UNIVERSITY OF SOUTHAMPTON

Immunotherapy of Murine B-cell Lymphoma Mediated by Agonistic Anti-CD40 Antibody: Role of Co-stimulation in Generation of $CD8^+$ CTL

Graham Roy Crowther, MBlochem.

Doctor of Philosophy

Cancer Sciences Division,
Faculty of Medicine, Health and Biological Sciences

March 2003

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
CANCER SCIENCES

Doctor of Philosophy

by Graham Roy Crowther

Agonistic monoclonal antibodies directed against murine CD40 can be used in experimental settings to induce anti-lymphoma T-cells that are able to effect primary tumour eradication *in vivo*. These anti-tumour CTL provide both protection against re-challenge with the original vaccinating tumour line and, furthermore, prevent relapse, thereby affecting long-term survival. Such therapeutic strategies are desirable in order to provide durable responses against human lymphomas without evolving adverse side-effects.

Here, we further investigate the mechanism by which anti-CD40 exerts its therapeutic activity and demonstrate that interactions via the TNFR family member 4-1BB contribute to the expansion of anti-tumour CTL and, hence, survival following immunotherapy of lymphoma; indeed, we show that all tumour-specific CTL generated during anti-CD40-induced immunotherapy express this molecule. Furthermore, we demonstrate for the first time that the murine lymphomas used in these studies invoke an immunological response prior to anti-CD40 administration which is characterised by the phenotypic maturation of DCs and differentiation of $CD8^+$ T-cells, as well as the accumulation of these cells at the site of tumour. Importantly, the kinetics and magnitude of these responses are augmented by administration of anti-CD40 thereby suggesting that this mAb exerts its therapeutic activity by the boosting of a pre-existing ineffectual adaptive immune response. Data shown here suggest that enhanced DC maturation following administration of anti-CD40 to tumour-bearing animals may contribute to this effect and, hence, support the hypothesis that anti-CD40 operates at the axis of the professional APC in order to affect the rejection of both CD40-positive and CD40-negative tumours.

In addition, we show that both $CD8^+$ T-cell lines and immortalised hybridomas derived from tumour-reactive $CD8^+$ CTL can be established *in vitro* from animals in remission following anti-CD40-induced immunotherapy. These provide a basis for the screening of a lymphoma-derived cDNA library in order to identify the tumour rejection antigens operating in this system.

List of Tables

Acknowledgements

I'd like to firstly thank my parents who have helped me and encouraged me always, despite my lack of appreciation at times. Thank you to the people in my church, house-group, and, especially, Duncan House for your continuing support. Thanks to all my mates (especially Dave, Jez, Loz, Garry and Claire) and Jen who have put up with me, prayed for me, and given me really good advice over the past four years.

I'd also like to thank Professor Martin Glennie, Dr. Aymen Al-Shamkhani, Dr. Ruth French, Alison Tutt, Dr. Claude Chan, Dr. Jamie Honeychurch, and all those working at the Cancer Sciences Division for their help and advice, especially Lisa, Kerry, Sam and Richard for assisting with my practical work. Thank you to Dr. Mark Cragg who has been, no exaggeration, a real inspiration and help.

I'd also like to thank Tenovus for the funding they have provided for this project.

Abbreviations

(i)DC	(Immature) dendritic cell
2-ME	2-mercapto-ethanol
7AAD	7-aminoactinomycin-D
Ad5E1	Human adenovirus type 5 early region 1
AICD	Activation-induced cell death
APC	Antigen presenting cell or Allophycocyanin
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine Serum Albumin
CB	Cathepsin B
Cdk2	Cyclin-dependent kinase 2
CDRs	Complementarity determining region
CFSE	Carboxy Fluorescein Succinimidyl Ester
CLIP	Class-II-associated Ii peptide
CM	Complete media
CSK	C-terminal Src kinase
CTL	Cytotoxic lymphocyte
CTLp	CTL precursors
DC-SIGN	DC-specific ICAM3-grabbing nonintegrin
EAE	Experimental allergic encephalitis
ELISA	Enzyme-Linked Immunsorbant Assay
ER	Endoplasmic reticulum
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FSC/SSC	Forward scatter/Side scatter
FTOC	Foetal thymic organ culture
GTE	Genetic targeting expression
HA	Haemagglutinin
HEL	Hen Egg Lysozyme
HPV16	Human papilloma virus type 16-derived
HSP	Heat-shock protein
hTERT	Human reverse transcriptase telomerase
ICOS	Inducible co-stimulator
ICTM	Iscove's complete tumour media
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
Ii	Invariant chains
IMDM	Iscove's modified Dulbecco's media
ITAM	Immunoreceptor tyrosine-based activation motif
LC	Langerhan cell
LPS	Lipopolysaccaride
mAb	Monoclonal antibody
MACS	Magnetically Assisted Cell Separation
MBP	Myelin basic protein
(p)MHC	(Peptide)-Major Histocompatibility complex
MLV	Murine leukaemia virus
MMTV	Mouse mammary tumour virus
MTOC	Microtubule organising centre
NP40	Nonidet P40
OVA	Ovalbumin
PAG	Glycosphingolipid-enriched microdomains
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-derived molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PE	Phycoerthyrin
PLC	Phospholipase C
PMA	Phorbol 12-myristidate 13-acetate
PPV-VLP	Recombinant porcine parvovirus-like particle
PRR	Pattern-recognition receptor
PS	Phosphotidylserine
PTK	Protein tyrosine kinase
RAG	Recombination activating gene
RT-PCR	Reverse transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
SEREX	Serological analysis of recombinant cDNA expression libraries
SMAC	Supramolecular activating complex
SPI-6	Serpin serine protease Inhibitor-6
STAg	Soluble Toxoplasma gondii extract
TAA	Tumour-associated antigens
TAE	Tumour antigen epitopes
T-bet	T box expressed in T-cells
TCR	T-cell receptor
T_H	T-helper
TIL	Tumour infiltrating lymphocyte
TLR	Toll-like receptor/ Toll/ILR homologous region
TNF(R)	Tumour necrosis factor (receptor)
TOC	Time of commitment
Tr	T-regulatory
TRA	Tumour rejection antigen
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

All multicellular organisms maintain structural homeostasis by appropriately balancing the rate of cellular proliferation with apoptotic cell death. Any disparity between these processes may therefore lead to aberrant cellular growth and the subsequent accumulation of a clonal neoplasm or tumour. Such tumourgenic dysfunction usually has a composite aetiology consisting of oncogene inheritance, viral manipulation of cellular survival, and/or mutagen exposure, *e.g.*, UV irradiation, chemical carcinogens. If unchecked by intracellular controls, transformed neoplastic cells may undergo bouts of mutations and selection thereby progressing the tumour from a benign state of non-invasiveness to a malignant cancer, *i.e.*, a tumour capable of traversing basal laminae, metastasising to secondary sites and evoking the production of dedicated capillaries (angiogenesis).

Currently in the UK, one third of people below the age of seventy-five are likely to develop cancer within their lifetime. Each year, one quarter of a million new cases of cancer are diagnosed and 140,000 people die of the disease. This accounts for approximately one quarter of the annual death toll in this country.

Traditional cancer control, consisting of surgery, radiotherapy, and chemotherapy, aims to sustain the increase in cancer survival over the past twenty years. Nonetheless, these techniques remain primitive in that they are incapable of fine distinction between normal and neoplastic tissue. Moreover, current treatment regimes often have adverse side effects that limit therapy and relapse often occurs due to the development of resistance. Thus, many cancers remain incurable, and alternative techniques are likely to be required in order to improve long-term survival. One possibility is immunotherapy – these regimes aim to circumvent the disadvantages encountered with conventional cancer treatment by either evoking or augmenting immunological anti-tumour responses.

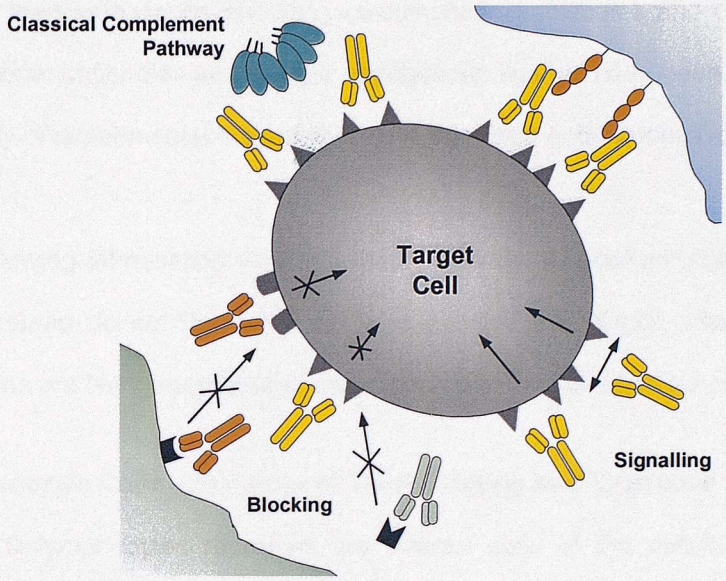


Figure 1.1. Possible Mechanisms by which Monoclonal Antibodies Evoke Therapeutic Activity

Therapeutic mAbs directed against tumour cells are primarily thought to work via the recruitment of effector systems such as Fc receptor-bearing phagocytes and the classical complement pathway (C1q is depicted), 1 and 2 respectively. Other mechanisms that may be at work include the blocking of soluble mediators (*e.g.*, cytokines), the blocking of receptors for these mediators and/or the blocking of cell-cell interactions, 3. Moreover, mAbs such as anti-idiotype and anti-CD20 may operate via the cross-linking of surface receptors that subsequently deliver intracellular signals that control cell division or affect apoptosis, 4. This effect is likely to be enhanced by hyper-crosslinking via Fc receptors [1].

Immunotherapeutic strategies can be categorised as either passive or active. Passive immunotherapy relies on the administration of antibodies, antibody derivatives (*e.g.*, radiolabeled antibodies) or effector cells in order to eradicate established tumours. Figure 1.1 outlines the mechanisms of antibody-mediated tumour killing known to occur *in vivo*. Of the successes, chimeric and humanised monoclonal antibodies (mAbs) such as anti-CD20 (Rituximab), anti-Her-2 (Trastuzumab), and anti-CD52 (Campath-1H) have all been shown to generate potentially useful clinical responses [1].

In contrast to this passive approach, active immunotherapy aims to incite the host immune system into generating effective anti-tumour responses. Manipulation of the immunological response in this way has been achieved experimentally by vaccination against specific

tumour antigens (*e.g.*, idiotype), exogenous cytokine administration, and transfection of tumour lines with genes encoding co-stimulatory molecules and cytokines. Additionally, monoclonal antibodies and soluble endogenous ligands have been used therapeutically in a variety of experimental tumour models to stimulate anti-tumour T-cells [2-5].

The following introduction aims to broadly discuss how cellular responses are generated, why tumours do not fully elicit these responses and, finally, what current experimental strategies are being employed in order to evoke effect anti-tumour responses.

