen presenting cells at the time of cognate interaction. The interaction between CD80 or CD86 and CD28 is considered one of the most important costimulatory pathways for T cell activation (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994).

Signalling through CD28 promotes T cell proliferation, increased cytokine secretion, upregulation of cytokine receptors and enhanced CD154 expression (Boussiotis *et al.*, 1994; Hathcock & Hodes, 1996). This has been demonstrated by a number of *in vitro* studies.

Transfectants expressing CD80 augment proliferation and IL-2 production by activated murine or human CD4 positive T cells (Boussiotis *et al.*, 1993; Freeman *et al.*, 1991; Galvin *et al.*, 1992; Linsley *et al.*, 1991; Norton *et al.*, 1992; Parra *et al.*, 1994). Similarly, CD28 mAb can enhance proliferation and cytokine secretion by human or murine T cells stimulated with CD2 mAb (Hassan *et al.*, 1995), CD3 mAb (Harding *et al.*, 1992) or PMA (Freeman *et al.*, 1991). CD28 mAb can effectively replace the costimulator activity normally provided by accessory cells (Harding *et al.*, 1992; Jenkins

et al., 1991). Blocking Fab fragments of CD28 mAb can inhibit the costimulatory activity of accessory cells, suggesting that this costimulation is mediated by CD28 signalling (Harding *et al.*, 1992). This inhibitory effect can be overcome by addition of IL-2. CD28 signalling appears to enhance IL-2 secretion by stabilising IL-2 mRNA (Powell *et al.*, 1998). Secretion of multiple other cytokines (Th1 and Th2) is also promoted by CD28 ligation (Harlan *et al.*, 1995).

T cells from CD28 deficient mice have an impaired proliferative response to mitogens, CD3 mAb, specific antigen and alloantigens (Green *et al.*, 1994; Lucas *et al.*, 1995; Shahinian *et al.*, 1993). IL-2 production and CD25 expression are also reduced in T cells from CD28 knockout mice (Shahinian *et al.*, 1993). The proliferative responses of T cells from CD28-deficient mice can be partially restored by treatment with exogenous IL-2. This suggests that the augmentation of T cell proliferation by CD28 signalling is partially, but not completely, mediated by enhanced production of IL-2 (Green *et al.*, 1994).

In certain circumstances, ligation of the TCR by the antigen-MHC complex in the absence of concurrent signalling through CD28 results in antigen-specific T cell anergy. This is characterised by long-term unresponsiveness to re-challenge with that specific antigen. This state of anergy can be overcome by large amounts of IL-2 (Boussiotis *et al.*, 1994). The ability to induce anergy by blocking CD28 signalling has been demonstrated *in vitro* and *in vivo*. T cell clones cultured with antigen or sub-mitogenic doses of CD3 mAb in the absence of antigen presenting cells expressing CD80 or CD86 respond poorly to subsequent antigenic exposure (Boussiotis *et al.*, 1994; Gimmi *et al.*, 1993; Harding *et al.*, 1992). This can be overcome by including CD28 mAb in the primary culture. T cell anergy can also be induced, despite the presence of functional antigen presenting cells, if binding by CD80 and CD86 is blocked by a fusion protein, CD152-Ig, during the primary culture of T cells with antigen (Gimmi *et al.*, 1993). Animal studies have demonstrated that blocking costimulatory signals provided by CD80 and CD86 using CD152-Ig can prevent rejection of transplanted human pancreatic islet cells in mice (Lenschow *et al.*, 1992) and leads to prolonged survival of cardiac allografts in rats

(Turka *et al.*, 1992). These observations have important implications for organ transplantation, tumour immunity and understanding autoimmune diseases.

Not surprisingly, blockade of the CD80/CD86-CD28 pathway interferes with T-dependent antibody responses, since effective activation of helper CD4 positive T cells cannot occur. CD152-Ig transgenic mice secrete soluble CD152-Ig which inhibits the binding of CD80 or CD86 to CD28 (Lane *et al.*, 1994). These mice have limited primary and secondary antibody responses to T-dependent antigens. Isotype switching, germinal centre formation and somatic mutation are all deficient in these mice. Similarly, in CD28 knockout mice, basal immunoglobulin levels are low, class switching is reduced in response to infection and secondary responses are decreased (Green *et al.*, 1994; Shahinian *et al.*, 1993). CD28 knockout mice also have no evidence of germinal centre formation or somatic hypermutation following antigenic challenge (Ferguson *et al.*, 1996). *In vivo* administration of CD86 mAb during the early phase of a T-dependent antibody response in mice reduces immunoglobulin production and abolishes germinal centre formation (Han *et al.*, 1995). Treatment with CD86 mAb later in the response does not prevent germinal centre formation but reduces the frequency of somatic mutations and interferes with memory B cell development (Han *et al.*, 1995). These effects may be at least partially mediated by the CD40-CD154 interaction since signalling through CD28 enhances and stabilises CD154 expression on T cells (de Boer *et al.*, 1993; Ding *et al.*, 1995; Johnson-Leger *et al.*, 1998; Klaus *et al.*, 1994).

CD28 signalling is also important for the generation of cytotoxic activity in CD8 positive T cells. CD152-Ig inhibits the induction of cytotoxic function in murine CD8 positive T cells (Guerder *et al.*, 1995). Transfection of murine cells bearing FcγRII with CD80 makes them susceptible to CD3 mAb redirected cytotoxicity mediated by resting human peripheral T cells (Azuma *et al.*, 1992). This can be blocked by $F(ab')_2$ fragments of CD28 mAb or CD80 mAb (Azuma *et al.*, 1992). Cells transfected with CD80 provide strong costimulatory signals for induction of cytotoxic activity by Staphylococcal

enterotoxin A-stimulated CD8 positive T cells (Parra *et al.*, 1997).

CD28 augments the expression of the intrinsic survival factor Bcl-xL in T cells activated with CD3 mAb and thus probably also plays a role in T cell survival (Boise *et al.*, 1995).

There has been much controversy as to whether the binding of CD80 or CD86 has a differential effect on the outcome of CD28 signalling. In particular, there is conflicting evidence concerning the role that these molecules may play in driving Th1 vs Th2 differentiation. This has been reviewed in detail (Greenfield *et al.*, 1998). In view of their different temporal expression (Boussiotis *et al.*, 1993; Freedman *et al.*, 1987; Hathcock *et al.*, 1994; Lenschow *et al.*, 1994; Lenschow *et al.*, 1993; Stack *et al.*, 1994; Valle *et al.*, 1991), dissociation kinetics (Linsley *et al.*, 1994) and the different CD28 signalling pathways triggered by each molecule (Slavik *et al.*, 1999), it seems likely that although they perform many similar functions they probably do have different roles.

This is supported by observations in CD80 and CD86 knockout mice. In response to intravenous or intraperitoneal immunisation with T-dependent antigens, CD86 deficient mice show markedly decreased or delayed specific antibody production of IgG1 and IgG2a isotypes compared to wild-type mice (Borriello *et al.*, 1997). In comparison, antibody secretion by CD80 deficient mice is only slightly reduced. After immunisation, CD86 deficient mice lack germinal centres in the spleen, whereas well-formed germinal centres are seen in the spleens of CD80 deficient mice (Borriello *et al.*, 1997). In contrast, immunisation of CD86 or CD80 deficient mice in the presence of complete Freund's adjuvant induces IgG1 and IgG2a responses comparable to wild-type mice (Borriello *et al.*, 1997). Double knockout mice (CD80 and CD86 deficient) do not produce IgG1 and IgG2a or form germinal centres, even in the presence of complete Freund's adjuvant (Borriello *et al.*, 1997). These results suggest that CD80 and CD86 have overlapping but distinct functions in T-dependent antibody responses.

1.2.7 Role of signalling via CD152

Early studies of CD152 function using CD152 mAb suggested that CD152 had a similar function to CD28. CD152 mAb appeared to act synergistically when co-immobilised with CD28 mAb in the presence of a TCR stimulus, to augment proliferation of CD4 positive T cells (Damle *et al.*, 1994; Linsley *et al.*, 1992).

However, more recent studies have suggested that signalling through CD152 down-regulates T cell activation (Linsley, 1995). It has been shown that although CD152 mAb increase T cell proliferation in a murine mixed lymphocyte reaction, similar results are also observed when Fab fragments of the CD152 mAb are used (Walunas *et al.*, 1994). This suggests that ligation of CD152 normally generates an inhibitory signal. In these experiments, this inhibitory signal was blocked by whole CD152 mAb or Fab fragments of CD152 mAb (Walunas *et al.*, 1994). When optimal costimulation (via CD28) and Fc cross-linking of CD152 mAb occur, CD152 mAb inhibit proliferation and IL-2 secretion by CD3-activated T cells (Krummel & Allison, 1996; Walunas *et al.*, 1994). In addition, the T cell proliferative response to CD3 stimulation is determined by the relative concentrations of CD28 and CD152 signals, implying that the magnitude of the T cell response to TCR engagement is determined by the balance of these two signals (Krummel & Allison, 1996). More recent studies indicate that CD152 signalling inhibits IL-2 production, IL-2 receptor expression and cell cycle progression of activated T cells (Krummel & Allison, 1996; Walunas *et al.*, 1996) and that IL-2 production is inhibited in a CD28 dependent fashion (Walunas *et al.*, 1996).

Further evidence that CD152 has a negative regulatory role, comes from observations in CD152 deficient mice which suffer a fatal lymphoproliferative disorder (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). Interestingly, CD152 deficient mice that also lack CD80 and CD86 expression do not develop lymphoproliferation (Mandelbrot *et al.*, 1999). Treatment with CD28 mAb reproduces the CD152 deficient phenotype in these triple knockout mice but has no effect on wild-type mice, indicating that negative signalling through CD152 can normally overcome signalling through CD28.

Interestingly, CD152 appears to play an important role in the differentiation of CD4 positive T cells into Th1 or Th2 secreting cells. Naive CD4 positive T cells from CD152 deficient mice differentiate into Th2 secreting cells when stimulated with antigen and wild-type antigen presenting cells, producing large amounts of IL-4, IL-5 and IL-10 (Oosterwegel *et al.*, 1999). Treatment of CD152/CD80/CD86 deficient mice with CD28 mAb *in vivo* also leads to Th2 dominated responses, whereas this is not observed in wild-type mice treated with CD28 mAb (Oosterwegel *et al.*, 1999). Thus CD152 signalling appears to normally limit the extent of Th2 differentiation.

A recent study has suggested that T cell anergy is induced by CD152 signalling (Perez *et al.*, 1997), in contrast to earlier studies which proposed that T cell anergy was induced by TCR ligation in the absence of CD28 signals (Boussiotis *et al.*, 1994; Gimmi *et al.*, 1993; Harding *et al.*, 1992). In the recent study, an *in vivo* model was used whereby mice expressing a transgenic TCR are treated with a large dose of antigen intraperitoneally to induce T cell tolerance (Perez *et al.*, 1997). It was shown that blocking CD80/CD86-mediated costimulation *in vivo* with CD152-Ig or blocking CD152 with mAb at the time of intraperitoneal injection of antigen, prevents the induction of T cell tolerance and leaves the T cells capable of responding to subsequent *in vitro* challenge with antigen (Perez *et al.*, 1997). It is suggested that in the complete absence of CD80 and CD86 expression, T cells will not receive CD28 signals and will therefore not respond to antigen (Perez *et al.*, 1997). When CD80 and CD86 are expressed at low levels or for a limited time they may preferentially bind to the high affinity CD152 molecules inducing anergy (Perez *et al.*, 1997). Higher or more prolonged expression of CD80 and CD86 will lead to signalling through CD28, resulting in T cell activation (Perez *et al.*, 1997).

1.2.8 Signalling pathways of CD28 and CD152

Cross-linking of CD28 on activated T cells causes phosphorylation of a tyrosine residue in the cytosolic domain of the CD28 molecule (June *et al.*, 1994). Phosphoinositide 3-

kinase then binds to this site and initiates signalling via tyrosine phosphorylation of phospholipase Cγ1 (June *et al.*, 1994). Recently it has been demonstrated that CD80 and CD86 differ in their ability to stimulate this pathway, possibly contributing to a difference in the functional outcome of CD28 ligation by each of these molecules (Slavik *et al.*, 1999).

CD152 is thought to inhibit T cell responses by directly antagonising CD28 signalling and also by inhibiting TCR signalling (Oosterwegel *et al.*, 1999). CD152 associates with the TCR complex ζ chain (Lee *et al.*, 1998). Cross-linking CD152 inhibits anti-CD3 induced phosphorylation of the TCR ζ chain, possibly mediated by the tyrosine phosphatase, SHP-2, which co-localises with CD152 (Lee *et al.*, 1998). Since phosphorylation of TCR ζ determines the threshold for T cell activation, it has been suggested that this may be the mechanism by which CD152 antagonises TCR signal transduction (Lee *et al.*, 1998).

1.2.9 CD80, CD86, CD28 and CD152 expression and function on human neonatal lymphocytes

A significantly higher proportion of neonatal T cells compared to adult T cells are positive for CD28 expression and the proportion of CD28 positive T cells declines throughout life (Hoshino *et al.*, 1993; Jennings *et al.*, 1994; McCloskey *et al.*, 1997; O'Gorman *et al.*, 1998). In normal individuals CD28 negative T cells fall predominantly within the CD8 positive subset (Azuma *et al.*, 1993b). CD8 positive CD28 negative T cells fail to proliferate in response to mitogens but possess cytotoxic activity, expand in response to viral infection (Haffar *et al.*, 1995; Lewis *et al.*, 1994) and express high levels of adhesion molecules (Azuma *et al.*, 1993b; Kern *et al.*, 1996). It has been suggested that they represent a population of terminally differentiated effector T cells (Azuma *et al.*,

1993b). There is also evidence that CD28 negative T cells may have a suppressor function (Freedman *et al.*, 1991).

CD28 expressed on neonatal T cells is functional, since signalling via CD28 using CD28 mAb at the time of activation can enhance proliferation and cytokine secretion by neonatal T cells from mice (Adkins *et al.*, 1994) and humans (Hassan *et al.*, 1995; King *et al.*, 1995; Servet Delprat *et al.*, 1996; Webb & Feldmann, 1995; Yang *et al.*, 1995).

At the commencement of the studies described in this thesis, there were no published reports which examined the expression and function of CD80, CD86 and CD152 on human neonatal lymphocytes.

1.3 CD40 and CD154

CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily (Stamenkovic *et al.*, 1989) and CD154 (previously known as CD40 ligand) is a member of the TNF family. Members of the TNF receptor family generally have roles in lymphocyte activation, survival or death. CD40 is found on B cells, antigen presenting cells and a wide variety of other cell types while CD154 is predominantly expressed by activated T cells (van Kooten & Banchereau, 1996).

1.3.1 Structure of CD40

CD40 is a 48 kDa phosphorylated glycoprotein and, like other members of the TNF receptor family, it has the structure of a typical type I transmembrane protein (Kehry, 1996; van Kooten & Banchereau, 1996). The CD40 molecule therefore consists of an extracellular segment, a transmembrane region and an intracellular tail. The extracellular

region contains four homologous repeating cysteine-rich domains (Kehry, 1996; van Kooten & Banchereau, 1996) (Fig. 1.4).

1.3.2 CD40 expression

1.3.2.1 Cell types

CD40 is expressed by all B cells from peripheral blood, tonsils and spleen, however it is not expressed on fully differentiated plasma cells (van Kooten & Banchereau, 1996). It can be detected on the majority of B cell precursors from adult and fetal bone marrow, many B lineage leukaemias and haematopoietic progenitors (van Kooten & Banchereau, 1996). CD40 is also expressed by human and murine T cells, and soluble CD40 can be detected bound to CD154 expressed by activated T cells (van Kooten *et al.*, 1994). A number of accessory cells, including monocytes (Alderson *et al.*, 1993), dendritic cells (McLellan *et al.*, 1996) and follicular dendritic cells (Vyth Dreese *et al.*, 1995) constitutively express CD40. Other cell types expressing CD40 include certain types of epithelium (including thymic epithelium), endothelium, fibroblasts and several other tissues (van Kooten & Banchereau, 1996; Vogel & Noelle, 1998).

CD40 is expressed on the majority of B cells in the germinal centre and in interfollicular areas (Vyth Dreese *et al.*, 1995). Monocytes and interdigitating dendritic cells in the follicle mantle zone and interfollicular areas show higher levels of CD40 expression than observed on B cells. Small clusters of extrafollicular T cells also express CD40 (Vyth Dreese *et al.*, 1995).

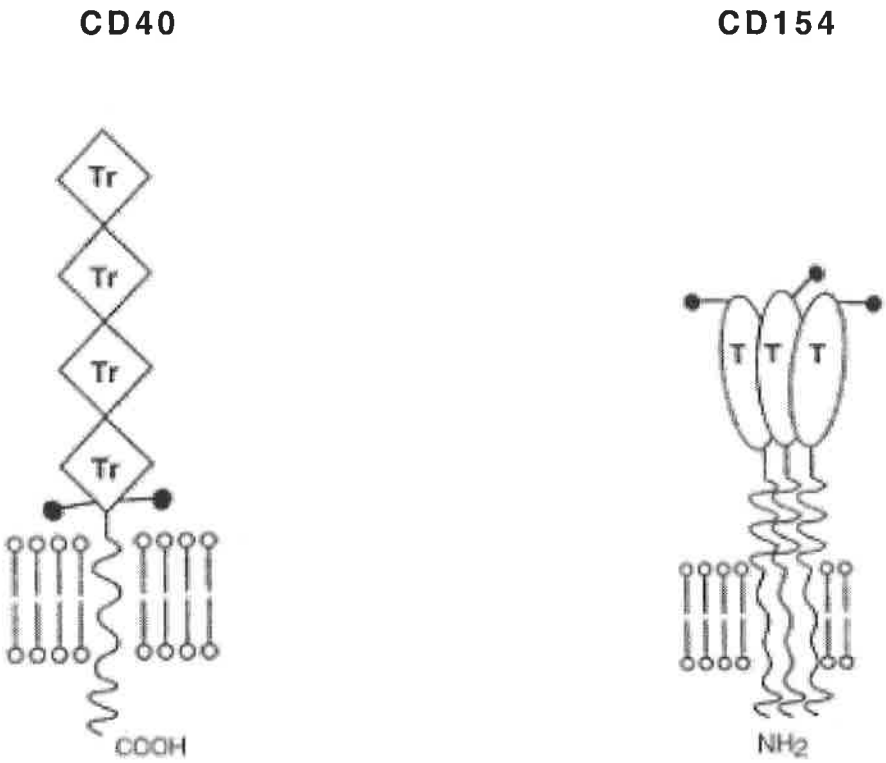


Fig. 1.4 Structure of CD40 and CD154 (Barclay *et al.*, 1997). CD154 is expressed on the surface of T cells as a trimer. (Tr = TNF receptor domain; T = TNF domain; sites of N-glycosylation are indicated by ●—).

1.3.2.2 Regulation on activated B cells

Upregulation of surface CD40 expression can be achieved by stimulating human tonsillar B cells with IL-4 (Valle *et al.*, 1989) or peripheral blood B cells with phorbol esters or anti-IgM (Ledbetter *et al.*, 1987). IFN-γ, anti-IgM, CD20 mAb or PMA induce CD40 mRNA expression by human tonsillar B cells (Stamenkovic *et al.*, 1989). A soluble form of CD40 can be detected in the supernatants of SAC-induced blasts (van Kooten *et al.*, 1994) or B cell lines (Bjorck *et al.*, 1994). However, soluble CD40 is not detectable in supernatants from cultures of activated tonsillar B cells or T-B cell co-cultures (van Kooten *et al.*, 1994).

1.3.3 CD40 signal transduction

The cytoplasmic tail of CD40 has no enzymatic domain. Signalling through CD40 results in phosphorylation and activation of multiple substrates (tyrosine protein kinases, serine/threonine specific kinases, phospholipase Cγ2 and phosphatidylinositol-3-kinase). This in turn leads to activation of transcription factors, including NF-κB (van Kooten & Banchereau, 1997). These effects are mediated by proteins that associate with the cytoplasmic domain of the CD40 molecule, known as TNF receptor associated factors (TRAFs) (Kehry, 1996; van Kooten & Banchereau, 1997; Vogel & Noelle, 1998). There are six members of the TRAF family and their recruitment to the CD40 receptor complex seems to be determined by other signals received by the B cell at the time of CD40 ligation (Kuhne *et al.*, 1997). This may provide an explanation for the various biological responses to CD40 engagement.

B cells at various stages of differentiation respond differently to CD40 stimulation (Gray *et al.*, 1997). Resting B cells proliferate strongly in response to CD40 ligation whereas

germinal centre B cells respond less dramatically. However, CD40 ligation delivers a potent survival signal to germinal centre B cells. It is believed that this reflects an alteration in the signal transduction pathway for CD40 during B cell differentiation. Supporting this theory is the observation of high levels of TRAF3 expression in germinal centre B cells and minimal levels in resting B cells (Gray *et al.*, 1997).

1.3.4 Structure of CD154

CD154 is a 33 kDa type II glycoprotein, consisting of a short cytoplasmic N-terminal domain, a transmembrane region and an extracellular domain (van Kooten & Banchereau, 1996) (Fig 1.4). Like other members of the TNF family, CD154 exists as a trimer (Hsu *et al.*, 1997). CD154 may exist on the cell surface as a heteromultimeric complex made up of full length CD154 associated with 2 shorter versions of the molecule (31 kDa and 18 kDa) (Hsu *et al.*, 1997; van Kooten & Banchereau, 1997).

1.3.5 Expression of CD154

1.3.5.1 Cell types

CD154 is not expressed on resting human T cells but is induced with activation on the majority of CD4 positive T cells and a small proportion of CD8 positive T cells (Lane *et al.*, 1992). A number of B cell lines and purified peripheral blood B cells stimulated with a phorbol ester and ionomycin also express CD154, although at lower levels than activated T cells (Grammer *et al.*, 1995). Interestingly, approximately 50% of unstimulated mouse splenic B cells express CD154 in the cytoplasm but not on the cell surface (Wykes *et al.*, 1998). Mast cells, basophils, eosinophils (Gauchat *et al.*, 1995), NK cells (Carbone *et al.*, 1997) and dendritic cells (Pinchuk *et al.*, 1996) also express

surface CD154, and CD154 mRNA has been detected in monocytes (van Kooten & Banchereau, 1996). In lymphoid tissue, CD154 is weakly expressed on a small proportion of CD4 positive T cells in the apical light zone and in the margins of the T cell zones (Casamayor Palleja *et al.*, 1995; Vyth Dreese *et al.*, 1995).

Reports as to the association of CD154 expression with CD45RA positive and CD45RO positive T cell subsets are conflicting. Several studies have demonstrated that CD154 is expressed equally on CD45RA positive and CD45RO positive (or CD45RA positive and CD45RA negative) CD4 T cells activated with phorbol ester and ionomycin (Lane *et al.*, 1992; Nonoyama *et al.*, 1995; Patel *et al.*, 1996). Another group has shown that CD154 is expressed at higher levels on purified CD45RA positive T cells compared with CD45RO positive T cells following stimulation with plate-bound CD3 mAb (Patel *et al.*, 1996). Yet another study has found that both the percentage of CD154 positive T cells and the level of CD154 expression is higher on CD45RA negative CD4 T cells than CD45RA positive CD4 T cells stimulated with PMA and ionomycin (Brugnoni *et al.*, 1994). Thus, there is no consistent evidence that CD154 expression is greater on CD45RO positive CD45RA negative 'memory' T cells.

1.3.5.2 Induction of CD154 on activated T cells

The magnitude, kinetics and stability of CD154 expression on T cells depend on the method of activation used (Castle *et al.*, 1993; Nusslein *et al.*, 1996; van Kooten & Banchereau, 1996). Activation of human peripheral blood T lymphocytes with PMA and ionomycin induces maximal expression of CD154 at 6-8 hours with no expression by 48 hours (Lane *et al.*, 1992; Nusslein *et al.*, 1996). Activation of T cells with plate-bound CD3 mAb induces surface expression that is relatively stable for at least 48-72 hours (Castle *et al.*, 1993; Miyashita *et al.*, 1997). Compared with phorbol ester and ionomycin, anti-CD3 stimulation induces only low levels of CD154 expression

(Casamayor Palleja *et al.*, 1995; Lane *et al.*, 1992; Nusslein *et al.*, 1996; Patel *et al.*, 1996; Splawski *et al.*, 1996). This is not due to inefficient stimulation of T cells by CD3 mAb, since all T cells can be induced to express CD25 in this system (Hermann *et al.*, 1993). On CD4 positive CD45RO positive human tonsil T cells activated by CD3 mAb or PMA and ionomycin, preformed CD154 is expressed on the cell surface within 15 minutes of activation (Casamayor Palleja *et al.*, 1995).

A soluble form of CD154 is expressed by CD4 positive T cells activated with superantigen and mature human Langerhans cells, or PMA and ionomycin (Graf *et al.*, 1995; Ludewig *et al.*, 1996; Pietravalle *et al.*, 1996). The soluble form is a truncated form of the CD154 molecule and exists as a trimer (Pietravalle *et al.*, 1996). It is not released by proteolytic cleavage of surface CD154, but is generated intracellularly by proteolytic processing of the full-length molecule (Graf *et al.*, 1995; Pietravalle *et al.*, 1996). The kinetics of soluble CD154 release parallel CD154 surface expression (Graf *et al.*, 1995). It does not interfere with the CD40-CD154 interaction but can down-regulate CD40 expression on Langerhans cells (Ludewig *et al.*, 1996). Soluble CD154 and IL-4 can induce proliferation of tonsillar B cells (Pietravalle *et al.*, 1996).

T cell CD154 expression is influenced by cytokines. CD154 expression on CD3-stimulated T cells is inhibited by cyclosporine A, but this can be overcome by a combination of IL-2 and IL-4 (Splawski *et al.*, 1996). Prostaglandin E_2, which inhibits IL-2 production, also dramatically reduces T cell CD154 expression. Once again, this can be reversed by supplemental IL-2 (Splawski *et al.*, 1996). Finally, a neutralising mAb to IL-2 and mAb to CD25 (IL-2 receptor) both partially inhibit CD154 expression on CD3-stimulated T cells (Splawski *et al.*, 1996). IL-12 also increases the level of CD154 expression on CD3-stimulated T cells and this results in enhanced helper activity for B cells (Peng *et al.*, 1998).

Accessory molecules also influence CD154 expression. Ligation of CD28 enhances CD154 expression induced by CD3 mAb (de Boer *et al.*, 1993; Ding *et al.*, 1995; Klaus

et al., 1994). However, CD154 expression can be induced on CD4 positive T cells from CD28 deficient mice, and agents which block the CD80/CD86-CD28 interaction only partially inhibit CD154 expression, suggesting that other costimulators may also influence CD154 induction (Ding *et al.*, 1995). It has been shown that expression of CD80 and ICAM-1 on antigen presenting cells increases the intensity and duration of CD154 expression induced on naive murine T cells (Jaiswal & Croft, 1997). Interestingly, this effect is not related to costimulatory signals delivered by these molecules, but rather due to enhanced adhesion allowing stronger, more prolonged signals via the T cell receptor (Jaiswal & Croft, 1997). Finally, stimulation through CD28 during CD3-stimulation of murine T cells appears to stabilise the expression of CD154 and make it more resistant to down-regulation in the presence of B cells (Johnson-Leger *et al.*, 1998).

1.3.5.3 Regulation of T cell CD154 expression by B cells

CD154 expression on peripheral blood CD4 positive T cells activated with CD3 mAb, superantigen or phorbol esters and ionomycin, is down-regulated in the presence of B cells or transfectants expressing CD40 (Hermann *et al.*, 1993; Ludewig *et al.*, 1996; Nusslein *et al.*, 1996; van Kooten *et al.*, 1994; Yellin *et al.*, 1994). The presence of monocytes or CD8 positive T cells has minimal effect on T cell CD154 expression (Yellin *et al.*, 1994). The effect is dependent on the ratio of CD40 positive cells to T cells, requires cell-cell contact and can be blocked with CD40 mAb (Castle *et al.*, 1993; Ludewig *et al.*, 1996; van Kooten *et al.*, 1994; Yellin *et al.*, 1994). This downmodulation is at least partly caused by receptor mediated endocytosis followed by lysosomal degradation (Yellin *et al.*, 1994). It has also been suggested that diminished expression of CD154 by T cells co-cultured with B cells results from blocking of the CD154 epitope by soluble CD40 released by B cells (van Kooten *et al.*, 1994).

Interestingly, although downmodulation of surface CD154 in the presence of B cells

occurs from the initiation of culture, T cells express similar amounts of CD154 mRNA when cultured in the presence or absence of B cells at 6 hours, but lower levels in the presence of B cells after 18 hours (van Kooten *et al.*, 1994). Similarly, downregulation of CD154 mRNA expression in T cells activated with superantigen in the presence of CD40 positive antigen presenting cells, can be inhibited by addition of blocking CD40 mAb at late time points but not in the first 8 hours of stimulation (Ludewig *et al.*, 1996).

The possibility that surface CD154 is downregulated by proteolytic cleavage is unlikely, since protease inhibitors do not block the downregulation and there is no significant parallel increase in soluble CD154 which would be expected if this was the case (Ludewig *et al.*, 1996). It has been demonstrated that activated B cells more effectively down-regulate CD154 expression by anti-CD3 stimulated CD4 positive T cells than resting B cells (Miyashita *et al.*, 1997). CD154 is rapidly re-expressed after removal of the B cells from culture (Miyashita *et al.*, 1997).

It is thought that this transient expression is a mechanism for tightly regulating lymphocyte activation, since T cells are incapable of providing contact-dependent signals to B cells following CD154 downmodulation (Yellin *et al.*, 1994). CD154 transgenic mice develop mononuclear cell infiltrates in many tissues and lymphoid tissue hypertrophy (Clegg *et al.*, 1997).

1.3.6 Signalling role and signal transduction pathway of CD154

Although its primary role is generally seen as being a ligand for CD40, there is evidence that CD154 also acts as a direct signalling molecule in T cells. Proliferation and IL-4 synthesis by purified peripheral blood CD4 positive T cells stimulated with plate-bound CD3 mAb and CD28 mAb are significantly enhanced by stimulating through CD154 with plate-bound mAb (Blotta *et al.*, 1996). Ligation of CD154 on resting human CD4 positive

T lymphocytes using CD40 positive transfectants promotes proliferation and secretion of IL-2 and IFN-γ induced by CD3 mAb (Cayabyab *et al.*, 1994). Enhancement of proliferation and cytokine secretion is also observed in cord blood T cells stimulated through CD154 (Blotta *et al.*, 1996; Cayabyab *et al.*, 1994). Production of IL-4 and IFN-γ by CD3-stimulated total lymph node cells from CD40 knockout mice is impaired compared to production by cells from wild type mice, and this can be partially overcome by adding CD40-Ig to cultures (Poudrier *et al.*, 1998). Cross-linking of CD154 on naive ($CD44^{lo}$) CD4 positive murine T cells stimulated with CD3 mAb and CD28 mAb results in increased production of IL-4 and reduced IFN-γ secretion (Poudrier *et al.*, 1998). Further evidence for a role for CD154 as a T cell signalling molecule comes from the observation that administration of soluble CD40 to CD40 knockout mice restores their ability to initiate the formation of germinal centres (van Essen *et al.*, 1995).

Recent studies have elucidated several signalling pathways triggered by ligation of T cell CD154. The first pathway results in activation of Jun-N-terminal kinase and p38 MAP kinases (Brenner *et al.*, 1997a). The second pathway induces tyrosine phosphorylation of phospholipase Cγ, release of inositol triphosphate and intracellular calcium and activation of protein kinase C (Brenner *et al.*, 1997b). Finally, signalling through CD154 activates the neutral sphingomyelinase, possibly by a novel signalling cascade (Koppenhoefer *et al.*, 1997). These signalling pathways may mediate the enhanced T cell proliferation and cytokine secretion described in other studies (Blotta *et al.*, 1996; Cayabyab *et al.*, 1994; Poudrier *et al.*, 1998), or may be involved in down-regulation of surface CD154 expression after ligation by CD40.

1.3.7 The role of CD40-CD154 interaction

1.3.7.1 Hyper-IgM syndrome

The importance of the CD40-CD154 interaction *in vivo* is highlighted by X-linked hyper-IgM syndrome, which results from defects of CD154 expression secondary to mutations of the CD154 gene (DiSanto *et al.*, 1993). A clinically similar condition occurs as a result of defective CD40 signal transduction (Conley *et al.*, 1994; Durandy *et al.*, 1997). Hyper-IgM syndrome is characterised by normal or elevated levels of circulating IgM, severely reduced IgG, lack of IgE and IgA, absence of germinal centres and failure to generate memory B cell responses (Callard *et al.*, 1993; Facchetti *et al.*, 1995; van Kooten & Banchereau, 1996). Immunisation of hyper-IgM patients with T-dependent antigens induces depressed primary and secondary responses entirely restricted to IgM (Nonoyama *et al.*, 1993). Analysis of immunoglobulin variable region genes from hyper-IgM patients demonstrates a reduction or absence of somatic mutations (Chu *et al.*, 1995; Razanajaona *et al.*, 1996). Patients are susceptible to bacterial and opportunistic infections (eg. Pneumocystis carinii, Cryptosporidium), and have an increased frequency of neutropaenia (Callard *et al.*, 1993). Interestingly, neutropaenia and opportunistic infections are not seen in hyper-IgM patients with normal CD154 expression and defective CD40 signal transduction (Durandy *et al.*, 1997). Hyper-IgM patients also have a high incidence of autoimmunity and lymphoproliferative disorders, possibly related to defective T cell development in the thymus (Callard *et al.*, 1993).

1.3.7.2 T-dependent antibody responses and isotype switching

Interaction between CD40 and CD154 appears to be crucial for T-dependent antibody responses to occur. Ligation of CD40 on B cells by CD40 mAb or CD154 has been shown by a number of studies to induce B cell proliferation, differentiation and

immunoglobulin secretion of all isotypes, as outlined below (van Kooten & Banchereau, 1996). CD40 mAb costimulate human B cell proliferation induced by anti-IgM mAb (Valle *et al.*, 1989). Human B cells from tonsil, spleen or peripheral blood cultured in the CD40 system (CD40 mAb presented by CD32 positive murine L cells) undergo long-lasting proliferation which is enhanced by the addition of IL-4 (Banchereau & Rousset, 1991). In the presence of IL-4 and IL-10, B cells in this system secrete antibodies of all isotypes and show features of plasma cell differentiation (Banchereau & Rousset, 1991). In human peripheral B cells stimulated with IL-4 and soluble CD40 mAb, IL-4 induces germline Cε transcription while CD40 engagement induces deletional switch recombination, mRNA production and IgE synthesis (Shapira *et al.*, 1992). The ligand for CD40, CD154, can also induce B cell proliferation and differentiation *in vitro*. Transfectants expressing human CD154 induce proliferation of tonsillar B cells and IgE secretion in the presence of IL-4 (Spriggs *et al.*, 1992). Membrane-bound recombinant CD154 induces murine B cell proliferation and polyclonal secretion of multiple isotypes in a similar manner to fixed, activated T cell clones (Grabstein *et al.*, 1993). Similarly, soluble CD154 and IL-10 induce antibody secretion of multiple isotypes by human splenic B cells (Urashima *et al.*, 1996).

The role of CD40 and CD154 in antibody responses and isotype switching can be demonstrated using mAb which block CD40-CD154 interactions. Specific antibody responses by purified murine B cells stimulated with fixed activated T cell clones can be inhibited with soluble CD40-Ig (Grabstein *et al.*, 1993). Blocking mAb to CD154 or CD40 inhibit T-dependent activation of peripheral blood B cells (via anti-CD3 activated T cells) and subsequent polyclonal B cell immunoglobulin production (all isotypes) (Nishioka & Lipsky, 1994; Splawski *et al.*, 1993). *In vivo* treatment of mice with blocking mAb to CD154 inhibits primary and secondary antibody responses to immunisation with erythrocytes and inhibits the expression of all immunoglobulin isotypes in the secondary response to soluble protein antigens (Foy *et al.*, 1994). Responses to a T-independent type 2 antigen, TNP-Ficoll, are not affected (Foy *et al.*,

1994).

Interestingly, signalling through CD40 has also been shown to enhance antibody responses to T-independent type 2 antigens in mice (Dullforce *et al.*, 1998; Snapper *et al.*, 1997). *In vivo* administration of CD40 mAb at the time of immunisation of adult mice with pneumococcal polysaccharide induces strong sustained IgG responses and long-term protective immunity (Dullforce *et al.*, 1998). The IgM response of neonatal murine B cells to anti-Ig dextran in the presence of IL-4 and IL-5 (a model for induction of T-independent type 2 responses) is enhanced to adult levels by costimulation with CD154 (Snapper *et al.*, 1997).

1.3.7.3 Germinal centre formation

The importance of the CD40-CD154 interaction for germinal centre formation is illustrated by the absence of germinal centres in hyper-IgM syndrome (Facchetti *et al.*, 1995). CD40 and CD154 knockout mice also lack germinal centres (Castigli *et al.*, 1994; Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994). A number of *in vitro* and animal studies have examined the role of these molecules in the generation of germinal centres. Co-ligation of surface IgM and CD40 on resting human B cells induces a partial germinal centre phenotype (Galibert *et al.*, 1996; Wheeler & Gordon, 1996). IL-2, IL-10 and CD154 transfectants induce strong proliferation of purified germinal centre B cells (Arpin *et al.*, 1997). When mice previously immunised with a T-dependent antigen are treated with blocking mAb to CD154, germinal centre formation is completely inhibited (Foy *et al.*, 1994; Han *et al.*, 1995). Interestingly, treatment of mice with soluble CD40-Ig does not prevent germinal centre formation, even though isotype switching and memory responses are disrupted (Gray *et al.*, 1994a). It is now recognised that germinal centre formation is probably dependent on bi-directional signals delivered during the CD40-CD154 interaction. Initiation of germinal centres appears to require CD154 signals

(van Essen *et al.*, 1995) whereas maintenance of germinal centres is probably dependent on CD40 signalling, since established germinal centres can be abrogated by treatment with CD154 mAb (Gray *et al.*, 1997; Han *et al.*, 1995). The means by which centroblasts receive adequate CD40 signals to maintain proliferation, in view of the small number of T cells in the dark zone of the germinal centre, is still not understood (Gray *et al.*, 1997).

1.3.7.4 B cell memory

As the germinal centre is the site where differentiation of memory B cells occurs (Klaus & Humphrey, 1977), it is not surprising that B cell memory is also critically dependent on the interaction between CD40 and CD154. Once again, this is illustrated by the failure of hyper-IgM patients to develop memory responses (Nonoyama *et al.*, 1993). Similarly, CD40 deficient and CD154 deficient mice fail to mount secondary responses to immunisation with T-dependent antigens (Kawabe *et al.*, 1994; Renshaw *et al.*, 1994). Adoptive transfer experiments in mice have shown that CD154 mAb or CD40-Ig administered at the time of immunisation interferes with memory B cell differentiation (Foy *et al.*, 1994; Gray *et al.*, 1994a).

In vitro experiments have shown that CD40 is important both for selection of germinal centre B cells expressing high affinity antibody and for differentiation of germinal centre B cells into memory B cells. Centrocytes are highly sensitive to apoptosis but can be rescued by signalling through surface immunoglobulin and CD40 (Casamayor Palleja *et al.*, 1996; Koopman *et al.*, 1997; Liu *et al.*, 1989). Initially, centrocytes bind to antigen complexes on the surface of intrafollicular dendritic cells (Koopman *et al.*, 1997; Liu *et al.*, 1989). Binding is determined by the affinity of surface immunoglobulin for antigen. Those centrocytes which express immunoglobulin with low affinity will not bind, and therefore undergo apoptosis (Gray *et al.*, 1996; Liu & Arpin, 1997). This allows selection of B cells expressing surface immunoglobulin with high affinity. Binding of

surface immunoglobulin to antigen alone does not provide a strong rescue signal, and additional signalling through CD40 is required for optimum survival (Liu *et al.*, 1989). Presumably, CD40 signalling occurs when centrocytes interact with germinal centre T cells (Gray *et al.*, 1996).

Selected B cells expressing high affinity immunoglobulin then differentiate either into memory cells or plasma cells. CD40 signalling also influences this process. When germinal centre B cells are cultured *in vitro* with IL-2, IL-10 and CD154 transfectants, proliferating B cell blasts differentiate into cells with characteristics of memory B cells (CD38 negative, CD20 positive) (Arpin *et al.*, 1995). When CD154 transfectants are removed and secondary culture is performed with IL-2 and IL-10 alone, germinal centre cells differentiate into CD38 positive CD20 negative immunoglobulin secreting cells (Arpin *et al.*, 1995).

1.3.7.5 Induction of costimulator activity on antigen presenting cells

CD40-CD154 interaction is essential for T cell activation in response to T-dependent antigens. In part, this effect is indirectly mediated, with CD40-CD154 interaction inducing upregulation of costimulator activity on antigen presenting cells, which then provide signals for T cell activation (Grewal *et al.*, 1996; Yang & Wilson, 1996). Upregulation of CD80 and CD86 on B cells and other antigen presenting cells is induced by CD40 ligation (Ranheim & Kipps, 1993; Roy *et al.*, 1995). Blocking mAb to CD40 significantly inhibit the induction of CD80 expression on human peripheral B cells cultured with anti-CD3 activated T cells (Ranheim & Kipps, 1993). CD80 expression is induced on normal and leukaemic B cells activated in the CD40 system (CD40 mAb bound to CD32-expressing L cells) (Ranheim & Kipps, 1993). CD154 mAb block the induction of CD80 and CD86 on murine B cells cultured with antigen-stimulated T cells (Roy *et al.*, 1995). The mixed lymphocyte reaction of T cells stimulated by allogeneic B cells is enhanced if the B cells are pre-activated by CD40 cross-linking (Ranheim &

Kipps, 1993). However, this effect can be abrogated by blocking with CD152-Ig which prevents the CD80/CD86-CD28 interaction (Ranheim & Kipps, 1993). Cross-linking CD40 on dendritic cells also induces CD80 and CD86 expression (McLellan *et al.*, 1996). Interestingly, CD40 ligation results in upregulation of CD80 and CD86 on memory B cells more rapidly and strongly than on naive B cells from human tonsils (Liu *et al.*, 1995). Signalling through CD40 can also induce the expression of other molecules with costimulatory activity on B cells and antigen presenting cells (Shinde *et al.*, 1996; Wu *et al.*, 1995). Not all these molecules have been identified as yet.

Recent studies suggest that induction of costimulatory activity on antigen presenting cells via the CD40-CD154 interaction is also the mechanism by which CD4 positive T cells provide help for cytotoxic T cell responses (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998).

1.3.8 Expression and function of CD40 and CD154 on human neonatal lymphocytes

CD40 expression on B cells from full-term neonates is equivalent to adult B cell expression (Durandy *et al.*, 1995). Long-term B cell lines can be generated from cord blood B cells in the CD40 system (Banchereau & Rousset, 1991). Signalling through CD40 on neonatal B cells in combination with supplemental cytokines can induce adult levels of B cell proliferation, but only limited secretion of antibodies of all isotypes, particularly of IgG, IgA (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1993). Reduced immunoglobulin secretion in response to stimulation via CD40 plus supplemental cytokines is also observed in IgD positive adult B cells (Durandy *et al.*, 1995; Splawski *et al.*, 1993).

A number of studies have shown that cord blood T cells express minimal amounts of

CD154 when activated with PMA and ionomycin (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). Reduced surface expression of CD154 correlates with reduced mRNA expression (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). Kinetics of CD154 surface expression and mRNA expression are similar in adult and neonatal T cells, with mRNA levels and membrane CD154 being lower at all time points in neonatal T cells (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). Priming of neonatal T cells increases expression of CD154 to adult levels on subsequent stimulation with PMA and ionomycin (Brugnoni *et al.*, 1994; Nonoyama *et al.*, 1995). Higher levels of CD154 expression can be induced on T cells from infants over 3 weeks of age, but expression is still lower than adult levels (Durandy *et al.*, 1995). Neonatal CD8 positive T cells do not express CD154 (Byun *et al.*, 1994; Durandy *et al.*, 1995; Nonoyama *et al.*, 1995) whereas 30% of adult CD8 positive T cells are CD154 positive.

Neonatal T cells can express CD154 at adult levels when activated with plate-bound CD3 mAb and supplemental IL-2 or IL-4 (Splawski *et al.*, 1996). CD154 expressed by neonatal T cells is functional, since the production of IgE and IgG4 by neonatal lymphocytes in this system can be blocked by CD40-Ig, which prevents the CD40-CD154 interaction. In addition, neonatal T cells activated by CD3 mAb induce CD86 expression on neonatal B cells, and this is also partially inhibited by blocking mAb to CD154.

1.4 Aims of this project

Young children, and particularly neonates, are highly susceptible to infection. This is a major cause of morbidity and mortality world-wide. The most effective means of preventing infection is immunisation. Immunisation leads to the presence of long-lived plasma cells that produce protective levels of circulating antibody, and memory B and T

cells capable of rapidly proliferating and differentiating into effector cells on re-exposure to the pathogen (Ahmed & Gray, 1996). Unfortunately, many vaccines have limited efficacy in the neonatal period. This is probably at least partly due to immaturity of the neonatal immune system.

The CD80/CD86/CD28/CD152 and CD40/CD154 families of costimulator molecules are known to play important roles in T-dependent antibody responses. As outlined above, these groups of molecules provide contact-mediated signals during interactions between T cells and B cells or other antigen presenting cells that regulate lymphocyte activation. Primary T-dependent antibody responses, isotype switching, germinal centre formation, development of memory B cells and affinity maturation are all dependent on costimulatory signals. This project examined the hypothesis that lymphocyte expression of costimulator molecules differs in neonates and young children compared with adults, with subsequent effects on the outcome of T-B cell interactions during T-dependent antibody responses. As a result, B cell and/or T cell activation may be sub-optimal, contributing to the limitations of T-dependent antibody responses observed in young children.

A greater understanding of the expression and function of costimulator molecules on neonatal lymphocytes may have implications for neonatal immunisation. Developing vaccine formulations which could promote costimulation of neonatal lymphocytes may enhance the immunogenicity of vaccines administered during this period. For example, using adjuvants that increase the expression of costimulator molecules on antigen presenting cells may promote CD4 positive T cell activation in neonates and may enhance antibody responses to T-dependent vaccine antigens.

Published studies suggest that there are some differences in the expression of costimulator molecules in the neonatal period. CD28 has been shown to be expressed on a higher percentage of neonatal T cells than adult T cells (Hoshino *et al.*, 1993; Jennings *et al.*, 1994; McCloskey *et al.*, 1997; O'Gorman *et al.*, 1998). Neonatal T cells are capable of responding to ligation of CD28, although T cell activation often remains sub-optimal (Hassan *et al.*, 1995; King *et al.*, 1995; Webb & Feldmann, 1995). There are no

reports investigating how CD28 expression is regulated on neonatal T cells during *in vitro* activation. No comparative studies have been done which examine the regulation of other members of the CD80/CD86/CD28/CD152 family of costimulator molecules on neonatal lymphocytes.

CD40 is expressed at adult levels on neonatal B cells (Durandy *et al.*, 1995) although there have been no studies examining the regulation of CD40 expression during activation of adult and neonatal B cells. Several studies have examined the regulation of CD154 on neonatal T cells in detail but the results are conflicting (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1996). Neonatal T cell expression of CD154 seems to be influenced by the method of *in vitro* stimulation used. The response of neonatal B cells to CD40 ligation is diminished compared to adult B cell responses, although comparable to that of naive adult B cells (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1993). It is possible that this is related to differential regulation of CD40 expression on naive B cells during activation.

The aim of this project was to compare the expression of these costimulator molecules (CD80, CD86, CD28, CD152 and CD40, CD154) on cord blood lymphocytes from human neonates and peripheral blood lymphocytes from young children with expression on adult peripheral blood lymphocytes. Differential expression of these molecules on lymphocytes from neonates and young children could be one explanation for the observed limitations of T-dependent antibody responses in early life.

CHAPTER 2.

MATERIALS AND METHODS

The materials and methods described in this chapter were used generally throughout these studies. More specific details are given in each chapter.

2.1 Monoclonal antibodies and staining reagents

All mAb and second step reagents were titrated to determine the concentration for optimal specific staining prior to use in these experiments. MAb produced from hybridomas in the laboratory were used as supernatants, purified mAb or biotinylated mAb and are shown in Table 2.1. MAb obtained from commercial sources are shown in Table 2.2. MAb from the Leucocyte Typing Workshops are shown in Table 2.3. Finally, other reagents used for immunofluorescence staining are shown in Table 2.4.

2.2 Buffers/Solutions

All chemicals used were of analytical standard.

2.2.1 Phosphate buffered saline (PBS)

PBS (pH 7.2 - 7.6; osmolarity 281 - 297) was prepared using 160 g NaCl, 4 g KCl, 23 g Na_2HP0_4, 4 g KH_2PO_4 and 20 litres of deionised water. Sodium azide (0.02%) and 1% filtered newborn bovine serum (Flow Laboratories, NSW, Australia; catalogue no. 29-121-54) were added to PBS used for washing cells for phenotyping experiments. Sterile PBS was used in the preparation of cells for functional experiments.

Table 2.1 MAb produced on site from hybridomas

mAb	Clone	Reference/Source	Form used
costimulator molecules			
CD40	G28/5	(Ledbetter *et al.*, 1987)	supernatant purified biotinylated
CD154	5c8	American Type Culture Collection (ATCC) (Lederman *et al.*, 1992)	purified biotinylated
positive/ negative controls			
IgG1 negative control	X63	IgG1 myeloma protein	supernatant biotinylated
IgG2a negative control	SAL5	IgG2a anti -Salmonella mAb, Dr L. Ashman	supernatant
IgG2b negative control	SAL4	IgG2b anti -Salmonella mAb, Dr L. Ashman	biotinylated
IgM negative control	TC12.6	IgM anti-HIV core protein, Dr A. Hohman	supernatant
CD25	7G7/B6	(Rubin *et al.*, 1985)	supernatant biotinylated
CD3	OKT3	ATCC	supernatant
calreticulin mAb (positive control for intracellular staining)	AD2	D. Beroukas	supernatant
cell purification/ cell culture			
CD19	FMC 63	Flinders Medical Centre	supernatant
CD14	FMC 32	Flinders Medical Centre	supernatant
glycophorin A mAb	10F7	ATCC (Bigbee *et al.*, 1983)	purified
CD3	OKT3	ATCC	purified

Table 2.2 Commercial antibodies

mAb	Company	Catalogue number	Clone	Isotype
costimulators				
CD80 (purified)	Pharmingen San Diego, CA.	33511A	BB1	IgM, κ
CD86 (purified)	Pharmingen	33401A	FUN-1	IgG1, κ
CD28 (purified)	Becton Dickinson San Jose, CA.	348040	L293	IgG1
CD152 (purified)	Pharmingen	34581A	BNI3	IgG2a, κ
biotin-CD80	Pharmingen	33512X	BB1	IgM, κ
biotin-CD86	Pharmingen	33432X	IT2.2	IgG2b, κ
biotin-CD28	Pharmingen	33742X	CD28.2	IgG1, κ
biotin-CD152	Pharmingen	34582X	BNI3	IgG2a, κ
CD154-PE	Pharmingen	33585X	TRAP1	IgG1, κ
CD154 (purified)	Pharmingen		TRAP1	IgG1, κ
lineage markers				
CD3-PE	Caltag San Francisco, CA.	MHCD0304		
CD3-FITC	Becton Dickinson	349201		
CD2-FITC	Becton Dickinson	347593		
CD19-PE	Caltag	MHCD1904		
CD19-FITC	AMRAD, Vic. Australia.	987122010		
CD19-Cy5/PE (CyChrome)	Pharmingen	30668X		
CD19-Cy5/PE (Quantum Red)	Sigma Chemical Co. St Louis, MO.	R4260		
CD14-PE	AMRAD	10HLEUK14E		
Simultest				
CD45-FITC/CD14-PE	Becton Dickinson	340040		
IgG1-FITC/IgG2a-PE (negative control)	Becton Dickinson	340041		
CD3-FITC/CD19-PE	Becton Dickinson	349211		
CD4-FITC/CD8-PE	Becton Dickinson	340039		
CD3-FITC/CD16-PE/ CD56-PE	Becton Dickinson	340042		
positive/ negative controls				
CD25-PE	Becton Dickinson	347647	2A3	IgG1
IgG2a-PE negative control	Becton Dickinson	349053		
biotin-IgM negative control (specific for TNP)	Pharmingen	33062X	G155-228	IgM, κ

Table 2.3 Leucocyte Typing Workshop antibodies

Marker	workshop code	Use
CD28	5T-083 5T-061 5T-051	surface staining of resting lymphocytes
p105	NL-141	positive control for intracellular staining
p53	NL-142	
pCNA	NL-143	
p120	NL-144	
p2.7	NL-145	

Table 2.4 Other reagents used during immunofluorescence staining

Name	Company	Catalogue number
biotinylated horse anti-mouse immunoglobulin	Vector, Burlingame, CA.	BA-2000
horse serum	Life Technologies, Gibco	200-6050
human immunoglobulin	Commonwealth Serum Laboratories, Vic. Australia.	11201
mouse serum	Sigma Chemical Co.	M5905
streptavidin-PE	Caltag Sigma Chemical Co.	SA 1004-4 S-3402
streptavidin-Cy5/PE (Tricolor)	Caltag	SA1006
streptavidin-Cy5/PE (Quantum Red)	Sigma Chemical Co.	S-2899
anti-mouse immunoglobulin-PE (DDAPE)	AMRAD	985052005
anti-mouse immunoglobulin-FITC (DDAF)	AMRAD	985051020
streptavidin-Cy3	Jackson Immunoresearch, West Grove, PN	

2.2.2 Phosphate buffer

0.1 M phosphate buffer (pH 7.0 - 8.5) was prepared by combining 19 ml of 1 M $NaH_2PO_4.H_2O$, 81 ml of 1 M $Na_2HPO_4.2H_2O$ and 900 ml of distilled water. This was used for coating magnetic beads. 0.02 M phosphate buffer was used as a binding buffer for the protein G column.

2.2.3 FACS fixative

FACS fixative (pH 7.3) was prepared by adding 10 g D-glucose, 13 ml formaldehyde (37-40%) and 2.5 ml 1M sodium azide to 500 ml of PBS. 500µl of fixative was added to each tube of cells which were then stored at 4°C until flow cytometric analysis could be performed.

2.2.4 Ammonium chloride lysing solution

A stock solution of hypotonic ammonium chloride (pH 7.2-7.4) was prepared from 8.26 g NH_4Cl, 1.0 g $NaHCO_3$ and 0.037 g sodium EDTA dissolved in 100 ml distilled water and stored at 4°C. This solution was diluted 1/10 in distilled water immediately prior to use, and approximately 2 ml was used to resuspend the pellet of cells requiring lysis. After standing for 10 minutes at room temperature, the cells were washed twice in PBS.

2.2.5 Permeabilisation solution

Permeabilisation solution consisted of 10 mmol/l HEPES, 0.1% saponin and 4% paraformaldehyde in distilled water. The solution was filtered (0.2 µm) and stored at

room temperature.

2.2.6 Cell culture medium

RPMI 1640 (Life Technologies, Gibco BRL, catalogue no. 31800-089) supplemented with 10% fetal calf serum (not heat inactivated, Commonwealth Serum Laboratories, Vic., Australia, catalogue no. 09702301), penicillin (100 i.u. / ml), streptomycin sulphate (100 µg/ml) and L-glutamine (2 mM) was used for all cell culture work and is referred to in this thesis as RF10.

2.3 Production of monoclonal antibodies from hybridoma cell lines

Hybridoma cell lines were cultured in RF10 supplemented with hypoxanthine (0.014 µg/ml) and thymidine (0.004 µg/ml). Cell lines were grown in 50 ml flasks (Nalge Nunc Int., Roskilde, Denmark; catalogue no. 147589) and split every 2-3 days when the medium became acidic (demonstrated by the yellow colour of the indicator). Cell suspensions were spun down and the supernatants were collected and stored at -20°C until required.

2.4 B cell lines

Raji and Ramos B cell lines were cultured in RF10. Cell lines were grown in 50 ml flasks (Nalge Nunc Int; catalogue no. 147589) and split every 2-3 days when the medium became acidic.

2.5 Cryopreservation

Hybridomas and B cells lines were stored in liquid nitrogen according to standard techniques. In preparation for storage, cells were centrifuged and resuspended at approximately 5×10^6 cells/ml in RPMI 1640 containing 50% fetal calf serum. An equal volume of 30% dimethyl sulfoxide (DMSO) in RPMI 1640 was then added. After gentle mixing, the cells were dispensed into sterile cryotubes (Nalge Nunc Int.), sealed and frozen slowly by placing in a polystyrene container at -80°C for at least 24 hours before transfer to liquid nitrogen. Cells that had been stored in liquid nitrogen were thawed rapidly in a 37°C waterbath. After thawing, an equal volume of RF10 was added dropwise over 5 minutes. The cells were left to stand for a further 5 minutes and then washed in RF10.

2.6 Purification of monoclonal antibodies using protein G column

All mAb purified from hybridoma supernatants (X63, 7G7/B6, G28/5, 5c8, SAL4, OKT3, 10F7) or ascites (X63, 10F7) were isolated using a protein G column (Protein G Sepharose 4 Fast Flow; Pharmacia, Uppsala, Sweden; catalogue no. 17-0618-01). Prior to purification, ascites was delipidised using SeroClear Reagent (Calbiochem, NSW, Australia, catalogue no. 437616). 1.5 parts of the ascitic fluid were added to 1 part SeroClear Reagent and mixed thoroughly. The mixture was then centrifuged at 3000*g* for 10 minutes and the clear ascites layer collected. The protein G column was attached to an LKB 8300 Uvicord II chromatography system. Absorbance at 280 nm was used to detect protein in the eluate. 0.02 M phosphate buffer was run through the column for approximately 20 minutes to pack the gel. Supernatant or ascites was then loaded onto the column and run through at approximately 35 ml per hour. The purified mAb was eluted using 0.1 M glycine-HCl (pH 2.5), collected directly into 1 M Tris-HCl (pH 9.0;

approximately 100µl/ml eluate) and dialysed overnight against PBS. The concentration of purified mAb was calculated from the absorbance at 280 nm by dividing by the extinction coefficient for mouse immunoglobulin (1.43) (Johnstone & Thorpe, 1987). Alternatively, the concentration of mAb was determined by Bradford assay (see 2.10).

2.7 Purification of goat anti-mouse immunoglobulin from polyclonal serum

Goat anti-mouse immunoglobulin was purified from polyclonal serum using a technique described by Steinbuch and Audran (1969). Serum was warmed to room temperature and adjusted to pH 4.8 by the dropwise addition of 1 M acetic acid. Octanoic acid was then added dropwise (7.4 ml (6.8 g) per 100 ml serum) over 10 minutes with vigorous stirring on a magnetic stirrer. After stirring for a further 30 minutes, the solution was centrifuged at 10 000*g* for 15 minutes. The supernatant was collected and filtered through a Whatman No. 1 filter to remove precipitate. pH was returned to neutral using 0.5 N NaOH. The purified immunoglobulin was then dialysed against PBS at 4°C for 48 hours.

2.8 Bradford assay

Bradford reagent was prepared by dissolving 50 mg Coomassie Brilliant Blue G in 25 ml 95% ethanol, adding 50 ml 85% (w/v) orthophosphoric acid and making the solution up to a final volume of 500 ml with distilled water. Various concentrations of bovine serum albumin (BSA) standard protein (Sigma Chemical Company, catalogue no. P7656) were made up in PBS. 100 µl of each dilution of BSA standard protein was added to 900µl of Bradford reagent, incubated for 5 minutes at room temperature and the absorbance was measured at 595 nm and used to construct a standard curve. The absorbance of various

dilutions of the mAb was determined using the same method. The standard curve was then used to calculate the concentration of the mAb.

2.9 Agarose gel electrophoresis

The purity of mAb recovered from the protein G column was assessed by agarose gel electrophoresis. Purity was indicated by the demonstration of a single band with γ mobility. The gel was made by adding 1% E.E.O. agarose to 0.075 M barbitone buffer (20.7 g barbitone, 4 g calcium lactate, 131.4 g sodium barbital, 2 g thimerosal in 10 litres deionised water; pH 8.6). The gel was melted, poured onto a Gelbond plate and the template applied to cast wells and tracks. MAb to be tested were loaded into the wells (2-4µl / well). Normal human serum (1µl / well) with bromophenol blue marker dye was run as a control. The plate was set up in the electrophoresis tank and current was applied at 200 V for 30 minutes. The plate was then stained for 15 minutes with Coomassie blue (2.5g Coomassie blue in 500 ml destaining solution - see below). After compressing the plate and allowing it to dry, the plate was destained for approximately 10 minutes (destaining solution: 45% v/v methanol, 10% v/v glacial acetic acid in deionised water) and then allowed to dry. Quantification of bands was performed using an Appraise Densitometer (Beckman).

2.10 Biotinylation of monoclonal antibodies

MAb purified from hybridoma supernatants were biotinylated based on the method described by Goding (1996). The mAb was dialysed overnight in 0.1 M $NaHCO_3$ (pH 8.5). The mAb needed to be between 1 mg/ml and 10 mg/ml concentration. EZ-link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) was dissolved in DMSO at 2-4 mg/ml. The biotinylating reagent was then added to the mAb so that the weight of the biotinylating

reagent was 1/10 of the total weight of the mAb to be labelled. The solution was incubated for 2 hours at room temperature with mixing. The biotinylated mAb was then dialysed against PBS overnight. Biotinylation was assessed by comparing staining using the biotinylated mAb and streptavidin-PE with staining using the unlabelled mAb and goat anti-mouse PE.

2.11 Specimens

Umbilical cord blood was collected by drainage of the transected cord at the time of delivery of healthy term neonates. Peripheral venous blood was collected from healthy children between 2 and 20 months of age enrolled in an immunisation trial and adult peripheral venous blood was obtained from laboratory volunteers. Cord blood specimens were kept at room temperature and were processed within 12 hours of collection for functional studies or within 18 hours for experiments using resting lymphocytes. All blood specimens were heparinised with preservative-free heparin and were obtained with the approval of the Ethics Committee of the Women's and Children's Hospital, Adelaide.

2.12 Lymphoprep separation

Blood was diluted in PBS (1/3 dilution for cord blood, 1/2 dilution for adult blood), overlaid on Lymphoprep (Nycomed, Oslo, Norway) (two volumes of diluted blood to one volume of Lymphoprep) and centrifuged at 1200*g* for 25 minutes at room temperature. The interface layer was collected, diluted at least 1/5 in PBS and centrifuged for 10 minutes at 600*g*. After one further wash, the mononuclear cells were resuspended in PBS. When a double Lymphoprep separation was performed to remove contaminating erythroid cells from cord blood mononuclear cells (Ridings *et al.*, 1996), the cells were resuspended in 10-15 ml of PBS, overlaid on Lymphoprep and the procedure was repeated as above.

2.13 Removal of erythroid cells from cord blood mononuclear cells using 10F7 mAb and magnetic beads

Contaminating erythroid cells were removed from cord blood mononuclear cells using the glycophorin A mAb, 10F7, and magnetic beads. This method was developed as part of this study and is described in detail in Chapter 3.

2.14 Depletion of B cells or monocytes from mononuclear cell preparations using magnetic beads

B cells were removed from mononuclear cell preparations under sterile conditions using magnetic beads coated with CD19 mAb (Dynabeads M450 CD19 (Pan B) Dynal, Oslo, Norway, catalogue no. 111.03) according to the manufacturer's instructions. Briefly, mononuclear cells were resuspended at approximately 10^7 cells/ml in RF10 in a 3.6 ml sealed test tube (Nalge Nunc Int., Roskilde, Denmark). Magnetic beads coated with CD19 mAb were washed 3 times in RF10 and added to mononuclear cells to give a bead: B cell ratio of 4:1. The cells were incubated with the beads for 30 minutes at 4°C with bi-directional mixing. The cell suspension was then placed in a Dynal magnetic particle concentrator for three minutes to retain the magnetic beads and B cells while the supernatant was aspirated. The supernatant was transferred to another tube which was placed in the magnetic particle concentrator for a further 3 minutes to remove any residual beads.

B cells or monocytes were removed from mononuclear cell preparations under sterile conditions using anti-mouse IgG coated magnetic beads (Dynabeads M450 Sheep anti-Mouse IgG, Dynal, catalogue no. 110.01) and CD19 mAb or CD14 mAb, respectively. Mononuclear cells were resuspended at approximately 2×10^7 cells/ml in FMC 63

supernatant (CD19 mAb) or FMC 32 supernatant (CD14 mAb) in a 3.6 ml sealed test tube (Nalge Nunc Int.). The cells were incubated for 30 minutes at 4°C with bi-directional mixing. After 2 washes in RF10, the cells were resuspended in RF10 at 10^7 cells/ml. Anti-mouse IgG coated magnetic beads (Dynal, catalogue no. 110.01) (previously washed 3 times in RF10) were added at a bead: target cell ratio of 4:1. After another 30 minute incubation at 4°C with bi-directional mixing, the beads and target cells were retained using a magnetic particle concentrator as described above, while the supernatant containing the B cell or monocyte-depleted mononuclear cells was collected.

After all depletions, cells were washed and resuspended in RF10 and the cellular composition was assessed using CD3-FITC, CD14-PE and CD19-Cy5/PE prior to culture.

2.15 Depletion of B cells and monocytes from mononuclear cell preparations using a nylon wool column

Adult and cord blood mononuclear cell fractions were depleted of B cells and monocytes by passage through a nylon wool column as previously described (Julius *et al.*, 1973). Scrubbed nylon wool fibre (Robbins Scientific, Mountain View, CA) was soaked in 0.5 N HCl overnight, washed several times in distilled water over 3 days and dried in a 37°C incubator. After teasing the wool apart, 2.5 g quantities were packed into 50 ml plastic luer-lock syringes and sterilised with ethylene oxide. A sterilised column was placed in a retort stand with a 3 way tap and a 20 G needle attached to the end of the barrel. 200 ml RPMI with 5% sterile newborn bovine serum (Flow Laboratories, NSW, Australia; catalogue no. 29-121-54) was poured through the column and air bubbles were removed by tapping the side of the column. The top of the column was covered with a petri-dish and the column was incubated at 37°C in 5% CO_2 for 1 hour. After incubation, 25 ml

warm RF10 was washed through the column. Mononuclear cells were resuspended at approximately 10^8 cells/ml and added dropwise to the column. They were then washed into the column by the dropwise addition of warm RF10. The column was again covered and incubated at 37°C in 5% CO_2 for 45 minutes. Cells were eluted by the dropwise addition of warm RF10, washed and resuspended for determination of yield, viability and composition.

2.16 Functional studies

Adult and cord blood mononuclear cells or B cell/monocyte depleted mononuclear fractions were cultured in RF10 at 2×10^6 cells/ml in flat-bottom 24 well plates (Nalge Nunc Int., Roskilde, Denmark) at 37°C in 5% CO_2.

2.17 Activation stimuli

2.17.1 PMA

PMA (phorbol 12-myristate 13-acetate) (Sigma Chemical Co., catalogue no. P-8139) was used at a final concentration of 10 ng/ml. The stock reagent was dissolved in DMSO at 20μg/ml and aliquots were stored at -20°C. On the day of use, one aliquot was thawed and diluted in RF10 to 1 μg/ml. 10 μl of this solution was then added to each ml of cells to achieve a final concentration of 10 ng/ml. Importantly, PMA was not frozen and thawed more than once. Activity was monitored by its ability to rapidly downregulate T cell surface CD4 expression (Anderson & Coleclough, 1993).

2.17.2 Ionomycin

Ionomycin (Sigma Chemical Co., catalogue no. I-0634) was dissolved in DMSO at 1 mg/ml, diluted in RF10 to 100 μg/ml and stored at 4°C. This was used to stimulate cells at a final concentration of 1 μg/ml.

2.17.3 Plate-bound CD3 mAb

Based on a method previously described (Hirohata *et al.*, 1988), sterile purified OKT3 antibody was diluted in 0.05 M Tris (pH 9.0) at 1 μg/ml, and 0.5 ml was added to each well of a 24 well plate (Nalge Nunc Int., Roskilde, Denmark). The plates were incubated for at least 4 hours at 37°C, then washed three times in sterile PBS before use. Plates coated with CD3 mAb were either used immediately or stored at -20°C for up to 1 month before use.

2.18 Mitomycin C treatment

Ramos and Raji cells were treated with mitomycin C prior to culture with B cell/monocyte depleted mononuclear cells. Mitomycin C (Kyowa, Japan; distributed by Bristol-Meyers-Squibb, NSW, Australia) was made up to a 1 mg/ml solution in RF10 and 250 μl was added to 10 ml cells (at 10^6 cells/ml) to give a final concentration of 25 μg/ml. Cells were incubated for 30 minutes at 37°C and then washed 3 times with RF10 before adding to mononuclear cells.

2.19 Immunofluorescence staining

In all studies, one adult and one cord blood or child blood specimen were processed in parallel. Staining was performed on 100 µl of whole blood or 5×10^5 mononuclear cells (at 1×10^7 cells/ml). Staining with isotype-matched negative controls was conducted in parallel. All incubations were conducted for 30 minutes on melting ice and two washes were performed after each stage of staining using PBS with 0.02% sodium azide and 1% newborn bovine serum. Whole blood was lysed after staining using FACS Lysing Solution (Becton Dickinson; catalogue no. 349202) according to the manufacturer's instructions. FACS Lysing Solution was diluted 1/10 in distilled water. 2 ml of the working solution was added to each tube and the cells were incubated in the dark at room temperature for 10 minutes, and then washed twice before flow cytometric analysis. Cells prepared by the whole blood lysis method or stained for CD154 expression were subject to flow cytometric analysis immediately after staining was completed. In other experiments, cells were either analysed immediately or fixed with FACS fixative for up to 48 hours before analysis.

Direct and indirect staining were performed according to standard techniques (Goding, 1996). Indirect staining used either purified primary mAb and anti-mouse PE or anti-mouse FITC, or biotinylated primary mAb and streptavidin conjugated second step reagents. For dual immunofluorescence staining, when PE- or FITC-conjugated anti-mouse immunoglobulin was used to detect the primary mAb, residual anti-mouse binding sites were blocked by adding mouse serum before staining with the directly conjugated mAb.

Three-stage high sensitivity staining was performed as described by Zola et al. (Zola *et al.*, 1990). Whole blood or mononuclear cells were incubated with the primary mAb. Fc receptors were blocked by incubating cells for 10 minutes at room temperature with 10 µl horse serum and 5 µl human immunoglobulin prior to staining with biotinylated horse

anti-mouse immunoglobulin. 5µl mouse serum was added to block residual anti-mouse binding sites before adding streptavidin-PE or streptavidin-Cy5/PE and directly conjugated lineage markers.

Intracellular staining was conducted as follows. Surface lineage markers were stained with directly conjugated mAbs and surface CD152 or CD154 was blocked with an unconjugated mAb. Mononuclear cells were then treated with FACS Lysing Solution (Becton Dickinson) (in the same manner as whole blood was treated to lyse erythrocytes). Following centrifugation, cells were incubated on ice for 10 minutes with 0.5 ml permeabilisation solution. After washing, intracellular CD152 or CD154 was then stained using biotinylated mAb followed by streptavidin-PE or streptavidin-Cy3. Fluorescence microscopy was performed with a Nikon Optiphot 2 fluorescence microscope using a G2A filter block (excitation filter 510-560 nm, dichroic mirror 580 nm, barrier filter 590 nm).

2.20 Flow cytometry

All data were recorded using a FACScan (Becton Dickinson). The machine was calibrated daily using CaliBRITE beads (Becton Dickinson, catalogue no. 349502). For mononuclear cells, 10000 events were counted for each tube. For whole blood, live gating on the lymphocyte population was performed and 10000 events in the lymphocyte gate were recorded. Data were analysed using Cellquest software (Becton Dickinson). Lymphocytes were gated based on light scatter properties and B and T lymphocytes were gated according to staining with directly conjugated lineage markers. Expression of CD80, CD86, CD152 and CD40 was unimodal and therefore the mean fluorescence intensity (MFI) of the whole B cell or T cell population was recorded. Expression of CD28 and CD154 was bimodal, therefore the MFI of the positive population and the percentage of positive T cells were examined.

2.21 Statistical analyses

Data were analysed using the Student's paired t-test (Chapter 3) or the Mann Whitney U-test (Chapters 4 - 6). Outliers were excluded from analysis if they were 1.5 × interquartile range from the upper or lower quartile (Moore & McCabe, 1992). $P < 0.05$ was considered significant.

CHAPTER 3.

REMOVAL OF ERYTHROID CELLS FROM UMBILICAL CORD BLOOD MONONUCLEAR CELL PREPARATIONS USING MAGNETIC BEADS AND A MONOCLONAL ANTIBODY AGAINST GLYCOPHORIN A

3.1 Introduction

Cord blood mononuclear cells (MC) are usually isolated using density centrifugation. Whereas adult peripheral blood MC are easily purified using this technique (Boyum, 1968), cord blood MC isolated in this way are contaminated with mature erythrocytes and nucleated erythroid precursors. The proportion of erythroid cells is variable but may exceed 50% of the total cell population and thus interfere with phenotypic, functional and mRNA analyses (Ridings *et al.*, 1996). On flow cytometric analysis, the red cell precursors cannot be excluded from lymphocyte gates based on light scatter properties and thus confound the interpretation of phenotypic data by reducing the proportions of lymphocyte sub-populations (Ridings *et al.*, 1996). In functional studies, the presence of large numbers of erythroid cells in cord blood MC preparations reduces the relative concentration of effector cells and therefore may physically interfere with intercellular interactions.

Hypotonic ammonium chloride effectively lyses the contaminating erythroid cells but also inhibits lysosomal function, interfering with cellular activities such as antigen processing (McCoy & Schwartz, 1988; Seglen, 1983). Ammonium chloride should therefore not be used to purify cord blood MC required for functional analyses. For the purposes of this chapter, 'purification' of cord blood MC refers to methods used for removing contaminating erythroid cells. Cord blood MC may be purified using a second density

separation prior to functional studies (Ridings *et al.*, 1996), but this method was not found to be effective when the number of contaminating erythroid cells was high. The aim of the studies described in this chapter was to develop a technique for effectively removing erythroid cells from cord blood MC preparations that would not interfere with cellular function, and could thus be used to prepare cord blood MC for *in vitro* studies.

This chapter describes a method for purifying cord blood MC using a monoclonal antibody against glycophorin A. Glycophorin A is the major sialoglycoprotein of the human erythrocyte membrane and is responsible for MN blood group activity (Hamaguchi & Cleve, 1972). It is specifically expressed on all erythrocytes and erythroid precursors (Ekblom *et al.*, 1985; Gahmberg *et al.*, 1978; Kudo *et al.*, 1994; Loken *et al.*, 1987; Rearden *et al.*, 1985). 10F7 is a mouse IgG1 κ monoclonal antibody which binds equally to M and N forms of human glycophorin A (Bigbee *et al.*, 1984; Bigbee *et al.*, 1983) inducing haemagglutination. Using anti-mouse immunoglobulin-coated magnetic beads and 10F7 mAb, contaminating erythrocytes and erythroid precursors could be effectively removed from the cord blood mononuclear fraction which had been isolated by density separation.

3.2 Materials and methods

3.2.1 Counting erythroid cells

Commonly used methods for counting erythroid cells (eg. Coulter counting, methylene blue stain) were unreliable due to the fact that nucleated red cell precursors are similar to lymphocytes in size and microscopic appearance, and have similar light scatter properties on flow cytometric analysis. Cord blood MC obtained after a single density separation were stained with a FITC-conjugated monoclonal antibody (mAb) against the leucocyte common antigen, CD45 (Prod. no. 340040, Becton Dickinson, San Jose, California) and analysed by flow cytometry. Erythrocytes and nucleated red cell precursors do not

express CD45 and therefore the number of contaminating cells could be calculated from the proportion of the unpurified cord blood MC preparation which was CD45 negative.

Alternatively, since erythroid cells are lysed by acetic acid, the difference between the total cell count in trypan blue and the total cell count in 1% acetic acid determined with a haemocytometer could be used to estimate the number of erythroid cells.

3.2.2 Removal of erythroid cells from cord blood mononuclear cell preparations using 10F7 mAb and sheep anti-mouse IgG coated magnetic beads

The volume of the unpurified cord blood MC suspension was adjusted so that the concentration of erythroid cells was approximately 10^7 cells/ml. Purified 10F7 mAb and sheep anti-mouse IgG coated magnetic beads (Dynabeads M-450, Prod. no. 110.01, Dynal, Oslo, Norway) were used to remove contaminating erythroid cells by the indirect technique described in the Dynal protocol. Briefly, the cells were incubated with 25 μg/ml 10F7 mAb in a 3.6 ml sealed test tube (Nalge Nunc Int., Roskilde, Denmark) with bi-directional mixing for 30 minutes. The cells were washed twice and resuspended at their original volume. Magnetic beads were added to the cell suspension at a bead to target (erythroid) cell ratio of 4:1, ensuring a minimum concentration of 10^7 beads/ml. Following a 15 minute incubation with bi-directional mixing, the cell suspension was placed in the Dynal magnetic particle concentrator for 3 minutes. Erythroid cells bound to the magnetic beads were retained at the side of the tube by the magnet while the supernatant was aspirated. The collected supernatant was placed in the magnetic particle concentrator for a further 3 minutes and then transferred to another test tube to ensure that any residual beads were removed. All washes and incubations were conducted at room temperature. When cells were to be stained immediately, all washes were done in PBS with 1% newborn bovine serum and 0.02% sodium azide. Cells intended for functional

studies were washed in RF10.

3.2.3 Coating magnetic beads with polyclonal goat anti-mouse immunoglobulin

As an alternative to sheep anti-mouse IgG coated Dynabeads, uncoated Dynabeads (kind gift of Professor John Ugelstad, 1984) were coupled with purified polyclonal goat anti-mouse immunoglobulin according to the Dynal protocol,, and proved equally effective for the purification of cord blood MC. Uncoated beads were washed 3 times in sterile PBS and resuspended in sterile 0.1 M phosphate buffer at 4×10^8 beads/ml. 2.4 mg of goat anti-mouse immunoglobulin was added per ml of beads and incubated for 15 minutes at 37°C with mixing. 50% BSA in PBS was then added to give a final concentration of 0.05% v/v, and the beads were incubated overnight at 37°C with mixing. After incubation, the beads were washed 3 times for 5 minutes at 4 °C with sterile PBS (plus 0.02% sodium azide, 0.1% BSA) and resuspended at 4×10^8 beads/ml in the same buffer.