1.1. Dendritic Cells – Initiators of T-cell Priming and Tolerance

T and B lymphocytes represent the effector cells of the cellular arm of the acquired immune system. As detailed in section 1.2, T-cells recognise unique combinations of peptides and major histocompatibility complexes (pMHCs) displayed on the surface of antigen presenting cells (APCs) via their clonotypic T-cell receptor (TCR). Induction or tolerisation of a T-cell response is predominantly orchestrated by a specialised set of professional APCs called dendritic cells (DCs). Dendritic cells represent a minor (1-2 % total cell number) heterogeneous cell population derived from bone-marrow haematopoietic stem cells. In the steady state, immature DCs (iDCs) reside in most peripheral tissues, notably at the environmental interface (*e.g.*, mucosae), and constitutively migrate through the blood, non-lymphoid tissues, lymphatics, and secondary lymphoid organs. Whilst in peripheral tissue, iDCs capture antigens and, subsequently, present peptides derived from these antigens on MHC class-I and –II molecules [6-13].

T-cell induction requires a terminal process of DC maturation that occurs in response to endogenous 'danger signals' (*e.g.*, heat-shock proteins (HSPs)) and/or pathogen-derived molecular patterns (PAMPs, *e.g.*, lipopolysaccaride (LPS)) in a context-specific and lineage-specific manner. DC maturation results in the quantitatively and kinetically distinct expression of co-stimulatory molecules and cytokines, as well as changes in endocytic capacity, morphology, migration and survival, all of which act to promote eventual interaction with T-cells. Therefore, in conjunction with non-lymphoid cells (*e.g.*, parenchymal cells), DCs can tailor the T-cell response to both the tissue-type and threat

encountered [14]. The resulting T-cell response may be categorised are either T_H1 or T_H2; T_H1 development protects against intracellular pathogens and is characterised by the activation of cytotoxic T-lymphocytes (CTL), macrophages and B-cells that produce complement-fixing or viral-neutralising immunoglobulin (Ig) G_{2a}; T_H2 responses, on the other hand, provide help for IgG1, IgE and IgA production and thereby aid protection against extracellular pathogens. As DCs lie at the centre of T-cell induction, understanding DC-mediated antigen presentation is crucial in evaluating how immunity may be raised against tumours. An overview of the T-cell response is given in figure 1.2.

The importance of DCs over other APCs during the priming of T-cell responses has recently been demonstrated by the selective ablation of DCs *in vivo* [15] - in transgenic mice that express human diptheria toxin receptor under the control of the murine CD11c promoter, injection of diptheria toxin results in the selective and transient depletion of DCs with the concomitant abrogation of responses towards *Listeria*, malaria parasites and OVA-loaded $\beta_2$microglobulin$^{-/-}$ cells (*c.f.*, cross-presentation, section 1.1.1.1).

The following sections discuss how DCs induce or tolerise T-cell responses, while subversion of DC activation by tumours is discussed in section 1.5.2.

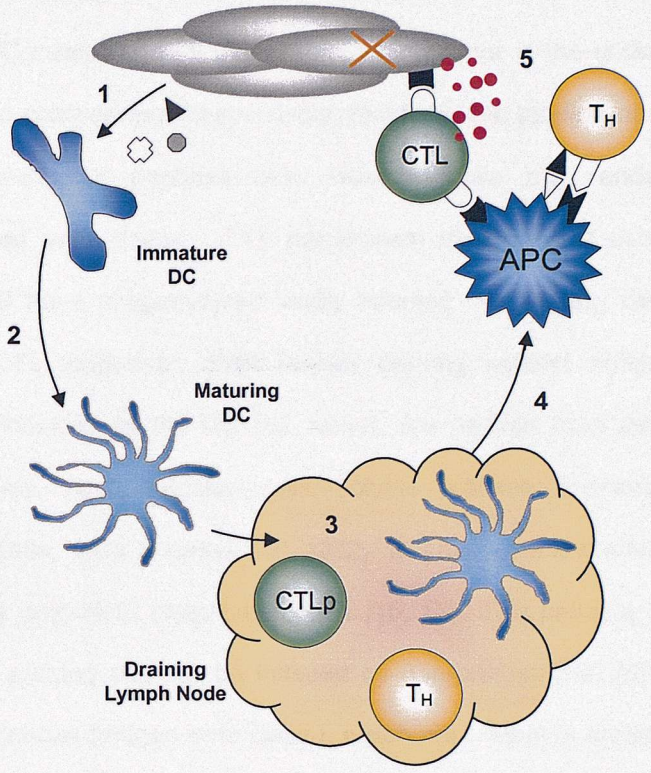


Figure 1.2. Overview of the T-cell Response

Immature tissue-resident or re-cycling DCs capture antigen from tissues and maturation is initiated by local pathogen-induced 'danger' signals, *e.g.*, IFNα, 1. Subsequently, maturing DCs migrate to and enter the local draining lymph node, 2. Here, DCs present class-I/II-restricted peptides derived from antigens captured in the tissues thereby initiating priming of $CD4^+$ T_H cells and, subsequently, $CD8^+$ CTL, 3. Signals derived from T_H cells upon antigen recognition (*e.g.*, CD40L) amplify DC maturation and promote T-cell priming. B-cells are also likely to be involved. Once primed, both T_H cells and CTL differentiate into effector cells and migrate to the inflammatory site (4) where local APCs are able to sustain the effector response, *e.g.*, CTL-mediated target cell lysis, 5.

1.1.1. Antigen Processing and Presentation

MHC molecules are categorised as either class-I or class –II. In all cell types, the segregation of MHC class-I and –II biogenesis, which occur in the endoplasmic reticulum (ER) and endocytic compartment respectively, results in the loading of both exogenously and endogenously-derived peptides onto class-II, while only endogenously-derived peptides are loaded onto class-I. This mechanism may prevent activated CTL killing unaltered cells that have phagocytosed virally infected neighboring cells. Nonetheless, class-I-restricted CTL responses often require priming against antigens that are not endogenously synthesised by the DC but, rather, are derived from exogenous sources, *e.g.*, microorgansims, virally infected cells, soluble proteins, immune complexes and apoptotic cells. Thus, DCs possess the ability to cross-present antigen derived from exogenous sources onto MHC class-I molecules [16, 17]. This pathway is also required in cases where CTL priming can not be initiated by non-professional APC. The ability to cross-present exogenous antigen onto class-I, which may result in cross-priming or cross-tolerance, is just one example of how DC-mediated antigen presentation differs from that in non-professional APCs.

1.1.1.1. Class-I-Restricted Antigen Presentation and Cross-presentation.

Presentation of endogenously-derived peptides by DCs occurs in a similar fashion to other cells - ubiquitination of newly synthesised endogenous proteins leads to proteolytic degradation and TAP-dependent transport of the derived peptides to the ER. Subsequently, an ER-resident chaperone loading complex containing, for example, tapasin, calnexin and calreticulin, catalyses the binding of these peptides to new class-I molecules [18]. Peptide-class-I complexes are then transported to the plasma membrane via the golgi. Dendritic cells do, nonetheless, present a unique array of class-I-restricted peptides due to the constitutive expression of the immunoproteosome components LMP2, LMP7 and PA28 [19], which results in the cleavage of endogenous proteins at distinct sites. Furthermore, DCs and mature B-cells possess a di-ubiquitin gene within the MHC locus that may participate in antigen presentation [20].

However, as stated above, in contrast to the class-I-restricted presentation of peptides derived from endogenous proteins, CTL priming does often require cross-presentation of exogenous-derived peptides and this pathway is prominent in DCs. Indeed, the majority of immune responses against allografts, tumour cells, or intracellular pathogens require cross-presentation by bone-marrow-derived APCs, from which DCs are derived [21-23]. For example, reconstitution of lethally irradiated $TAP^{+/+}$ mice with TAP-deficient bone marrow fails to rescue vaccinia-specific CTL responses, whereas reconstitution with $TAP^{+/+}$ bone marrow does (see below).

Macrophages are also able to cross-present exogenous antigens but, compared with DCs, are less efficient at this process as demonstrated in models of human influenza infection [24] and mouse *Salmonella* infection [25], as well as during the cross-presentation of ovalbumin (OVA) or immune complexes [26, 27]. The inefficiency of cross-presentation by macrophages is likely to be due to the lack of a specific endosome-to-cytosol transportation mechanism (see below [27, 28]), the different receptors utilised by DCs and macrophages for cell engulfment, and the superiority of DCs in stimulating naïve T-cells. [17] Nonetheless, macrophages may cross-present antigen in some cases, as demonstrated *in vitro* [28]. Interestingly, until recently, it was unclear as to which DC subset was responsible for cross-presentation *in vivo* (see section 1.1.1.1).

Antigens may access the cross-presentation pathway in DCs via all routes of antigen capture, but some of these may also mediate DC maturation. For example, FcR-mediated internalisation of immune complexes [29], opsonised liposomes [30], and opsonised dead cells [31] enhance cross-presentation and often induce DC maturation. [32] Similarly, HSPs mediate efficient cross-presentation and DC maturation *in vivo* [33-36]. Whether receptor-mediated internalisation of antigen enhances cross-presentation by concentrating these antigens within DCs or by specifically targeting antigen to the endocytic compartment is unknown. Nonetheless, two specific mechanisms have been proposed for cross-presentation [37]. Castellino *et al.* directly visualised class-I-peptide complexes in the endosomal compartment of macrophages by using an OVA-H-2K-specific mAb [38]. This

observation suggests that, due to low endosomal pH, peptides generated from exogenous sources may exchange with bound peptides present on recycling class-I molecules. This mechanism must thus occur in a TAP-independent manner. However, cross-presentation has often been shown to be TAP-dependent *in vivo* (*c.f.*, reconstitution with TAP-deficient bone marrow), suggesting that internalised antigens are able to traverse the endocytic membrane, enter the cytosol and participate in classical class-I peptide generation. Indeed, DCs have a transportation pathway whereby molecules of 3-20kDa in size can enter the cytoplasm from the endosomal compartment [27]. Furthermore, this pathway is specific for internalised antigens (the lysosomal resident proteins cathepsin D and β-hexosaminidase are not transported) and, as expected, is blocked by proteosome inhibitor. Other cells, such as thymic epithelia, also cross-present soluble OVA in a TAP-dependent manner, but the process is very inefficient in these cells. [39]. Which class-I loading pathway, endosomal or ER-restricted, will predominate is likely to be dictated by the antigen, the cell type, and the route of internalization [32]. Interestingly, Castellino *et al.* also demonstrated that the orientation of peptide binding to HSP70 determines whether the pMHC complexes formed appeared in the endosomal compartment or the ER [38].

The involvement of cross-presentation in the priming of T-cell responses and induction of T-cell tolerance is discussed in sections 1.3.1. and 1.4.3. The following section briefly summarises class-II-restricted antigen presentation in DCs, as a detailed analysis of this topic is outside the scope of this thesis.

1.1.1.2. Class-II-Restricted Antigen Presentation.

In APCs other than DCs, three newly synthesised class-II molecules associate with a trimer of invariant chains (Ii) in the ER. These nonamers are subsequently translocated via the golgi to the endocytic/lysosomal compartment. Once in an acidic environment, Ii is degraded by proteases of the cathepsin family, leaving only class-II-associated Ii peptide (CLIP) in the peptide-binding groove. Subsequently, two nonpolymorphic MHC class-II molecules, HLA-DM/H2-M and HLA-DO/H2-O (in human/mouse), catalyse the exchange of CLIP for peptides derived from exogenous proteins. Class-II dimers complexed with

peptide are then translocated to the cell surface. Alternatively, internalised class-II-peptide complexes can exchange peptides in the endocytic compartment [40].

DCs specifically regulate the formation of peptide-MHC class-II complexes such that, in contrast to other APCs, surface peptide-class-II complexes are only efficiently presented after maturation. Thus, class-II-Ii nonamers are diverted from the secretory pathway following exit from the *trans*-golgi network and subsequently accumulate in late endosomes/lysosomes. A fraction of peptide-loaded, CLIP-loaded or empty class-II dimers do however reach the plasma membrane. Nonetheless, iDCs only transiently and inefficiently present peptide-class-II complexes. Interestingly, expression of surface HLA-DM/H2-M suggests that empty class-II molecules may be loaded by exogenous peptides directly at the cell surface and, indeed, this may be achieved *in vitro* [41].

Several mechanisms are responsible for the inefficiency of class-II-restricted presentation by iDCs. Firstly, antigen degradation in iDCs is impaired due to the inhibited activity of several proteases including cathepsin B and L [42]. A splice variant of Ii, Iip41, may mediate this inhibition [43]. The obstructed antigen degradation in iDCs results in the retention of both antigen and class-II in lysosomal compartments for one to two days [44, 45]. Secondly, few class-II molecules are available to bind peptide in iDCs. In some bone-marrow-derived murine DCs, class-II haplotypes with strong affinity for Ii remain inaccessibly in lysosomes associated with a partially degraded form of Ii, Iip10 [46]; this is due to the high activity of cystatin C, a protease inhibitor that prevents cathepsin S from degrading Iip10 [46, 47]. Thirdly, empty or associated class-II complexes that do reach the plasma membrane are rapidly internalised.

Upon DC maturation (see section 1.1.3), all these aspects of antigen presentation are co-ordinately modified resulting in a rapid increase in the number of class-II-peptide complexes presented. Class-II synthesis is transiently increased simultaneously with a sustained increase in protease activity, the latter being achieved on re-localisation to a more acidic compartment [42]. This is followed by the down-regulation of class-II synthesis

which focuses the repertoire of peptides displayed to those encountered prior to maturation. This is supplemented by the down modulation of phagocytosis and macropinocytosis, as well as receptor-mediated endocytosis via a select number of receptors (*e.g.*, FcR, DEC205), which further restricts presentation to those peptides that are derived from peripherally encountered antigens. Overall, the number of available peptides and empty class-II molecules simultaneously increases, thus pMHC complexes are rapidly formed following maturation [44]. These complexes subsequently appear in specialised non-lysosomal class-II vesicles, segregated from H2-M and Ii, but together with class-I and B7-2 [45]. Class-II-vesicles subsequently fuse with the plasma membrane resulting in the co-ordinate delivery of T-cell ligands to the cell surface and thereby promoting the formation of an immunological synapse (see section 1.2.3).

An additional point to be noted is that anti-inflammatory cytokines are able to interfere with class-II presentation in DCs. Notably, IL-10 is able to increase endosomal pH, thus inhibiting protease activity and decreasing the number of peptides available to bind class-II [42]. Thus, the cytokine milieu present on receiving a maturation signal is a crucial factor in determining the ensuing T-cell response.

1.1.2. Antigen Internalisation

Antigen acquisition by DCs is achieved by phagocytosis, macropinocytosis and receptor-mediated endocytosis. Receptor-mediated endocytosis occurs via clatherin-coated pits, which form on the recruitment of clatherin lattices to the cytoplasmic tail of the endocytic receptor once ligand is bound. Generally, endocytic receptors are also able to mediate phagocytosis.

Immature DCs express a variety of endocytic receptors such as FcγRI-III which mediate internalisation of immune complexes [48, 49] and the complement receptors CR3 and 4 [50]. Immature DCs also bind and internalize HSPs, which are thus able to act as antigenic peptide chaperones (see section 1.1.3.1), *e.g.*, HSPs-96, -90, and -70 as well as calrecticulin were shown to bind the α2-macroglobulin receptor CD91 on iDCs, thereby

leading to the re-presentation of bound peptide. [51] Similarly, Toll-like receptor (TLR) 4 has been shown to bind HSP60, while CD14 is a co-receptor for HSP70 (*c.f.*, LPS signaling via TLR4, section 1.1.3.1) [52, 53].

Apoptotic corpses also serve as a source of antigen for iDCs [11, 54, 55]. Interestingly, CD36 and the integrin $\alpha v\beta 5$ are both involved in the phagocytosis of apoptotic corpses by human iDCs, [56] while CD36 is not required for this process in mice [57, 58]. Nonetheless, current data show that an array of different molecular flags exposed on apoptotic cells are bound by receptors on iDCs. This process may occur directly or indirectly via adaptor molecules, *e.g.*, direct binding of exposed phosphotidylserine (PS) residues by the PS receptor and indirect binding via components of the complement cascade (iC3b, C1q). Initial tethering of apoptotic cells, by surface CD14 or $\beta 2$ integrins, is thought to precede cytoskeletal re-organisation and the resulting phagocytosis [59]. Indeed, $\alpha v\beta 5$ integrin is known to recruit the CrkII/Dock180/Rac1 signaling complex, thereby orchestrating cytoskeleton re-organisation. Moreover, functional ablation of the *C.elegans* CrkII and Rac homologs impairs apoptotic cell clearance [60]. The plethora of different molecules involved in apoptotic cell engulfment suggests that his process is tightly regulated and may lead to a variety of different functional outcomes for the iDC, *i.e.*, tolerance induction or priming of a T-cell response.

Immature DCs additionally endocytose antigen via the carbohydrate-binding C-type lectins such as the macrophage mannose receptor, DEC205, and the low-affinity IgE receptor CD23. DEC205 is exclusively expressed on $CD8\alpha^+$ DCs and possesses a unique translocation motif that results in its accumulation in late endosomes and, thus, co-localisation with MHC class-II in iDCs (see section 1.1.1.2). As an aside, DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) and langerin are two other type II lectins expressed on interstitial DCs and Langerhan cells (LCs) respectively [32]. DC-SIGN is known to function during the formation of the T-cell-DC synapse via its interaction with ICAM3 and during DC migration via interactions with ICAM2 [61]. Conversely, the function

of langerin is unknown. Interestingly, HIV-1 binds DC-SIGN, thus utilising this integrin to infect DC, migrate to lymph nodes and, subsequently, *trans*-infect T-cells [62].

Immature DCs are also able to phagocytose a myriad of different bacteria. Indeed, iDCs open tight junctions in gut epithelial monolayers, project dendrites into the apical side of the epithelia and phagocytose gut bacteria [63]. This process may be important in the induction of tolerance towards gut flora (see section 1.4.3).

Unlike other cell types, iDCs constitutively internalise large volumes of extracellular fluid by macropinocytosis [64]. Like phagocytosis, macropinocytosis is actin-dependent and, therefore, reliant upon Rho family GTPases. The expression of aquaporins 3 and 7 in DCs is thought to prevent excess fluid accumulation due to macropinocytosis [65].

1.1.3. Dendritic Cell Maturation

In response to two types of signals, PAMPs and/or indirect indicators of infection (*e.g.*, HSPs, immune complexes, type I interferons), iDCs initiate a terminal developmental program known as maturation. Maturation results in (1) increased peptide class-II presentation with a concomitant decrease in the rate of antigen up-take, (2) increased expression of co-stimulatory molecules (*e.g.*, B7-1, 4-1BBL), (3) elevated or *de novo* production of cytokines (*e.g.*, mature IL-18, -12, -10), (4) production or increased expression of DC survival and stimulatory molecules (*e.g.*, TRANCE, CD40), (5) production of chemokines and chemokine receptors, (6) migration to T-cell-stimulating areas of secondary lymphoid organs and (7) the extension of 'dendritic' processes which may increase opportunities for interactions with T-cells [6, 7, 9, 10, 13, 32, 66-68]. Overall, DC maturation converts iDCs into potent T-cell stimulators and, thereby, largely dictates the balance between T-cell priming and T-cell tolerance. Thus, as expected, this process is tightly regulated. The following section aims to discuss the role of a select number of receptors in eliciting DC maturation and what happens during DC maturation.

1.1.3.1. Receptors that Mediate Dendritic Cell Maturation

Four families of surface pattern-recognition receptors (PRRs) are known to initiate *de novo* DC maturation: (1) TLRs, (2) cytokine receptors, (3) Fc receptors, and (4) sensors of cell death or stress. Activation of DC via indirect mechanisms allows the sensing of pathogen-induced cellular stress and, furthermore, may allow a tissue-specific context to be added to the maturation process. TLRs are a major family of receptors that are involved in this process. Throughout different phyla, TLRs have a conserved architecture consisting of extracellular hypervariable leucine-rich repeats, a cysteine-rich juxtamembrane spacer, and an intracellular signaling Toll/ILR homologous region (TLR) [69, 70]. The extracellular leucine-rich repeats appear to be responsible for ligand binding, with different TLRs recognising distinct pathogen-derived products, *e.g.*, TLR2 mediates immunological responses against yeast and gram-positive bacteria, whereas TLR4 seems to recognise LPS and gram-negative bacteria. [71-73] Similarly, responses to CpG-containing oligonucleotides are mediated via TLR9 [74-76]. Co-receptors may be involved in TLR-ligand interaction, *e.g.*, LPS-binding protein transfers LPS to soluble or GPI-anchored CD14 which then allows the interaction of LPS with a TL4-MD-2 complex [77]. Human TLR4 alone is incapable of sensing LPS, and MD-2 seems to convey this property upon the receptor via physical association. The mAb MTS510, which binds the murine TLR4-MD-2 complex but not TLR4 alone, has been used to show expression of the TLR4-MD-2 complex on peritoneal macrophages *in vivo* [78]. Furthermore, MTS510 abolishes LPS-induced tumour necrosis factor (TNF)α production by peritoneal macrophages, thus verifying that it is indeed the TLR4-MD-2 complex, and not lone TLR4, which senses and signals the presence of LPS *in vivo*.

In addition to co-receptor usage, other complexities exist in the TLR system. Firstly, there may be redundancy between TLR family members, *e.g.*, traditionally LPS-unresponsive $lps^{d/d}$ mice can still respond to LPS at high doses or if LPS challenge is pre-empted with IFNγ [79]. Secondly, $TLR4^{-/-}$ macrophages are not only unresponsive to LPS and gram-negative bacteria, but also to lipoteichoic acid, which is only found in gram-positive bacteria [80]. This observation suggests that TLRs bind structurally related ligands rather than

ligands of common microbial origin. Finally, Polly Matzinger convincingly argues that TLRs may have actually evolved to bind endogenous ligands and that microorganisms manipulate this system to their advantage by producing LPS and other PAMPs [81-83]. Indeed, $CD14^{-/-}$ mice are less susceptible to toxic shock induced by gram-negative bacterial infection than their wild-type counterparts [84]. It is therefore likely that TLRs bind endogenous ligands that are produced or exposed in response to danger. There is an increasing body of evidence to support this view, *e.g.*, CD14 binds ICAM-3 on apoptotic cells and promotes the induction of tolerance to apoptotic cell antigens via the production of immunosuppressive cytokines [53, 85] and, as mentioned, HSP60 has been suggested as the endogenous ligand of TLR4 [52]. This molecule is exposed on necrotic cells and is responsible for the increased immunogenicity of syngeneic tumour lines following transfection of a suicide gene [86]. Further links between cellular death and immunogenicity have been made as necrotic, as opposed to primary or apoptotic, cells have been shown to stimulate the maturation of DCs *in vitro* [87].