3.2.4 Yield and viability

The yield was determined by calculating the number of viable mononuclear cells (based on trypan blue exclusion) using a haemocytometer. Viability was assessed by staining 10^6 mononuclear cells with 5 µl of propidium iodide (2 µg/ml) for 5 minutes at room temperature and analysing by flow cytometry.

3.3 Results

3.3.1 Purity of cord blood mononuclear cells treated with 10F7 mAb and magnetic beads

Cord blood MC prepared by a single Lymphoprep separation were contaminated by variable numbers of erythrocytes and erythroid precursors, which could exceed 50% of the total cell population. A second Lymphoprep separation did not give adequate purity when the level of erythroid contamination was high (Fig. 3.1). The purity of cord blood MC (as assessed by expression of the leucocyte common antigen, CD45) was comparable and consistently high after treatment with magnetic beads or ammonium chloride (Table 3.1). Purity was lower and more variable following two Lymphoprep separations (mean percentage of CD45 positive cells: 75.5% (standard deviation 29.9%) for 4 specimens of cord blood MC purified by this technique). A large proportion of the contaminating erythroid cells lay within the lymphocyte gate (Fig. 3.1).

Table 3.1
Comparison of ammonium chloride lysis and magnetic beads for removing erythroid cells from cord blood MC preparations.

	NH_4Cl Lysis	Magnetic Beads	t test (P)
Purity n=13	97.9% (3.3)	97.6% (4.9)	0.85
Yield n=5	4.3 x 10^6/ml (1.8)	3.5 x 10^6/ml (1.3)	0.07
Viability n=5	96.9% (2.7)	97.2% (2.4)	0.40

Specimens of cord blood MC recovered after a single Lymphoprep separation were divided and treated by each of these methods to remove contaminating erythroid cells. Purity was assessed by staining with CD45-FITC and is recorded as the percentage of the total cell population which was CD45 positive. Yield is recorded as the number of viable mononuclear cells recovered per ml of cord blood. Viability was assessed by staining with propidium iodide and is recorded as the percentage of non-staining cells. The values shown represent means and standard deviations (in brackets).

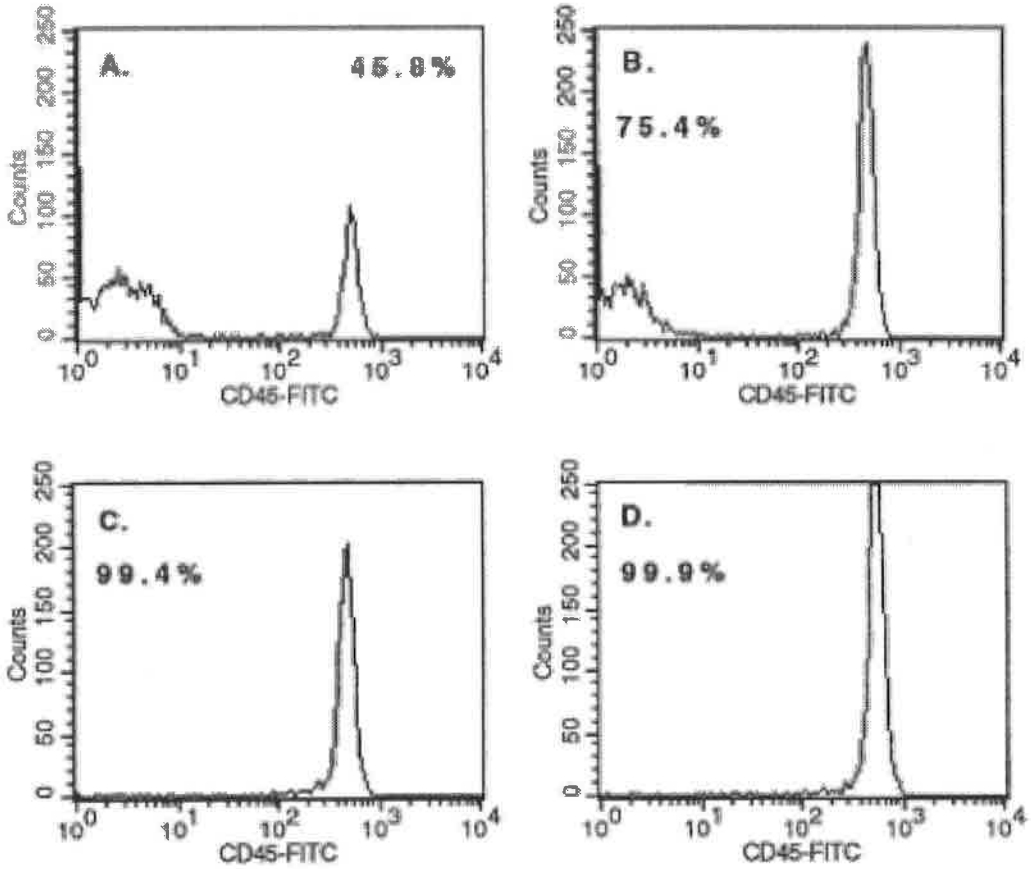


Fig. 3.1 Purity of cord blood MC treated by each of the methods indicated to remove contaminating erythroid cells. The cord blood MC recovered after a single Lymphoprep separation were divided and either left untreated **(A)**, subjected to a second Lymphoprep separation **(B)**, lysed with ammonium chloride **(C)** or treated with 10F7 mAb and magnetic beads **(D)**. The purity of each preparation was then assessed by staining with CD45-FITC. The percentage of CD45 positive cells in the lymphocyte gated population is indicated in each histogram. These histograms are from one specimen of cord blood MC (representative of 13 specimens).

3.3.2 Yield and viability

The final yield was expressed as the number of viable mononuclear cells recovered per ml of cord blood. The yield tended to be slightly lower after purification using magnetic beads compared with ammonium chloride lysis (Table 3.1).

The viability was expressed as the percentage of unstained cells. Viability was similar after both techniques (Table 3.1).

3.3.3 Composition of cord blood mononuclear cells after purification with magnetic beads or lysis

The relative size of mononuclear cell subpopulations in cord blood MC was compared after removal of contaminating erythroid cells using ammonium chloride lysis or 10F7 mAb and magnetic beads (Fig. 3.2). The proportion of CD19 positive lymphocytes was significantly lower ($P < 0.01$) after purification of cord blood MC with magnetic beads compared with ammonium chloride lysis. Conversely, the proportion of CD3 positive lymphocytes was significantly lower ($P < 0.05$) after purification of cord blood MC by lysis than after purification with magnetic beads. No significant differences were found for any of the other subpopulations examined.

Interestingly, the standard deviations for the proportions of CD19 positive, CD3 positive and CD3 positive, CD4 positive cells after purification of cord blood MC by ammonium chloride lysis were higher than after purification by magnetic beads (Fig. 3.2). The variability in the relative proportions of CD19 positive and CD3 positive cells in cord blood MC after lysis was evident when data from individual cord blood MC specimens were examined (Table 3.2). In specimen number 4, the results obtained after ammonium chloride lysis were quite unusual, with 27.7% B cells (CD19 positive), 23.0% T cells (CD3 positive), 32.8% monocytes (CD14 positive) and 36.2% NK cells. The same cord blood MC specimen purified with magnetic beads was composed of 11.6% B cells,

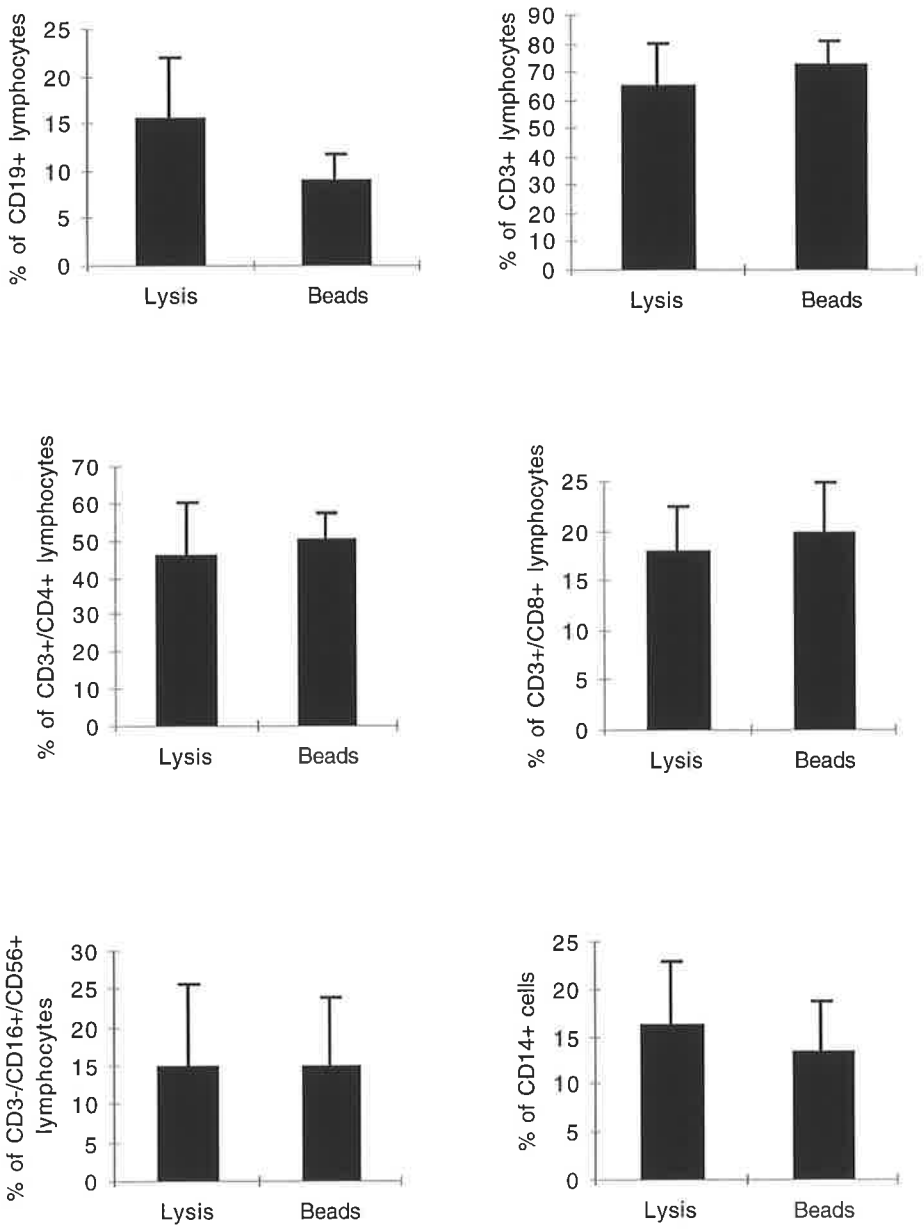


Fig. 3.2 Composition of cord blood MC treated by lysis or magnetic beads to remove contaminating erythroid cells. Each specimen of cord blood MC recovered after a single Lymphoprep separation was divided and treated with ammonium chloride or 10F7 mAb and magnetic beads to remove contaminating erythroid cells. Mononuclear subpopulations were assessed by staining with directly conjugated monoclonal antibodies to the markers indicated. Data are presented as mean values plus standard deviation (error bars) for 13 specimens of cord blood MC. The proportion of CD19 positive cells was significantly lower ($P < 0.01$) in cord blood MC purified with 10F7 and magnetic beads. The proportion of CD3 positive cells was significantly lower in cord blood MC purified by ammonium chloride lysis ($P < 0.05$). There were no significant differences between cord blood MC treated with lysis and cord blood MC treated with magnetic beads for the other cell subpopulations examined.

66.5% T cells, 15.5% monocytes and 18.3% NK cells. Purification of the same cord blood MC specimen by a second Lymphoprep separation (96.7% CD45 positive) yielded results comparable to those observed after treatment with magnetic beads (8.7% B cells, 64.0% T cells, 16.4% monocytes and 8.1% NK cells). The reduction of T cells in this cord blood MC specimen during ammonium chloride lysis affected both subsets: 10.4% CD3 positive, CD4 positive and 11.8% CD3 positive, CD8 positive cells.

Table 3.2

Relative proportions of CD19 and CD3 positive lymphocytes in cord blood MC treated with lysis or 10F7 mAb and magnetic beads to remove contaminating erythroid cells.

Specimen number	CD19 positive		CD3 positive	
	Lysis	Magnetic Beads	Lysis	Magnetic Beads
1	7.9%	5.5%	86.0%	85.1%
2	20.0	12.4	57.9	69.1
3	10.6	9.1	70.8	63.9
4	27.7	11.6	23.0	66.5
5	13.1	8.9	70.7	76.2
6	11.0	9.2	77.8	81.2
7	14.2	6.9	63.2	76.2
8	11.0	9.6	51.6	53.2
9	14.9	11.8	77.5	79.5
10	13.2	6.3	70.4	74.0
11	8.6	3.6	68.1	68.5
12	23.5	9.0	65.9	77.9
13	26.0	13.3	66.1	75.8
Mean	15.5%	9.0%	65.3%	72.9%
Standard deviation	6.6	2.9	15.5	8.5

Each specimen of cord blood MC recovered after a single Lymphoprep separation was divided in two and treated with ammonium chloride or 10F7 mAb and magnetic beads to remove contaminating erythroid cells. Each row represents a single specimen of cord blood MC. The proportion of positively staining cells is expressed as a percentage of the gated lymphocyte population.

3.3.4 *In vitro* activation of cord blood mononuclear cells purified with 10F7 mAb and magnetic beads

It was necessary to ensure that lymphocytes from cord blood MC purified with 10F7 mAb and magnetic beads would respond to activation *in vitro*, and that non-specific activation of cultured cells would not be induced by the purification process.

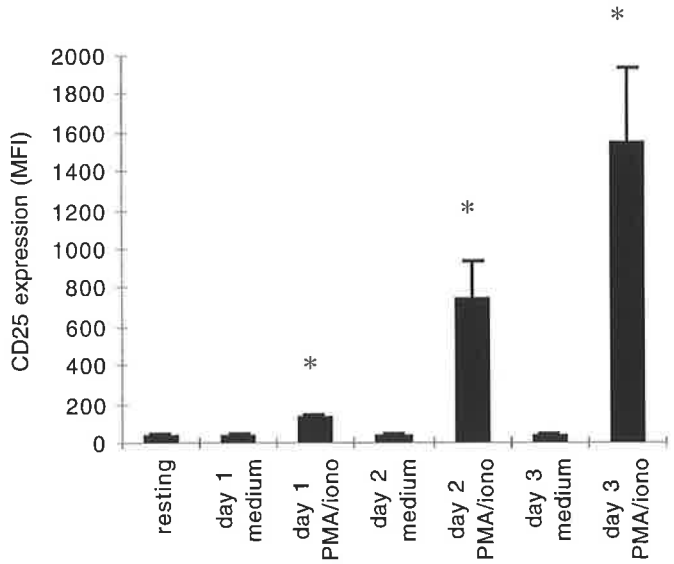
Purified cord blood MC were cultured for up to 3 days in medium alone or stimulated with PMA and ionomycin. The expression of the activation marker, CD25 (IL2Rα), on both B cells and T cells was assessed by flow cytometric analysis. All B and T lymphocytes were CD25 positive after 24 hours of stimulation. As can be seen from Fig. 3.3, the MFI of CD25 expression was significantly upregulated on B and T lymphocytes over three days of activation. There was no significant increase in CD25 expression on lymphocytes cultured in medium alone, when compared with expression on resting cells.

When purified cord blood MC were stimulated for three days with PMA and ionomycin or plate-bound CD3 mAb, there was significant upregulation of CD40 expression on B lymphocytes (Fig. 3.4). This illustrates not only that cord blood lymphocytes treated with magnetic beads can respond to direct mitogenic stimulation (PMA and ionomycin) but that indirect activation of B lymphocytes by CD3-stimulated T lymphocytes (a model for T-dependent B cell activation) is also functional.

3.4 Discussion

The aim of this chapter was to develop an effective technique for removing erythrocytes from cord blood MC which would not interfere with lymphocyte function. This would assist in the preparation of whole mononuclear cell preparations and purified cell populations derived from cord blood for use in *in vitro* studies.

A.



B.

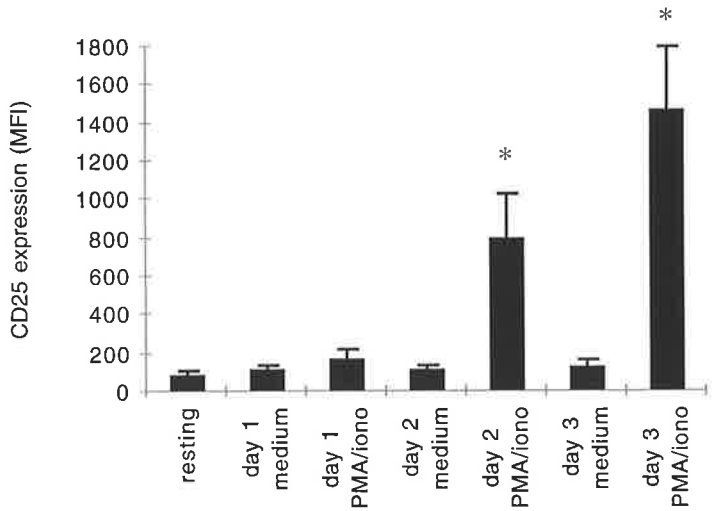


Fig. 3.3 *In vitro* activation of cord blood MC purified with 10F7 mAb and magnetic beads. Purified cord blood MC were cultured in medium alone or stimulated with PMA and ionomycin. Cells were harvested after 1, 2 or 3 days of culture and the expression of CD25 on B lymphocytes **(A)** and T lymphocytes **(B)** was examined. Cells were stained with 7G7/B6 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-PE. B cells were identified by staining with directly conjugated CD19 mAb and T cells were identified as CD19 negative and CD2 positive with directly conjugated mAb. The mean values plus standard deviations (error bars) are shown for four specimens of cord blood MC. Significant differences ($P < 0.05$) in the levels of CD25 expression between cells cultured in medium alone and stimulated cells are indicated by asterisks.

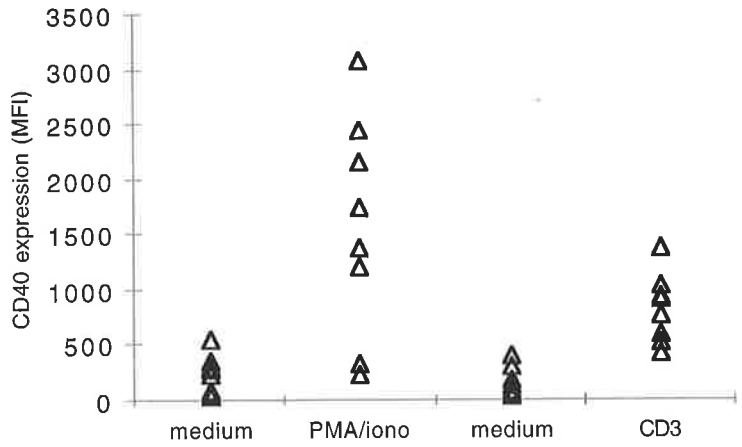


Fig. 3.4 *In vitro* activation of cord blood MC purified with 10F7 mAb and magnetic beads. Purified cord blood MC were cultured in medium alone, or stimulated with PMA and ionomycin, or plate-bound CD3 mAb. Cells were harvested after 3 days of culture and the expression of CD40 on CD19 positive B lymphocytes was examined. Cells stimulated with PMA and ionomycin were stained with CD40 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE and cells activated in the CD3 system were stained with biotin-CD40 mAb and streptavidin-PE. The points represent values for individual specimens of cord blood MC ($n = 8$ for PMA/ionomycin stimulation and $n = 9$ for CD3 stimulation). There was significant upregulation of CD40 expression on B lymphocytes activated with PMA and ionomycin ($P < 0.01$) or indirectly via CD3-stimulated T lymphocytes ($P < 0.01$) compared with those cultured in medium alone.

It has previously been suggested that a second Lymphoprep separation is an effective method of removing contaminating erythroid cells from cord blood MC (Ridings *et al.*, 1996). This technique was useful when contamination of cord blood MC with erythroid cells was low, but was ineffective when contamination levels were high. Using the anti-glycophorin A monoclonal antibody, 10F7, and magnetic beads coated with sheep anti-mouse IgG or polyclonal goat anti-mouse immunoglobulin, purity equivalent to that achieved with hypotonic ammonium chloride lysis could be attained without compromising the yield or viability of the mononuclear cells recovered. Since glycophorin A is expressed in high density on erythroid cells (Anstee, 1981), successful purification could be accomplished using the minimum recommended number of magnetic beads.

In comparing the composition of cord blood MC after purification using lysis or magnetic beads, there was a significant difference in the proportion of B cells, with lower numbers being found in cord blood MC purified with magnetic beads. The occurrence of low level cross-reactivity between goat or sheep anti-mouse IgG coated Dynabeads and human B cells has been noted by the manufacturers (Dynal product information) and it is possible that this may cause selective depletion of B cells in cord blood MC treated with magnetic beads.

Conversely, the proportion of CD3 positive T lymphocytes was significantly lower after purification of cord blood MC by lysis compared with magnetic beads. The sporadic occurrence of extreme variations in the composition of cord blood MC treated with ammonium chloride could not be readily explained and was not pursued further, since it was the objective of this study to identify an alternative to ammonium chloride lysis. It is interesting that a comparative study of whole blood lysis methods found that ammonium chloride-based commercial lysing solutions selectively deplete CD4 positive T cells in some circumstances (Bossuyt *et al.*, 1997). Another study that compared Ficoll-Hypaque separation with the whole blood lysis method using ammonium chloride, noted that the proportion of CD19 positive cells is lower after Ficoll-Hypaque separation, whereas the

proportion of CD3 positive cells and the CD4 positive subset is lower when the whole blood lysis method is used (Tamul *et al.*, 1994). It was suggested that this indicates a selective loss of B cells during Ficoll-Hypaque separation and that the lower proportion of CD3 positive and CD4 positive T cells after whole blood lysis is due to the higher proportion of B cells present, causing a relative reduction in the number of T cells. An alternative interpretation is that depletion of T cells by lysis with ammonium chloride causes a relative increase in the proportion of B cells in these specimens and this may also explain the findings in the current study. Essentially, there is no way to establish which of these methods for isolating cord blood MC causes the most significant alterations to the composition of the final cell population recovered as there is no standard technique with which to compare. Research workers using magnetic beads for purification should be aware that some depletion of B cells may occur, but that the losses are unlikely to be as substantial as suggested by the results obtained from comparison with ammonium chloride lysis.

Activation of cord blood MC purified with magnetic beads using PMA and ionomycin induced upregulation of the activation markers CD25, on B and T lymphocytes, and CD40 on B lymphocytes. There was no evidence of non-specific activation of unstimulated cells. Background staining with negative control antibodies was low, indicating that there was no interference by residual magnetic beads or anti-mouse immunoglobulin with the flow cytometric analysis. Plate-bound CD3 mAb were used as a model for T-dependent B cell activation and induced significant upregulation of CD40 expression on B lymphocytes, indicating that T-B cell interactions remain functional after treatment with magnetic beads. These results are in accordance with previous findings which show that even when cells are positively selected using magnetic beads, they remain in the resting state after isolation and are capable of proliferating and differentiating in response to *in vitro* stimulation (Funderud *et al.*, 1990; Hansel *et al.*, 1989).

Prior to purification with 10F7 mAb and magnetic beads, cord blood MC could be

subjected to a second Lymphoprep separation (Ridings *et al.*, 1996) to reduce the number of contaminating erythroid cells. In this way, a smaller quantity of magnetic beads was required for the final purification which improved cost-effectiveness, particularly when using large volumes of blood or when the number of contaminating erythroid cells was high.

In conclusion, using the anti-glycophorin A monoclonal antibody, 10F7, and magnetic beads provides a simple method of removing contaminating erythroid cells from preparations of cord blood MC without using hypotonic ammonium chloride lysis. This is of particular relevance for *in vitro* functional studies using mononuclear or purified cell populations derived from umbilical cord blood. This method was used to remove erythroid cells from contaminated cord blood MC fractions in all the experiments described in this thesis.

CHAPTER 4.

EXPRESSION OF THE COSTIMULATOR MOLECULES, CD80, CD86, CD28 AND CD152 ON LYMPHOCYTES FROM NEONATES AND YOUNG CHILDREN

4.1 Introduction

As described in Chapter 1, T-dependent antibody responses by neonatal B cells *in vivo* and *in vitro* are often of lower magnitude than antibody responses of adult B cells and are largely restricted to IgM, with minimal production of down-stream isotypes (Andersson *et al.*, 1981; Durandy *et al.*, 1990; Gathings *et al.*, 1981; Hayward & Cosyns, 1994; Miyawaki *et al.*, 1981; Splawski *et al.*, 1991; Splawski & Lipsky, 1991; Tosato *et al.*, 1980). Neonates also have a limited ability to form immunologic memory to some antigens (Di Sant'Agnese, 1950) and lack somatic mutations in their immunoglobulin genes (Ridings *et al.*, 1997). Binding of CD80 and CD86 on B cells to CD28 and CD152 on T cells regulates T cell activation during T-dependent antibody responses (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994). Observations in CD28 knockout mice and CD152-Ig transgenic mice indicate that CD28 signals are important for antibody responses to T-dependent antigens, isotype switching, germinal centre formation, differentiation of memory B cells and somatic hypermutation (Ferguson *et al.*, 1996; Green *et al.*, 1994; Lane *et al.*, 1994; Shahinian *et al.*, 1993). It is therefore suggested that this pathway may differ in the human neonate and young child, giving rise to some of the reported limitations of humoral immunity in early life.

To date, there have been few studies comparing the expression and function of CD80, CD86, CD28 and CD152 on neonatal lymphocytes and adult peripheral blood lymphocytes. A higher proportion of T cells express CD28 in cord blood than in adult peripheral blood (Hoshino *et al.*, 1993; Jennings *et al.*, 1994; McCloskey *et al.*, 1997;

O'Gorman *et al.*, 1998) and this is believed to reflect the absence of a population of CD8 positive CD28 negative effector T cells in neonates (Azuma *et al.*, 1993b). There have been studies showing that *in vitro* activation of human cord blood CD4 positive T cells by CD3 mAb, CD2 mAb or mitogens can be enhanced by CD28 ligation using CD28 mAb (Hassan *et al.*, 1995; King *et al.*, 1995; Servet Delprat *et al.*, 1996; Webb & Feldmann, 1995). However, these *in vitro* observations may not reflect events *in vivo*. Different levels of expression of CD80 and CD86, the ligands for CD28, on neonatal B cells could affect the degree of CD28 cross-linking and hence the magnitude of the CD28 signal delivered to neonatal T cells. Similarly, differential neonatal T cell expression of CD152, which downregulates T cell activation, could influence the outcome of T cell-B cell interactions in neonates. The ability of cord blood T cells to respond to *in vitro* CD28 ligation therefore does not indicate that this pathway operates in the same way in neonates as in adults. Information about the expression of all these costimulator molecules on neonatal lymphocytes is needed.

The initial aim of the studies described in this chapter was to compare the expression of the four members of this family of costimulator molecules, CD80, CD86, CD28 and CD152, on resting cord blood lymphocytes with expression on resting adult peripheral blood lymphocytes. The expression of CD80 and CD86 on B lymphocytes, and CD28 and CD152 on T lymphocytes were examined.

Having examined costimulator expression on cord blood lymphocytes, the expression of these molecules was examined during early childhood. During the first 2 years of life, events such as weaning, primary exposure to infectious agents and immunisation may critically affect the development of the immune system. The expression of CD80, CD86 and CD28 on resting peripheral blood lymphocytes from children in this age group was assessed. As CD152 was not detected on resting adult or cord blood T cells, it was not examined in young children.

The regulation of CD80, CD86, CD28 and CD152 expression on adult and cord blood

lymphocytes during *in vitro* activation was then examined. Young children could not be included in these studies, as large volumes of blood were required which could not be obtained from children in this age group. Preliminary experiments were conducted using different activation systems, including PHA, PMA, PMA and ionomycin, IL-4, IL-4 and anti-IgM beads and plate-bound CD3 mAb. PMA and ionomycin were selected to provide strong mitogenic stimulation of B and T lymphocytes (Berry *et al.*, 1989) (Callard *et al.*, 1992) and plate-bound CD3 mAb was used as a method of inducing receptor-mediated T cell activation and as a model for T-dependent B cell activation (Hirohata *et al.*, 1988). Using these two different activation systems, the kinetics of CD80, CD86, CD28 and CD152 expression on adult and cord blood lymphocytes were examined. Finally, intracellular expression of CD152 was compared on adult and cord T cells, since it has been demonstrated that this is primarily an intracellular molecule (Leung *et al.*, 1995; Vyth Dreese *et al.*, 1995).

4.2 Results

4.2.1 Expression of CD80, CD86, CD28 and CD152 on resting lymphocytes

The expression of CD80, CD86 and CD28 was compared on resting lymphocytes from adults, young children (2-20 months of age) and cord blood from neonates. The whole blood method of staining was used. For each experiment, one adult specimen was processed in parallel with one specimen of cord blood or peripheral blood from a child. CD80 and CD86 on B cells or CD28 on T cells were detected by 3-stage high sensitivity immunofluorescence using purified mAb, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE. B cells and T cells were identified using directly conjugated mAb to CD19 and CD3, respectively. CD152 was not expressed on resting T cells. CD152 expression was therefore not examined in this initial series of experiments on resting lymphocytes.

In all the experiments described in this chapter, the MFI of CD80 or CD86 staining on the whole B cell population and the MFI of CD152 on the whole T cell population were recorded, as expression of these molecules was unimodal. CD28 expression was bimodal, therefore the MFI of CD28 expression on CD28 positive T cells and the percentage of T cells expressing CD28 were both examined.

CD80

When compared with the level of background staining using an isotype-matched negative control antibody, TC12.6, there was no significant expression of CD80 on resting adult (n = 12) or cord blood (n = 14) B cells (Fig. 4.1). Similarly, there was no significant expression of CD80 on resting B cells from adults (n = 20) or children (n = 19) when compared with background staining using the negative control mAb, X63 or TC12.6.

CD86

The MFI of CD86 expression was not significantly different between resting adult (n = 12) and cord blood (n = 14) B cells (P = 0.98) (Fig. 4.2A, Fig. 4.2B). However, the MFI of CD86 expression was significantly higher on resting B cells from children (n = 19) compared with resting adult B cells (n = 19) ($P < 0.05$) (Fig. 4.2A, Fig. 4.2C).

CD28

The MFI of CD28 expression was not significantly different between resting cord blood T cells (n = 21) and resting adult T cells (n = 17) (P = 0.93) (Fig. 4.3A, Fig. 4.3B) or between resting T cells from adults (n = 22) and children (n = 20) (P = 0.05) (Fig. 4.3A, Fig. 4.3C). However, the proportion of resting T cells that expressed CD28 was significantly higher in cord blood ($P < 0.01$) (Fig. 4.3B) and peripheral blood from children ($P < 0.01$) (Fig. 4.3C) than in adult peripheral blood.

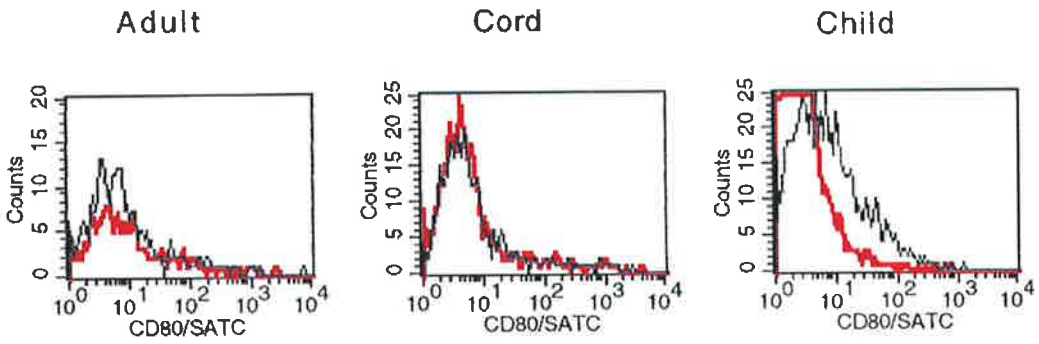


Fig. 4.1 No significant expression of CD80 was demonstrated on resting B cells from cord blood or peripheral blood of adults or young children, using high sensitivity staining. Representative histograms from one adult, one cord blood specimen and one child (57 weeks old) are shown. Staining with CD80 mAb (**red**) and the negative control mAb, TC12.6 (**black**), is illustrated. The child specimen was not processed on the same day as the adult and cord blood specimens. Whole blood was stained with purified CD80 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE. B cells were identified with directly conjugated mAb to CD19.

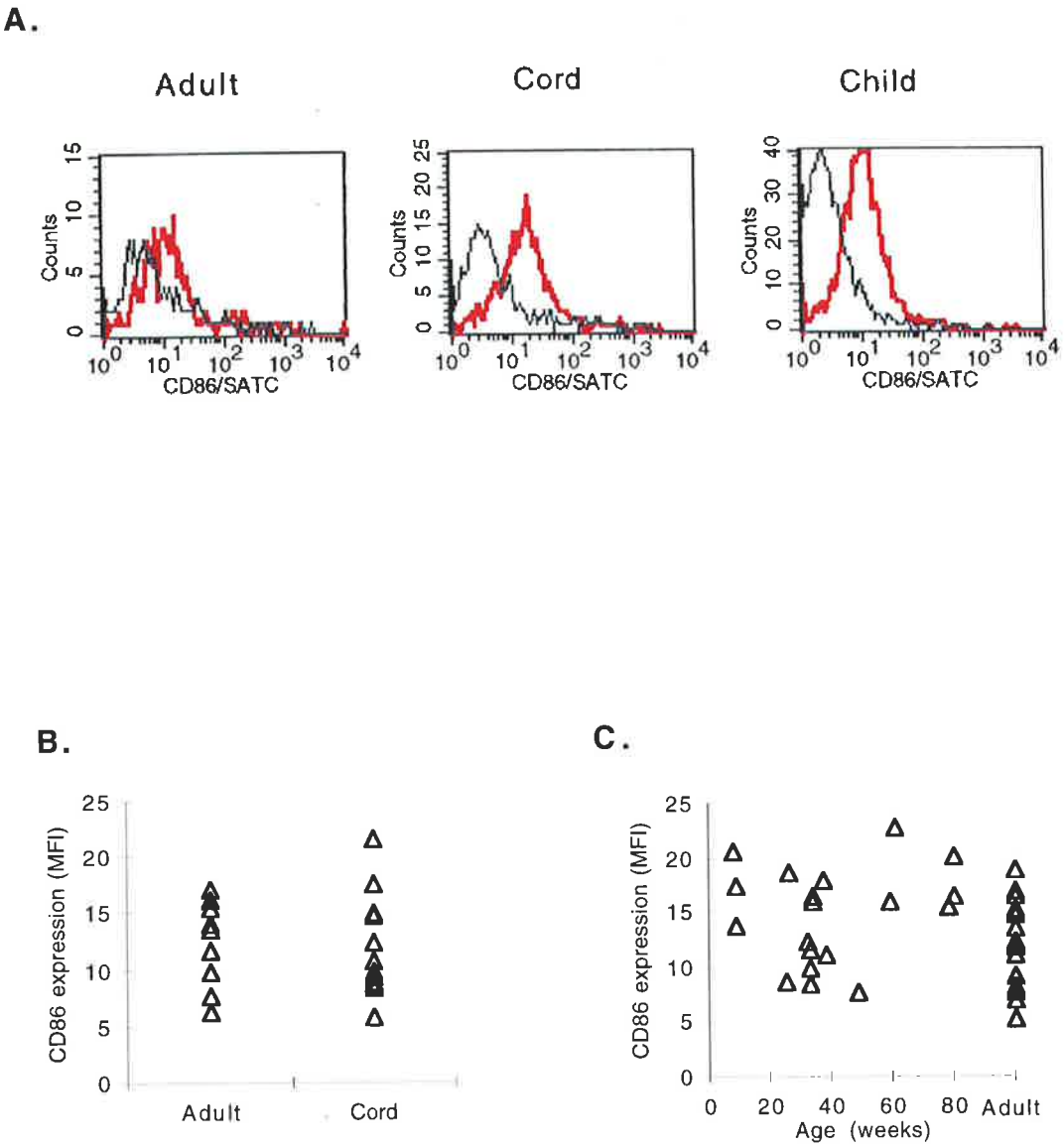


Fig. 4.2 A. CD86 was expressed by resting B cells from cord blood and peripheral blood of adults and young children. Representative histograms from one adult, one cord blood specimen and one child (57 weeks old) are displayed. Staining with CD86 mAb (**red**) and the negative control mAb, X63 (**black**) is shown. The child specimen was not processed on the same day as the adult and cord blood specimens. **B.** There was no significant difference between adult (n = 12) and cord blood (n = 14) B cells in the MFI of CD86 staining. **C.** The MFI of CD86 expression was significantly higher on B cells from children (n = 19) compared with adult B cells (n = 19) ($P < 0.05$). Each point represents an individual specimen. In all experiments, whole blood was stained with purified CD86 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE. B cells were identified with directly conjugated CD19 mAb.

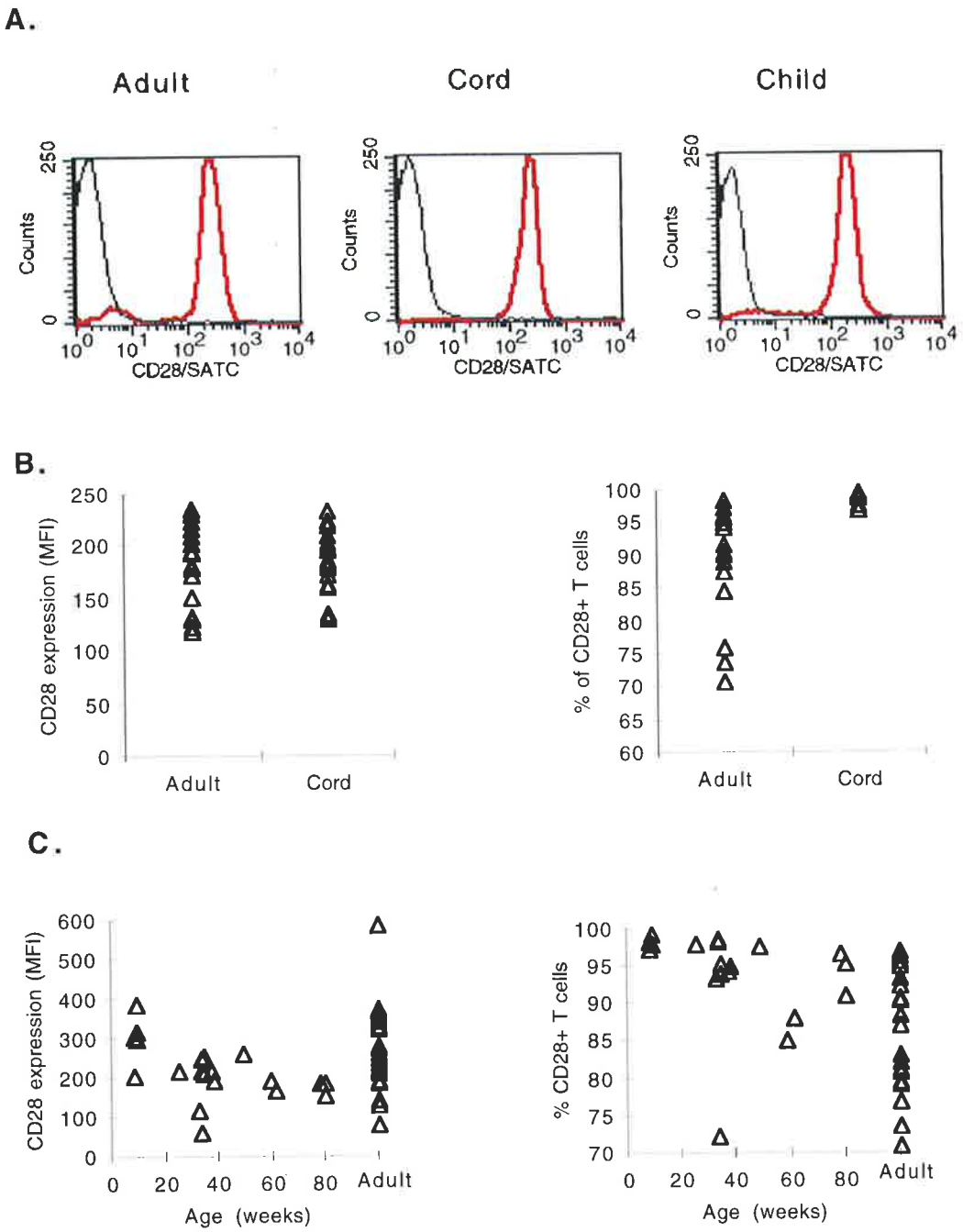


Fig. 4.3 **A.** CD28 was expressed by a large proportion of adult T cells and the majority of T cells in cord blood and peripheral blood from young children. These histograms from one adult, one cord blood specimen and one child (57 weeks old) are representative. Staining with CD28 mAb (**red**) and the negative control mAb, X63 (**black**) is illustrated. The child specimen was not processed on the same day as the adult and cord blood specimens. **B.** A significantly higher percentage of cord blood T cells (n = 21) expressed CD28 compared to adult T cells (n = 17) ($P < 0.01$), although the MFI of CD28 expression was similar. **C.** A significantly higher percentage of T cells from young children (n = 20) expressed CD28 compared with adult T cells (n = 22) ($P < 0.01$), although the MFI of CD28 expression was similar on T cells from adults and children. Each point represents an individual specimen. Whole blood was stained with purified CD28 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE in all of the experiments shown. T cells were identified with directly conjugated CD3 mAb.

CD152

CD152 was not expressed on resting T cells (Fig. 4.4) and was therefore not examined in this series of experiments on unstimulated lymphocytes.

4.2.2 Expression of CD80 and CD86 on activated cord blood B lymphocytes

4.2.2.1 Stimulation with PMA and ionomycin

Adult peripheral blood MC and cord blood MC were stimulated for three days in the presence or absence of PMA and ionomycin. The expression of CD80 and CD86 on B cells was assessed using the high sensitivity method with purified mAb against CD80 and CD86, biotinylated anti-mouse immunoglobulin and streptavidin-PE. For each experiment, one adult specimen was processed in parallel with one cord blood specimen. B cells were identified using directly conjugated mAb to CD19. B cell activation was indicated by upregulation of the activation marker, CD25 (Fig. 4.5).

CD80

There was significant induction of CD80 expression on adult ($P < 0.01$) and cord blood B cells ($P < 0.01$) after 3 days of stimulation with PMA and ionomycin (Fig. 4.6A). There was no significant difference in the MFI of CD80 expression on activated cord blood B cells ($n = 8$) compared with activated adult B cells ($n = 8$) ($P = 0.50$) (Fig. 4.6A). Kinetics of CD80 expression were similar on adult ($n = 4$) and cord blood B cells ($n = 4$) (Fig. 4.6B), with detectable CD80 expression on B cells from adult and cord blood after 2 days of stimulation.

CD86

CD86 expression was significantly increased on activated adult ($n = 8$) ($P < 0.01$) and activated cord blood B cells ($n = 8$) ($P < 0.01$) compared with unstimulated cells (Fig.

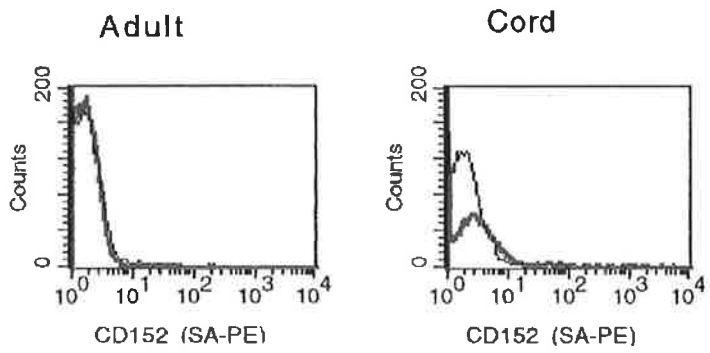


Fig. 4.4 CD152 was not expressed by resting T lymphocytes from adult (n = 4) or cord blood (n = 4), as illustrated in these representative histograms. Mononuclear cells were stained using purified CD152 mAb, anti-mouse immunoglobulin and streptavidin-PE. T cells were identified with directly conjugated CD2 mAb. Staining with CD152 mAb (**red**) and the negative control mAb, SAL5 (**black**), is shown.

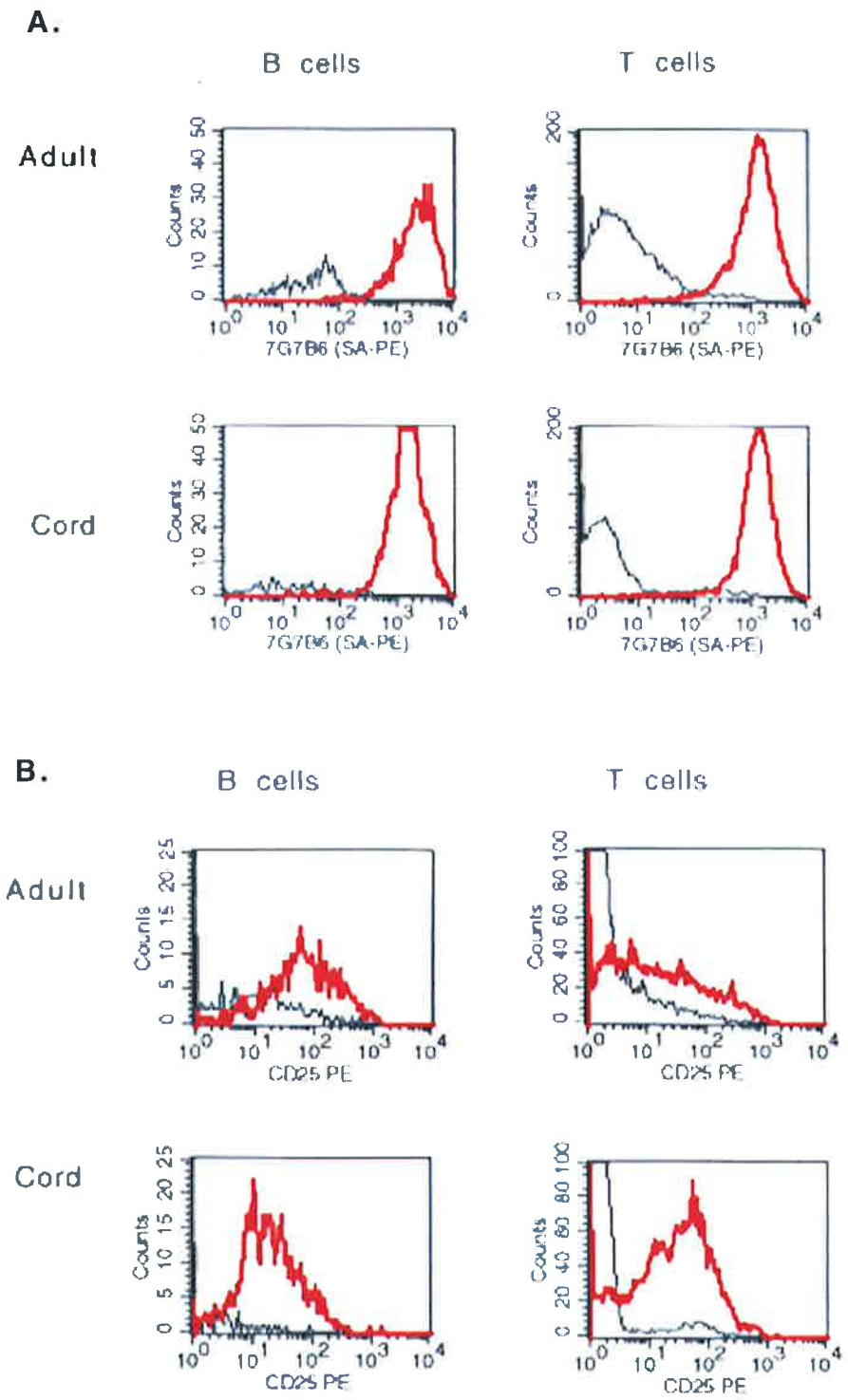


Fig. 4.5 Upregulation of CD25 on adult and cord blood B and T cells stimulated with PMA and ionomycin **(A)** or plate-bound CD3 mAb **(B)** was used as a positive control for lymphocyte activation. Mononuclear cells cultured in the presence (**red**) or absence (**black**) of PMA and ionomycin were stained with 7G7/B6 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-PE. Mononuclear cells cultured in the presence (red) or absence (black) of CD3 mAb were stained with CD25-PE. B cells were identified by staining with directly conjugated CD19 mAb and T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. CD25 expression was assessed in all activation experiments.

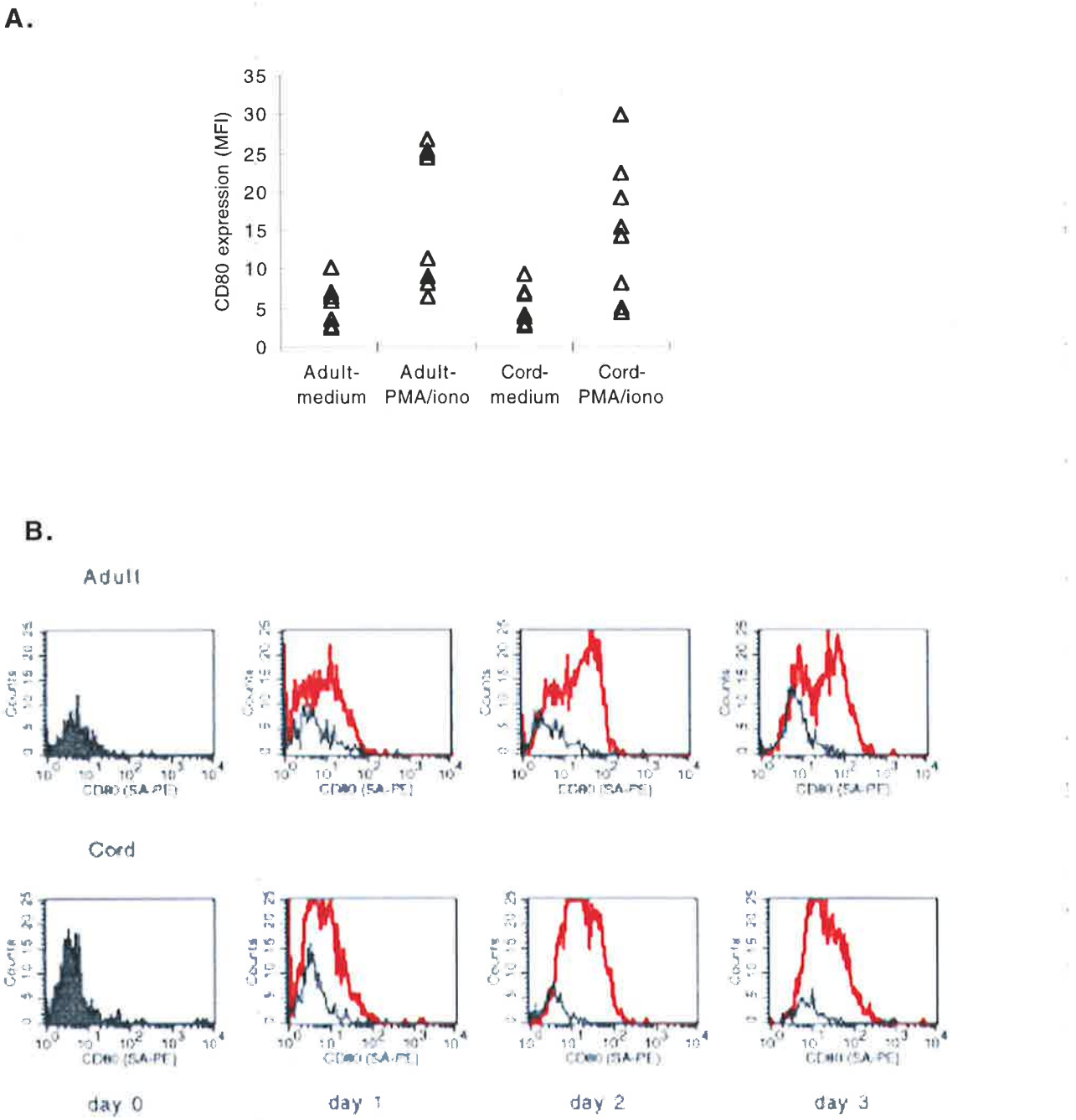


Fig. 4.6 A. CD80 was significantly upregulated on adult and cord blood B cells stimulated for 3 days with PMA and ionomycin compared with unstimulated cells. Mononuclear cells from adult and cord blood, cultured in parallel in the presence or absence of PMA and ionomycin, were stained with CD80 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE. B cells were identified with directly conjugated CD19 mAb. There was no significant difference in the MFI of CD80 expression between activated adult (n = 8) and activated cord blood B cells (n = 8). Each symbol represents one experiment. **B.** The kinetics of CD80 expression were similar on adult and cord blood B cells during the activation period. These histograms are from one adult and one cord blood specimen processed in parallel and are representative of four experiments. Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with PMA and ionomycin (**red**) are shown.

4.7A). The MFI of CD86 expression did not differ significantly between activated cord blood B cells and activated adult B cells (P = 0.33) (Fig. 4.7A). There was no difference in the kinetics of CD86 expression on adult B cells (n = 4) compared with cord blood B cells (n = 4) (Fig. 4.7B). CD86 was upregulated on both adult and cord blood B cells after 1 day of stimulation with PMA and ionomycin. Interestingly, the MFI of CD86 expression also increased slightly on unstimulated cord blood B cells cultured in medium in the absence of stimulation (Fig. 4.7B).

4.2.2.2 Stimulation with plate-bound CD3 mAb

T-dependent B cell activation was modelled by stimulating adult peripheral blood MC and cord blood MC with plate-bound CD3 mAb for 3 days. B cell activation in this system was indicated by upregulation of the activation marker, CD25 (Fig. 4.5). One cord blood specimen and one adult specimen were processed in parallel for each experiment. The expression of CD80 and CD86 on B cells was assessed using biotinylated mAb detected with streptavidin-PE. B cells were identified using directly conjugated mAb to CD19.

CD80

There was significant expression of CD80 on cord blood B cells (n = 8) ($P < 0.01$) and adult B cells (n = 8) ($P < 0.05$) following 3 days of activation in the CD3 system (Fig. 4.8A). There was no significant difference in the MFI of CD80 expression between activated adult B cells and activated cord blood B cells (P = 0.33) (Fig. 4.8A). There was no difference between adult and cord blood B cells in the kinetics of CD80 expression during 3 days of stimulation in the CD3 system (n = 4) (Fig. 4.8B). CD80 expression was evident on adult and cord blood B cells after 2 days of activation in the CD3 system.

CD86

After 3 days of stimulation in the CD3 system, the level of CD86 expression was

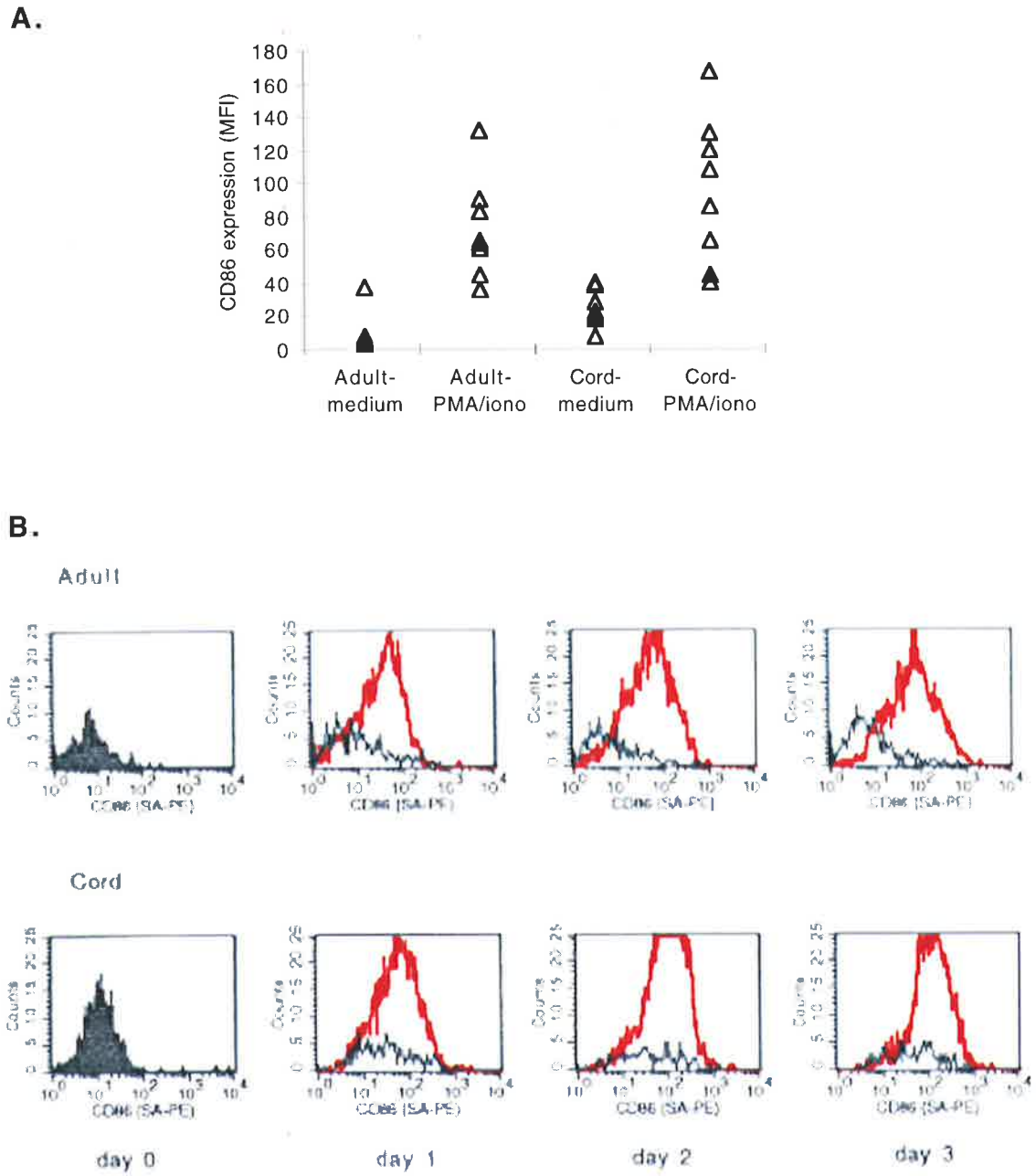


Fig. 4.7 A. CD86 was significantly upregulated on adult and cord blood B cells after 3 days of activation with PMA and ionomycin. Mononuclear cells from adult and cord blood, cultured in parallel in the presence or absence of PMA and ionomycin, were stained with CD86 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE. B cells were identified with directly conjugated CD19 mAb. The MFI of CD86 expression was similar on activated adult (n = 8) and activated cord blood (n = 8) B cells. Each symbol represents one experiment. **B.** Adult and cord blood B cells showed similar kinetics of CD86 expression during 3 days of activation. Histograms for one adult specimen and one cord blood specimen that were processed in parallel are displayed (representative of four similar experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with PMA and ionomycin (**red**) are shown.

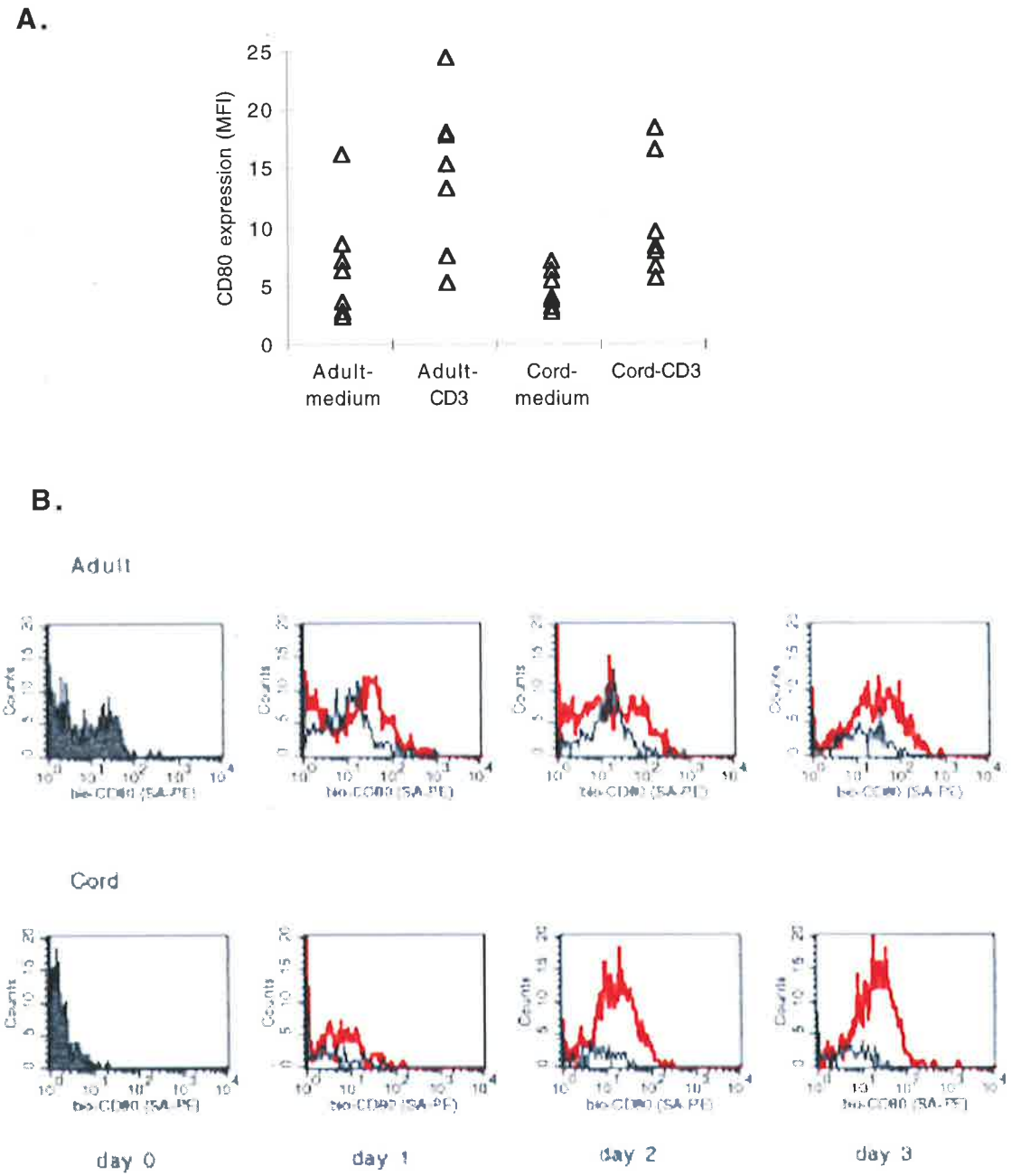


Fig. 4.8 A. CD80 was upregulated on adult and cord blood B cells activated for 3 days in the CD3 system. Mononuclear cells from adult and cord blood were cultured for 3 days in parallel in the presence or absence of plate-bound CD3 mAb, and then stained with biotin-CD80 mAb and streptavidin-PE. B cells were identified with directly conjugated CD19 mAb. The MFI of CD80 expression was similar on adult B cells (n = 8) and cord blood B cells (n = 8) after stimulation. Each symbol represents one experiment. **B.** Adult and cord blood B cells showed similar kinetics of CD80 expression during 3 days of activation in the CD3 system. Histograms for one adult specimen and one cord blood specimen that were processed in parallel are displayed (representative of four experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated in the CD3 system (**red**) are shown.

upregulated on activated adult (n = 8) ($P < 0.01$) and activated cord blood B cells (n = 8) ($P < 0.01$) compared with unstimulated cells (Fig. 4.9A). There was no significant difference in the MFI of CD86 expression between activated adult and activated cord blood B cells (n = 8) ($P = 0.10$) (Fig. 4.9A). Kinetics of CD86 expression were similar on cord blood B cells (n = 4) and adult B cells (n = 4) during 3 days of stimulation in the CD3 system (Fig. 4.9B). An increase in the MFI of CD86 expression on adult and cord blood B cells was demonstrated after 1 day of stimulation.

4.2.3 Expression of CD28 and CD152 on activated cord blood T lymphocytes

4.2.3.1 Stimulation with PMA and ionomycin

Adult peripheral blood MC and cord blood MC were cultured for up to 3 days in the presence or absence of PMA and ionomycin. In each experiment, one adult peripheral blood specimen was processed in parallel with one cord blood specimen. CD28 and CD152 were detected using the high sensitivity staining method with purified mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. (CD3 is downregulated by PMA and therefore was not used as a lineage marker). T cell activation was indicated by upregulation of the activation marker, CD25 (Fig. 4.5).

CD28

There was a significant increase in the percentage of T cells expressing CD28 in activated adult peripheral blood MC (n = 7) ($P < 0.01$) and activated cord blood MC (n = 8) ($P < 0.01$) compared with cells cultured in medium alone (Fig. 4.10A). Whereas in the absence of stimulation, the percentage of T cells expressing CD28 was higher in cord blood MC compared with adult peripheral blood MC ($P < 0.05$), there was no significant difference between activated adult peripheral blood MC and activated cord blood MC in the percentage of CD28 positive T cells ($P = 0.07$) (Fig. 4.10A).

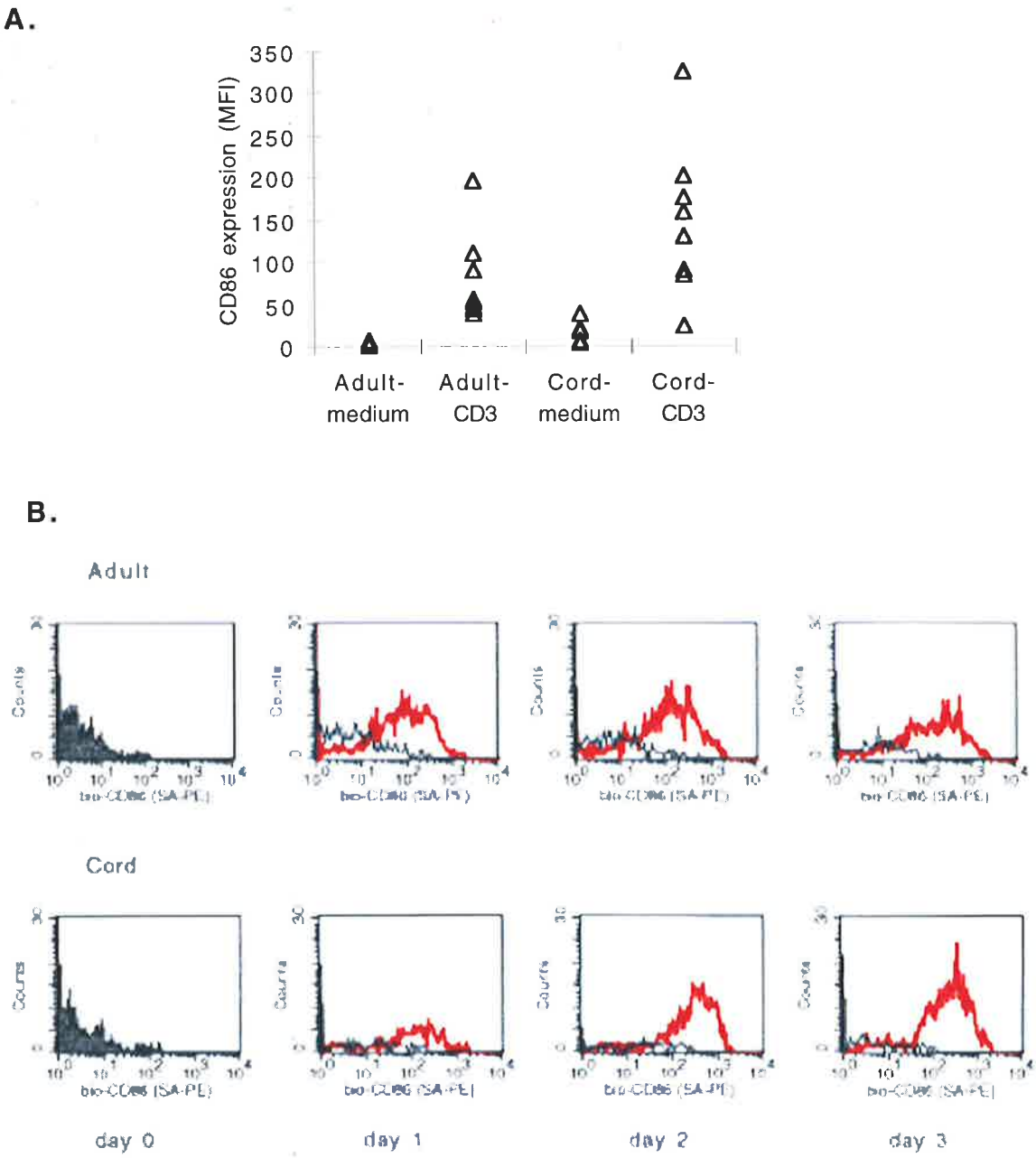


Fig. 4.9 A. CD86 was upregulated on adult and cord blood B cells cultured in the CD3 system for 3 days. Adult and cord blood mononuclear cells, cultured in parallel in the presence or absence of plate-bound CD3 mAb, were stained with biotin-CD86 mAb and streptavidin-PE. B cells were identified using directly conjugated mAb to CD19. There was no significant difference in the MFI of CD86 expression between activated adult (n = 8) and activated cord blood (n = 8) B cells. Each symbol represents one experiment. **B.** The kinetics of CD86 expression were similar on adult and cord blood B cells. One adult and one cord blood specimen that were processed in parallel are shown (representative of four experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated in the CD3 system (**red**) are included.

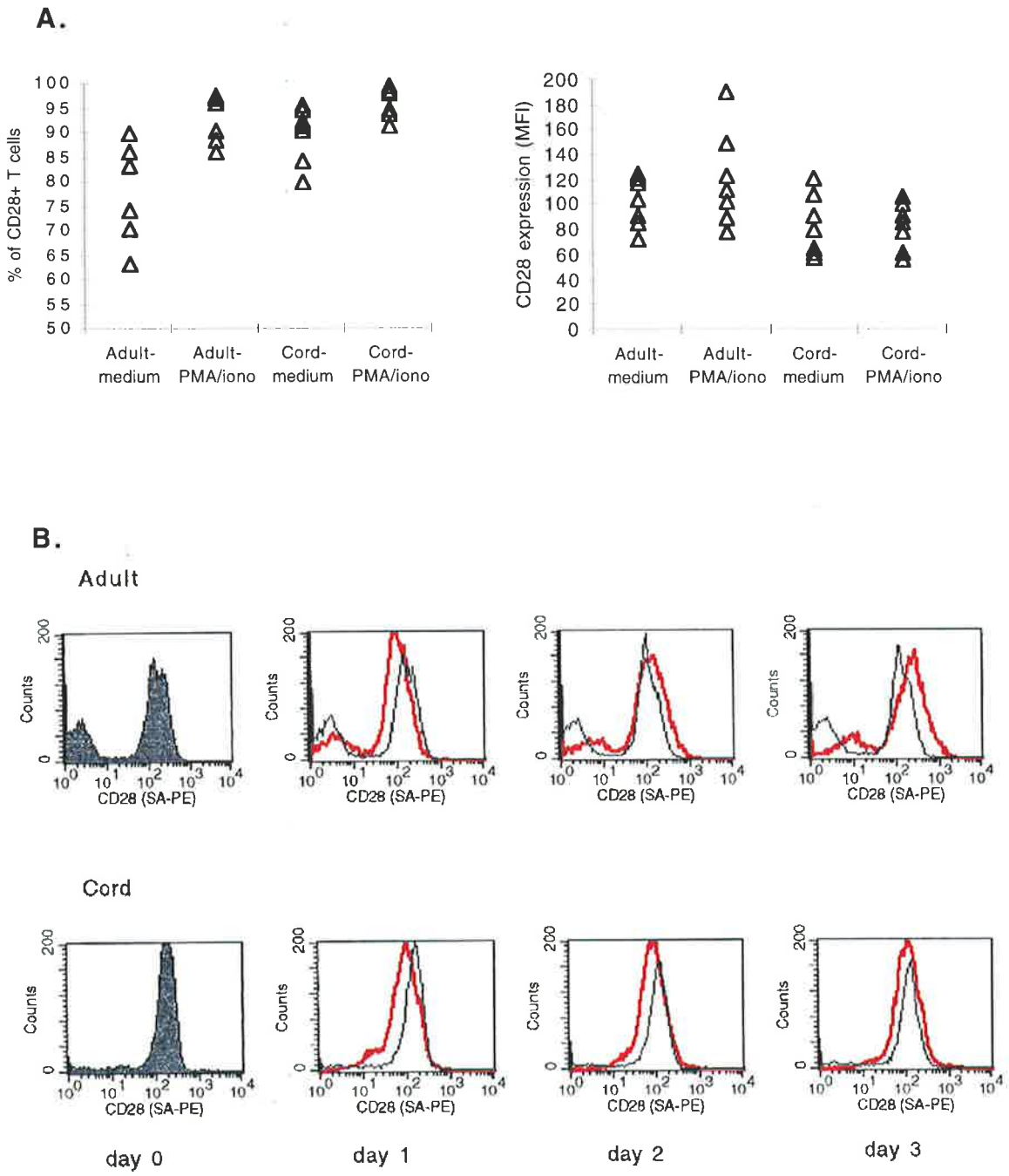


Fig. 4.10 **A.** The percentage of T cells expressing CD28 increased significantly in adult peripheral blood MC (n = 7) and cord blood MC (n = 8) stimulated with PMA and ionomycin for 3 days. Mononuclear cells from adult and cord blood, cultured in parallel in the presence or absence of PMA and ionomycin, were stained with purified CD28 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. The MFI of CD28 expression on adult or cord blood T cells did not increase with 3 days of activation. Each symbol represents one experiment. **B.** The kinetics of CD28 expression are illustrated by one adult specimen and one cord blood specimen (representative of four experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with PMA and ionomycin (**red**) are shown.

After 3 days of culture there was no difference in the MFI of CD28 expression between stimulated and unstimulated T cells from adult blood ($P = 0.54$) or cord blood ($P = 0.88$) (Fig. 4.10A).

The kinetics of CD28 expression were examined during 3 days of stimulation ($n = 4$). In all 4 experiments, the percentage of adult T cells expressing CD28 increased within 1 day of stimulation, whereas the percentage of CD28 positive T cells in activated cord blood MC was only higher than in unstimulated cord blood MC following 3 days of stimulation (Fig. 4.10B). Interestingly, the MFI of CD28 expression on both adult and cord blood T cells decreased after the first day of stimulation with PMA and ionomycin. After 3 days of activation, the MFI of CD28 expression on stimulated T cells increased to a level comparable to that observed on resting T cells.

CD152

There was significant induction of CD152 expression on activated T cells from adult ($n = 9$) ($P < 0.01$) and cord blood ($n = 9$) ($P < 0.05$) after 3 days of stimulation with PMA and ionomycin (Fig. 4.11A). CD152 expression on activated adult T cells was significantly higher than on activated cord blood T cells ($P < 0.01$) (Fig. 4.11A). The kinetics of CD152 expression were examined during 3 days of activation. There was significant upregulation of CD152 on adult ($n = 4$) and cord blood ($n = 4$) T cells after 2 days of stimulation, with no apparent differences in kinetics (Fig. 4.11B).

4.2.3.2 Stimulation with plate-bound CD3 mAb

Plate-bound CD3 mAb was used to induce receptor-mediated T cell activation. T cell activation was indicated by upregulation of the activation marker, CD25 (Fig. 4.5). The expression of CD28 and CD152 on T cells was assessed using biotinylated mAb detected with streptavidin-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. (CD3 could not be used to identify T cells because residual

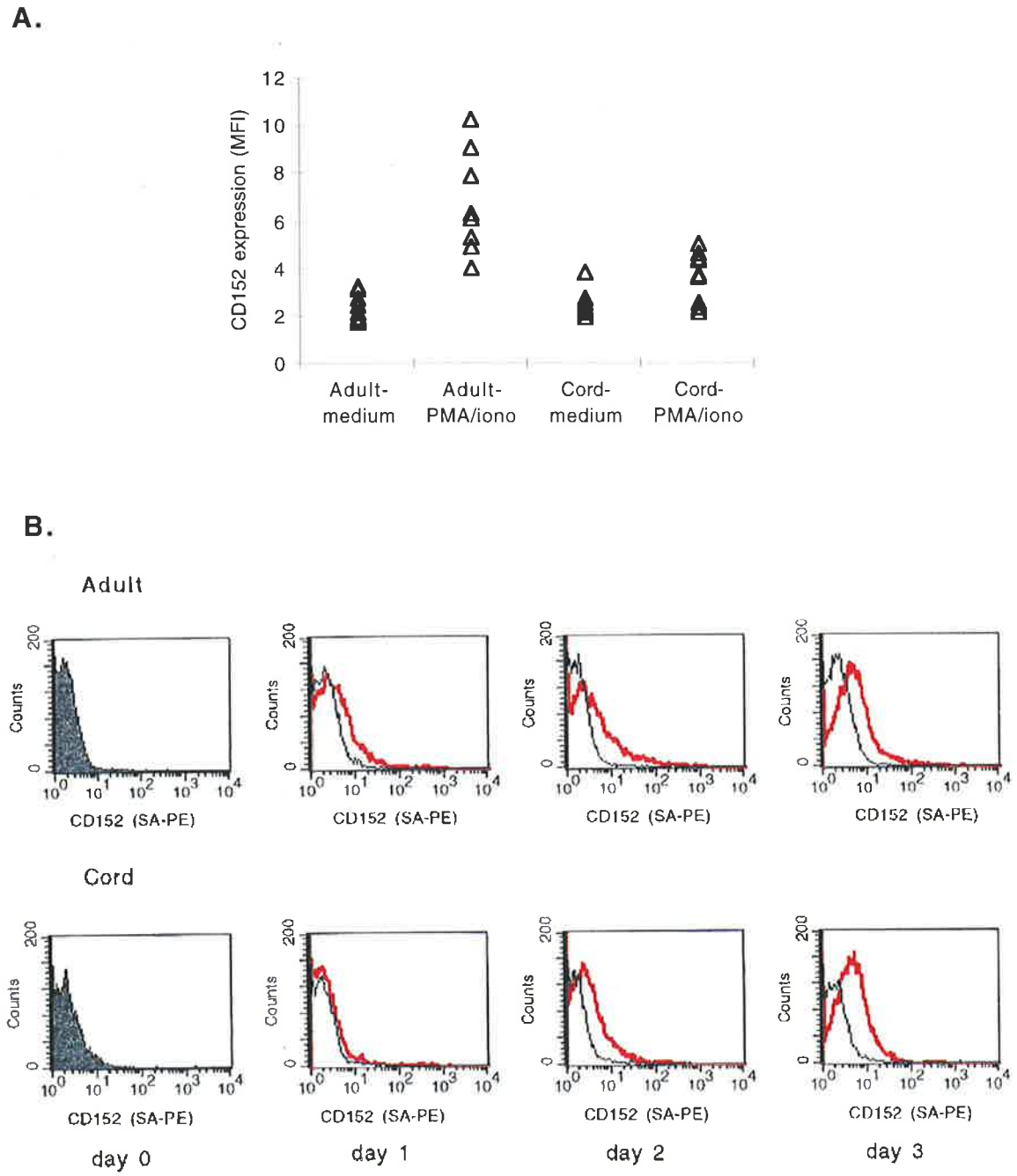


Fig. 4.11 **A.** CD152 expression was induced on adult (n = 9) and cord blood (n = 9) T cells stimulated with PMA and ionomycin for 3 days. Adult and cord blood mononuclear cells, cultured in parallel in the presence or absence of PMA and ionomycin, were stained with purified CD152 mAb, biotinylated anti-mouse immunoglobulin and streptavidin-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. Each symbol represents one experiment. **B.** Adult and cord blood T cells showed similar kinetics of CD152 expression. One adult and one cord blood specimen are displayed and are representative of four similar experiments. Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with PMA and ionomycin (**red**) are shown.