Additionally, HSPs released during necrotic cell death are able to activate DCs as well as chaperone antigenic peptides. [88, 89] HSPs are constitutively loaded with endogenous peptides in the ER and are up-regulated during periods of cell stress. Following primary or secondary necrotic, but not apoptotic, cell death, HSPs are released and thus become available for internalisation [35, 85, 89, 90]. Thus, blocking HSP receptors such as CD91 prevents cross-presentation via HSPs [91], while, as stated above, stable transfection of HSP-encoding cDNA into murine tumour lines has been used to increase immunogenicity [86].

It is possible that secreted exosomes – 50-100 nm vesicles derived from endosomal multivesicular bodies – act as HSP carriers, and thus antigen vehicles. Indeed, it has recently been shown that exosomes are selectively enriched in the HSP, hsc73 [92] and that isolated DC-derived exosomes may be used to prime CTL responses against murine tumours [93]. Similarly, human melanoma cell line-derived exosomes transfer Mart-

1/MelanA$_{27-35}$ antigen to monocyte-derived DCs that are subsequently able to cross-present to Mart-1-specific CTL clones *in vitro* [94].

Other potential DC activators include ATP and UTP which activate DCs via purinergic receptors, thereby acting as signals of aberrant cell lysis [95]. Similarly, IFNα, TNFα, IL-1, and mature IL-18 released from tissues in response to double-stranded RNA or caspase-1 activation are able to induce DC activation [96]. Moreover, third party cells may orchestrate DC maturation, *e.g.*, MIC-A/-B non-classical MHC molecules are upregulated on tissues following cellular stress; these molecules serve as ligands for δγ T-cells that are able to produce TNFα, GM-CSF, MIP1α/β and RANTES. Subsequently, it is conceivable that these mediators may activate DC [13].

The above examples, together with those described during cell clearance in section 1.1.2, demonstrate the level of complexity utilized by the immune system in order to decipher the meaning of cell stress and death, as well as the recognition of pathogen-derived products. These mechanisms, integrated by the DC, provide a context in which to elicit the appropriate T-cell response.

1.1.3.2. CD40

CD40 is constitutively expressed on DCs and, as will be discussed, acts to potentiate DC maturation initiated by PAMPs and endogenous signals. CD40 is a type I transmembrane glycoprotein of the TNF receptor (TNFR) superfamily which also includes molecules such as Fas, CD27, CD30, 4-1BB and OX-40. [97, 98] The extracellular portion of TNFR family members consists of three to four stacked domains stabilised by conserved cysteine bridges, thereby forming a rod-like protein. On the other hand, CD40L is a type II glycoprotein that spontaneously forms three-fold symmetrical homotrimers analogous to TNFα.

Initially thought to be restricted to activated CD4$^+$ T-helper (T_H) cells, CD40L is now known to be expressed on mast cells, basophils, eosinophils, platelets, B-cells, NK cells, human

blood DCs, double-negative γδ T-cells, and subsets of αβ $CD8^+$ T-cells. CD40 itself is expressed by a variety of cell types and exerts differing functions upon ligation on these cells. Some examples are given in table 1. Notably, CD40-CD40L interactions promote proliferation, differentiation and immunoglobulin production by B-cells in germinal centres. CD40-induced Ig isotype switching is skewed by the cytokine milieu with, for example, IL-10 favouring production of IgG_1 and IgG_3. Patients suffering from X-linked hyper-IgM syndrome, which is characterised by point mutations and deletions in the CD40L gene, display defective isotype switching and concomitant susceptibility to infection requiring antibody-mediated clearance [98]. CD40 ligation on B-cells promotes cytokine production (*e.g.*, IL-6, IL-10, and TNFα), as well as up-regulation of adhesion and co-stimulatory molecules such as ICAM-1, B7-1 and B7-2. Thus, $CD40L^{-/-}$ $CD4^+$ T_H cells are unable to induce B7 up-regulation on B-cells [99, 100].

Table 1.
Consequences of *in vitro* CD40 activation, phenotype of $CD40(L)^{-/-}$ mice and effect of anti-CD40L *in vivo*

Expression of CD40	
Cell Type	**Functional Consequences**
T-cells	Proliferation, CD25 expression, cytokine production, memory cell generation
Monocytes	Cytokine secretion, NO production, production of metalloproteases
Dendritic cells	Growth and survival, elevated co-stimulatory molecules, cytokine production
$CD34^+$ precursors	Proliferation, development into dendritic cells
Pre-B-cells	Proliferation, CD23 expression
Naïve mature B-cells	Proliferation, differentiation, isotype switching
Germinal centre B-cells	Proliferation, differentiation, Fas expression, Isotype switching
Plasma cells	IL-6 production
Eosinophils	Enhanced survival, GM-CSF production
Endothelial cells	Upregulation of CD54, CD62E, CD106
Thymic epithelial cells	GM-CSF production
Kidney epithelial cells	IL-6, IL-8, MCP-1, RANTES
Keratinocytes	Enhanced expression of CD54, Bcl-x, IL-8 secretion
Carcinomas	Growth inhibition
Fibroblasts/Synoviocytes	Growth; cytokine production
Phenotype of $CD40(L)^{-/-}$ mice	
Model	**Impact of Defect**
Leishmania infection	Reduced resistance to infection; more severe lesions
LCMV	Reduced humoral response and primary CTL response to some epitopes; reduced secondary CTL response
EAE	No disease development
Anti-CD40L treatment	
Model	**Impact**
Graft-versus-host disease	Inhibits donor allospecific T_H cells
Non-obese diabetic mice	Prevents insulitis and diabetes when treated at week 3-4
Transplantation models	Prolonged allograft or xenograft survival, *e.g.*, heart, kidney and pancreatic islets
Murine thyroiditis	Prevents priming of thyroglobulin-specific T-cells

It has long been appreciated that T_H activity is required not only for the induction of thymus-dependent antibody production but also for the induction of CTL responses (see table 1 for some examples of the defects observed in CD40(L)$^{-/-}$ mice and the effects of anti-CD40L in murine experimental disease models). Indeed, patients with hyper-IgM syndrome also display enhanced susceptibility to opportunistic infections such as *Pneumocytis carinii* pneumonia. Exposure of CTL precursors (CTLp) to antigen in the absence of T_H activity often leads to functional tolerance, *e.g.*, Qa1-specific alloreactive T-cells may be tolerised against Qa1-disparate skin allografts by prior exposure to Qa1 allodeterminants in the absence of T-help [101]. Furthermore, early experiments demonstrated that T_H cells and CTLp must bind antigen on the same APC in order for CTL priming to occur [102], thus explaining epitope linkage within antigenic proteins. This observation raised two possibilities by which T_H cells could act to induce CTL responses (figure 1.3). Initially, it was proposed that T_H cells induce CTL responses by providing cytokines such as IL-2 in the vicinity of the APC presenting the linked epitopes (figure 1.3a) [102]. Nonetheless, a model in which T_H cells 'license' iDCs to prime CTL responses (figure 1.3b) is now accepted as matured DCs are able to replace the need for CD4$^+$ T-cell help, *e.g.*, B6 female class-II$^{-/-}$ mice are unable to mount an anti-HY response (*i.e.*, anti-'male') when injected with male DC unless these cells have been treated with anti-CD40 [103-106]. Within this situation rare antigen-specific T_H cells would not have to interact with similarly rare DC-CTLp conjugates, and the activity of a few T_H cells may be amplified by the 'licensing' of several DC.

Several studies have demonstrated that the licensing effect of activated T_H cells may be attributed to the up-regulation of CD40L on these cells following epitope recognition, followed by the subsequent binding of CD40 on professional APCs and the licensing of these cells to prime CTLp. Inoculation of C57BL/6 (H-2^b) mice with allogeneic BALB/c (H-2^d) mouse embryo cells transformed by human adenovirus type 5 early region 1 (Ad5E1) induces Ad5E1-specific CTL, which must therefore occur due to cross-presentation of Ad5E1-derived peptide(s) [107]. Both depletion of CD4$^+$ T_H cells prior to tumour inoculation and mAb-mediated blockade of CD40L following inoculation abolishs this response [103].

Furthermore, administration of anti-CD40 mAb following T_H depletion or in the absence of class-II (B6 I-A^b knock-out hosts) restores CTL priming [103]. Importantly, this restoration of CTL priming by anti-CD40 mAb occurs in the absence of B-cells, thus suggesting that professional APCs, such as DCs, are responsible for CTL priming following CD40-induced licensing. Indeed, in these studies, bone-marrow DCs were shown to express higher levels of B7-2 following anti-CD40 administration [108].

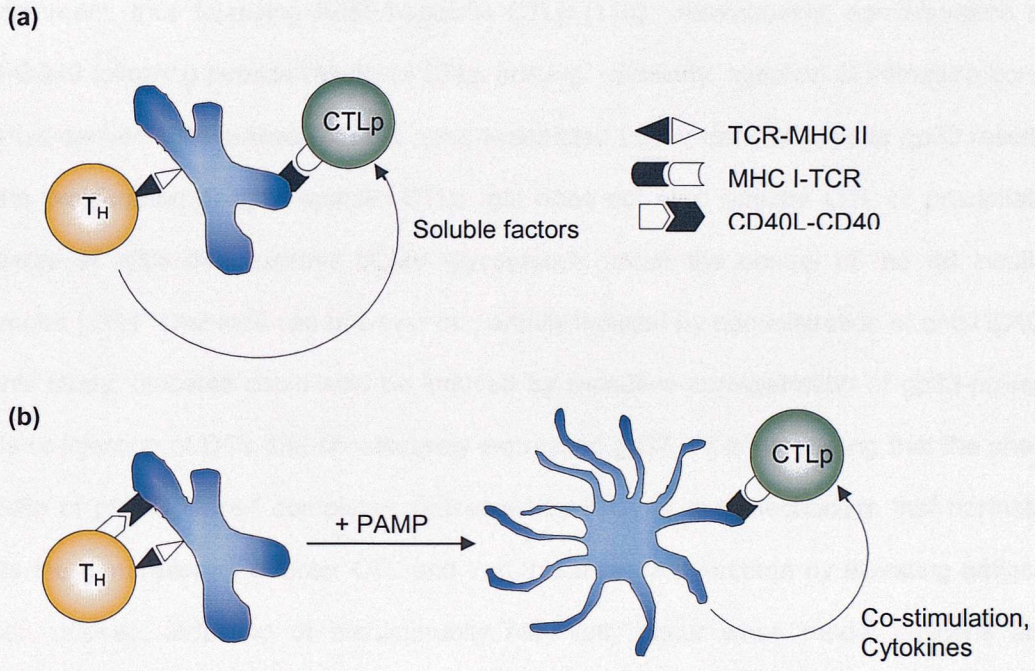


Figure 1.3. Alternative Mechanisms of T-help for CTL Priming

T_H-mediated induction of primary CTL responses was initially proposed to occur via the deliver of cytokines to CTLp following class-II epitope binding, (a). It is now appreciated that T-help is affected by the licensing of DCs via T_H-derived molecules, such as CD40L. In this way, T_H-derived signals amplify PAMP-initiated DC maturation, (a).