OKT3 bound to surface CD3 after culture would interfere with binding of the directly conjugated CD3 mAb).

CD28

Although the percentage of CD28 positive T cells tended to be higher in adult peripheral blood MC (n = 8) after stimulation with CD3 mAb compared with unstimulated cells, this difference was not significant (P = 0.08). A significantly higher percentage of activated cord blood T cells expressed CD28 (n = 8) (P < 0.01) compared with unstimulated cells (Fig. 4.12A). The percentage of CD28 positive T cells was significantly higher in activated cord blood MC than in activated adult peripheral blood MC (P < 0.01) (Fig. 4.12A).

The MFI of CD28 expression increased significantly on activated cord blood T cells (P < 0.01) but there was no significant increase in the MFI of CD28 expression on activated adult T cells (P = 0.23) compared with cells cultured in medium alone (Fig. 4.12A). The MFI of CD28 expression on activated cord blood T cells was significantly higher than expression on activated adult T cells (P < 0.01) (Fig. 4.12A).

Examining the kinetics of CD28 expression during stimulation with CD3 mAb showed a small increase in the percentage of cord blood T cells (n =3) expressing CD28 after 3 days of activation (Fig. 4.12B), whereas the MFI of cord blood T cell CD28 expression increased from Day 1 of activation.

CD152

There was low but significant upregulation of CD152 on CD3-stimulated adult T cells (P < 0.05) (n = 6) (Fig. 4.13A). However, there was no significant induction of CD152 expression on activated cord blood T cells (n = 6) (P = 0.24) compared to background staining on cells cultured in medium alone (Fig. 4.13A). Examining the kinetics of CD152 expression on adult T cells (n = 3) over 3 days showed upregulation of expression by day 2 (Fig. 4.13B).

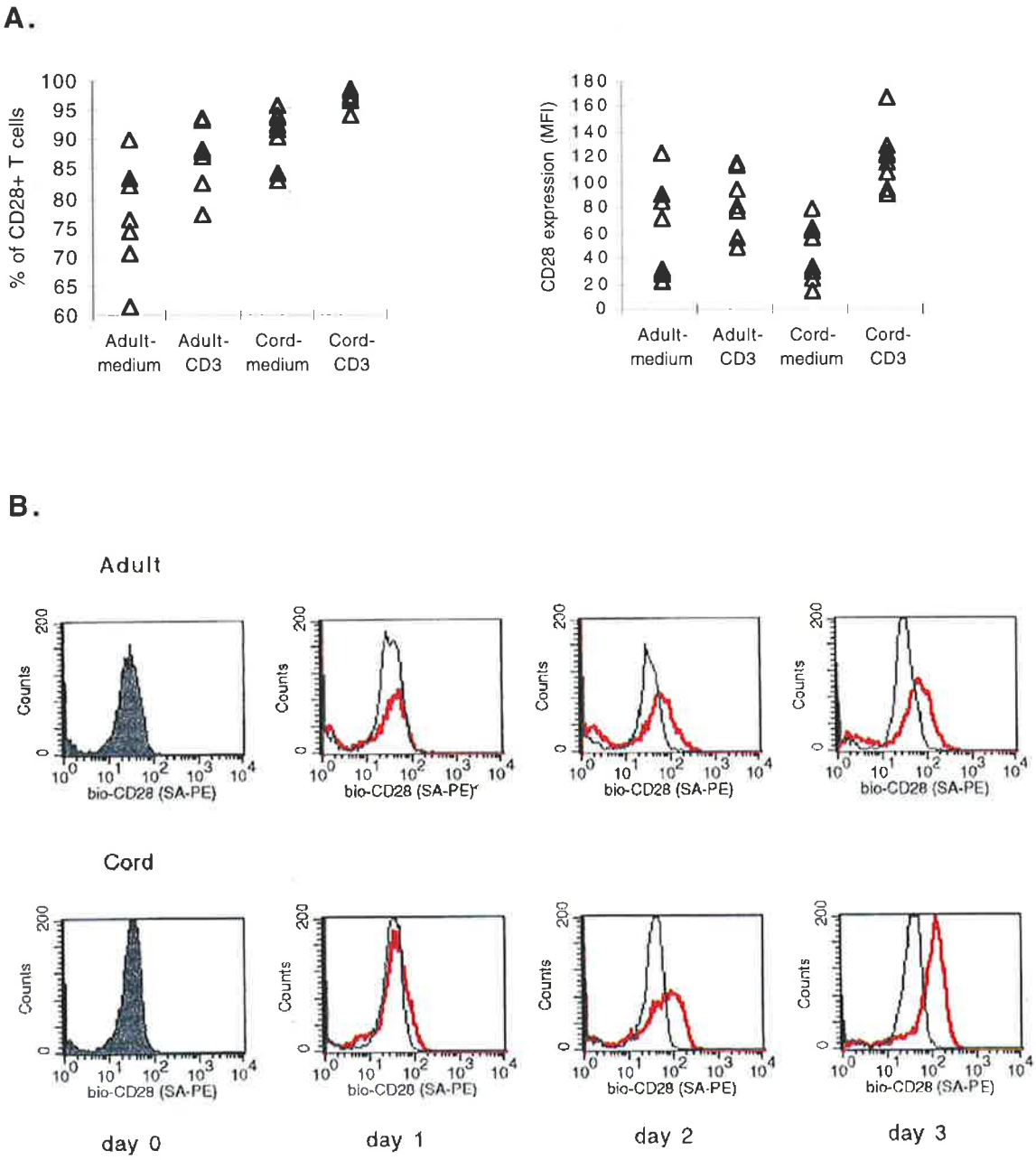
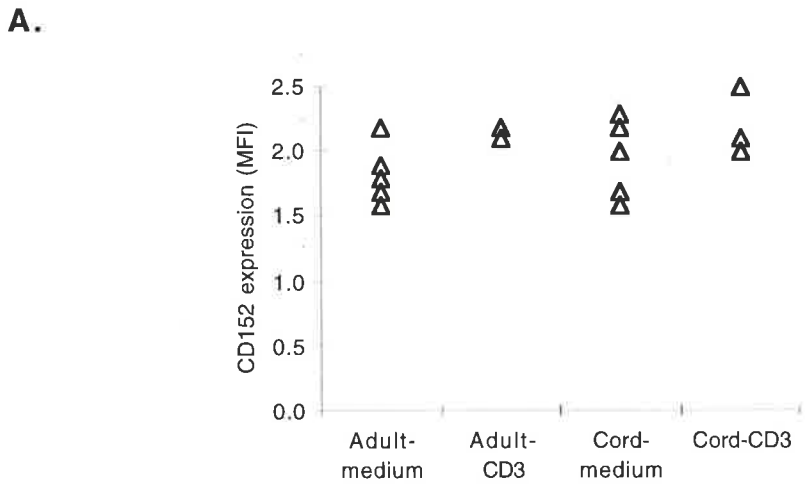


Fig. 4.12 **A.** The percentage of CD28 positive T cells and the MFI of CD28 expression increased significantly in cord blood MC (n = 8) but not adult peripheral blood MC (n = 8) activated with CD3 mAb for 3 days. Adult and cord blood mononuclear cells were cultured in parallel in the presence or absence of plate-bound CD3 mAb, and then stained with biotin-CD28 mAb and streptavidin-PE. T cells were identified as CD2 positive and CD19 negative using directly conjugated mAb. Compared to activated adult peripheral blood MC, activated cord blood MC showed a significantly higher percentage of T cells expressing CD28 ($P < 0.01$), and the MFI of CD28 expression was higher on activated cord blood than activated adult T cells ($P < 0.01$). Each symbol represents one experiment. **B.** The kinetics of CD28 expression on adult and cord blood T cells are illustrated (representative of three experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with CD3 mAb (**red**) are shown.



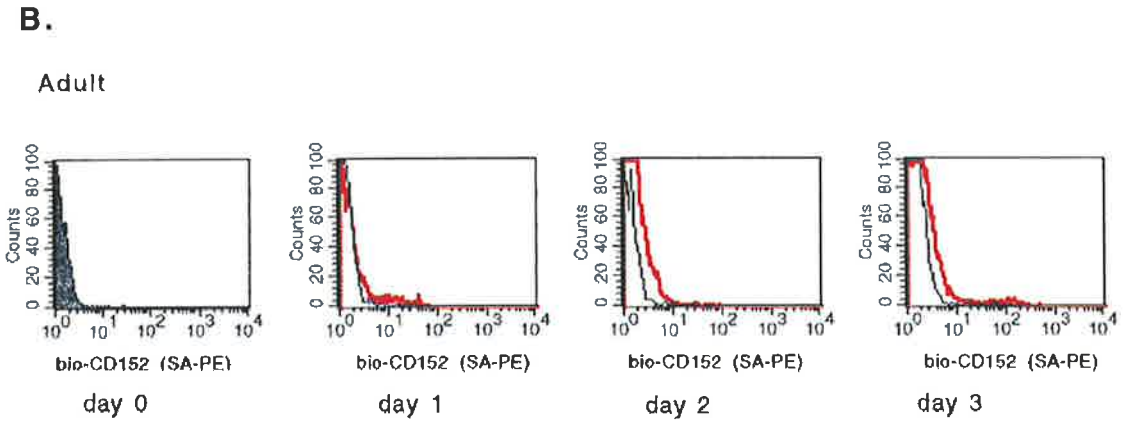


Fig. 4.13 A. Significant CD152 expression was induced on adult T cells (n = 6) but not cord blood T cells (n = 6) after 3 days of CD3 stimulation. Adult and cord blood mononuclear cells were cultured in parallel in the presence or absence of plate-bound CD3 mAb, and then stained with biotin-CD152 mAb and streptavidin-PE. T cells were identified as CD2 positive and CD19 negative using directly conjugated mAb. Each symbol represents one experiment. **B.** The kinetics of CD152 expression for one adult specimen are illustrated (representative of three experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with CD3 mAb (**red**) are shown.

4.2.4 Surface vs Intracellular CD152

Surface and intracellular expression of CD152 were examined on adult and cord blood T cells stimulated for 3 days with PMA and ionomycin. Staining for surface and intracellular CD152 was conducted in parallel on each specimen. CD152 was detected using a biotinylated mAb and streptavidin-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. MAb against nuclear antigens (p105, p53, pCNA, p120, p2.7) were used as positive controls for intracellular staining. These were detected using goat anti-mouse-PE (DDAPE). Unconjugated CD152 mAb were used to block surface CD152 molecules before staining intracellular CD152 with biotin-CD152 mAb. Using standard indirect staining rather than high sensitivity staining, there was no detectable upregulation of surface CD152 on activated cord blood T cells ($n = 5$) ($P = 0.22$) whereas there was significant induction of CD152 expression on the surface of activated adult T cells ($n = 5$) ($P < 0.01$) compared with unstimulated cells (Fig. 4.14A, Fig. 4.14B). Positive intracellular staining for the nuclear antigens was demonstrated (Fig. 4.14A). Intracellular CD152 was expressed at significantly higher levels compared with surface CD152 by both adult ($P < 0.01$) and cord blood T cells ($P < 0.01$) (Fig. 4.14A, Fig. 4.14B). Similar levels of intracellular CD152 were found in activated adult and cord blood T cells ($P = 0.69$) (Fig. 4.14A, Fig. 4.14B).

4.3 Discussion

The results of these studies showed that cord blood B cells express adult levels of CD80 and CD86. This suggests that neonatal B cells should be capable of delivering costimulatory signals to T cells via CD28 and CD152. Interestingly, the MFI of CD86 expression was significantly higher on peripheral blood B cells from young children than adult peripheral blood B cells. This may reflect *in vivo* upregulation of B cell CD86 expression during a period of high immune stimulation (ie. weaning, immunisation, primary exposure to infection).

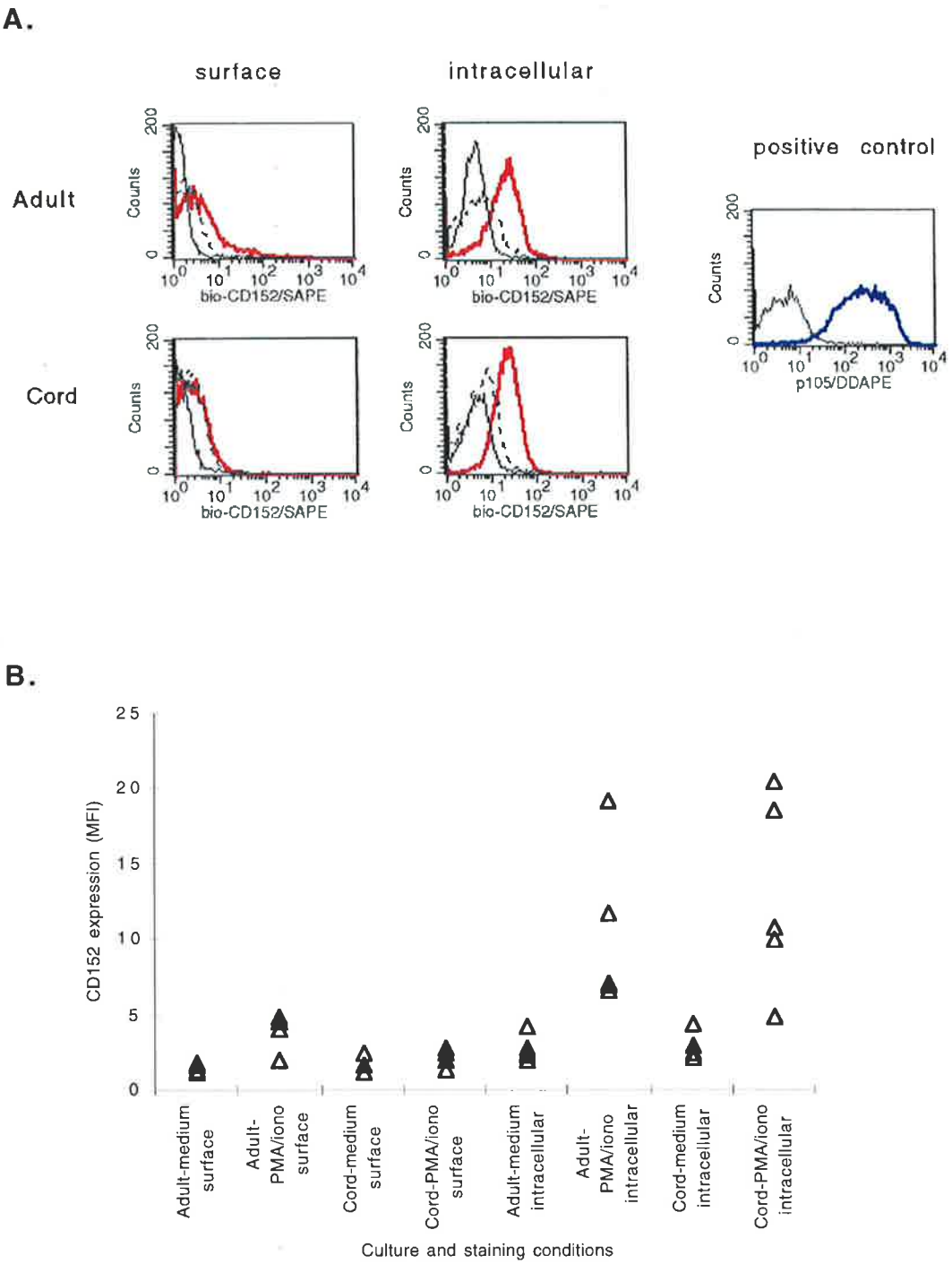


Fig. 4.14 **A.** Surface and intracellular CD152 expression on adult and cord blood T cells cultured in the presence (**red**) or absence (**black**) of PMA and ionomycin for 3 days. Concurrent surface (**black**) and intracellular (**blue**) staining of adult cells with mAb against the nuclear antigen, p105, demonstrated effective intracellular staining. Surface and intracellular staining of activated cells with the negative control mAb are also shown (dashed line). Histograms for one adult and one cord blood specimen are shown and are representative of five experiments. **B.** Intracellular CD152 expression was significantly higher than surface CD152 expression on adult (n = 5) and cord blood (n = 5) T cells. Adult and cord blood T cells expressed similar levels of intracellular CD152. There was significant surface expression of CD152 on activated adult but not activated cord blood T cells. Each symbol represents one experiment.

The proportion of resting T cells that expressed CD28 was higher in cord blood and peripheral blood from children than in adult peripheral blood. This is consistent with results from a previous study which showed that the proportion of CD28 positive T cells declines throughout life (Hoshino *et al.*, 1993). In normal individuals CD28 negative T cells fall predominantly within the CD8 positive subset (Azuma *et al.*, 1993b). CD8 positive CD28 negative T cells fail to proliferate in response to mitogens but possess cytotoxic activity, expand in response to viral infection (Haffar *et al.*, 1995; Lewis *et al.*, 1994) and express high levels of adhesion molecules (Azuma *et al.*, 1993b; Kern *et al.*, 1996). It has been suggested that they represent a population of terminally differentiated effector T cells which would explain their low frequency in neonates and young children (Azuma *et al.*, 1993b).

Following stimulation with PMA and ionomycin, the percentage of CD28 positive adult T cells increased to a level similar to that seen in neonates. This may reflect induction of CD28 on CD28 negative T cells in adults, or preferential expansion of the CD28 positive population. This latter explanation would be consistent with reports that CD28 negative T cells proliferate poorly in response to many forms of mitogenic stimulation (Azuma *et al.*, 1993b; Haffar *et al.*, 1995; Lewis *et al.*, 1994).

Interestingly, activation with PMA and ionomycin initially decreased the MFI of CD28 expression on both adult and cord blood T cells, and after 3 days of activation there was no difference in the level of CD28 expression between stimulated and unstimulated T cells from adult or cord blood. Down-regulation of CD28 mRNA expression within 6 hours of stimulation with PMA and ionomycin has been reported previously (Lindsten *et al.*, 1993). In addition, surface expression of CD28 on activated T cells is transiently downregulated by ligation (Linsley *et al.*, 1993). Since the whole mononuclear cell population was stimulated in the present study, down-modulation of CD28 expression may have resulted from the interaction of CD28 positive T cells with activated B cells expressing high levels of CD80 and CD86.

Neither the percentage of cells expressing CD28 nor the MFI of CD28 expression changed significantly on adult T cells stimulated with plate-bound CD3 mAb, whereas there was a significant increase in the percentage of CD28 positive cord blood T cells and the MFI of CD28 expression on activated cord blood T cells. Adult T cells were obviously responsive to CD3 stimulation, based on upregulation of the activation marker, CD25, so it appears that CD28 expression on cord blood T cells stimulated through the T-cell receptor complex may be differentially regulated.

CD28 on neonatal T cells is functional, since signalling via CD28 can enhance proliferation and cytokine secretion by murine (Adkins *et al.*, 1994) and human (Hassan *et al.*, 1995; King *et al.*, 1995; Kuiper *et al.*, 1994; Webb & Feldmann, 1995) neonatal T cells stimulated *in vitro* with mitogens, CD3 mAb or CD2 mAb. Naive CD45RO negative T cells from adults require a higher density of CD28 cross-linking than memory CD45RA negative T cells to induce proliferation and IL-2 secretion (Kuiper *et al.*, 1994). It is possible that the higher levels of CD28 expression on activated cord blood T cells compared with activated adult T cells demonstrated in the present study may allow increased cross-linking to provide the level of signalling required for effective activation of naive neonatal T cells.

The MFI of surface CD152 expression was lower on activated cord blood T cells compared with activated adult T cells stimulated with PMA and ionomycin. Since CD152 operates as a negative regulator of T cell activity (Krummel & Allison, 1995; Krummel & Allison, 1996; Walunas *et al.*, 1996; Walunas *et al.*, 1994), this may have implications for the activation of neonatal T cells. It is believed that the balance of signals from CD28 and CD152 ligation regulates the response of T cells to T cell receptor ligation (Krummel & Allison, 1995), therefore lower expression of CD152 may allow a stronger costimulatory signal through CD28 to activate neonatal T cells. Alternatively, since CD152 expression is CD28-dependent (Lindsten *et al.*, 1993; Walunas *et al.*, 1994), lower expression of CD152 may reflect altered signalling via CD28 on neonatal T lymphocytes.

Low expression of CD152 on adult T cells and absent expression on cord blood T cells stimulated with plate-bound CD3 mAb suggests that this method of stimulation may be insufficient to induce high levels of surface CD152 expression. It should be noted, however, that CD152 was stained using a high sensitivity technique for cells stimulated with PMA and ionomycin, whereas standard indirect immunofluorescence was performed when CD3 stimulation was used.

It is now recognised that CD152 is expressed only at low levels on the cell surface and is predominantly an intracellular molecule (Waterhouse *et al.*, 1996). Parallel staining of surface and intracellular CD152 in adult and cord blood T cells confirmed this. Interestingly, the level of intracellular CD152 in activated cord blood T cells was similar to levels seen in activated adult T cells, even though surface expression on cord blood T cells was low or absent. This suggests that neonatal T cells are capable of producing CD152 at adult levels, but that transport of CD152 to the cell surface and cell surface expression may be differentially regulated.

The balance of CD28 and CD152 signals may regulate the differentiation of CD4 positive T cells into Th1 and Th2 secreting cells. The differentiation of naive murine T cells into Th2 secreting cells is dependent on CD28 signalling (Rulifson *et al.*, 1997; Schweitzer & Sharpe, 1998). Similarly, repetitive stimulation of human CD4 positive T cells from cord blood with soluble CD3 mAb and CD28 mAb promotes their development into cells which preferentially secrete IL-4 and IL-5 (Yang *et al.*, 1995). Secretion of IL-4 and Il-5 by CD4 positive neonatal T cells is enhanced, and production of IFN-γ is slightly reduced if the T cells are primed in the presence of antigen presenting cells expressing increased levels of CD80 (Yang *et al.*, 1995). In contrast, signals through CD152 may limit Th2 differentiation (Oosterwegel *et al.*, 1999). Naive CD4 positive T cells from CD152 deficient mice differentiate into Th2 secreting cells when stimulated with antigen and wild-type antigen presenting cells, producing large amounts of IL-4, IL-5 and IL-10 (Oosterwegel *et al.*, 1999). Treatment of CD152/CD80/CD86 deficient mice with CD28 mAb *in vivo* also leads to Th2 dominated responses, whereas this is not observed in

wild-type mice treated with CD28 mAb (Oosterwegel *et al.*, 1999). Observations in the current study, of higher levels of CD28 expression and lower levels of CD152 expression on activated cord T cells compared to adult T cells, may suggest an explanation for the Th2 deviation of neonatal CD4 positive T cells.

In conclusion, it appears that there are several differences in the way the expression of this family of costimulatory molecules is regulated on lymphocytes from neonates and young children compared with adult lymphocytes. However, these differences do not explain the reported limitations of neonatal antibody responses. CD80, CD86 and CD28 are all expressed at adult levels or higher on cord blood lymphocytes, suggesting that this pathway is operative in the human neonate. Since the T cell response is regulated by the balance of signals received from CD28 and CD152 (Krummel & Allison, 1995), high levels of CD28 expression and lower surface expression of CD152 on cord blood T cells may indicate that neonatal T cells are more capable of responding to activation by antigen presenting cells. The CD80/CD86-CD28 pathway has been shown to play an important role in the activation of naive lymphocytes (Hassan *et al.*, 1995). Contrary to expectations, it is possible that the regulation of CD28 and CD152 on neonatal T cells is specialised to promote T cell activation.

CHAPTER 5.

CD40 AND CD154 EXPRESSION ON LYMPHOCYTES FROM NEONATES AND YOUNG CHILDREN

5.1 Introduction

CD40 and its ligand, CD154, play a critical role in primary T-dependent antibody responses, isotype switching, induction and maintenance of germinal centres, affinity maturation and memory B cell differentiation (Banchereau *et al.*, 1994; Callard *et al.*, 1993; Foy *et al.*, 1994; Gray *et al.*, 1996; Gray *et al.*, 1994b; Splawski *et al.*, 1993). Altered activity of this pair of costimulator molecules in neonates and young children could contribute to limitations of T-dependent antibody responses in this age group.

CD40 expression by neonatal B cells has been reported to be equivalent to adult B cell expression (Durandy *et al.*, 1995). However, there have been no studies of the regulation of CD40 on activated neonatal B cells. Neonatal B cells have a diminished capacity to secrete antibodies of downstream isotype in response to CD40 ligation, although a similarly poor response is seen in naive adult B cells stimulated via CD40 (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1993). This may be related to differential regulation of CD40 expression on naive B cells during activation and the studies described in this chapter examined this possibility. CD40 expression was first examined on resting B cells from cord blood and peripheral blood of adults and young children. The regulation of CD40 expression on adult and cord blood B cells during *in vitro* activation was then investigated. PMA and ionomycin were used to provide a strong mitogenic B cell stimulus (Berry *et al.*, 1989; Callard *et al.*, 1992) and stimulation of mononuclear cells with plate-bound CD3 mAb was used to model T-dependent B cell activation (Hirohata *et al.*, 1988).

Several studies have examined the induction of CD154 on activated neonatal T cells, however, the results are conflicting (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1996). Neonatal T cell expression of CD154 appears to be influenced by the method of *in vitro* stimulation used. Neonatal T cells express minimal amounts of CD154 when activated with PMA and ionomycin (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). In contrast, it has been shown that neonatal CD4 positive T cells activated with plate-bound CD3 mAb and supplemental IL-2 or IL-4 can express CD154 at similar levels to adult CD4 positive T cells activated with plate-bound CD3 mAb alone (Splawski *et al.*, 1996). The studies described in this chapter compared the induction of CD154 expression on adult and cord blood T cells stimulated with either PMA and ionomycin or plate-bound CD3 mAb.

In view of the documented influence of B cells on surface CD154 expression by T cells (Hermann *et al.*, 1993; Ludewig *et al.*, 1996; Nusslein *et al.*, 1996; van Kooten *et al.*, 1994; Yellin *et al.*, 1994), cord blood T cell CD154 expression was compared to adult T cell expression in whole mononuclear cell fractions or B cell/monocyte depleted mononuclear fractions stimulated with CD3 mAb.

In all studies, CD154 expression was examined at multiple time-points to assess whether the kinetics of expression differed between adult and cord blood T cells.

Reports as to the association of CD154 expression with CD45RA positive and CD45RO positive T cell subsets are conflicting (Brugnoni *et al.*, 1994; Lane *et al.*, 1992; Nonoyama *et al.*, 1995; Patel *et al.*, 1996). Since different periods of stimulation were used in each of these studies, different kinetics of CD154 expression on the two subsets could account for the conflicting results and experiments were performed in the current study to investigate this.

5.2 Results

5.2.1 Expression of CD40 on resting cord blood B cells and peripheral blood B cells from young children

The expression of CD40 on resting B cells from cord blood and peripheral blood of children between 2 and 20 months of age was compared with expression on unstimulated adult peripheral blood B cells. The whole blood method of staining was used. CD40 was detected by 3-stage high sensitivity immunofluorescence using G28/5 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE. B cells were identified using directly conjugated mAb to CD19.

All B cells from adults, children and cord blood expressed CD40 (Fig. 5.1A). CD40 was expressed at high levels on cord blood B cells from a small number of neonates, however, in the majority of neonates (n = 21), the MFI of B cell CD40 expression was comparable with levels seen on adult B cells (n = 16) (Fig. 5.1A, Fig. 5.1B). There was no significant difference (P = 0.42) in the MFI of CD40 expression between resting B cells from adults (n = 20) and B cells from young children (n = 20) (Fig. 5.1A, Fig. 5.1C).

5.2.2 Expression of CD40 on activated cord blood B lymphocytes

5.2.2.1 Stimulation with PMA and ionomycin

To compare the regulation of CD40 expression on adult and cord blood B cells during *in vitro* activation, mononuclear cells were cultured in the presence or absence of PMA and ionomycin. The expression of CD40 on B cells was assessed by high sensitivity staining using G28/5 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-PE. B cells were identified using directly conjugated mAb to CD19. After 3 days of culture, there were significant differences in the MFI of CD40 expression between unstimulated

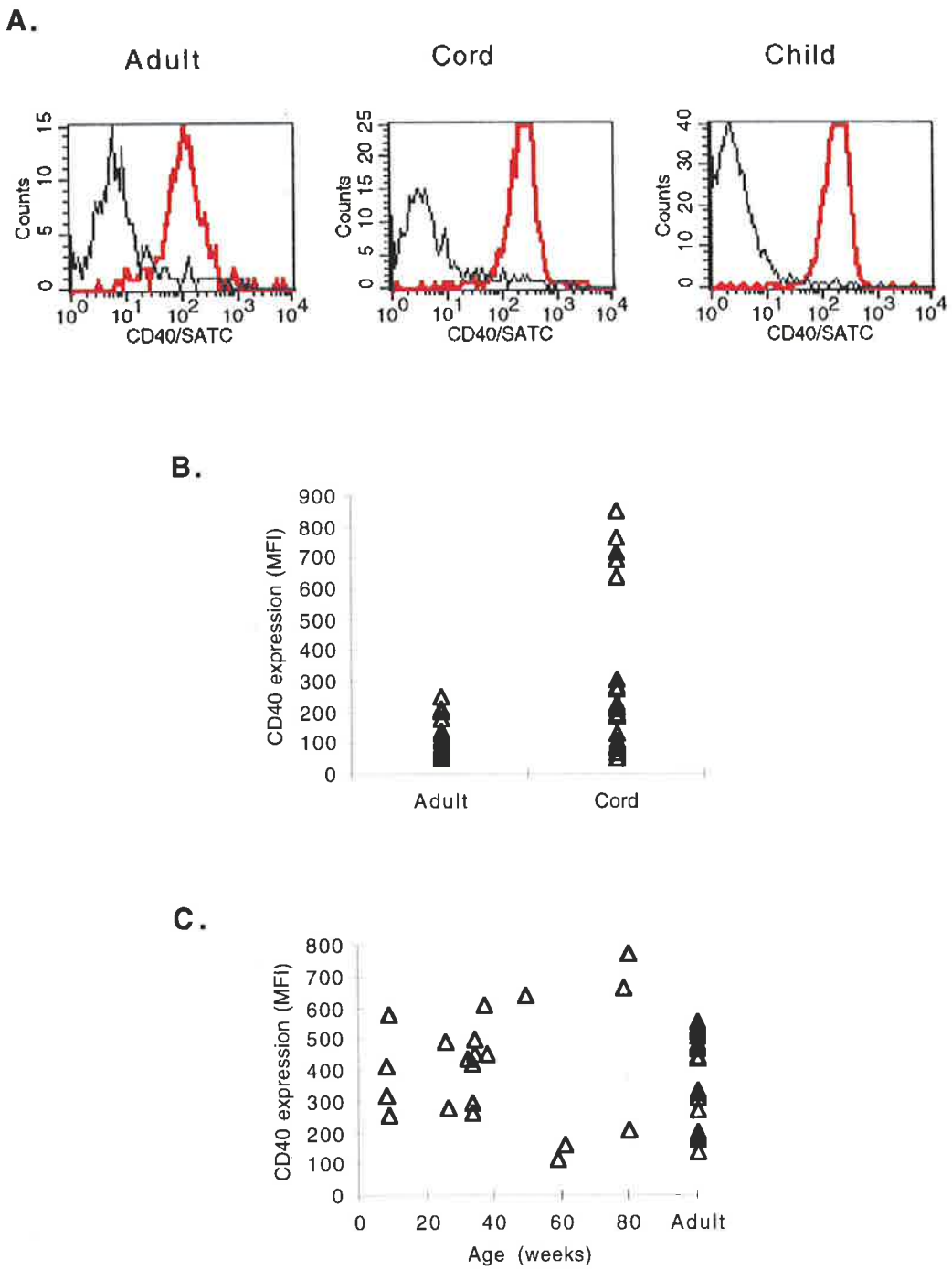


Fig. 5.1 A. CD40 was expressed on all resting B cells from cord blood and peripheral blood of adults and young children. Representative histograms from one adult, one cord blood specimen and one child (57 weeks old) are shown. Staining of B cells with CD40 mAb (**red**) and the negative control mAb, X63 (**black**), is illustrated. The child specimen was not processed on the same day as the adult and cord blood specimens. **B.** CD40 expression was similar on resting adult B cells (n = 16) and resting B cells from most cord blood specimens (n = 21). **C.** There was no difference in the MFI of CD40 expression on resting B cells from children (n = 20) compared with resting adult B cells (n = 20). Each point represents one specimen. In all these experiments, whole blood was stained with G28/5 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-Cy5/PE. B cells were identified with directly conjugated mAb to CD19.

and activated B cells in cultures of adult peripheral blood MC (n = 8) ($P < 0.01$) and cord blood MC (n = 10) ($P < 0.01$) (Fig. 5.2A). The MFI of CD40 expression was significantly higher ($P < 0.05$) on activated cord blood B cells compared with activated adult B cells (Fig. 5.2A). Interestingly, the MFI of CD40 expression on B cells from 2 adults was extremely high (specimen 1 - medium: 2924.2, PMA/ionomycin: 8477.2; specimen 2 - medium: 452.4, PMA/ionomycin: 6578.0). The individuals were apparently healthy at the time of venipuncture. These specimens were excluded from the overall analysis.

The kinetics of CD40 expression on adult (n = 3) and cord blood (n = 4) B cells were examined during 3 days of stimulation with PMA and ionomycin (Fig. 5.2B). This demonstrated upregulation of CD40 expression on cord blood B cells by day 2 of stimulation with further upregulation by day 3. Interestingly, the MFI of CD40 expression on adult B cells decreased after 1 day of stimulation with PMA and ionomycin relative to CD40 expression on unstimulated or resting adult B cells. There was not a similar decrease in the MFI of CD40 expression observed on stimulated cord blood B cells at day 1. Generally adult B cells showed only a small increase in the MFI of CD40 expression by day 2 and failed to achieve the levels of CD40 expression seen on cord blood B cells after 3 days of stimulation.

5.2.2.2 Stimulation with plate-bound CD3 mAb

T-dependent B cell activation was modelled using plate-bound CD3 mAb. B cell activation in this system was confirmed by upregulation of the activation marker, CD25. The expression of CD40 on B cells was assessed using biotin-CD40 mAb (G28/5) and streptavidin-PE. B cells were identified using directly conjugated mAb to CD19. After 3 days of culture, the MFI of CD40 expression was significantly upregulated on activated adult (n = 10) ($P < 0.01$) and activated cord blood B cells (n = 10) ($P < 0.01$) compared to unstimulated B cells (Fig. 5.3A). There was no significant difference between the MFI

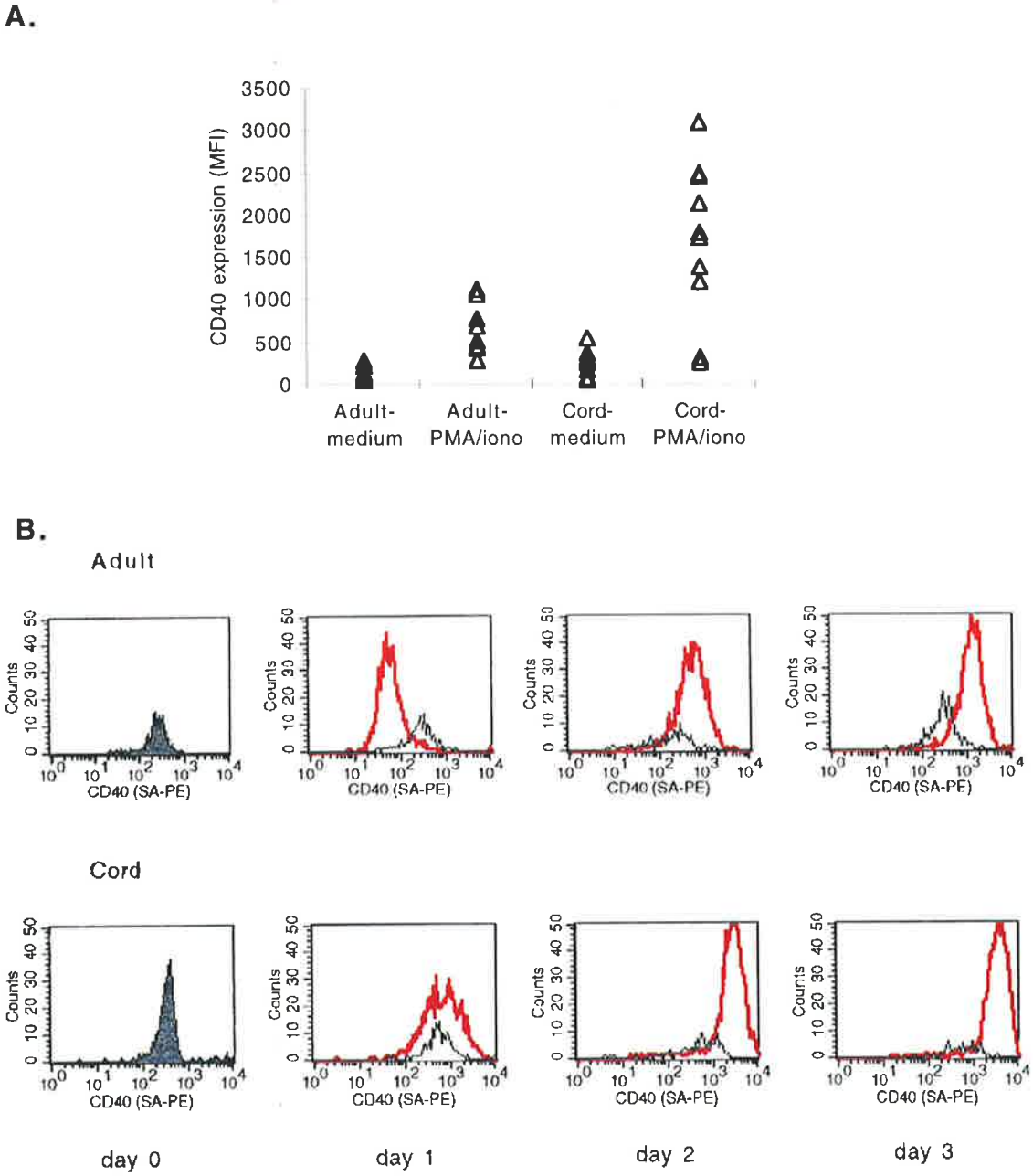


Fig. 5.2 **A.** CD40 expression was significantly upregulated on adult and cord blood B cells after 3 days of activation with PMA and ionomycin. Adult and cord blood mononuclear cells were cultured in the presence or absence of PMA and ionomycin and then stained with G28/5 supernatant, biotinylated anti-mouse immunoglobulin and streptavidin-PE. B cells were identified with directly conjugated mAb to CD19. The MFI of CD40 expression was significantly higher ($P < 0.05$) on activated cord blood B cells ($n = 10$) compared with activated adult B cells ($n = 8$). Each symbol represents one experiment. **B.** The kinetics of B cell CD40 expression during 3 days of activation are shown for one adult specimen and one cord blood specimen that were processed in parallel (representative of 3 adult specimens and 4 cord blood specimens). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated with PMA and ionomycin (**red**) are shown.