CD40-mediated activation of DCs is also responsible for overcoming peptide-induced T-cell tolerance. In some cases peptide emulsified in IFA or CFA has been shown to induce protective T-cell immunity against viral challenge, *e.g.*, vaccination of mice with a human papilloma virus type 16-derived (HPV16) epitope provides protection against HPV16-expressing tumour cells [109]. Nonetheless, other peptide 'vaccines' are known to induce tolerance, *e.g.*, The Ad5E1-derived peptide $Ad5E1A_{234-243}$ diffuses rapidly from IFA emulsion and is presented systemically, outside the adjuvant-induced inflammatory environment, thus tolerising Ad5E1-specific CTLp [110]. Alternatively, administration of anti-CD40 following peptide results in CTLp priming. Similarly, injection of immature bone marrow-derived DCs pulsed with the class-I-restricted LCMV-derived peptide gp33 results in the proliferation of gp33-specific CTLp, but does not elicit effector CTL or precipitate diabetes in mice that express LCMV glycoprotein under the control of the rat insulin promoter [111]. Diabetes can however be partially induced by administration of anti-CD40. In this study, diabetes could also be induced by repetitive administration of gp33-pulsed iDCs or injection of DCs that constitutively expressed gp33, thus suggesting that the short half-life of pMHC class-I complexes presented by iDCs is one mechanism that normally limits the generation of effector CTL and that this may be overcome by elevating antigen dose. Indeed, induction of autoimmunity may only occur when model antigens are expressed systemically at high levels (*e.g.*, 1 μg/mg protein) and central tolerance is by-passed by injection of high avidity transgenic T-cells [112, 113].

In the above study of autoimmune diabetes, anti-CD40 was only able to partially induce diabetes when co-administered with gp33-pulsed iDCs. In contrast, co-administration of LCMV p13-specific $CD4^+$ T_H cells with gp33/p13-pulsed iDCs resulted in severe diabetes. These data suggest that additional T_H-derived factors, other than CD40L, promote DC licensing. Indeed, other members of the TNF family, such as LIGHT and TRANCE, are expressed on activated T_H cells and may contribute to DC-mediated CTLp priming by increasing DC survival (*e.g.*, TRANCE-R ligation increases Bcl-xL expression in DC) [114], elevated secretion of pro-inflammatory cytokines and/or up-regulating MHC, adhesion and co-stimulatory molecules, *e.g.*, LIGHT ligates herpes virus entry mediator on DCs resulting

in partial DC maturation and enhancing priming of MelanA-specific CTL in conjunction with anti-CD40 without altering cytokine secretion [115]. Furthermore, 4-1BB-4-1BBL interactions were initially thought to have co-stimulatory activity solely towards T-cells (see section 1.1.3.4). However, it has recently been shown that 4-1BB is expressed on murine iDCs and that ligation of this molecule results in cytokine production (IL-6 and IL-12) [116] and up-regulation of B7 [117]. In this case, 4-1BB may be ligated on DCs *in vivo* by binding 4-1BBL expressed on adjacent APCs [118] and, interestingly, the varied effects of systemic anti-4-1BB administration (discussed in section 1.1.3.4) may not only be attributed to T-cell co-stimulation, but also to DC activation.

It seems likely therefore that members of the TNF receptor superfamily other than CD40 may account for at least some of the CTL responses that occur in a CD40-independent manner, and, furthermore, that TNF/TNFR member pairs act at multiple levels during the priming and effector phases of a T-cell response. Indeed, administration of anti-CD40 has not only been shown to overcome tolerance in the CD8 compartment, but it has also been shown to aid T_H priming, *e.g.*, $CD4^+$ class-II-restricted haemagglutinin (HA)-specific transgenic T-cells may be tolerised by exposure to either the cognate epitope (administered in PBS) or renal carcinoma cells expressing HA, whereas co-administration of anti-CD40 results in priming of the transgenic T-cells [119]. The fact that T_H tolerisation occurs in the absence of CD40 ligation on DCs suggests that the initial encounter of antigen is insufficient to endow T_H cells with full DC-licensing capabilities. In explanation of this, CD40-CD40L interactions are now appreciated to simply act to amplify pathogen-derived DC activating signals, thus allowing the ensuing T-cell response to be dictated by the initial pathogen recognition event(s) (see section 1.1.4); importantly, PAMP recognition by DCs does induce partial maturation, notably, up-regulation of CD40 and this event may render DC more susceptible to T_H cell function thereby preventing T_H tolerisation upon antigen encounter [120, 121].

In this model of DC maturation, two observations have to be explained. Firstly, why does anti-CD40 administration alone, either *in vitro* or *in vivo*, result in full DC maturation and

concomitant T-cell priming in most experimental models to date? Several possibilities for this exist. Tissue injury or stress sustained at the injection site may result in initial DC activation via proteoglycan or polysaccharide degradation products as well as other factors detailed in section 1.1.3.1 [122, 123]. Similarly, *in vivo* or *in vitro*-maintained tumour cell lines may activate DCs by a variety of mechanisms, *e.g.*, elevated HSP expression, [86] presence of virus (as is often the case, *e.g.*,) [124, 125] and/or the presence of transfection vectors carrying model antigens. Additionally, injected 'immature' DCs may be partially matured due to *in vitro* culture and manipulation [87]. Importantly, mAb preparations are often contaminated with LPS due to preparation techniques (*e.g.*, protein A column) and, indeed, when endotoxin-free anti-CD40 is administered systemically DC maturation does not occur [120]. Secondly, why are DCs licensed *in vitro* with PAMP-containing products, such as LPS and poly-I:C, able to prime CTL [126]? This effect may be explained by either the PAMP encountered or, more likely, the dose used. Certain PAMP-containing reagents such as STAg, LPS and poly-I:C appear to promote DC maturation further towards a T-cell priming state than others, *e.g.*, mycobacterial purified protein derivative [121]. Alternatively, it is likely that the level of *in vitro* stimuli used to test the DC-maturing capacities of these products is non-physiological and that, *in vivo*, T-cell-derived feedback signals are required in order to elicit full DC maturation and, possibly, prolong IL-12 production for T-cell survival following priming (*c.f.*, section 1.1.3.6). Indeed, sub-optimal doses of super-antigen only induce T-cell proliferation if both LPS and anti-CD40 are administered *in vivo* [127].

Together, the data outlined in this section places CD40-CD40L interactions at the pivotal point of DC activation that, subsequently, is able to determine the outcome of antigen encounter by T-cells. The following section outlines the function of co-stimulatory molecules during T-cell priming following DC licensing.

1.1.3.3. Co-stimulation

As well as elevated biogenesis of class-II-peptide complexes, maturation also induces the up-regulation of adhesion and co-stimulatory molecules that act to sustain, diversify and/or amplify the ensuing T-cell response. Specifically, members of the B7 family of co-stimulatory molecules influence the T-cell response by binding a variety of constitutively expressed and inducible CD28-like molecules on T-cells; [128-130] these interactions are often indispensable if successful priming is to occur. Furthermore, members of the TNF receptor family, such as 4-1BBL, are up-regulated following DC activation and participate in bi-directional signalling with T-cells [118].

All B7-like and CD28-like molecules are type I transmembrane glycoproteins of the immunoglobulin superfamily. B7-1 (CD80) and B7-2 (CD86) both bind CD28 and CTLA-4. While CD28 is constitutively expressed on T-cells and facilitates activation, CTLA-4 is expressed following activation and acts to inhibit the T-cell response. The expression of B7-1 and -2 vary with B7-2 being expressed/up-regulated within hours of APC activation, while B7-1 is only induced after three to four days. Moreover, B7-1 and –2 have distinct binding properties – B7-1 binds CD28 and CTLA-4 with higher affinity that does B7-2 and, furthermore, B7-1 and –2 bind distinct determinants on these ligands [131]. Despite these differences, B7-1 and –2 seem to support T-cell activation equally [132]; some disease models do describe differing roles for B7-1 versus B7-2, but these are often disputed, *e.g.*, [133, 134]. Interestingly, B7-CD28 interactions appear to be essential for the homeostasis of $CD4^{+}25^{+}$ T-regulatory cells (section 1.4.3) [135]. Conversely, the re-activation of memory T-cell is B7-independent (*c.f.*, raft expression) [136].

Four new B7-like molecules have recently been identified: PDL1 and 2, B7 related protein 1 (B7-RP1), and B7 homolog 3 (B7-H3) (table 2) [129, 137]. B7-RP1 has a unique expression pattern compared to that of B7-1 and B7-2 in that it is expressed on non-lymphoid tissues. Human DCs express B7-RP1, but expression is down-regulated by TNFα and LPS, thereby suggesting a distinct role in regulating T-cell function [138]. Indeed, B7-RP1 does not bind either CD28 or CTLA-4, but, rather, has been shown to bind

inducible co-stimulator (ICOS), another CD28 family member [139, 140]. Interestingly, the *de novo* expression of ICOS by naïve T-cells is largely B7-dependent, as expression is inhibited if T-cell activation occurs in the presence of B7-2 blocking mAb [141]. Thus, effects that have been directly attributed to CD28-mediated co-stimulation may, at least in part, be ICOS-dependent responses.

Table 2.
The B7 Family of Co-stimulatory Ligands

B7 Family Member	Expression	Receptor(s)	Function
B7-1 and B7-2	Predominantly lymphoid expression; induced on monocytes, DCs and B cells	CD28 CTLA-4	Priming of naive T-cells, IL-2 production Inhibition of T-cell responses
B7-RP1 (B7h, B7-H2)	Lymphoid and non-lymphoid expression (heart, kidney, testis, lung); LPS-inducible in non-lymphoid tissue; induced on monocytes and DCs	ICOS	Enhancement of cytokine production in peripheral tissue without biasing
PD-L1 (B7-H1)	Lymphoid and non-lymphoid expression (heart, lung, kidney); induced on monocytes by IFNγ	PD-1	Inhibits T-cell proliferation and cytokine production
PD-L2 (B7-DC)	Lymphoid and non-lymphoid expression (heart, lung, kidney, pancreas, liver); predominantly DC expression	PD-1	Inhibits T-cell proliferation and cytokine production and/or enhances IFNγ and IL-2 production
B7-H3	Lymphoid and non-lymphoid expression (heart, kidney, testis, lung, liver); induced on monocytes and DCs	?	IFNγ, TNFα and IL-8 production

In vitro, ICOS operates independently of CD28 to promote IFNγ, TNFα, IL-4, IL-10, IL-13, IL-5, GM-CSF but, importantly, not IL-2 production [139]. Thus, ICOS is likely to be important for sustaining an effector T-cell response at peripheral sites without effecting clonal proliferation or cytokine skewing. Indeed, studies in ICOS-deficient mice, and *in vivo* blockade of B7-RP1-ICOS signalling, has revealed a role for ICOS in both T_H1- and T_H2-mediated responses [142]. Concurrently, engineered expression of B7-RP1 in J558 plasmacytoma enhances tumour immunogenicity in a B7-dependent manner [143].

PD-L1 and 2 are also members of the B7 superfamily that are constitutively expressed on non-lymphoid tissues, *e.g.*, placenta, heart, lung and kidney [144, 145]. Moreover, PD-L1 and 2 are constitutively expressed on DCs and are up-regulated following exposure to LPS

[137]. The T-cell ligand for the PD-L1 and 2, PD-1, is strongly induced upon T-cell activation and subsequent PD-L-PD ligation acts to attenuate IL-2, IL-10, TNFα and IFNγ production [144]. Furthermore, PD-1-deficient mice on a B6 and BALB/c background develop glomerulonephritis [146] and dilated cardiomyopathy [147] respectively. These data suggest that, together with CD28 and CTLA-4, PD-L-PD interactions may also be involved in the maintenance of peripheral tolerance. However, PD-L2 (also termed B7-DC) was originally described to be predominantly expressed on DCs and induce IL-2/IFNγ production from CD3-stimulated $CD8^+$ T-cells *in vitro* [148]. The reason for this discrepancy has yet to be resolved.