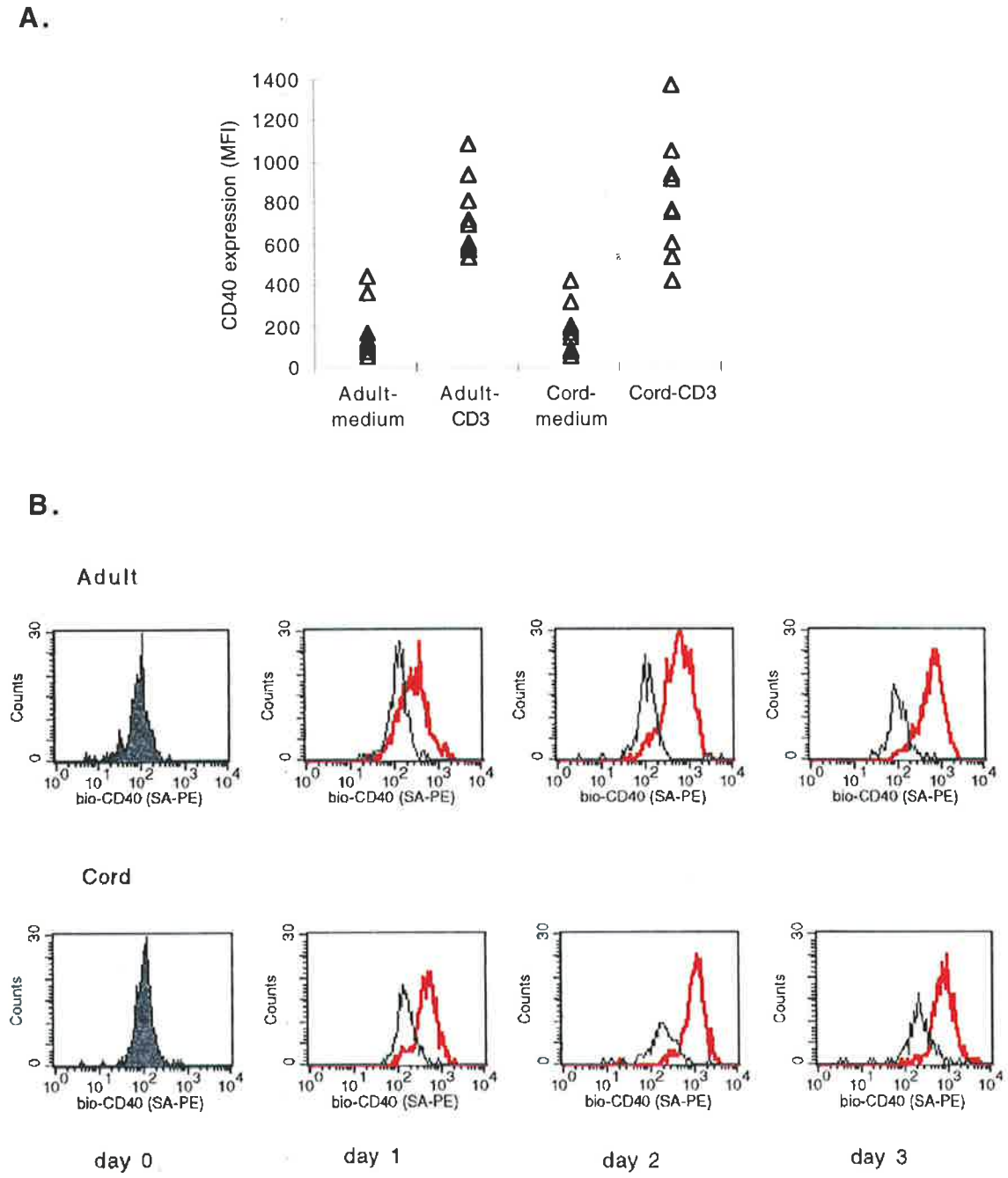


Fig. 5.3 A. CD40 expression was upregulated on adult and cord blood B cells cultured in the CD3 system for 3 days. Adult and cord blood mononuclear cells cultured in parallel in the presence or absence of plate-bound CD3 mAb were stained with biotin-CD40 mAb and streptavidin-PE. B cells were identified with directly conjugated mAb to CD19. There was no significant difference in the MFI of CD40 expression between activated adult (n = 10) and activated cord blood (n = 10) B cells. Each symbol represents one experiment. **B.** The kinetics of CD40 expression were similar for adult and cord blood B cells. One adult and one cord blood specimen that were processed in parallel are shown (representative of five experiments). Uncultured cells (shaded), cells cultured in medium alone (**black**) and cells stimulated in the CD3 system (**red**) are illustrated.

of CD40 expression on activated adult B cells and activated cord blood B cells in this system (P = 0.25) (Fig. 5.3A).

The kinetics of CD40 expression were examined on adult (n = 5) and cord blood (n = 5) B cells stimulated in the CD3 system. There was significant upregulation of CD40 on both activated adult ($P < 0.05$) and cord blood B cells ($P < 0.05$) from day 1 of stimulation with similar kinetics of expression on adult and cord blood B cells throughout the period of activation (Fig. 5.3B).

5.2.3 Expression of CD154 on activated cord blood T lymphocytes

5.2.3.1 Stimulation with PMA and ionomycin

Adult and cord blood MC were cultured for up to 3 days in the presence or absence of PMA and ionomycin and the kinetics of T cell CD154 expression were examined. CD154 was detected with a PE-conjugated mAb and T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. (CD3 is downregulated by PMA and therefore was not used as a lineage marker). The percentage of T cells expressing CD154 and the MFI of CD154 expression on the positive population were equivalent on cord blood (n = 3) and adult (n = 3) T cells after 6 hours of stimulation with PMA and ionomycin (Fig. 5.4A, Fig. 5.4B). Both the MFI and the percentage of CD154 positive T cells declined more rapidly in the activated cord blood MC compared with adult peripheral blood MC (Fig. 5.4B). Whereas CD154 expression on adult T cells was not completely downregulated until 72 hours, there was minimal CD154 expressed on cord blood T cells 48 hours after the initiation of stimulation.

5.2.3.2 Stimulation with plate-bound CD3 mAb

Adult peripheral blood MC and cord blood MC were cultured for up to 3 days in the

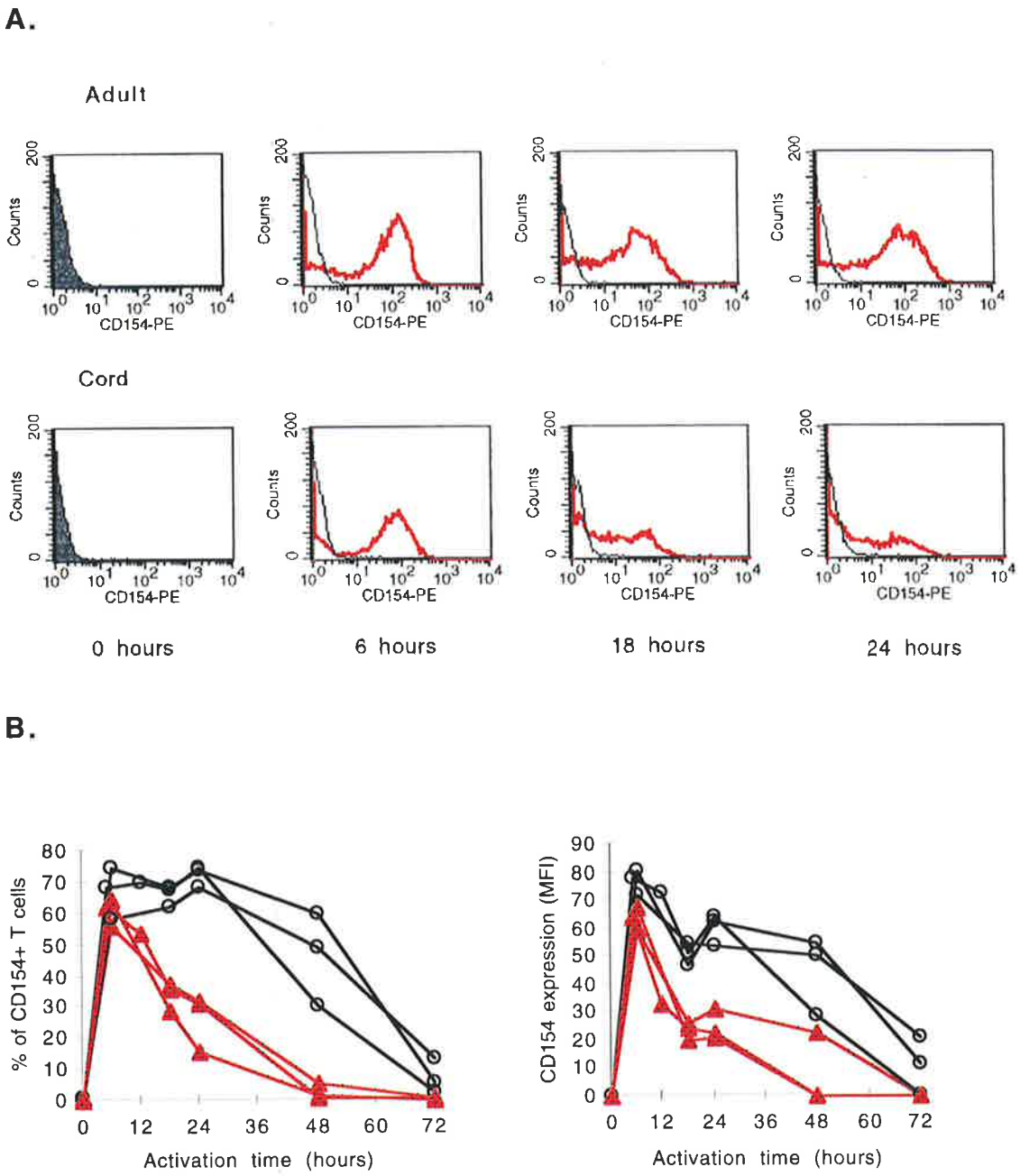


Fig. 5.4 **A.** Expression of CD154 on adult and cord blood T cells after stimulation of adult peripheral blood MC and cord blood MC with PMA and ionomycin. Staining with CD154-PE is shown for uncultured (shaded), unstimulated (**black**) and stimulated (**red**) T cells. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. These results are from one adult and one cord blood specimen processed in parallel and are representative of three experiments. **B.** Adult and cord blood T cells showed different kinetics of CD154 expression. Adult and cord blood MC were cultured with PMA and ionomycin for up to 72 hours and the level of CD154 expression was assessed at regular time intervals. Three adult specimens (**circles**) and three cord blood specimens (**triangles**) were examined. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

presence or absence of plate-bound CD3 mAb and T cell CD154 expression was examined over time. CD154 was stained with CD154-PE and T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. (CD3 could not be used to identify T cells because residual OKT3 bound to surface CD3 after culture would interfere with binding of the directly conjugated CD3 mAb). In one experiment, one specimen of adult peripheral blood MC and one cord blood MC specimen were stimulated in parallel with PMA and ionomycin and CD3 mAb. This demonstrated that the MFI of CD154 expression and the percentage of CD154 positive T cells induced by CD3 stimulation were much lower compared with expression induced by PMA and ionomycin (Fig. 5.5). A series of three experiments was performed, in each of which one specimen of adult peripheral blood MC and one specimen of cord blood MC were stimulated with CD3 mAb. In each of these experiments, the percentage of T cells expressing CD154 after 6 hours of stimulation with CD3 mAb was considerably higher in cord blood MC than in adult peripheral blood MC (Fig. 5.6A, Fig. 5.7A). The MFI of CD154 expression was low and did not differ between adult and cord blood T cells after 6 hours of CD3-stimulation (Fig. 5.7A). Subsequently, CD154 expression was rapidly downregulated on cord T cells, with minimal expression after 24 hours of stimulation, whereas expression on adult T cells was more sustained. There was no consistent difference in the MFI of CD40 expression on cord blood (n = 2) and adult (n = 2) B cells from the same CD3-stimulated mononuclear cell cultures (Fig. 5.6B).

5.2.3.3 Stimulation of B cell/monocyte-depleted cell preparations with plate-bound CD3 mAb

Subsequently, mononuclear cell preparations were depleted of B cells and monocytes to eliminate the influence of CD40 positive cells on CD154 expression by T cells. The composition of the B cell/monocyte depleted cell preparations recovered was as follows (mean +/- standard deviation): B cells 1.0% (+/- 0.5), monocytes 0.5% (+/- 0.2) and T cells 71.1% (+/- 8.8) (n = 8) with no significant difference in the composition of adult

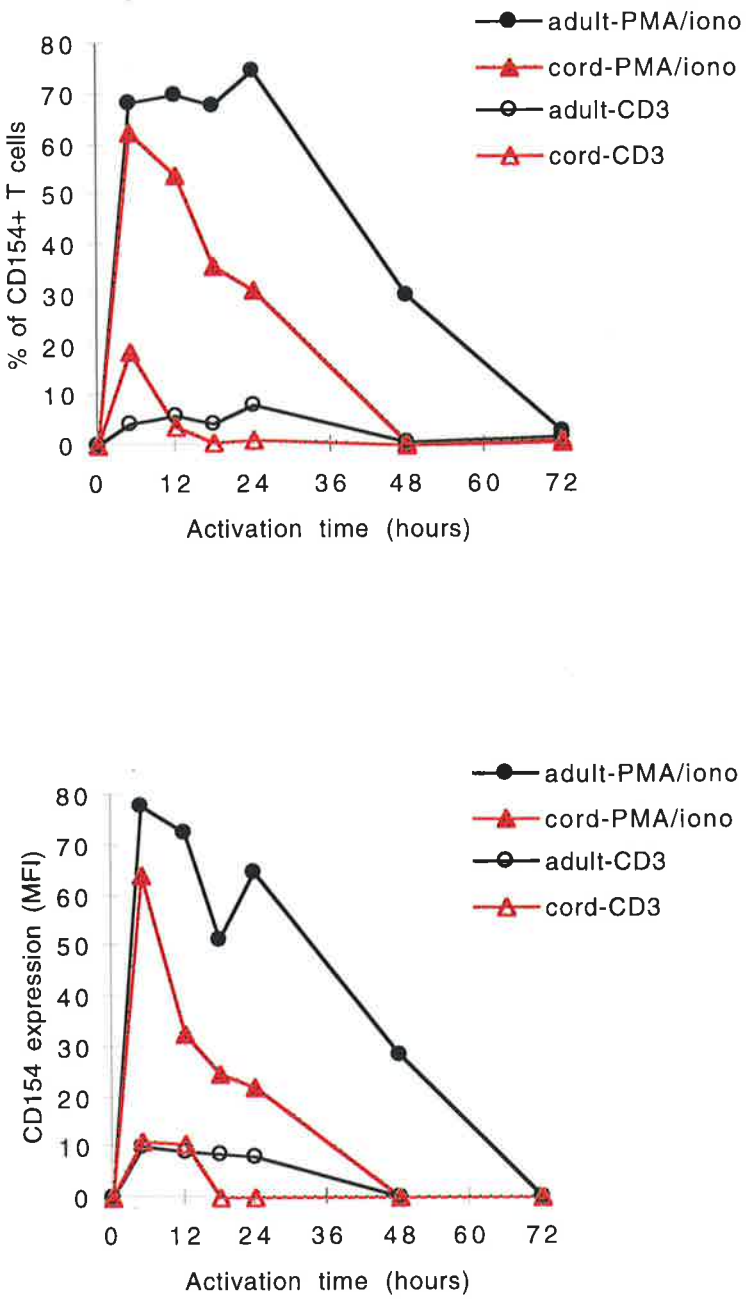


Fig. 5.5 Expression of CD154 was higher on T cells stimulated with PMA and ionomycin compared with CD3 mAb. Mononuclear cells from one adult and one cord blood specimen were stimulated in parallel with PMA and ionomycin or CD3 mAb. Cells were stained with CD154-PE and T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

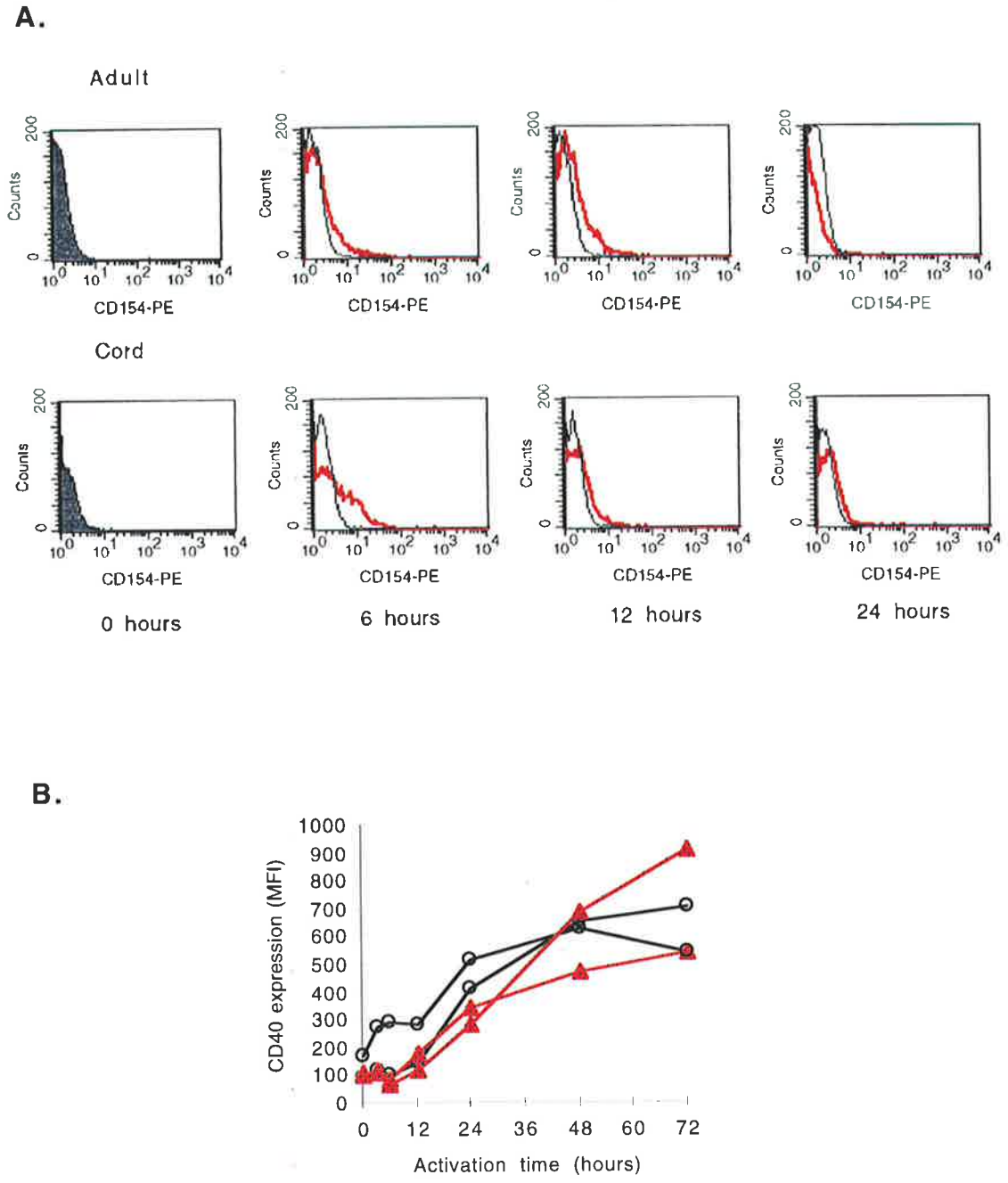


Fig. 5.6 **A.** Expression of CD154 on adult and cord blood T cells after stimulation of adult peripheral blood MC and cord blood MC with plate-bound CD3 mAb. Staining of the gated T cell population with CD154-PE is shown for uncultured cells (shaded), cells cultured in medium alone (**black**) and CD3-stimulated (**red**) cells. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. These results are from one adult and one cord blood specimen and are representative of three experiments. **B.** Expression of CD40 on adult and cord blood B lymphocytes from the same cultures of CD3-stimulated mononuclear cells. Cells were stained with biotin-CD40 mAb and streptavidin-PE. B cells were identified using directly conjugated CD19 mAb. Results from two adult (**circles**) and two cord blood specimens (**triangles**) are shown.

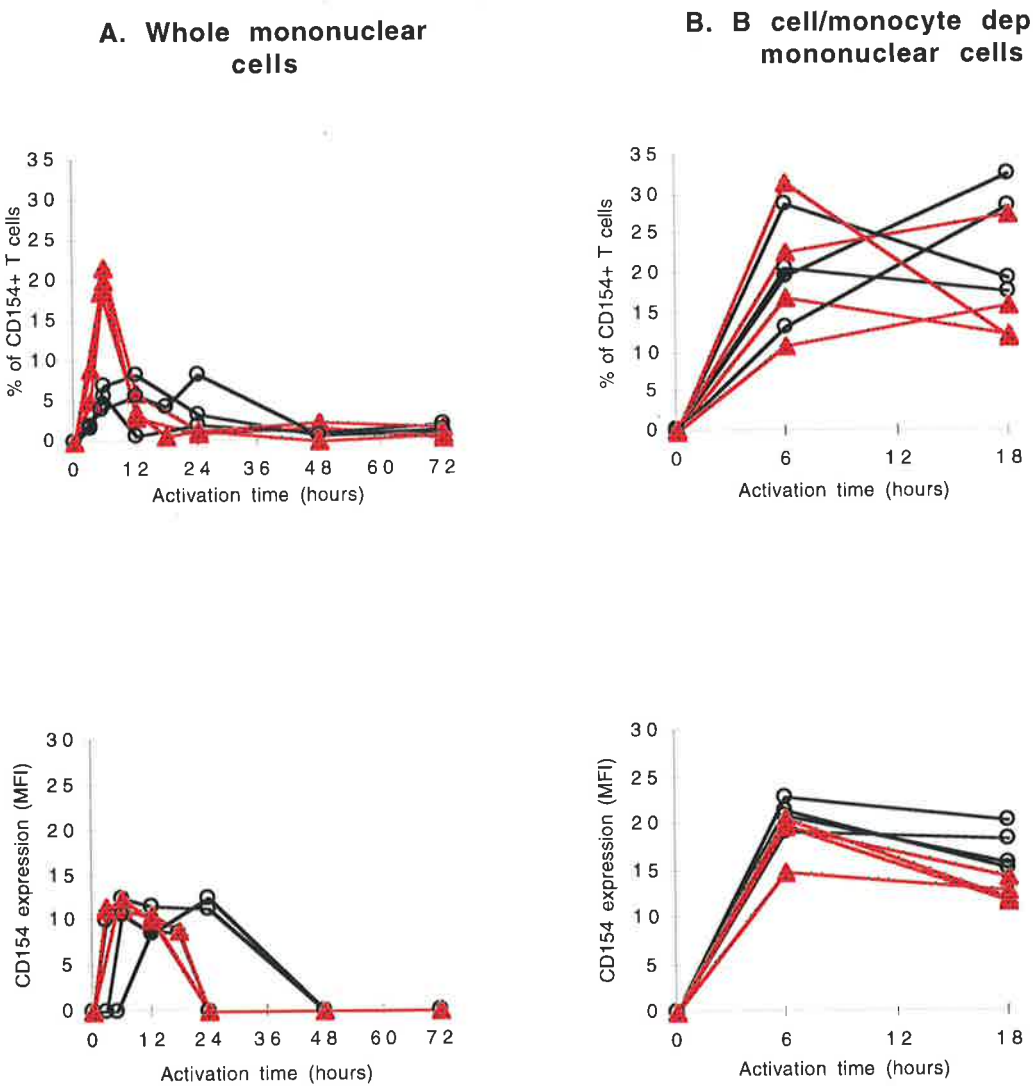


Fig. 5.7 Adult (**circles**) and cord blood (**triangles**) T cell CD154 expression during stimulation with CD3 mAb was influenced by the presence or absence of B cells and monocytes. Adult and cord blood whole MC **(A)** or B cell/monocyte depleted MC (obtained by passage of mononuclear cells through a nylon wool column) **(B)** were cultured with plate-bound CD3 mAb. Cells were stained with CD154-PE at regular time intervals. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. For the experiments with whole MC, three adult and three cord blood specimens were used. For the experiments with B cell/monocyte depleted MC, four adult and four cord blood specimens were examined. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown. Note that the MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

and cord blood preparations. The remaining non-T cells were NK cells (CD3 negative, CD16 positive, CD56 positive). As these cells do not express CD40 and therefore should not influence CD154 expression by T cells, they were not removed from the B cell/monocyte-depleted cell preparations. Adult or cord blood B cell/monocyte depleted MC were cultured in the presence or absence of plate-bound CD3 mAb and T cell CD154 expression was examined at various time-points. CD154 was stained with CD154-PE and T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb.

When B cell/monocyte depleted MC from adult (n = 4) and cord blood (n = 4) were stimulated for 6 hours with plate-bound CD3 mAb, the MFI of CD154 expression and the percentage of T cells expressing CD154 tended to be higher (Fig. 5.7B) compared with the levels of CD154 expression observed in previous experiments where whole MC were stimulated (Fig. 5.7A). There was no consistent difference in the percentage of adult and cord blood T cells expressing CD154 or the MFI of CD154 expression after 6 hours of CD3-stimulation (Fig.5.7B). After 18-24 hours of stimulation, the percentage of CD154 positive T cells and the MFI of CD154 expression tended to be slightly lower in B cell/monocyte depleted MC from cord blood than adult blood (Fig. 5.7B, Fig. 5.8). After B cell/monocyte depleted MC from adult (n = 2) and cord blood (n = 2) had been stimulated with CD3 mAb for 2-3 days, CD154 expression was similar on cord blood T cells and adult T cells (Fig. 5.8).

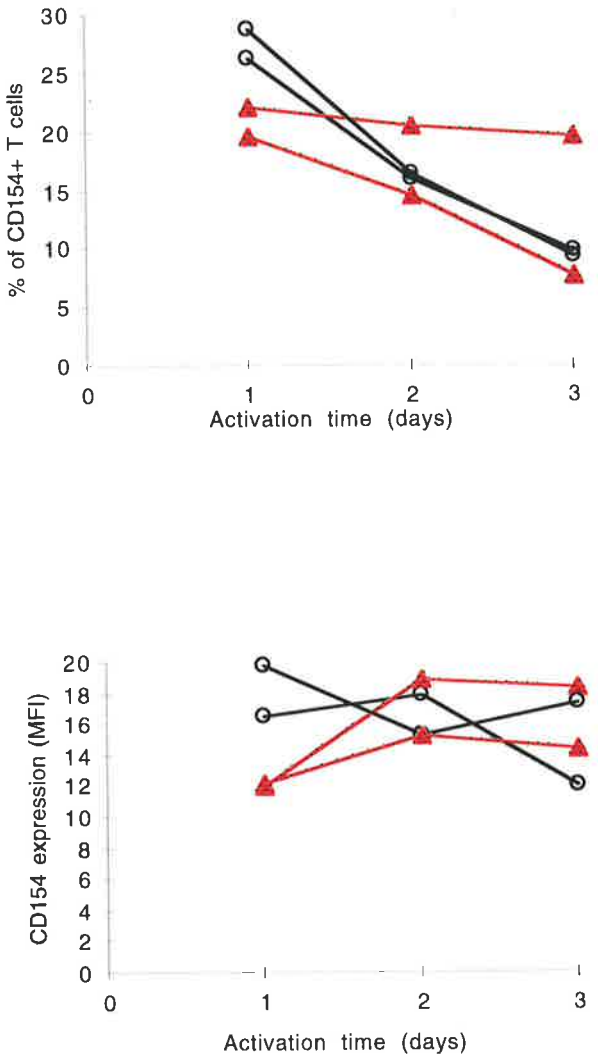


Fig 5.8 Expression of CD154 on adult and cord blood T cells after 1, 2, or 3 days of stimulation of adult and cord blood B cell/monocyte depleted MC with plate-bound CD3 mAb. B cell/monocyte depleted MC were cultured with plate-bound CD3 mAb and CD154 expression was assessed daily by staining with CD154-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. Two adult (**circles**) and two cord blood specimens (**triangles**) were examined. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown.

5.2.4 CD154 expression on CD45RA positive and CD45RO positive T cell subsets

Adult peripheral blood MC were cultured for 6 or 24 hours in the presence or absence of PMA and ionomycin and the expression of CD154 on CD45RA positive and CD45RO positive T cell subsets was examined. CD154 was stained using biotin-CD154 mAb and streptavidin-Cy5/PE. T cell subpopulations were identified using directly conjugated mAb to CD3 and CD45RA or CD45RO. Only those T cells that stained brightly for CD45RA or CD45RO were counted as CD45RA positive or CD45RO positive, respectively. CD154 was expressed on a marginally higher proportion of CD45RO positive T cells than CD45RA positive T cells (Table 5.1). The proportion of either CD45RA positive or CD45RO positive T cells that expressed CD154 did not change between 6 and 24 hours. There was no consistent difference in the MFI of CD154 expression between CD45RA positive and CD45RO positive subsets (Table 5.1). There was not a consistent difference in the MFI of CD154 expression between CD45RA positive T cells stimulated for 6 hours and those stimulated for 24 hours.

Table 5.1 The kinetics of CD154 expression on adult CD45RA positive and CD45RO positive T cell subpopulations stimulated with PMA and ionomycin.

		CD45RA positive T cells percentage of cells expressing CD154 (MFI)	**CD45RO positive T cells** percentage of cells expressing CD154 (MFI)
6 hours	Adult 1	54.8 (54.5)	ND
	Adult 2	ND	ND
	Adult 3	67.8 (79.6)	73.0 (87.8)
24 hours	Adult 1	53.0 (73.4)	ND
	Adult 2	58.8 (72.4)	74.2 (83.9)
	Adult 3	61.5 (74.2)	71.6 (70.3)

Adult peripheral blood MC (n = 3) were stimulated with PMA and ionomycin and stained with biotin-CD154 mAb plus streptavidin-Cy5/PE, and either CD45RA-PE and CD3-FITC or CD45RO-FITC and CD3-PE at the indicated times. ND = not done.

Adult peripheral blood MC were cultured for up to 24 hours in the presence or absence of plate-bound CD3 mAb and the kinetics of CD154 expression on CD45RA positive and CD45RA negative T cell subsets were examined. CD154 was stained using biotin-CD154 mAb and streptavidin-Cy5/PE. T cell subpopulations were identified using CD2-FITC and CD45RA-PE. The kinetics of CD154 expression during 24 hours of CD3-stimulation were similar on CD45RA positive and CD45RA negative T cell subsets (Fig. 5.9). A slightly higher percentage of CD45RA negative T cells expressed CD154 in both specimens at all time points.

5.3 Discussion

CD40 expression by resting neonatal B cells has been reported to be equivalent to adult B cell expression (Durandy *et al.*, 1995). Similarly, in the present study, the MFI of CD40 expression on resting cord blood B cells from the majority of neonates was comparable with levels seen on adult B cells. CD40 expression on peripheral blood B cells from young children between 2 and 20 months of age was also comparable to adult levels of expression.

The significance of the high levels of CD40 expression observed on cord blood B cells from a small group of neonates is unknown. These neonates were all apparently healthy. There have been no *in vivo* studies to investigate whether upregulation of CD40 expression on human peripheral blood B cells occurs during infection. In any case, subclinical infection would be unlikely to occur at this frequency in seemingly healthy neonates. It is possible that upregulation of CD40 may be induced by the physiological events during parturition. This could be investigated further by examining the clinical details of the pregnancy, delivery and health of neonates in relation to CD40 expression on cord blood B cells. Unfortunately, detailed clinical information was unavailable as part of this study. It would also be interesting to compare CD40 expression on cord blood B cells from healthy neonates and neonates with documented intra-uterine infection. The

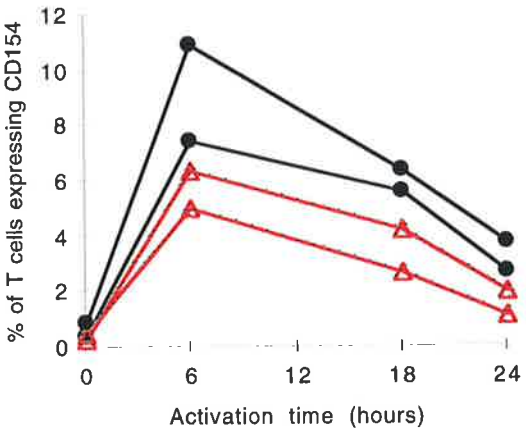


Fig. 5.9 The kinetics of CD154 expression were similar on CD45RA positive and CD45RA negative T cell subpopulations from adult peripheral blood MC (n = 2) stimulated with CD3 mAb. Adult peripheral blood MC were stimulated for up to 24 hours with plate-bound CD3 mAb. Cells were stained with biotin-CD154 mAb and streptavidin-Cy5/PE, CD45RA-PE and CD2-FITC at the time-points as indicated. The percentages of CD45RA positive (**triangles**) or CD45RA negative (**circles**) T cells that expressed CD154 are shown.

observation of very high levels of CD40 expression on B cells from 2 healthy adult controls also suggests that B cell CD40 expression may be increased *in vivo* in the absence of clinical infection. It is interesting that B cells from these two adults showed greater upregulation of CD40 expression in response to *in vitro* stimulation.

There have been no previous studies of the regulation of CD40 expression on neonatal B cells during activation. In the present study, the degree of CD40 upregulation on activated cord blood B cells was dependent on the method of stimulation used. These results suggest that although neonatal B cells are capable of upregulating CD40 to high levels if adequately stimulated (eg. with PMA and ionomycin), this probably does not happen in a physiological setting in response to T-dependent antigens (modelled by plate-bound CD3 mAb).

Ligation of CD40 on neonatal B cells in combination with supplemental cytokines can induce adult levels of B cell proliferation but only limited secretion of antibodies of all isotypes, particularly IgG and IgA (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1993). From the results of the present study, it is clear that the limited responsiveness of neonatal B cells to CD40 ligation is not attributable to reduced CD40 expression. There may be differences in the CD40 signalling pathway in neonatal B cells, or alternatively, downstream factors may be responsible for reduced immunoglobulin secretion and isotype switching in neonatal B cells in response to CD40 ligation.

Previous reports have variously suggested that CD154 expression by activated neonatal T cells is absent, reduced or equivalent to levels of expression by adult T cells (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1996). This appears to depend on the *in vitro* stimulus used. Previous studies have shown that when neonatal T cells are stimulated with PMA and ionomycin, CD154 expression is reduced or undetectable (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1996), but adult levels of

CD154 expression are induced on neonatal T cells stimulated with plate-bound CD3 mAb (Splawski *et al.*, 1996). It is therefore surprising that in the present study, cord blood T cells were initially capable of expressing adult levels of CD154 in response to stimulation with PMA and ionomycin, even though this expression was not as stable as adult T cell CD154 expression.

In the current study, the percentage of cord T cells expressing CD154 and the MFI of CD154 expression on cord T cells was similar to expression in adult specimens after 6 hours of stimulation with PMA and ionomycin. However, CD154 expression on cord blood T cells was rapidly downregulated, whereas adult T cells exhibited more stable expression. It is possible that CD154 mRNA transcription may be differentially regulated in neonatal T cells activated with PMA and ionomycin. It has been shown that CD154 mRNA expression by purified neonatal T cells stimulated with PMA and ionomycin is lower than adult T cell CD154 mRNA expression at all time points during the first 8 hours of stimulation (Nonoyama *et al.*, 1995). However, this does not explain the difference in kinetics of CD154 surface expression on cord blood T cells observed in the present study.

Both the percentage of CD154 positive T cells and the MFI of CD154 expression were markedly lower when mononuclear cells were stimulated with plate-bound CD3 mAb compared to PMA and ionomycin. Low expression of CD154 on T cells stimulated with CD3 mAb rather than phorbol esters and ionomycin has also been reported in other studies (Casamayor Palleja *et al.*, 1995; Lane *et al.*, 1992; Patel *et al.*, 1996; Splawski *et al.*, 1996). It is interesting that CD154 expression is comparable on adult and neonatal T cells stimulated with plate-bound CD3 mAb (Splawski *et al.*, 1996) but when PMA and ionomycin are used for stimulation, the high level of expression induced on adult T cells cannot be sustained by neonatal T cells.

It has been demonstrated that CD154 expression on adult peripheral blood CD4 positive T cells activated with CD3 mAb, superantigen, or phorbol esters and ionomycin, is rapidly downregulated in the presence of B cells or transfectants expressing CD40 (Hermann *et*

al., 1993; Ludewig *et al.*, 1996; Miyashita *et al.*, 1997; van Kooten *et al.*, 1994; Yellin *et al.*, 1994). This downregulation is CD40-dependent, and is partly caused by receptor-mediated endocytosis followed by lysosomal degradation (Yellin *et al.*, 1994). The presence of CD40 positive cells can also induce downregulation of CD154 mRNA expression, although this does not occur until 12-18 hours after the start of stimulation (Ludewig *et al.*, 1996; van Kooten *et al.*, 1994). Downregulation of T cell CD154 expression by B cells probably explains the lower levels of CD154 expression on T cells in adult and cord blood whole MC cultures compared with B cell/monocyte depleted MC cultures observed in the current study.

The kinetics of T cell CD154 expression differed between adult and cord blood MC stimulated with CD3 mAb. This difference in kinetics was not evident when mononuclear cell preparations were depleted of B cells and monocytes prior to stimulation. This suggests that B cells (and/or monocytes) differentially regulate the kinetics of CD154 expression on cord blood T cells stimulated with CD3 mAb.

In the present study, the MFI of CD40 expression was not consistently different between adult and cord blood B cells in mononuclear cell cultures stimulated for 3, 6 or 12 hours with CD3 mAb. The different kinetics of cord blood T cell CD154 expression in CD3-stimulated cultures of whole MC could not therefore be explained by altered levels of CD40 expression on cord blood B cells. It has been demonstrated previously that activated adult B cells are more effective than resting B cells at inducing down-modulation of CD154 expression on CD3-stimulated CD4 positive T cells (Miyashita *et al.*, 1997). This is believed to occur by a mechanism that is independent of the level of B cell CD40 expression (Miyashita *et al.*, 1997). It is possible that delayed activation of naive cord blood B cells results in a delay in their ability to down-modulate CD154 expression on cord blood T cells. Alternatively, delayed down-modulation of cord T cell CD154 expression in the presence of B cells and monocytes may reflect the immaturity of the T cells.

CD45RA is expressed mainly by naive T lymphocytes whereas CD45RO expression is

considered a marker of T cells previously exposed to antigen (Akbar *et al.*, 1988). Reports as to the association of CD154 expression with CD45RA positive and CD45RO positive T cell subsets are conflicting (Brugnoni *et al.*, 1994; Lane *et al.*, 1992; Nonoyama *et al.*, 1995; Patel *et al.*, 1996). In view of the different kinetics of CD154 expression demonstrated on neonatal and adult T cells, the possibility that the kinetics of CD154 expression may differ between CD45RA positive and CD45RO positive T cells was investigated. Although a marginally higher percentage of CD45RO positive T cells were CD154 positive, there was no evidence that the kinetics of CD154 differed between 'naive' CD45RA positive and 'memory' CD45RO positive T cells, regardless of the method of activation used.

The hypothesis underlying the studies described in this chapter was that the differences observed in the neonatal antibody response compared to the adult response may be attributable to differences in the regulation of CD40 and/or CD154 expression on neonatal lymphocytes. Although cord blood B cells from neonates were capable of expressing adult levels of CD40, the kinetics of CD154 expression on cord blood T cells differed from adult patterns of expression. Cord T cells activated with PMA and ionomycin showed more transient expression of CD154 than adult T cells. It was also found that the interaction of cord blood T cells and B cells/monocytes had a differential effect on the kinetics of cord blood T cell CD154 expression during CD3 stimulation. This may reflect immaturity of neonatal T cells and/or B cells and monocytes, or may represent a specialisation for this stage of development. Different kinetics of CD154 expression in the neonate could influence signalling of neonatal B cells through CD40 and/or signalling of the neonatal T cells through CD154.

CHAPTER 6.

DOWN-REGULATION OF CD154 EXPRESSION ON ADULT AND CORD BLOOD T CELLS IN THE PRESENCE OF B CELLS

6.1 Introduction

The following studies investigated the differential downregulation of CD154 expression on adult and cord blood T cells in the presence of B cells. The aim was to establish whether the delayed downregulation of CD154 expression on cord blood T cells in the presence of B cells demonstrated in the Chapter 5 was T cell-dependent or B cell-dependent. In order to do this, it was necessary to develop a method of inducing downregulation of CD154 on T cells without using autologous B cells. Using such a method, downregulation of CD154 could be directly compared on adult and cord blood T cells. If downregulation of CD154 was still regulated differently on cord blood T cells compared to adult T cells, this would suggest that delayed downregulation of CD154 on cord blood T cells in the presence of B cells was T cell-dependent. If the delayed downregulation of cord blood T cell CD154 expression was B cell-dependent, a method of inducing CD154 downregulation that did not use autologous B cells would be expected to induce a similar response in adult and cord blood T cells.

Prior to these experiments, it was necessary to determine whether the higher levels of CD154 expression on activated T cells in B cell/monocyte-depleted MC preparations, compared with the level of expression on activated T cells in whole peripheral blood MC preparations, were attributable to depletion of B cells and/or depletion of monocytes. It has previously been reported that downregulation of T cell CD154 expression is induced by B cells but minimally affected by monocytes (Yellin *et al.*, 1994), which seems surprising in view of the fact that monocytes constitutively express CD40 (Alderson *et al.*, 1993). These studies therefore examined CD154 expression on adult T cells in

cultures of whole MC, B cell-depleted MC, monocyte-depleted MC or B cell/monocyte-depleted MC stimulated with CD3 mAb.

The next aim was to establish that the downregulation of T cell CD154 expression in the presence of B cells was, as previously reported, a result of receptor-mediated endocytosis (Yellin *et al.*, 1994). Endocytosis of CD154 would be indicated by a simultaneous reduction in the level of surface CD154 expression and an increase in intracellular CD154 when activated T cells were cultured with CD40 positive cells. This should be able to be inhibited by interfering with cytoskeletal mechanisms using reagents such as cytochalasin B. A lower level of intracellular CD154 in cord blood T cells activated in the presence of B cells, together with higher surface CD154 expression, would suggest reduced endocytosis of surface CD154 by cord blood T cells.

Depending on whether the differential regulation of CD154 expression on cord blood T cells in the presence of B cells was found to be B cell-dependent or T-cell dependent, the final objective was to further assess characteristics of cord blood B cells or cord blood T cells which could be responsible for this phenomenon.

6.2 Results

6.2.1 The influence of B cells and monocytes on the expression of CD154 by adult T cells

In Chapter 5, it was shown that removal of B cells and monocytes from MC preparations (using nylon wool) prior to CD3 stimulation, resulted in higher levels of CD154 expression on activated T cells. It was suggested that the low expression of CD154 on T cells in CD3-stimulated whole MC fractions was due to the presence of CD40 positive B cells. B cells have been previously shown to induce endocytosis of CD154 on the surface of T cells (Yellin *et al.*, 1994). However, nylon wool also removes monocytes from MC preparations and these cells also express CD40. Prior to investigating the influence of B

cells on T cell CD154 expression it was therefore necessary to exclude the possibility that monocytes may influence T cell CD154 expression.

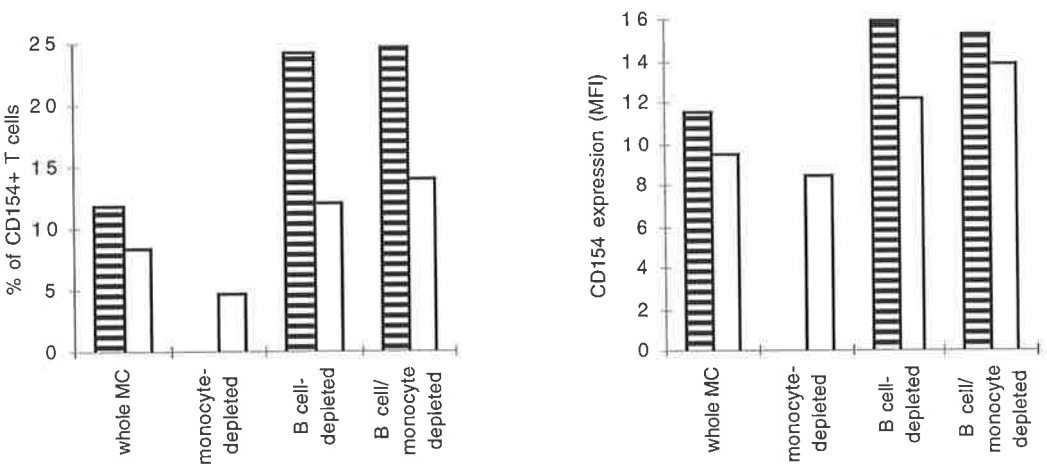
In the first experiment (experiment 1), mononuclear cells isolated from one specimen of adult peripheral blood were divided and used whole, passed through nylon wool to deplete B cells and monocytes, or treated with CD19 mAb-coated magnetic beads to remove B cells. For the second specimen of adult peripheral blood (experiment 2), one aliquot of mononuclear cells was also treated with CD14 mAb and magnetic beads to remove monocytes. Fractions from each specimen were stimulated with plate-bound CD3 mAb for 5-6 hours.

The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression were increased similarly in B cell/monocyte-depleted MC and in B cell-depleted MC, compared with the level of CD154 expression when whole mononuclear cells were stimulated (Fig. 6.1A). In contrast, there was no increase in the percentage of CD154 positive T cells or the MFI of T cell CD154 expression in monocyte-depleted MC compared to whole MC. Therefore, despite the fact that monocytes expressed surface CD40 (Fig. 6.1B), they appeared to have minimal influence on the downregulation of T cell CD154 expression.

6.2.2 Downregulation of T cell CD154 could not be induced by cross-linking with mAb

In the rest of this chapter, B cell/monocyte-depleted MC recovered by passage of MC through a nylon wool column will be referred to as enriched T cells. The next experiments attempted to induce downregulation of adult T cell surface CD154 expression by cross-linking with mAb. Enriched T cells from adult peripheral blood (n = 2) were stimulated overnight with plate-bound CD3 mAb, then incubated on melting ice with biotin-CD154 mAb (5c8) (sodium azide free) followed by streptavidin-PE (sodium azide free). As a positive control for endocytosis, unstimulated T cells were stained with

A.



B.

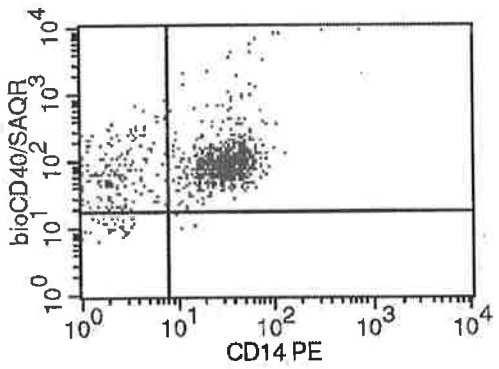


Fig. 6.1 A. T cell CD154 expression was enhanced by the depletion of B cells but not monocytes from adult peripheral blood mononuclear cells stimulated with CD3 mAb. Monocytes were depleted from mononuclear fractions using CD14 mAb and anti-mouse IgG coated-magnetic beads, B cells were depleted using CD19 mAb and anti-mouse IgG coated-magnetic beads or magnetic beads coated with CD19 mAb, and both B cells and monocytes were depleted using a nylon wool column. T cell CD154 expression induced by CD3 stimulation was compared on these fractions and whole peripheral blood MC. Cells were stimulated with plate-bound CD3 mAb for 6 hours (expt 1 - hatched) or 5 hours (expt 2- white) and T cells were dual stained with CD154-PE and CD2-FITC. **B.** Monocytes from peripheral blood were positive for CD40 expression. Adult peripheral blood mononuclear cells were dual stained with CD14-PE and biotin-CD40 mAb detected by streptavidin-Cy5/PE. Cells within the monocyte gate (based on light scatter properties) are shown.

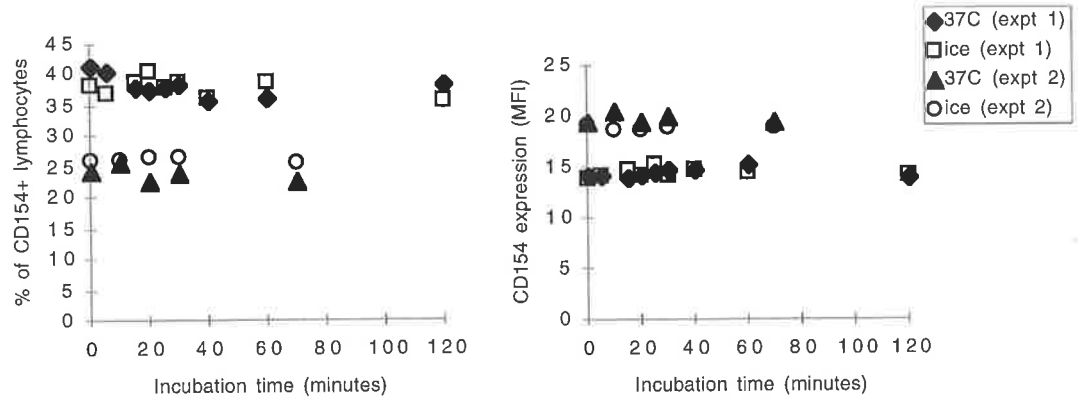
OKT3 and FITC-conjugated anti-mouse immunoglobulin (experiment 2 only). After staining, cells were divided and either incubated on ice or at 37°C. At various time-points, cells were analysed for CD154 or CD3 staining by flow cytometry.

There was no downmodulation of surface CD154 expression on lymphocytes incubated at 37°C or on ice after cross-linking with biotin-CD154 mAb and SA-PE (Fig. 6.2A). In contrast, when surface CD3 was cross-linked by OKT3 and FITC-conjugated anti-mouse immunoglobulin under the same experimental conditions, the MFI of CD3 expression (but not the percentage of CD3 positive lymphocytes) was down-modulated on lymphocytes incubated at 37°C but not on lymphocytes incubated on ice (Fig. 6.2B). Thus cross-linking CD154 using CD154 mAb was insufficient to induce downregulation of T cell surface CD154 expression.

6.2.3 Downregulation of T cell CD154 expression in the presence of chronic lymphocytic leukaemia (CLL) cells

CLL cells were used as a source of CD40 positive cells to induce downregulation of T cell CD154 expression (Fig. 6.3A). Enriched T cells from adult peripheral blood (n = 2) were stimulated overnight with plate-bound CD3 mAb. CLL cells were added to enriched T cells at a ratio of 4:1 (based on cell numbers determined by counting with a haemocytometer). Cells were then incubated at 37°C or on ice for 1-2 hours. The number of enriched T cells and the volume of medium in each well was consistent between enriched T cells incubated in the presence of CLL cells, and enriched T cells incubated alone. After incubation, cells were washed and resuspended in cold PBS (with 0.02% sodium azide and 1% newborn bovine serum) and stained with CD154-PE and CD2-FITC on melting ice. When enriched T cells were incubated with CLL cells at 37°C, the percentage of T cells expressing CD154 was lower than when enriched T cells were

A.



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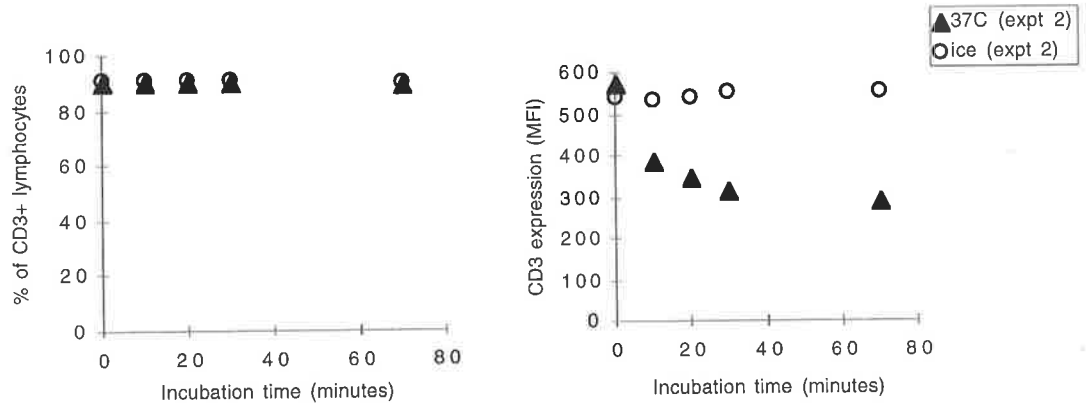
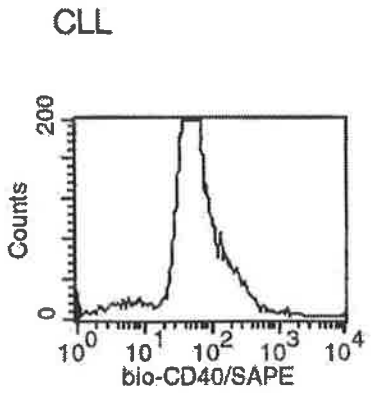


Fig. 6.2 Cross-linking surface CD154 on CD3-stimulated T cells using biotin-CD154 mAb and streptavidin-PE did not induce endocytosis of CD154 **(A)**. In contrast, cross-linking CD3 using OKT3 and FITC-conjugated anti-mouse immunoglobulin induced downregulation of surface CD3 expression **(B)**. Enriched T cells were stimulated overnight with plate-bound CD3 mAb. Cells were stained on ice and then incubated at 37°C or on ice. At various time-points, cells were taken out and analysed for CD154 or CD3 expression. The percentage of lymphocytes expressing CD154 or CD3 and the MFI of CD154 or CD3 expression are shown.

A.



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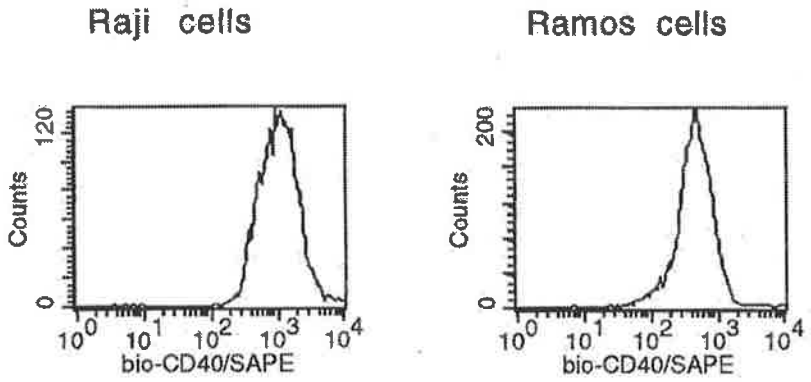

Fig. 6.3 **A.** CLL cells used to induce down-modulation of T cell surface CD154 expression were positive for CD40 expression. CLL cells were stained with biotin-CD40 mAb and streptavidin-PE. **B.** B cell lines, Raji and Ramos, used to induce downregulation of T cell surface CD154 expression, were positive for CD40 expression. Raji and Ramos cells were stained with biotin-CD40 mAb and streptavidin-PE.

incubated at 37°C in the absence of CLL cells (Table 6.1). However, there was also a reduction in the percentage of T cells expressing CD154 after incubation with CLL cells on ice, even in the presence of sodium azide (experiment 2).

Table 6.1 CD154 expression was downregulated on activated T cells incubated with CLL cells.

	expt 1 incubation: 1 hour	**expt 2** incubation: 2 hours (sodium azide added to cells on ice)
37°C no CLL	21.3% (14.8)	6.9% (13.3)
37°C + CLL	8.6% (9.7)	2.9% (0)
ice no CLL	19.3% (14.6)	7.2% (14.3)
ice + CLL	13.0% (11.8)	4.6% (12.9)

Enriched T cells from adult blood (n = 2) were stimulated with CD3 mAb overnight and then incubated in the presence or absence of CLL cells at 37°C or on ice as shown. Cells were then washed and stained with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression (brackets) are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

6.2.4 Inducing downmodulation of T cell CD154 expression using B cell lines

The B cell lines, Raji and Ramos, were used as a consistent and readily available source of CD40 positive cells to induce downregulation of T cell CD154 expression (Fig. 6.3B). Enriched T cells from adult peripheral blood were stimulated overnight with plate-bound CD3 mAb. Raji or Ramos cells were added to enriched T cells at the ratios indicated, and cells were incubated at 37°C or on ice for various time periods as shown. After incubation, cells were washed in cold PBS (plus 0.02% sodium azide and 1% newborn bovine serum) and stained with CD154-PE and CD2-FITC on melting ice. Both Raji and Ramos B cell lines were equally capable of inducing downregulation of T cell CD154

expression when incubated with activated enriched T cells at 37°C for 2 hours (Table 6.2).

Table 6.2 Raji and Ramos cells both induced downregulation of CD154 expression on activated T cells.

	37°C no B cell line	37°C + Raji	37°C + Ramos	ice no B cell line	ice + Raji	ice + Ramos
expt 1 T cell: B cell = 1:3 incubation: 2 hours	13.0% (17.9)	0.3% (0)	0.5% (0)	16.3% (16.0)	1.9% (0)	1.1% (0)

Enriched T cells from adult peripheral blood (n = 1) were stimulated overnight with CD3 mAb and incubated with Raji or Ramos cells at 37°C or on ice as indicated. Cells were then washed and stained with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression (brackets) are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

However, there was also downregulation of CD154 on activated adult T cells incubated with Raji or Ramos cells on ice. To a large extent, the reduction of T cell CD154 expression was not energy-dependent, being evident within 15 minutes of addition of the B cell line, regardless of whether the subsequent incubation was at 37°C or on ice in the presence of sodium azide (Table 6.3).

Table 6.3 Raji cells induced downregulation of CD154 expression on activated T cells at 37°C and on ice.

	37°C no B cell line	37°C + Raji	ice/ azide no B cell line	ice/ azide + Raji
expt 2	**15 min:**	**15 min:**	**15 min:**	**15 min:**
T cell: B cell = 1:2	17.8%	2.5%	13.9%	3.6%
incubation: as shown	(13.7)	(0)	(14.3)	(8.6)
	2 hrs:	**2 hrs:**	**2 hrs:**	**2 hrs:**
	19.5%	0.6%	18.6%	3.3%
	(25.1)	(0)	(24.7)	(19.8)

Enriched T cells from adult blood (n = 1) were stimulated overnight with CD3 mAb and incubated for up to 2 hours with Raji cells at 37°C or on ice (plus 0.02% sodium azide). Cells were stained with CD154-PE and CD2-FITC after 15 minutes and 2 hours of incubation. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression (brackets) are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

These initial experiments were conducted using a high ratio of B cells to enriched T cells. Previously published studies have shown that the down-modulation of CD154 expression on T cells is influenced by the ratio of B cells to T cells in a dose-dependent manner (van Kooten *et al.*, 1994; Yellin *et al.*, 1994). In the current study, titrating the ratio of enriched adult T cells to Raji cells showed that when the ratio of enriched T cells to Raji cells was increased, there was a slight increase in the percentage of CD154 positive T cells in cultures incubated on ice, but minimal change in percentage of T cells expressing CD154 in cultures incubated at 37°C (Table 6.4 expt 1). This suggests that the ratio of enriched T cells to B cells may have a greater impact on the energy-independent 'blocking effect' than on endocytosis. This was further supported by results obtained using a 'physiological' T cell: B cell ratio of approximately 9:1 (Table 6.4 expt 2). At this ratio, there was a greater reduction of CD154 expression on T cells incubated with Raji cells at 37°C (presumably endocytosis) and a lesser decrease when cells were incubated on ice in the presence of sodium azide (the 'blocking effect').

Table 6.4 Downregulation of T cell CD154 expression in the presence of Raji cells was dependent on the ratio of enriched T cells to Raji cells.

	Ratio of enriched T cells: Raji cells	37°C	ice/azide
expt 1	no Raji cells	14.1% (10.7)	16.8% (11.3)
	T cells: Raji cells = 1:1	0.3% (0)	1.5% (0)
	T cells: Raji cells = 2:1	0.3% (0)	3.0% (8.7)
	T cells: Raji cells = 4:1	1.1% (0)	4.1% (8.0)
expt 2	no Raji cells	16.9% (23.4)	21.5% (25.7)
	T cells: Raji cells = 9:1	4.3% (19.8)	12.4% (20.5)

Enriched T cells from adult peripheral blood (n = 2) were stimulated overnight with CD3 mAb and then incubated with Raji cells at 37°C or on ice (plus 0.02% sodium azide) for 1 hour at the ratios indicated. Cells were then stained with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression (brackets) are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

To ensure that the 'blocking' effect was not caused by a soluble factor produced by the B cell line which inhibited the binding of mAb to CD154, activated enriched T cells from adult peripheral blood were resuspended in Ramos supernatant or PBS prior to staining with CD154-PE. Neither the percentage of T cells staining positively for CD154 (16.2% in supernatant compared to 17.3% in PBS) nor the MFI of CD154 expression (23.5 in supernatant compared to 24.3 in PBS) was reduced on T cells resuspended in Ramos supernatant. This indicates that the 'blocking' effect could not be explained by the presence of a soluble factor produced by the B cell line.

Two further experiments were conducted where enriched T cells from adult peripheral blood were stimulated for 5 or 6 hours with plate-bound CD3 mAb and then incubated with Ramos or Raji cells for up to 2 hours either at 37°C or on ice with sodium azide.

Both these experiments showed a clear reduction of CD154 expression on T cells in the presence of B cells at 37°C, with a lesser reduction of CD154 expression on T cells incubated with B cells on ice (Fig. 6.4). It was decided that this was sufficient evidence for an energy-dependent process resulting in down-modulation of T cell surface CD154 expression to proceed with further experiments, keeping in mind that there was possibly a component of the reduced expression which may be caused by an energy-independent process eg. blocking.

In the next experiment, the aim was to determine whether the low level of T cell CD154 expression observed when adult peripheral blood MC were stimulated with plate-bound CD3 mAb could be reproduced by stimulating enriched adult T cells in the presence of a B cell line. Mitomycin-treated Raji cells and enriched adult T cells were cultured with plate-bound CD3 mAb for up to 24 hours. T cell CD154 expression was assessed at various time points by dual staining with CD154-PE and CD2-FITC. As can be seen in Fig. 6.5, stimulation of enriched T cells with CD3 mAb in the presence of Raji cells resulted in a reduced percentage of T cells expressing CD154 and a lower MFI of CD154 expression. This pattern of T cell CD154 expression was similar to that observed previously when whole mononuclear cells from adult blood were stimulated with CD3 mAb.

6.2.5 Mechanism of downregulation of T cell CD154 expression in the presence of B cells

Having established that surface CD154 expression could be downregulated on adult T cells by incubation or culture in the presence of a B cell line, it was necessary to show that this was in fact due to endocytosis of CD154, as has been previously suggested (Yellin *et al.*, 1994). The aim of these experiments was to demonstrate an increase in intracellular CD154 expression concurrent with a decrease in surface CD154 expression when enriched T cells were activated in the presence of B cells. Enriched T cells were

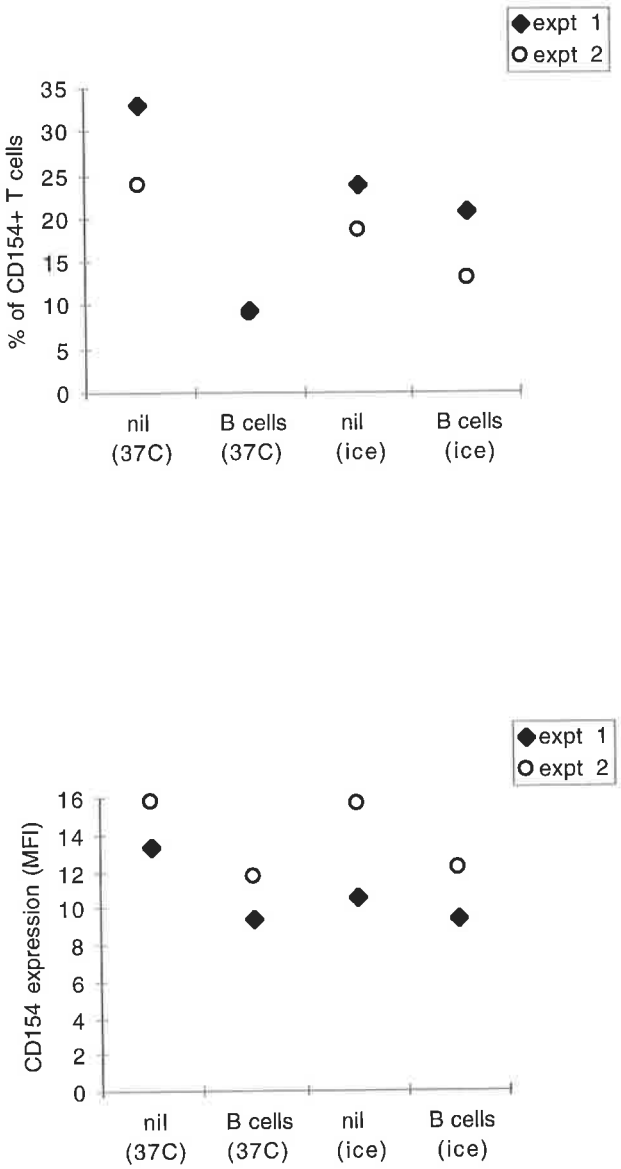

Fig. 6.4 CD154 expression on CD3-stimulated enriched T cells from adult peripheral blood after a 1 hour incubation with Raji cells (expt 1) or a 30 minute incubation with Ramos cells (expt 2). Enriched T cells were stimulated for 5-6 hours with plate-bound CD3 mAb. Raji or Ramos cells were added (enriched T cells : B cells = 9:1) and cells were incubated at 37°C or on ice (plus sodium azide 0.02%). Cells were dual stained with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown.

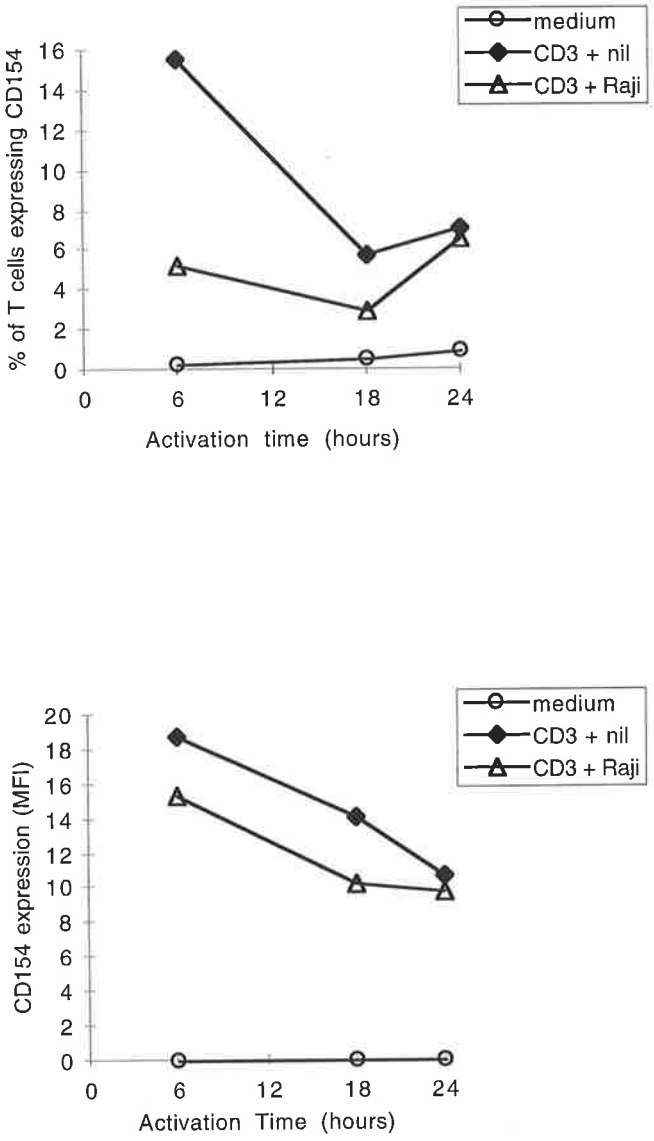

Fig. 6.5 The presence of Raji cells during T cell stimulation with CD3 mAb resulted in low levels of T cell CD154 expression. Enriched T cells from adult peripheral blood (n = 1) were cultured in medium alone, stimulated with CD3 mAb or stimulated with CD3 mAb in the presence of mitomycin-treated Raji cells (enriched T cells : B cells = 9:1). Cells were cultured for up to 24 hours and T cell CD154 expression was assessed at various time points by dual staining with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 and the MFI of CD154 expression are shown. The MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

stimulated for 5 hours with plate-bound CD3 mAb in the presence or absence of mitomycin-treated Ramos cells. Staining for surface and intracellular CD154 was conducted in parallel using CD154-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. A mAb against the cytoplasmic molecule, calreticulin (AD2 - detected using PE-conjugated goat anti-mouse immunoglobulin), was used as a positive control for intracellular staining. Surface CD154 molecules were blocked with unconjugated CD154 mAb (TRAP 1) before performing intracellular staining with CD154-PE.

Although intracellular staining with AD2 was positive, no intracellular CD154 was detected in T cells stimulated in either the absence or the presence of Ramos cells (Fig. 6.6). A reduction in the percentage of T cells expressing surface CD154 and decreased MFI of surface CD154 expression were observed on T cells stimulated in the presence of Ramos cells (Fig. 6.6).

6.2.6 CD154 expression on adult and cord blood T cells in the presence of autologous B cells or a B cell line

The overall aim of the studies described in this chapter was to investigate the differential regulation of CD154 expression on cord blood T cells in the presence of B cells. Having established a method for inducing downregulation of adult T cell CD154 expression that did not require autologous B cells, the next objective was to compare the downregulation of CD154 expression on cord blood T cells and adult T cells induced by a B cell line. Adult and cord blood MC, enriched T cells or enriched T cells plus mitomycin-treated Ramos cells were stimulated for 6 hours with plate-bound CD3 mAb. T cell CD154 expression was then assessed by dual staining with CD154-PE and CD2-FITC. There was no difference in the percentage of adult and cord blood T cells expressing CD154 when stimulated in the presence of Ramos cells (Fig. 6.7).

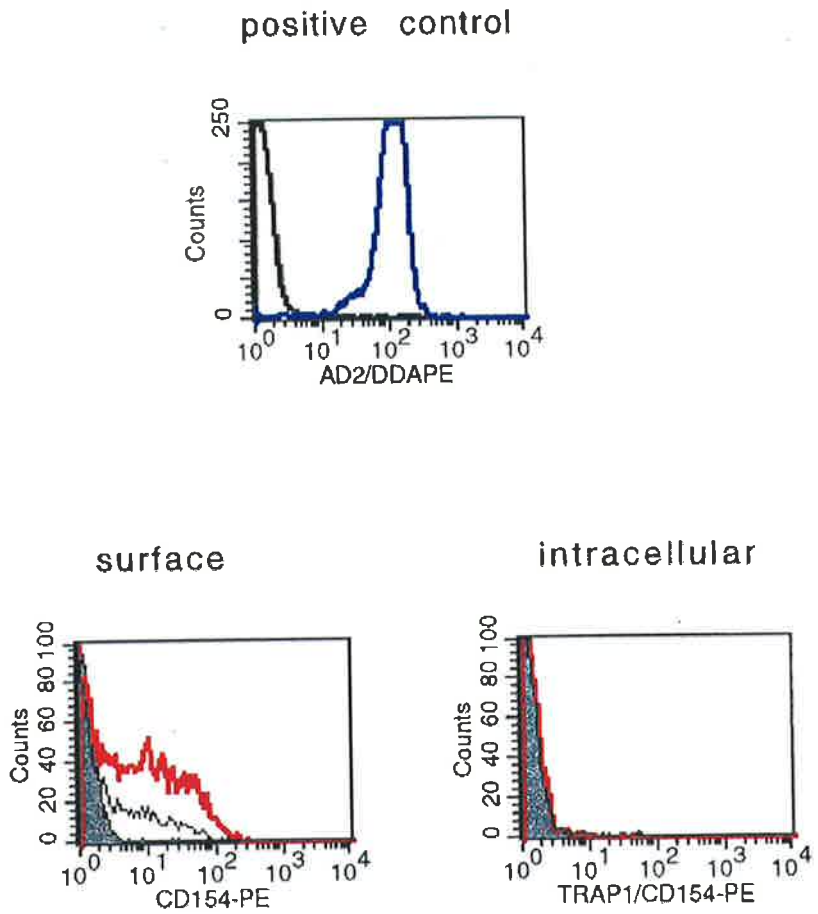

Fig. 6.6 T cells did not contain intracellular CD154 after CD3-stimulation in the presence of Ramos cells. Enriched T cells were cultured for 5 hours in medium alone (shaded) or with plate-bound CD3 mAb in the presence (**black**) or absence (**red**) of mitomycin-treated Ramos cells. Cells from each treatment group were stained for surface and intracellular CD154 in parallel using CD154-PE. T cells were identified as CD19 negative and CD2 positive using directly conjugated mAb. Concurrent surface (**black**) and intracellular (**blue**) staining of enriched T cells with mAb against the intracellular molecule calreticulin (AD2) showed the intracellular staining technique was effective.

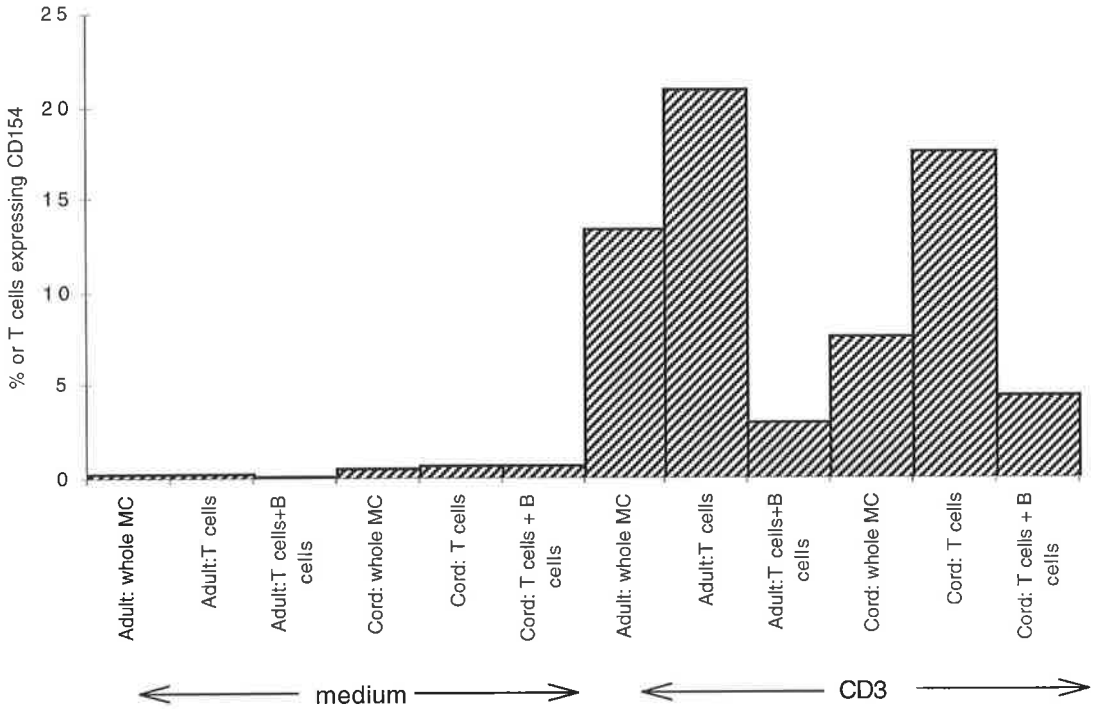

Fig. 6.7 CD3-stimulation of adult (n = 1) and cord blood (n = 1) T cells in the presence of Ramos cells or autologous B cells. Adult and cord blood MC, enriched T cells or enriched T cells plus Ramos cells were cultured in medium alone or with plate-bound CD3 mAb for 5 hours. T cell CD154 expression was assessed by dual staining with CD154-PE and CD2-FITC. The percentage of T cells expressing CD154 is shown.

In the series of experiments described in Chapter 5, a higher percentage of T cells expressed CD154 in cultures of cord blood MC compared with cultures of adult peripheral blood MC after 6 hours of stimulation with CD3 mAb (Fig. 6.8A). However, in the current experiment, the percentage of CD154 positive T cells was actually lower in cord blood MC than adult peripheral blood MC, despite the fact that similar experimental conditions were used (Fig. 6.7). A further experiment was performed where adult (n = 1) and cord blood (n = 1) mononuclear cells were stimulated for 6 hours with plate-bound CD3 mAb, to determine whether the original observations described in Chapter 5 could be replicated (Fig. 6.8B). The percentage of CD154 positive T cells in activated cord blood MC was again slightly lower than in cultures of activated adult peripheral blood MC, following 6 hours of CD3-stimulation (Fig. 6.8B).