B7-H3 is directly related to B7-RP, but this molecule is constitutively expressed on iDCs and co-stimulates $CD4^+$ and $CD8^+$ T-cells to proliferate and produce IFNγ. The use of B7-H3-Ig fusion protein has shown that B7-H3 ligand is up-regulated following T-cell activation but, at present, the identity of this molecule is unknown [137].

1.1.3.4. 4-1BB-Mediated Co-stimulation

As well as B7 and B7-like co-stimulatory molecules, T-cell priming and differentiation is reliant on other co-stimulatory pathways such as those mediated via 4-1BB [108, 149, 150]. 4-1BB is a member of the TNFR superfamily and is expressed on antigen-activated T-cells, IL-2/-15-activated NK cells and constitutively on murine DCs [116-118, 151]. Complementary to this, 4-1BBL is expressed on anti-IgM-activated B-cells, LPS-stimulated macrophages and DCs [117, 152, 153]. Activation of DC by anti-CD40 mAb results in the up-regulation of 4-1BBL, but diminished 4-1BB expression [117].

Ligation of 4-1BB on T-cells operates independently of CD28 at optimal antigen densities [153-155], and can enhance both primary [156, 157] and secondary [158, 159] $CD4^+$ or $CD8^+$ T-cell responses by promoting proliferation, survival and cytokine production [160]. These effects may be due, at least in part, to elevated IL-2 production and expression of the anti-apoptotic factor, Bcl-xL [161]. Notably, 4-1BB ligation does not promote the

development of cytotoxicity *in vivo*, suggesting that only certain effector functions are regulated via this pathway [162].

Interestingly, 4-1BBL$^{-/-}$ mice were used to demonstrate that 4-1BB-4-1BBL interactions play a role in allograft rejection and influenza clearance, but not during the generation of T-cell responses against LCMV, unless antigen is at sub-optimal levels [158, 163]. Similarly, anti-4-1BB mAb and transfection of 4-1BBL into tumour lines promotes tumour eradication (in models of melanoma, sarcoma, lymphoma, mastocytoma and plasmacytoma) with the concomitant generation of anti-tumour CTL [3, 4, 108, 164-167]. A recent study using carcinogen-induced fibrosarcoma demonstrated that anti-4-1BB mAb affected complete remission of this tumour in an IFNγ- and CD8-dependent, but CD4- and NK-cell-independent, manner [168]. Moreover, Miller *et al.*, found that anti-4-1BB-mediated tumour eradication requires the presence of CD40, suggesting therefore that DC licensing via CD40L is required in order to allow 4-1BB expression on T-cells. Indeed, in this study, diminished efficacy was observed if anti-4-1BB was administered at early time points following tumour inoculation. Accordingly, Diehl *et al.* demonstrated that CD28-mediated co-stimulation is required for 4-1BB expression on T-cells if antigen is limiting, and that this dependency is reduced as antigen concentration is increased [108]. Thus, a temporal segregation exists between 4-1BB and CD28-mediated co-stimulation. This fact is further apparent in murine models of influenza infection, where the absence of 4-1BBL leads to diminished NP-specific CTL late in the primary response and a concomitant decrease in the secondary response to virus (either due reduced primary clonal burst size, or decreased survival within the memory pool) [169].

Overall, co-stimulatory molecules thus act in a spatially and temporally regulated fashion to modulate the priming, expansion and differentiation (*i.e.*, T_H1-like versus T_H2-like) of the ensuing T-cell response. Furthermore, these effects will differ depending on the antigen experience of any given responding cell, as the levels of co-stimulatory ligand expressed on T-cells may alter following antigen exposure, *e.g.*, memory T-cells constitutively express ICOS [140, 170].

1.1.3.5. Migration of Dendritic Cells

A critical feature of DC lineage cells that allows highly efficient antigen presentation *in vivo* is their migratory capacity, both in the steady state and following pathogen encounter. As mentioned in section 1.1, iDCs constitutively patrol peripheral tissues and enter the lymphatics via vessels draining these tissues, thereby allowing pathogen surveillance and the maintenance of peripheral tolerance during tissue turn-over. The factors that regulate DC migration are largely those that promote maturation, *e.g.*, TLR ligands such as CpG-containing DNA, HSP gp96, TNFα and CD40L [68]. Some of these factors are likely to act in a linear sequence within the same pathway as, for example, CD40L and LPS both elicit TNFα production which is known to be the mediator that affects DC migration following exposure to these stimuli [171]. Competence for migration is accompanied by phenotypic changes such as the down-regulation of E-cadherin that allows detachment from neighbouring keratinocytes, the expression and/or activation of adhesion molecules including CD44 and $\alpha_6\beta_1$ integrin, induction of matrix metalloprotease-9 and, importantly, the expression of the chemokine receptor CCR7 [68, 172].

The replacement of the inflammatory chemokine receptors CCR1 and CCR5 with CCR7 is a pivotal switch that allows DCs to bind and respond to the lymphoid chemokines CCL19 (ELC, MIP-3β) and CCL21 (SLC, 6-Ckine) [173]. The importance of CCL19 for DC migration is indicated by the fact that neutralising mAbs directed against this chemokine inhibit LC migration from the skin following contact sensitisation [174]. Chemotaxis to CCL19, but not to CCL21, is licensed by cysteinyl leukotriene lipids ($LTC/D/E_4$) that are secreted by multidrug resistance proteins. CCL21 is expressed as two distinct species, CCL21Ser and CCL21Leu [68] with CCL21Leu being constitutively expressed by lymphatic endothelium, while CCL21Ser is expressed in the lymph node. *Plt* mice are unable to produce CCL21Ser, but retain the ability to produce CCL21Leu; these mice display defective DC migration following contact sensitisation, suggesting that lymph node-derived chemokine is important in this process and that CCL21Leu is insufficient to mediate mass migration of DC [175]. Nonetheless, constitutive expression of CCL21Leu indicates that

this chemokine serves as a CCR7 ligand under steady state conditions. Indeed, CCR7 is expressed by LCs in the steady state and phagocytosis of apoptotic cells is known to induced expression of this receptor [176]. Perhaps this latter effect is mediated by CD40L expressed in apoptotic cells [177].

1.1.3.6. Interleukin-12

Bioactive IL-12 (p75) is a heterodimeric cytokine that is pivotal during T_H1 development following DC maturation [178, 179]. IL-12 consists of disulphide-bonded p40 and p35 subunits that are encoded by separate genes. As such, p35 and p40 are differentially regulated; classically p35 is constitutively expressed in most cell types whereas p40 is inducible, nonetheless, murine DCs constitutively express p40 while p35 is induced upon activation [180]. Notably, p40 and p35 must be synthesised within the same cell for p75 production to be achieved [181].

Until recently, IL-12 was thought to be secreted relatively quickly after initial pathogen recognition and preceding DC-T-cell interaction, thus acting as a bridge between innate and adaptive immunity. However, p40 is secreted prior to IL-12 and is able to bind subunits other than p35 thereby eliciting novel functions prior to p75 synthesis [182]; and, furthermore, IL-12 production is dependent upon T-cell-derived signals (*e.g.*, CD40L, Class-II engagement and IFNγ) and is thus considered to sustain and/or perpetuate the T_H1 response as part of a positive feedback loop [183].

Thus, murine epidermal LCs and DCs produce p40 in the absence of p75 when exposed to *Leishmania major* and splenic p40 mRNA is detectable two hours following systemic exposure to LPS, whereas p35 expression is only increased after approximately 12 hours [184, 185]. Additionally, DCs and monocytes secrete 10-1000-fold excess of p40 compared with p75 and the expression of p40 and p35 often occurs at different anatomical sites [178], further suggesting that p40 is expressed in the absence of bioactive IL-12 *in vivo*. Interestingly, p40 is able to dimerise with itself or, alternatively, p19 to form IL-23 which has similar but distinct properties to IL-12 [182]. Furthermore, experimental

situations under which neither p35 or p19 are expressed in conjunction with p40 have suggested that as-yet unidentified p40-binding subunits may exist [186].

Both p40 homodimers and IL-23 are able to bind the p40-specific β1 chain of the IL-12 receptor but are unable to bind the signalling β2 chain [187]. This suggests that an alternative receptor subunit is likely to exist that allows IL-23 signalling and that, in light of the fact that excess p40 is produced in the absence of IL-12, p40 homodimers are likely to act as competitive antagonists of IL-12. It is possible, however, that p40 is not secreted in a homodimeric form and, therefore, that only a minor population of homodimers may act as IL-12 antagonists.

Together, these data suggest that p40 is secreted prior to IL-12 production as an early response to pathogens and that, like other homologous proteins, p40 acts as a shuttle molecule by binding a variety of subunits that are then able to differentially influence the ensuing T-cell response. Indeed, initial secretion of p40, unlike IL-12, is independent of T-cell-derived signals, *e.g.*, splenocytes from $CD40L^{-/-}$ mice secrete p40 in response to STAg without eliciting IL-12 [188].

The T-dependent nature of IL-12 production suggests that this cytokine acts late in the response against pathogens, as initial pathogen encounter usually occurs in areas devoid of large T-cell numbers. Thus, DCs are likely to secrete p40 following initial pathogen encounter, migrate to the T-cell areas of secondary lymphoid organs where, not only CD40L, but also class-II engagement by TCR on T_H cells and IFNγ appear to be important for eliciting IL-12 production, *e.g.*, DCs synthesis p35 upon mAb-induced or TCR-induced class-II cross-linking [189] and $IFN\gamma^{-/-}$ mice respond to bacteria by producing p40, but not IL-12 [190]. These data therefore suggest that DC require interaction with T_H1-differentiated T-cells in order to produce IL-12, however, DCs are now known to be able to produce IFNγ themselves in an IL-12-dependent manner suggesting that autocrine signalling may occur [183]. Similarly, IL-12 and IL-23 are able to elicit IL-12 production by murine splenic DCs that are known to constitutively express both β chains of the IL-12

receptor [182]. Thus, IL-12 may form part of a positive feedback loop – class-II epitope recognition by T_H cells on pathogen-educated DCs in the T-cell areas leads to CD40L up-regulation and IL-12 receptor expression on T_H cells thus, potentially, resulting in the production of IL-12 from the DCs that may elicit IFNγ from both the DC and the T_H cell. This positive feedback mechanism may drive T_H1 differentiation and require B-cell-derived IL-10 and IL-6, which is induced by IL-12 itself, for cessation [191]. Alternatively, DC may have encountered PAMPs that prescribe T_H2 development and, therefore, CD40 ligation may result in IL-10 secretion (see section 1.4.3).