6.2.7 Effect of CD3 mAb concentration on CD154 expression on adult and cord blood T cells stimulated in the presence of autologous B cells

As a new batch of purified CD3 mAb (OKT3) was used for stimulation of adult peripheral blood MC and cord blood MC in experiments in this chapter, it was possible that the inconsistency in results could be due to variation in the concentration of batches of CD3 mAb. The concentration of the batch of CD3 mAb used in earlier work had been determined using UV absorbance at 280 nm, whereas the concentration of the current batch was determined by the more accurate Bradford assay. Unfortunately, none of the original batch of CD3 mAb remained to enable its concentration to be determined more accurately.

Titration experiments were performed to assess the influence of CD3 mAb concentration on T cell CD154 expression after 6 hours of stimulation. In these two experiments it was obvious that, whereas the percentage of adult T cells expressing CD154 was consistent between specimens (n = 2), there was great variability between cord blood specimens (n = 2) in the results obtained (Fig. 6.9). This variability was not related to the

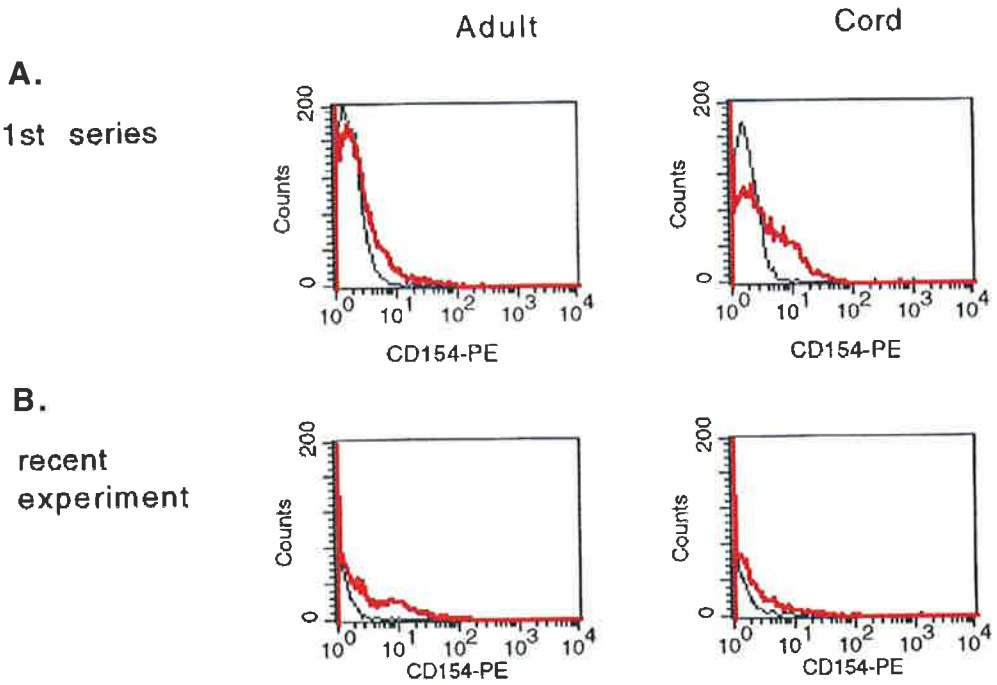

Fig. 6.8 CD154 expression on adult and cord blood T cells stimulated for 6 hours with plate-bound CD3 mAb. Unlike the series of experiments described in chapter 5 (representative histograms shown from one adult and one cord blood specimen) **(A)**, in the current experiment **(B)**, the percentage of CD154 positive T cells was not higher in cord blood MC (n = 1) than adult peripheral blood MC (n = 1). Adult peripheral blood MC or cord blood MC were cultured for 6 hours with plate-bound CD3 mAb. Staining with CD154-PE is shown for stimulated (**red**) and unstimulated (**black**) cells. T cells were identified by staining with CD2-FITC.

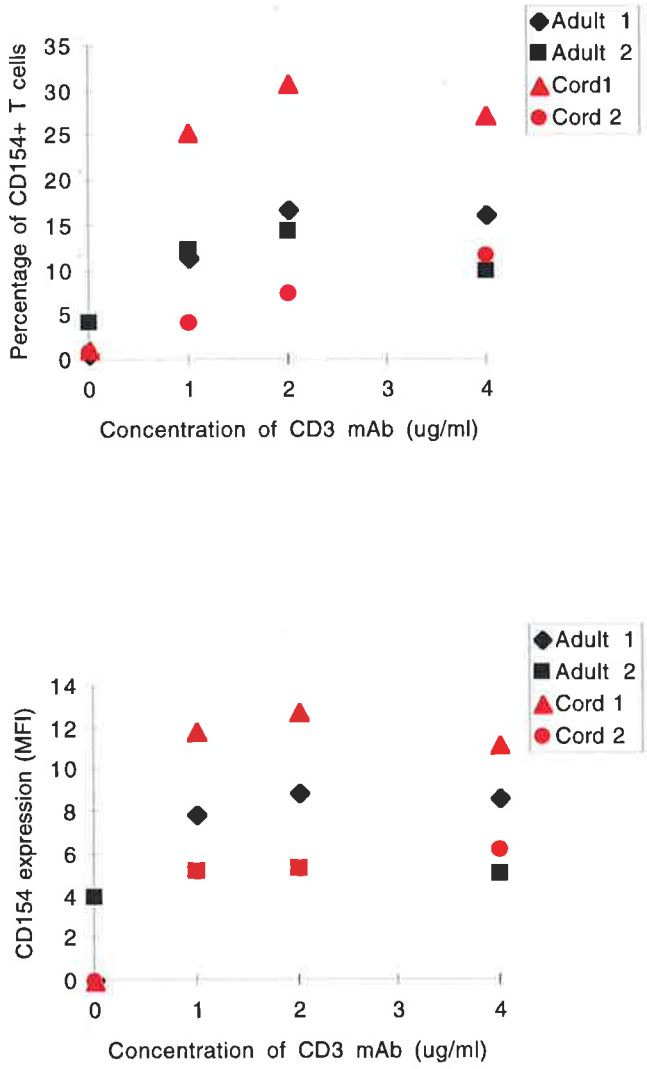

Fig. 6.9 Titration of CD3 mAb concentration used for stimulation. Adult peripheral blood MC and cord blood MC were stimulated for 5 hours with different concentrations of plate-bound CD3 mAb (OKT3). Cells were dual stained with CD154-PE and CD2-FITC. Two adult and two cord blood specimens were examined. The percentage of T cells expressing CD154 and the MFI of T cell CD154 expression are shown. Note that the MFI was recorded as zero if the percentage of CD154 positive T cells was less than 3%.

concentration of CD3 mAb used for stimulation. As these two experiments were performed during the same week, using the same mAb and reagents for staining, these factors could also be excluded as possible sources of variability.

6.3 Discussion

This chapter established that the downregulation of adult T cell CD154 expression in CD3-stimulated mononuclear cell cultures was largely due to the presence of B cells and minimally affected by the presence of monocytes, even though these cells are also CD40 positive. This is consistent with previously reported results (Yellin *et al.*, 1994). Thus, although endocytosis of CD154 occurs as a result of interaction with CD40 (Yellin *et al.*, 1994), B cells may supply other necessary signals which are not provided by monocytes.

Endocytosis of surface CD154 could not be induced by cross-linking with CD154 mAb. This suggests that either the CD154 mAb could not signal as effectively as CD40 molecules or that other membrane-associated factors were required.

CLL cells and B cell lines were able to induce down-modulation of adult T cell CD154 expression when added to CD3-stimulated enriched T cells and incubated at 37°C. Interestingly, there was also a reduction in staining for CD154 expression when incubations were carried out on ice, even in the presence of the metabolic inhibitor, sodium azide. This energy-independent 'blocking' effect occurred within 15 minutes of adding B cells to T cells and was not caused by a soluble factor released from B cells. This contrasts with conclusions drawn in a previous study (van Kooten *et al.*, 1994), where it was demonstrated that incubation of T cells with supernatant from a B cell line results in positive staining of T cells for CD40 and inhibition of CD154 staining. It is possible that the CLL cells, Ramos or Raji cells cause steric hindrance which prevents staining of the surface CD154 by mAb. In spite of this, these results clearly showed that at lower B cell: T cell ratios, the downregulation of T cell CD154 expression is partially

due to an energy-dependent process. This is consistent with the findings of Yellin *et al.* (1994) who showed that the CD154 down-modulation is partially caused by receptor-mediated endocytosis.

In the current study, the failure to demonstrate intracellular CD154 in T cells stimulated in the presence of Ramos cells, despite down-modulation of surface CD154 expression, may have resulted from rapid degradation of CD154 after it had been endocytosed. Inhibitors of endosomal acidification cause an accumulation of intracellular CD154 in T cells incubated with B cells (Yellin *et al.*, 1994), supporting this suggestion. In future experiments, degradation could be inhibited and intracellular CD154 may then be evident. This was not pursued further for reasons explained below.

The aim of this chapter was to investigate the differential downregulation of CD154 expression on cord blood T cells in the presence of B cells. Having established a method of inducing downregulation of surface CD154 on T cells without using autologous B cells, the downregulation of CD154 on adult and cord blood T cells in the presence of a B cell line was then compared. The aim was to determine whether the differential downregulation of CD154 expression on cord blood T cells in the presence of B cells was T cell-dependent or B-cell dependent. Unfortunately, the original difference observed in the percentage of cord blood T cells expressing CD154 in the presence of B cells could not be consistently reproduced and this was probably related to variability between cord blood specimens. This may have arisen from numerous factors related to the neonate and/or the delivery process. Access to detailed clinical information was not available for the purposes of this study, therefore possible clinical factors contributing to the variability between cord blood specimens could not be followed up. In addition, cord blood samples became difficult to obtain and pursuing these experiments was no longer feasible. Thus, unfortunately, these experiments were discontinued.

CHAPTER 7.

GENERAL DISCUSSION

Human neonates and young children have a high incidence of infectious diseases which are a cause of high morbidity and mortality, particularly in developing countries (World Health Organization, 1998). This susceptibility to infection is partially related to the relative immaturity of the immune system in early life. Innate and adaptive immune responses (including humoral immunity and cell mediated immunity) are limited in the neonate and mature gradually during early childhood (Adkins, 1999; Kovarik & Siegrist, 1997; Kovarik & Siegrist, 1998; Quie, 1990; Wilson, 1986).

This project focused on T-dependent antibody responses in neonates and young children. T-dependent antibody responses in neonates and infants are of lower magnitude, are induced more slowly and are of more limited duration than antibody responses in adults and older children (Halsey & Galazka, 1985; Kovarik & Siegrist, 1998; Stoll *et al.*, 1993 Lee, 1991 #524). Isotype switching (Stoll *et al.*, 1993 Lee, 1991 #524), affinity maturation (Ridings *et al.*, 1997) and development of immunologic memory (Di Sant'Agnese, 1950) occur to only a limited extent in neonates.

In vitro studies indicate that limitations of neonatal T-dependent antibody responses are related to immaturity of neonatal B cells and neonatal CD4 positive T cells. While neonatal B cells have a reduced capacity to produce antibodies of downstream isotype, even in the presence of adequate T cell help, neonatal CD4 positive T cells produce lower levels of a number of cytokines compared with adult CD4 positive T cells (Hayward & Cosyns, 1994; Lewis *et al.*, 1991; Pastorelli *et al.*, 1990; Splawski & Lipsky, 1991) and have a limited ability to support the differentiation of adult or neonatal B cells into antibody secreting cells (Andersson *et al.*, 1981; Hayward & Lawton, 1977; Miyawaki *et al.*, 1981; Splawski *et al.*, 1991; Tosato *et al.*, 1980; Yachie *et al.*, 1995).

During T-dependent antibody responses, effective activation of both B and T

lymphocytes depends on the interaction of costimulator molecules and their ligands (Janeway & Bottomly, 1994; Parker, 1993). These molecules deliver contact-mediated signals at the time of cognate interaction between T cells and B cells that regulate lymphocyte activation. Differential expression of costimulator molecules on B and T lymphocytes in early life could affect the outcome of T-B cell interactions during T-dependent antibody responses. Altered lymphocyte expression of costimulator molecules could thus contribute to the limitations of T-dependent antibody responses that have been observed in young children.

This project examined the expression of two groups of costimulator molecules on lymphocytes from neonates and young children. CD80, CD86, CD28 and CD152, and CD40 and CD154, are widely regarded as the most important groups of costimulator molecules for regulating T cell activation and B cell activation, respectively (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994; van Kooten & Banchereau, 1996).

The importance of CD28 signalling during T-dependent antibody responses is demonstrated in CD28 knockout mice, which have low basal immunoglobulin levels, diminished secondary responses and reduced isotype switching (Green *et al.*, 1994; Shahinian *et al.*, 1993). CD28 knockout mice also lack germinal centres and somatic mutations (Ferguson *et al.*, 1996).

The important role of the CD40-CD154 interaction in T-dependent antibody responses is evident in patients with hyper-IgM syndrome who lack expression of functional CD154. Hyper-IgM patients have antibody responses restricted to IgM, lack germinal centres, fail to generate memory B cell responses and have reduced or absent somatic mutations (Callard *et al.*, 1993; Facchetti *et al.*, 1995; van Kooten & Banchereau, 1996).

There are obvious parallels between the roles of these costimulator molecules and the limitations of T-dependent antibody responses in human neonates. Therefore, it was logical to propose that costimulator molecules from these two families may be

differentially expressed on neonatal lymphocytes. In support of this hypothesis, several studies had been published at the time that this project was commenced, that reported lower levels of CD154 expression on neonatal T cells compared with adult T cells stimulated with PMA and ionomycin. At that time, there were no studies examining the expression of any other costimulator molecules on neonatal lymphocytes during *in vitro* activation. In addition, the induction of neonatal T cell CD154 expression had not been studied in other activation systems.

Early in this project, difficulties were encountered in the preparation of cord blood mononuclear cells (MC) for culture. Cord blood MC isolated by density centrifugation are often contaminated with erythroid cells (Ridings *et al.*, 1996). A second density separation has been suggested as a method for removing contaminating erythroid cells from cord blood MC fractions in preparation for functional studies (Ridings *et al.*, 1996). However, in the present study, it was found that contaminated cord blood MC could only be adequately purified using this technique when the level of contamination with erythroid cells was low. A more effective method of removing contaminating erythroid cells from cord blood MC was developed in this project using 10F7, a mAb which binds to glycophorin A, and anti-mouse immunoglobulin coated magnetic beads.

It was necessary to determine whether treating cord blood MC with magnetic beads would affect the composition of the cells recovered or affect the ability of the lymphocytes to respond to *in vitro* stimulation. A comparative study was performed which examined the cellular composition of cord blood MC after ammonium chloride lysis (traditionally used to remove contaminating erythroid cells) or treatment with magnetic beads. This indicated that treatment with magnetic beads may selectively deplete B cells. However, the depletion of B cells by treatment with magnetic beads was probably not as marked as suggested in this study, since MC treated with ammonium chloride possibly had a relatively higher number of B cells due to selective depletion of T cells.

There is no standard method for preparing uncontaminated cord blood MC against which the functional responses of cord blood MC treated with magnetic beads could be

compared. However, cord blood lymphocytes treated with 10F7 mAb and magnetic beads showed upregulation of activation markers in response to *in vitro* stimulation with PMA and ionomycin or plate-bound CD3 mAb. The fact that the majority of cord blood MC were treated with magnetic beads after Lymphoprep separation, whereas adult MC were isolated by Lymphoprep separation alone, represents one potential source of artefact in the functional studies in this project. However, all of the costimulator molecules examined (with the exception of surface CD152) showed similar expression on adult and cord blood lymphocytes under at least one set of *in vitro* activation conditions, suggesting that the purification process used to remove contaminating erythroid cells from cord blood MC did not interfere with the subsequent functional responses of cord blood lymphocytes.

No studies have previously examined the regulation of CD80, CD86, CD28 and CD152 expression on human neonatal lymphocytes activated *in vitro*. The interaction between CD80 or CD86 and CD28 is considered one of the most important costimulatory pathways for T cell activation (Greenfield *et al.*, 1998; Hathcock & Hodes, 1996; June *et al.*, 1994). Signalling through CD28 promotes T cell proliferation, increased cytokine secretion, upregulation of cytokine receptors and enhanced CD154 expression (Boussiotis *et al.*, 1994; Hathcock & Hodes, 1996). In contrast, signalling through CD152 downregulates T cell activation and may play a role in inducing T cell anergy (Krummel & Allison, 1996; Perez *et al.*, 1997; Walunas *et al.*, 1996; Walunas *et al.*, 1994). It is believed that the balance of signals delivered by CD28 and CD152 regulates T cell activation (Krummel & Allison, 1996). There is also recent evidence that CD28 and CD152 signalling influence the differentiation of CD4 positive T cells into Th1 vs Th2 secreting cells (Oosterwegel *et al.*, 1999).

In view of their important role in regulating T cell activation, this project explored the hypothesis that differential expression of CD80, CD86, CD28 or CD152 on neonatal lymphocytes may alter the costimulatory signals delivered to neonatal T cells. This in turn could affect the ability of neonatal CD4 positive T cells to differentiate into effective

helper cells for T-dependent antibody responses.

Table 7.1. Summary of CD80 and CD86 expression on B cells from cord blood and peripheral blood from children compared with expression on adult peripheral blood B cells (based on experiments described in Chapter 4). Entries in bold indicate significant differences.

	CD80 expression on B cells	CD86 expression on B cells
adult vs cord unstimulated	not expressed on resting B cells	similar on adult and cord B cells
adult vs children unstimulated	not expressed on resting B cells	**higher expression on B cells from children**
adult vs cord (stimulation with PMA/ionomycin)	similar on activated adult and cord B cells	similar on activated adult and cord B cells
adult vs cord (stimulation with CD3 mAb)	similar on activated adult and cord B cells	similar on activated adult and cord B cells

CD80 was not expressed on resting B cells isolated from cord blood or peripheral blood of adults or children (2-20 months of age) (Table 7.1). However, CD80 was induced on B cells when mononuclear cells from adult or cord blood were activated with PMA and ionomycin or plate-bound CD3 mAb for 3 days. The kinetics and MFI of CD80 expression were similar on adult and cord blood B cells. CD86 was constitutively expressed on resting B cells from cord blood or peripheral blood of children and adults. Higher levels of CD86 expression on resting B cells from children compared with adult B cells may reflect upregulation of CD86 expression *in vivo*, although this would need to be investigated further. During *in vitro* activation, CD86 expression was upregulated to similar levels on adult and cord blood B cells. Since B cells from cord blood and peripheral blood of young children expressed adult levels of CD80 and CD86, they should be capable of delivering costimulatory signals to T cells via CD28 and CD152.

Table 7.2. Summary of CD28 and CD152 expression on T cells from cord blood and peripheral blood from children compared with expression on adult peripheral blood T cells (based on experiments described in Chapter 4). Entries in bold indicate significant differences.

	CD28 expression on T cells	CD152 expression on T cells
adult vs cord unstimulated	**higher percentage of CD28 positive T cells in cord blood**	not expressed on resting T cells
adult vs children unstimulated	**higher percentage of T cells from children expressed CD28**	not expressed on resting T cells
adult vs cord (stimulation with PMA/ionomycin)	similar percentage of CD28 positive T cells after activation no increase in MFI of CD28 expression on activated adult or cord T cells	**lower level of surface expression on cord T cells** similar levels of intracellular CD152
adult vs cord (stimulation with CD3 mAb)	**higher percentage of CD28 positive T cells in cord blood;** **higher MFI of CD28 expression on cord T cells**	**low level expression on adult T cells;** **not expressed on cord T cells**

A higher percentage of resting cord blood T cells and peripheral blood T cells from children expressed CD28 compared to adult peripheral blood T cells (Table 7.2). The MFI of CD28 expression on cord T cells or T cells from children was not significantly different compared to adult T cell expression. The differences observed in the percentage of T cells expressing CD28 are consistent with previous findings that the proportion of circulating CD28 positive T cells declines throughout life (Hoshino *et al.*, 1993).

Although the percentage of T cells expressing CD28 increased in adult and cord blood MC stimulated with PMA and ionomycin, there was no increase in the MFI of CD28 expression (Table 7.2). A similar percentage of activated adult and activated cord T cells were CD28 positive after stimulation with PMA and ionomycin. There was no significant increase in the percentage of adult T cells expressing CD28 or the MFI of CD28

expression on adult T cells following stimulation with CD3 mAb. In contrast, the percentage of CD28 positive T cells and the MFI of CD28 expression increased on cord T cells stimulated with CD3 mAb. Thus, expression of CD28 on CD3-stimulated cord T cells was higher than levels of CD28 expression on CD3-stimulated adult T cells.

CD152 was expressed at lower levels on the surface of cord T cells than adult T cells after stimulation with PMA and ionomycin (Table 7.2). When adult and cord blood MC were stimulated with CD3 mAb, no CD152 was detected on cord T cells and only low level expression was observed on adult T cells. It was therefore surprising to observe similar levels of intracellular CD152 in adult and cord T cells stimulated with PMA and ionomycin. These results suggested that neonatal T lymphocytes may be capable of producing adult levels of CD152 but that transport to the cell surface and surface expression may be regulated differently.

Higher levels of CD28 expression and lower levels of surface CD152 expression on activated neonatal T cells compared with activated adult T cells may mean that neonatal T lymphocytes are more capable of responding to stimulation by antigen presenting cells. This was an unexpected finding, and does not provide an explanation for the reported limitations of antibody secretion, isotype switching, memory B cell differentiation and affinity maturation in human neonates. It is possible that the altered levels of CD28 and CD152 expression represent a specialisation of neonatal T cells to partially compensate for the relative deficiency of T cells in neonates. In addition, higher expression of CD28 and reduced surface expression of CD152 on neonatal T lymphocytes may ensure that the balance of costimulatory signals favours activation of naive neonatal T lymphocytes.

There is also some evidence that CD28 and CD152 signals regulate the differentiation of CD4 positive T cells into Th1 and Th2 secreting cells. Differentiation of Th2 type cells seems to be dependent on CD28 signalling (Rulifson *et al.*, 1997; Schweitzer & Sharpe, 1998; Yang *et al.*, 1995), whereas CD152 signalling limits the differentiation of Th2 secreting cells (Oosterwegel *et al.*, 1999). Increased expression of CD28 and lower expression of surface CD152 on neonatal T cells could therefore potentially favour the

development of Th2 secreting cells and may be one explanation for neonatal T lymphocytes being skewed towards production of Th2 cytokines (Adkins, 1999; Demeure *et al.*, 1995; Shu *et al.*, 1994). Although this is not of direct relevance to T-dependent antibody responses in neonates, it could have implications for the development of effective vaccines against intracellular pathogens and viruses, which require the induction of Th1 cytokine responses (Kovarik & Siegrist, 1998).

CD40 and CD154 provide important signals for B cell differentiation during T-dependent antibody responses. Isotype switching, germinal centre formation, memory B cell differentiation and affinity maturation are all dependent on CD40 signalling (van Kooten & Banchereau, 1996). Germinal centre formation also requires T cell signalling via CD154 (van Essen *et al.*, 1995). These molecules have been the focus of much attention in relation to the reported limitations of neonatal humoral immunity. At the time this project was commenced, four studies had been published indicating that CD154 expression was reduced or absent on neonatal T cells stimulated with PMA and ionomycin (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). CD40 expression by resting neonatal B cells was reported to be similar to expression by adult B cells (Durandy *et al.*, 1995). There had been no investigation of the regulation of CD40 expression on neonatal B cells during *in vitro* activation. Similarly, there had been no studies of CD154 expression on neonatal T cells using other methods of *in vitro* stimulation. The aim of this project was to examine the regulation of CD40 expression on activated neonatal B cells and to further study the regulation of neonatal T cell CD154 expression during *in vitro* activation.

Table 7.3. Summary of CD40 and CD154 expression on cord blood lymphocytes and peripheral blood lymphocytes from adults and children (based on experiments described in Chapter 5). Entries in bold indicate significant differences in magnitude of expression (CD40) or qualitative differences in kinetics (CD154).

	CD40 expression on B cells	**CD154 expression on T cells**
adult vs cord unstimulated	similar on B cells from adults and majority of cord blood specimens	not expressed on resting T cells
adult vs children unstimulated	similar on B cells from adults and children	not expressed on resting T cells
adult vs cord (stimulation with PMA/ionomycin)	**higher expression on cord B cells**	**different kinetics of expression on adult and cord T cells**
adult vs cord (stimulation with CD3 mAb)	similar on adult and cord B cells	WHOLE MONONUCLEAR CELLS: **different kinetics of expression on T cells from some cord specimens** B CELL/MONOCYTE DEPLETED: similar expression on adult and cord T cells

In the majority of cord blood specimens, B cell CD40 expression was similar to adult levels of expression. High levels of CD40 expression were observed on B cells in a small group of cord blood specimens and may have reflected upregulation of CD40 expression *in vivo*, possibly related to events during parturition. As yet, there have been no *in vivo* studies of the regulation of B cell CD40 expression, so this explanation remains speculative. Expression of CD40 was similar on resting peripheral blood B cells from adults and young children.

When adult and cord blood MC were activated with PMA and ionomycin, CD40 expression was upregulated on adult and cord blood B cells. The MFI of CD40 expression on activated cord blood B cells was higher than the MFI of CD40 expression on activated adult B cells. In contrast, CD40 expression was similar on activated adult and cord blood B cells in cultures of CD3-stimulated adult and cord blood MC. Ligation

of CD40 on neonatal B cells in combination with supplemental cytokines can induce adult levels of B cell proliferation but only limited secretion of antibodies of all isotypes, particularly IgG and IgA (Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1993). From the results of the present study, it is clear that the limited responsiveness of neonatal B cells to CD40 ligation is not attributable to reduced CD40 expression. There may be differences in the CD40 signalling pathway in neonatal B cells, or alternatively, downstream factors may be responsible for reduced immunoglobulin secretion in response to CD40 ligation.

T cell CD154 expression was compared in adult and cord blood MC cultures stimulated with PMA and ionomycin. In contrast to the published reports (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995; Splawski *et al.*, 1996), the current study showed that the level of CD154 expression was similar on cord blood and adult T cells at early time points, but that CD154 expression subsequently declined more rapidly on cord blood T cells than adult T cells.

The regulation of T cell CD154 expression was also examined on adult and cord blood MC stimulated with CD3 mAb. During these studies, Splawski et al. (Splawski *et al.*, 1996) published their results showing that neonatal T cells could express adult levels of CD154 if stimulated with CD3 mAb and supplemental cytokines. Therefore, the current study was developed further to compare adult and cord blood T cell CD154 expression induced by PMA and ionomycin with expression induced by CD3 mAb. Both the percentage of CD154 positive T cells and the MFI of T cell CD154 expression in adult and cord blood MC stimulated with CD3 mAb were much lower compared with CD154 expression induced by PMA and ionomycin. These observations indicate that CD154 expression can be induced at higher levels on a greater proportion of adult and neonatal T lymphocytes when signalling through the TCR complex is bypassed, and protein kinase C and intracellular calcium release are directly stimulated using PMA and ionomycin, respectively. However, this maximal level of CD154 expression cannot be sustained in neonatal T lymphocytes. This may be related to limitations of CD154 mRNA transcription

in neonatal T lymphocytes.

In the first series of experiments comparing adult and cord blood T cell CD154 expression in mononuclear cells stimulated with CD3 mAb, a higher percentage of CD154 positive T cells was found in cord blood MC compared with adult MC after 6 hours of stimulation.

T cell expression of CD154 is downregulated in the presence of CD40 positive cells, such as B cells (Hermann *et al.*, 1993; Ludewig *et al.*, 1996; Nusslein *et al.*, 1996; van Kooten *et al.*, 1994; Yellin *et al.*, 1994). This is partially attributable to receptor-mediated endocytosis (Yellin *et al.*, 1994). Thus, the next series of experiments examined T cell CD154 expression in cultures of adult and cord blood MC which had been depleted of B cells and monocytes prior to CD3 stimulation. The MFI of CD154 expression and the percentage of T cells expressing CD154 tended to be higher in adult and cord blood MC which had been depleted of B cells and monocytes, consistent with downregulation of T cell CD154 expression in the presence of B cells and/or monocytes. Adult and cord T cells showed similar patterns of CD154 expression when mononuclear cells were depleted of B cells and monocytes prior to CD3 stimulation. These results suggested that differential downregulation of CD154 on cord blood T cells in the presence of B cells and/or monocytes may account for the different kinetics of CD154 expression on cord T cells in whole MC cultures stimulated with CD3 mAb.

The downregulation of T cell CD154 expression in the presence of B cells and/or monocytes was investigated further. It was shown that, although monocytes expressed CD40, they contributed minimally to the downregulation of T cell CD154 expression which appeared to be mainly caused by B cells. This suggested that factors in addition to expression of CD40 may be required on cells capable of inducing downregulation of T cell CD154 expression.

In an attempt to find a method of inducing downregulation of T cell CD154 expression without using autologous B cells, T cells previously stimulated with CD3 mAb were

incubated with CLL cells, or the B cell lines, Raji or Ramos. This revealed that the downregulation of CD154 expression on T cells incubated with B cells was partly due to an energy-dependent process that could be inhibited by incubating the cells on ice in the presence of sodium azide. However, some downregulation of T cell CD154 expression still occurred when incubations were performed under these conditions. This suggested that the reduced expression of CD154 on T cells incubated with B cells was also partially attributable to an energy-independent process. This was not caused by a soluble factor produced by B cells interfering with staining of CD154. It is suggested that this energy-independent process may result from CD40 positive cells physically interfering with the binding of CD154 mAb to the T cell CD154 molecules. This would be consistent with the observation that the effect was dependent on the ratio of B cells to T cells. A monoclonal antibody that binds to non-receptor parts of the CD154 molecule could be used to ascertain whether this hypothesis is correct.

The aim of the final series of experiments was to compare CD154 expression on adult and cord T cells stimulated with CD3 mAb in the presence of autologous B cells, a B cell line or in the absence of B cells. The first experiment showed that adult and cord T cell CD154 expression was higher on T cells stimulated in the absence of B cells, and that T cell CD154 expression was downregulated by endogenous or exogenous B cells.

However, whereas the first series of experiments comparing CD154 expression on adult and cord blood T cells from cultures of CD3-stimulated MC showed a higher percentage of cord blood T cells expressing CD154 after 5-6 hours of stimulation, in this latter experiment, a slightly higher percentage of T cells were CD154 positive in the adult whole MC culture than in the cord blood whole MC culture. In three further experiments where adult and cord blood whole MC were stimulated with CD3 mAb for 5-6 hours, one cord blood specimen had a higher percentage of T cells expressing CD154 after 6 hours of CD3 stimulation, whereas two other specimens showed an adult-like pattern of T cell CD154 expression. This was not related to the concentration of CD3 mAb used for stimulation, and could not be traced to experimental conditions. This suggests that

physiological factors, possibly related to parturition, influence the regulation of CD154 expression on neonatal T cells. The frequency of cord blood MC which showed a higher proportion of CD154 positive T cells after 5-6 hours of CD3 stimulation (four of a total of seven cord blood specimens examined) indicates that this phenomenon is not likely to be related to undetected intrauterine infection.

Overall, the small differences observed in the levels of cord T cell CD154 expression at different time points may have subtle effects on the signals delivered to B cells via CD40 or alternatively may affect the delivery of T cell signals via CD154. However, it is unlikely that these differences in neonatal T cell CD154 expression fully account for the limitations of T-dependent antibody responses, isotype switching and memory B cell differentiation observed in neonates. This conclusion is supported by the findings of Splawski *et al.* (1996) who showed that the CD154 expressed by neonatal T cells is capable of delivering signals to B cells.

This project had a number of design limitations, many of which were unavoidable. Because human material was being used, the experiments were conducted according to the availability of specimens. As a result, the series of experiments were often conducted over a long period of time. The child peripheral blood specimens, for example, were collected from children enrolled in a longitudinal immunisation study, thus specimens from older children were collected at a later date than specimens from younger children. (Note that all specimens were collected from different children and sequential specimens were not taken from the same child.) This meant that the levels of costimulator molecule expression could not be compared between children of different ages as the effects of time could not be controlled for. Cord blood specimens were of limited availability and therefore different batches of reagents were used for *in vitro* stimulation and for immunofluorescence staining during the course of each series of experiments. This inevitably would have increased the variability of data collected. Consequently, small differences between adult and neonatal lymphocytes in the level of costimulator expression may not have been detected. However, differences likely to be of biological

significance should have been identified.

In this study, umbilical cord blood was used as being representative of neonatal peripheral blood. This is routinely done in studies of the neonatal immune system because cord blood is the only source of large volumes of neonatal blood. Almost all the studies of neonatal lymphocytes cited in this thesis used cord blood lymphocytes rather than peripheral blood lymphocytes from neonates. However, there is evidence that it may not be valid to assume that cord blood is identical to neonatal peripheral blood. Significant differences have been demonstrated in lymphocyte subpopulations between umbilical cord blood and peripheral venous blood collected from the same neonates 5 days after birth (Raes *et al.*, 1993). It is possible that phenotypic and/or functional characteristics of neonatal peripheral blood lymphocytes may differ from those of cord blood lymphocytes. Currently, it is not possible to perform studies like those described in this project using the small volumes of peripheral venous blood that can be collected from neonates. However, the fact that observations from cord blood studies may not truly reflect what happens in neonatal peripheral blood should be considered.

Following on from this, the possibility of contamination of cord blood specimens by maternal leucocytes cannot be excluded. Use of a highly sensitive polymerase chain reaction technique to detect maternal DNA in DNA extracted from cord blood cells suggests that all cord blood samples are contaminated by maternal cells, although the absolute numbers of contaminating cells are small (Petit *et al.*, 1995). It is estimated that maternal cells represent one in 10^4 - 10^5 of nucleated cells in whole cord blood samples (Petit *et al.*, 1995). This low level contamination is unlikely to occur during collection of cord blood, but probably results from minor materno-fetal haemorrhage during delivery or even transmission of maternal cells to the fetus during pregnancy (Petit *et al.*, 1995). Although this number of contaminating maternal cells would be too small to interfere with phenotyping experiments on resting cord blood lymphocytes, the presence of even small numbers of maternal cells could theoretically affect differentiation of cord blood lymphocytes during functional experiments. Cytokine secretion by maternal T cells or

induction of a mixed lymphocyte reaction may affect the regulation of costimulator molecules on neonatal lymphocytes. Variable contamination with maternal cells could be one explanation for the observed variations in expression of CD154 on CD3-stimulated neonatal T cells.

Unfortunately, access to normal human lymphoid tissue is difficult at the best of times and almost impossible in the neonatal age group. In this project, as in many studies of immune responses in humans, *in vitro* cultures of peripheral blood lymphocytes have been used to model events which would normally occur in the specialised micro-environment of secondary lymphoid organs. Peripheral blood lymphocytes are trafficking from one lymphoid tissue to another (Dudley & Wiedmeier, 1991) and may differ functionally from lymphocytes found in lymphoid organs. Similarly, polyclonal activation with mitogens or plate-bound CD3 mAb has been used instead of antigen-specific lymphocyte activation. The regulation of costimulator molecules on lymphocytes responding to antigen-specific activation *in vivo* may differ from that observed in the experiments described here.

Mononuclear cells were used for these *in vitro* studies rather than purified lymphocyte populations in order to establish an activation system as physiologically relevant as possible. However, the composition of adult peripheral blood MC is different from that of umbilical cord blood MC which could account for some of the differences observed between the two groups (Beck & Lam Po Tang, 1994; Hannet *et al.*, 1992; Motley *et al.*, 1996). Although the absolute number of T cells is higher in neonates than adults, the percentage of T cells (CD4 positive and CD8 positive) is lower in cord blood (Beck & Lam Po Tang, 1994; Hannet *et al.*, 1992; Motley *et al.*, 1996). As a result, the level of CD4 T cell help available to B cells may be relatively less in cultures of cord blood MC. In addition, using whole MC makes it difficult to determine which cell population is primarily responsible for any differences observed between cultures of adult and cord blood cells. For example, differential expression of a B cell marker in cord blood MC stimulated with plate-bound CD3 mAb could be related to intrinsic immaturity of the cord

blood B cells or to altered cytokine secretion by cord blood T cells. Conversely, reduced expression of a particular marker on cord blood T cells may reflect a decreased capacity of antigen presenting cells in cord blood to provide essential accessory signals.

The studies described in this thesis did not examine expression of these groups of costimulator molecules on antigen presenting cells such as monocytes and dendritic cells. CD40, CD80 and CD86 are all expressed on antigen presenting cells (Alderson *et al.*, 1993; Azuma *et al.*, 1993a; McLellan *et al.*, 1996; Vyth Dreese *et al.*, 1995). Differential expression of these molecules on antigen presenting cells in neonates could affect neonatal T cell activation, thereby contributing to the limited ability of neonatal CD4 positive T cells to support B cell differentiation during T-dependent antibody responses. This would be worth investigating but was beyond the scope of this study. Likewise, the expression of CD80, CD86 and CD40 on T cells and CD152 and CD154 on B cells was not examined in this study. Once roles for these molecules on these cell types are defined, it would be interesting to examine their expression on neonatal lymphocytes.

What conclusions can be drawn about the implications of these results for understanding T-dependent antibody responses in the human neonate? Looking first at CD80, CD86, CD28 and CD152, the results from this study suggest that regulation of these molecules on neonatal lymphocytes may even be specialised to promote T cell activation. Therefore, this does not provide an explanation for reduced cytokine secretion by neonatal CD4 positive T cells and their limited ability to support B cell immunoglobulin secretion. CD40 expression was similar or higher on neonatal B cells compared with adult B cells. Although the kinetics of CD154 expression on neonatal T cells differed from the kinetics of adult T cell CD154 expression, adult levels of CD154 expression could be induced on neonatal T cells under certain activation conditions. Therefore, differences in the expression of CD40 and CD154 on neonatal lymphocytes could not account for the limitations of antibody production, isotype switching, development of immunologic memory and affinity maturation in neonates. Overall, this study does not support the hypothesis that differential expression of either of these groups of costimulator molecules

on neonatal lymphocytes is responsible for the relative deficiencies of T-dependent antibody responses in neonates and young children.

With the exception of CD152 (the negative regulator of T cell activation), all of the costimulator molecules examined in this project were expressed on the surface of neonatal lymphocytes at adult levels under at least one set of *in vitro* activation conditions. It thus seems reasonable to conclude that neonatal T and B lymphocytes should be capable of delivering and responding to costimulatory signals. However, neonatal lymphocytes may in fact require higher levels of costimulatory signals for activation, possibly because the duration of T-B cell interactions is limited in neonates due to the reduced expression of adhesion molecules (Gerli *et al.*, 1993; Sanders *et al.*, 1988). There is also evidence that naive lymphocytes require more costimulation than memory lymphocytes for activation (Kuiper *et al.*, 1994). Further studies could examine the signalling pathways activated by ligation of costimulator molecules on the surface of neonatal lymphocytes. Limited functional responses of neonatal lymphocytes, despite adult levels of expression of costimulator molecules, may be related to differences in the intracellular signalling pathways. It would also be worthwhile examining the regulation of these costimulator molecules on neonatal lymphocytes in response to cytokines. A greater understanding of the regulation and function of costimulator molecules on neonatal lymphocytes could lead to the development of vaccine formulations that promote costimulation of neonatal lymphocytes and thus potentially enhance the efficacy of vaccines administered in the newborn period.

APPENDIX

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