IL-12 is now also appreciated to also be important following initial priming of T_H1 cells. For example, C57BL/6 and $RAG^{-/-}$ mice reconstituted with splenocytes from C57BL/6 mice that have survived primary *L.major* infection display increased resistance to infection whereas reconstituted $p40^{-/-}$ mice do not [192]. Furthermore, $p40^{-/-}$ mice exposed to *Toxoplasma gondii* are able to overcome acute infection if treated with recombinant IL-12 but, nonetheless, succumb to infection after withdrawing IL-12 [193]. Thus, IL-12 seems important, not just for the establishment of a T_H1 response, but also for the maintenance of these responses. This effect may be due to one or a combination of possible mechanisms, *e.g.*, increased proliferation and diminished susceptibility to Fas-induced apoptosis, renewal of T_H1 cells from undifferentiated precursors, prevention of T_H2 conditions by negative feedback and expression of chemokine receptors on T-cells (*e.g.*, CCR5) [179, 194].

1.1.4. Dendritic Cell Lineage and Plasticity

The induction of T-cell responses by DCs during both T-cell tolerance and priming may be accounted for by either DCs being endowed with unique properties by differing external stimuli or, alternatively, by unique DC lineages performing defined functions. In fact, both points appear to be true, to a greater or lesser extent, depending on the circumstance, *i.e.*, distinct DC lineages respond to individual external stimuli in a unique fashion, thereby differentially impacting the T-cell response.

In the mouse, splenic DCs are classically subdivided into $CD8\alpha^+$ ($DEC205^+CD11b^-$) and $CD8\alpha^-$ ($DEC205^-CD11b^+$) lineages [195]. Originally, $CD8\alpha^+$ DCs were thought to arise from early thymic lymphoid precursors, while $CD8\alpha^-$ DCs were presumed to be myeloid in origin [196]. However, several lines of evidence demonstrate that this is not the case - the thymus has been shown not to be the origin of $CD8\alpha^+$ DCs [197], $CD8\alpha^-$ DCs express CD8α on *in vitro* activation [198], and, most strikingly, purified myeloid progenitors gave rise to $CD8\alpha^+$ DCs upon adoptive transfer into mice [199]. Thus, CD8 homodimer expression is not a reliable marker of DC ontogeny.

Extensive efforts to assign different functions to CD8α-positive and -negative DC subsets have been made. Intravenous injection of either subset results in similar priming of T-cell responses, but subcutaneous injection of antigen-loaded $CD8\alpha^+$ DCs induces T_H1 responses, whereas $CD8\alpha^-$ DCs prime T_H2 responses in this system [200, 201]. Equally confusing, administration of soluble or cell-associated OVA results in efficient cross-presentation of OVA-derived class-I-restricted peptides by $CD8\alpha^+$, but not $CD8\alpha^-$, DCs; however, class-II derived peptides were efficiently presented by the $CD8\alpha^-$ DCs [202, 203]. Conversely, the opposite results were obtained using lysozyme as a model antigen [204].

It has been proposed that any differences observed in the ability of CD8α-positive and –negative DCs in eliciting T_H1 versus T_H2 T-cell responses can be attributed to differential IL-12 production [9]. $CD8\alpha^+$ DCs may secrete more bioactive IL-12 than their $CD8\alpha^-$ counterparts under some circumstances and, indeed, IL-12 p70 is exclusively produced by this subset after intravenous administration of both soluble *Toxoplasma gondii* extract (STAg) and anti-CD40 mAb [120]. Nonetheless, both $CD8\alpha^+$ and $CD8\alpha^-$ DCs can produce IL-12 p70 in response to other stimuli, *e.g.*, heat-killed *Brucella abortus* [205]. These apparently contradictory data may be explained by the different PAMPs present within different pathogens and the expression of unique PRRs in the DC lineages. Concordantly, human DC1s (which broadly resemble $CD8\alpha^-$ murine DCs) and DC2s (plasmocytoid DCs resembling other murine DC subsets, *e.g.*, $CD11c^{lo}$) express unique TLRs, with DC1

expressing TLR 2, 4, 5 and 8, while DC2 express TLRs 7 and 9 [206]. Despite this, different DC lineages clearly retain a large element of plasticity in response to different stimuli; for example, murine DCs elicit IL-4 production following exposure to hyphae, but not yeast-phase *Candida* [207]. Furthermore, human DC2, which are classically associated with T_H2 induction, are able to make IL-12 in response to CpG and CD40 ligation or viral infection [9].

A recent study by Edwards *et al.* further demonstrates DC plasticity – purified $CD8\alpha^+4^-$ and $CD8\alpha^-4^-$ murine splenic CD40L-stimulated DCs were shown to produce IL-12 p70 in response to STAg, CpG and *Mycobacterium tuberculosis* extract, whereas exposure to yeast cell walls (Zymosan) and *S.pombe* elicited IL-10 production from these cells [121]. Additionally, IL-12 and IL-10 production was only significantly induced following CD40 ligation, thus, further indicating that T-cell derived signals act as neutral amplifiers of cytokine production which is primarily instructed by initial pathogen recognition. Notably, $CD8\alpha^-4^+$ DCs were selectively unable to produce IL-12 p70 in this study, thereby suggesting that some DC populations may retain specialised functions. Edwards *et al.* further demonstrated that pattern recognition leading to IL-10 production is independent of the TLR adaptor molecule MyD88, thus, the identity of the PRR in this case is unknown.

Intriguingly, as mentioned above, murine $CD8\alpha^+$ DCs have recently been shown to derive from a $CD8\alpha^-$ population – purified $Ly5.2^+$ $CD8\alpha^-$ DCs were intravenously injected into $Ly5.1^+$ recipients and their phenotype followed by ex-vivo FACS on days 1 to 4 following transfer. Four days post-transfer approximately 70 % of the $Ly5.2^+$ DC displayed a $CD8\alpha^+$ $DEC205^+$ phenotype suggesting that $CD8\alpha^+$ DC represent a mature form of $CD8\alpha^-$ DC [199]. Indeed, systemic LPS and STAg administration results in the translocation of splenic DCs from the marginal zone, bordering the red and white pulps, to the periarteriolar lymphoid sheath (PALS) which is enriched with T and B lymphocytes, a transition that is associated with an increase in the expression of DEC205 [199, 208]. Furthermore, *in vivo* exposure to recombinant porcine parvovirus-like particles (PPV-VLPs) results in the maturation of $CD8\alpha^-4^-$ DCs into a $CD8\alpha^+4^-$ $DEC205^+$ population accompanied by a

concomitant decrease in the parental population [209]. In this study, 90 minutes after PPV-VLP injection only $CD8\alpha^-$ DCs were able to present virus-derived antigen *in vitro*, but 15 hours post-injection $CD8\alpha^+$ DCs were solely able to elicit T-cell proliferation. Importantly, *in vivo*, $CD8\alpha^-$ DCs presenting antigen early after PPV-VLP exposure are unlikely to be able to stimulate T-cells as they are only present in the splenic marginal zone. Moreover, the shift from $CD8\alpha^-$ to $CD8\alpha^+$ DCs does not occur in $Rag^{-/-}$ mice and is therefore likely to be T-cell dependent. Together, these data point to the fact that $CD8\alpha^+$ DC stimulate T-cells in the PALS and are derived from a $CD8\alpha^-$ DC population following translocation from the marginal zone. These data, therefore, explain (1) why $CD8\alpha^-$ DC may prime T_H1 responses following *in vitro* antigen pulsing and adoptive transfer, (2) why $CD8\alpha^+$ DC predominantly produce IFNγ and IL-12, and (3) why $CD8\alpha^+$ DC are often the DC subset that is demonstrated to have endocytosed antigen [57, 120, 203, 210]. The function of CD8α on DCs is presently unknown, but the lack of CD8α mRNA transcripts in these DC suggests that de novo synthesis does not occur [209].

In humans, DC plasticity is further demonstrated by the fact that monocyte-derived DCs (DC1) matured by IFNγ, LPS, CpG-containing oligonucleotides or dsRNA polarise T-cells towards a T_H1 phenotype, whereas a maturation signal delivered by IL-1, prostaglandin E_2, TGFβ, or FAS ligation favours T_H2 development [211]. This effect is again mediated by IL-12 production from the maturing DC – T_H1-polarising stimuli induce transient IL-12 (8-16 hours) production from DCs, whereas T_H2 polarising stimuli do not, thus, T_H2 conditions develop due to IL-4 secreted by activated $CD4^+$ T-cells [211]. After initial IL-12 production, DCs become refractory to further stimulation, thereby limiting adverse immunopathology and restricting T_H1 priming to only recently stimulated DCs [6]. The factors responsible for this phenomenon are unknown, but the fact that IL-12 production is paralysed following initial synthesis suggests that the kinetics of DC stimulation are important in determining the characteristics of the instructed T-cell response [208]. Indeed, the presence of 'exhausted' DCs expressing low concentrations of antigen at the closing stages of a T_H1 response may be important during memory cell generation [6].

The tissue-specific and/or induced cytokine milieu present at the point of DC activation further modulates IL-12 production, and thus the T_H1-T_H2 balance. Thus, IFNγ promotes IL-12 production following exposure to LPS or CD40L, while IL-10 has a pan suppressive effect and TGFβ selectively inhibits IL-12 production following LPS stimulation [6]. Furthermore, $CD11c^+$ DCs purified from Peyer's patches, but not from the spleen, preferentially induce IL-4/-10-producing cells *in vitro* [212]. Finally, the DC-to-T-cell ratio is critical during T-cell polarisation, with high DC-to-T-cell ratios favouring T_H1 development and visa versa [213].

Overall, the type of T-cell-mediated response to any given pathogen or antigen may be dictated by a whole host of factors including DC lineage, PAMPs, cytokine/tissue microenvironment, kinetics of DC stimulation and the DC-to-T-cell ratio. Therefore, in the future, it may be critical to standardise the experimental conditions under which different DC subsets are examined.

1.2. Antigen Recognition by the T-cell Receptor

1.2.1. Binding of Peptide-MHC

TCRs recognise antigen in the form of peptides bound to MHC class-I and -II molecules on APCs. MHCs contain a peptide-binding groove formed by a β sheet 'floor' and a α helical 'wall' structure [214]. Polymorphic residues line the interior of the groove and form interactions with the peptide backbone, thus promoting promiscuous peptide binding. Conversely, select residues within the groove form bonds with anchor residues of the peptide side chains, thus conveying epitope selectivity. Interactions with the TCR occur across the face of the pMHC complex in a diagonal orientation, such that the TCR α chain aligns with the N-terminus of the presented peptide and the β chain with the C-terminus. This diagonal binding is structurally forced by a twist in the β sheet floor of the peptide-binding groove that, subsequently, causes peaks of α helices to protrude from the MHC face. TCRs specific for MHC class-II-restricted peptides have a more constrained docking topology than TCRs specific for class-I-restricted peptides. This phenomenon this also

structurally dictated and is thought to direct co-receptor engagement and, thus, lineage commitment during the final stages of thymic selection [215, 216] (see section 1.4.1).

TCR interaction with pMHC results in CDRs 1 and 2 mainly interacting peripherally with MHC residues, whereas the centrally placed CDR 3 from both α and β chains has most contact with the peptide. Thus, the most polymorphic region of the TCR aligns with the most polymorphic region of the pMHC complex.

1.2.2. Signal Transduction from the TCR

Signalling through the TCR is mediated via association with the CD3 complex (δ, γ, ζ, and two ε chains). One of the first biochemical consequences of TCR binding is activation of Lck (a Src family protein tyrosine kinase (PTK)) via the CD45-mediated de-phosphorylation of a C-terminal tyrosine. This event is initiated on the redistribution of the receptor complex with the lipid bilayer, as will be discussed in section 1.2.3. Active Lck subsequently phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic tail of the CD3 complex. Subsequently, binding of the SH2 domains within ZAP70 (a Syk family PTK) to these phosphorylated ITAMs juxtaposes ZAP70 and Lck, thereby allowing the phosphorylation of ZAP70. This definitive event endows the TCR-CD3 complex with PTK activity and results in the phosphorylation of numerous proteins and the activation of second-messenger cascades, *e.g.*, phospholipase C (PLC)-γ1, Vav (a guanosine triphosphate exchange factor involved in cytoskeletal reorganisation) and Tec family PTKs. Notably, Tec family kinases appear to be important in regulating cytokine production as mice deficient in the Tec PTK Itk are unable to mount T_H2 oriented responses due to defective IL-4 production, whereas mice deficient in other Tec members (Rlk and Txk) are selectively deficient in IFNγ production [217, 218].

Down-regulation of TCR-mediated signalling is controlled at multiple levels. Firstly, Lck not only activates ZAP-70 kinase but also SHP1 phosphatase which is able to counteract positive signalling via PTKS. Secondly, on binding B7, CTLA-4 is able to recruit SHP2 phosphorylase to the TCR complex. Thirdly, C-terminal Src kinase (CSK) is able to down-

regulate Lck activity if recruited to the active TCR complex. Finally, PTKs are able to activate Cbl ubiquitin-3 ligase which has been shown to down-modulate TCR-mediated signalling [219, 220]. Loss of ubiquitin activity via mutation results in sustained TCR-mediated signalling and cellular transformation [221]. It is unclear as to what elements of the TCR signalling complex are targeted for Cbl-mediated degradation.

1.2.3. The Immunological Synapse and Lipid Rafts

TCR signalling is now understood to occur with the concomitant formation of an immunological synapse [222-224]. These structures consist of ordered concentric protein arrays within the T-cell membrane that characteristically contain a metastable TCR-enriched central supramolecular activating complex (cSMAC) and a peripheral SMAC (pSMAC), into which LFA1-ICAM1 pairs are organised. Synapse formation allows the heterogeneous partitioning of inhibitory and excitatory signalling components, thus conveying exceptionally high control over TCR-mediated signalling, *e.g.*, the microtubule organising centre (MTOC) and PLC-θ are re-distributed to the synapse. Furthermore, the synapse structure allows high binding avidity to be achieved via multimolecular interactions, thus allowing high affinity interactions (*e.g.*, ICAM1-LFA1) to stabilise low affinity interactions (*e.g.*, TCR-MHC) and providing more scope for serial binding [222]. Indeed, TCR-mediated signalling is thought to be a continuous process during which individual TCRs engage pMHC, signal and subsequently disengage. Thus, disruption of the cytoskeleton or antigen removal results in the immediate cessation of signalling without synapse collapse [225]. Modulation of signalling transduction is achieved in part by continuous recycling of TCR-CD3 complexes and other signalling components (such as ζ, ZAP70 and Lck) from the synapse. This process occurs in an antigen-dose and time-dependent manner [226]. Thus, agonistic peptides induce rapid (1-2 hours) TCR internalisation, however, this is stabilised after approximately 6 hours by TCR synthesis and/or recycling. TCR down-regulation therefore serves to determine the length of antigen exposure and optimise signalling over a wide range of antigen concentrations, as long as the synapse is in place. This prolonged model of synapse formation has however been

challenged by the suggestion that naïve T lymphocytes only form transient, but sequential, interactions with APCs *in vivo*; this point is discussed in section 1.3.1.

Synapse formation occurs simultaneously with changes in lymphocyte morphology on APC encounter. Thus, before APC encounter, lymphocytes adopt a crawling state with the nucleus pushed to the leading edge of the cell and the cytoplasm concentrated in a trailing handle-like 'uropod' projection [227]. In this state the lymphocyte is highly polarised, *e.g.*, chemokine receptors, such as CCR2 and CCR5, which mediate migration up chemokine gradients, are distributed to the leading edge of the T-cell [228, 229]. Initial, antigen-independent encounters with the APC are termed 'pre-synaptic' and mediated by, for example, LFA1 and CD2 [230, 231]. Subsequently, specific TCR-pMHC interaction conveys a stop signal to the T-cell via the release of intracellular calcium. The magnitude of this signal correlates with the agonistic nature of the encountered peptide [232]. On initial peptide binding, microclusters (<1um) of TCR-MHC form at the T-cell-APC interface and these further coalesce within 5 to 10 minutes to form a TCR-CD3 cSMAC [233]. The formation of higher order structures is directly reliant on the present of agonistic peptide, cytoskeletal activity, co-receptor activity and calcium influx [234, 235].

Initial re-organisation is thought to occur via the 'tethering' of TCR-CD3 complexes into ordered sphingolipid- and cholesterol-enriched membrane rafts, which can subsequently act as platforms for signalling [233]. Under resting conditions, the TCR-CD3ζ complex is only weakly associated with rafts, while TCR-MHC engagement causes the translocation of the TCR into rafts domains, thus initiating formation of a supramolecular signalling complex [236]. Indeed, the importance of raft association during TCR-mediated signalling is demonstrated by the dual acylation of Lck that targets this PTK to lipid rafts; mutations that convey deficiencies in acylation ablate TCR-mediated signalling [237].

Synapse organisation involves the spatial partitioning of a number of molecules over time. For example, CD43, which has cell-cell repulsion properties, is actively excluded from the T-cell-APC interface by moesin [238]. Furthermore, CD45 is excluded, possibly to protect

active PTKs and ITAMs from dephosphorylation, and because its large size would inhibit intimate TCR-MHC interaction. Indeed, size exclusion is thought to be a passive mechanism that shapes re-organisation [222]. CD45 is nonetheless able to activate TCR signalling by dephosphorylating small fractions of Lck at raft edges in an antigen-independent manner [239]. Conditions that are inhibitory to TCR-mediated signalling also occur in the synapse, *e.g.*, CSK is constitutively linked to the phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), thus, upon TCR engagement and translocation into rafts, CSK is able to inhibit signalling.

Lipid rafts present within the synapse are also involved in controlling co-stimulation-mediated signal amplification. CD28 promotes the targeting of both intracellular and surface rafts to the synapse thereby increasing the local concentration of PTKs and adaptors, thus stabilising TCR-induced phosphorylation [240]. The mechanism by which this is achieved is obscure, but it is thought to involve the actin cytoskeleton and act via Vav-mediated signalling [241]. Interestingly, experimentally induced recruitment of rafts to the synapse, by cross-linking raft components, can induce amplification of TCR-induced signalling in a similar manner to CD28-mediated co-stimulation [240]. Thus, the efficiency by which TCR engagement is coupled to downstream signal transduction pathways can be varied by the amount of rafts available. Indeed, memory and effector T-cells express higher quantities of rafts than do naïve T-cells, thereby accounting for the lower dependence of these cells on co-stimulation or prolonged antigen exposure. This property of effector and memory T-cells is also due to constitutive re-distribution of Lck to the plasma membrane [242].

1.3. Induction of a T-cell Response

1.3.1. Priming

Naïve $CD4^+$ T-cells require a prolonged (6-30 hours) period of TCR-mediated stimulation in order to commit to proliferation [243]. This time of commitment (TOC) varies between antigen-experienced and naïve cells largely due to the co-stimulatory requirements of these cells and the differential distribution of Lck. Similarly, the TOC for primed $CD8^+$ T-cells is less than that for naïve cells but, in contrast to $CD4^+$ T-cells, the TOC of naïve $CD8^+$ cells is estimated to be two to four hours.

How is prolonged antigen exposure, in order to reach the TOC, achieved *in vivo*? Two non-contradictory mechanisms have been proposed – the monogamous interaction model suggests that single DC-T-cell conjugates are formed and stabilised by a prolonged immunological synapse. In contrast, studies *in vitro* using a collagen matrix have suggested that DC-T-cell interactions are transient and that T-cells migrate between DCs sequentially [244]. In this sequential model, T-cells would thus sense and integrate stimuli over time. Synapse formation could occur during these dynamic interactions, but the synapse would have to be more transient and motile than originally suggested. Alternatively, conditions that favour synapse formation may exist in T-cell areas of secondary lymphoid organs, *e.g.*, T-cells are shielded from collagen in lymph nodes by stromal cells [245]. Immunological synapse-like structures (CD43 excluded) were recently visualised between DC and T-cells directly ex-vivo in whole explanted lymph nodes [246]. Prolonged T-cell-DC association was observed in this system (> 15 hours), however, the imaging method used was unable to allow visualisation of the T-cell zones within the lymph node, thus, the existence of sequential interactions between DCs and T-cells preceding stable conjugate formation can not be ruled out. Nonetheless, TCR-induced signalling may be observed prior to synapse formation, suggesting that synaptic structures allow vectorial secretion rather than acting as signalling platforms [247].

DC-T-cell interactions additionally serve to terminate priming by, for example, class-II cross-linking [248] (see next section). Nonetheless, other molecules expressed by

antigen-stimulated T-cells such as TRANCE (or RANK receptor) are able to promote DC survival and increase T-cell stimulatory capacity, thereby favouring T-cell expansion [249, 250]. Together with T-cell-derived inhibitory signals, such interactions are likely to have a crucial effect upon the dynamics of T-cell priming *in vivo.*

Once committed to proliferation, $CD4^+$ T-cells require further stimulation via the TCR in the presence of either IL-12 or IL-4 in order to differentiate into T_H1 or T_H2 'effector memory' cells respectively [251, 252]. $CD4^+$ T-cells that do not receive this continued stimulation after the TOC are termed 'central memory' cells; these cells do not develop effector functions but retain the chemokine receptor CCR7 and L-selection (CD62L), thereby maintaining the ability to home to lymph nodes (*c.f.*, section Chapter 3 for CD62L). 'Central memory' cells thus represent an unpolarised population of T-cells that are able to rapidly generate a new wave of effector cells following secondary rechallenge.

Both central and effector memory-like populations have also been identified in the CD8 compartment [253], but some reports suggest that $CD8^+$ T-cells do not require TCR-mediated stimulation after commitment to proliferation [254]. Indeed, suboptimal antigen doses have been shown to reduce the number of naïve antigen-specific $CD8^+$ T-cells induced to proliferate *in vivo*, but the cells that do respond divide 7 to 10 times and differentiate in an antigen-independent/IL-2-dependent manner [254]. This programmed response by $CD8^+$ T-cells thus results in the production of effector and memory cells following initial antigen encounter and ensures that, under conditions where antigen is limiting, memory cells can be generated. Nonetheless, $CD8^+$ T-cells require persistent antigen in order to continue proliferating, thereby elevating the primary clonal burst size and increasing the frequency of antigen-specific cells in the memory pool [252].

The greater antigen dependency of $CD4^+$ memory cell generation may therefore be explained by the intrinsically lower potential of these cells to proliferate [255], as higher numbers of divisions are required in order to acquire new cellular phenotypes, *i.e.*, to remodel chromatin [256] and acquire memory phenotype. Both $CD4^+$ and $CD8^+$ memory

T-cells are therefore currently viewed as effector precursors that have undergone sufficient divisions in order to acquire a memory phenotype, but have not terminally differentiated into effector cells. Indeed, DCs presenting low concentrations of antigen, that entertain only brief interactions with T-cells, and that are incapable of producing IL-12 due to prior exposure to LPS, preferentially induce $CCR7^{+}$ non-polarised central memory cells [257]. These conditions are thought to emulate the late stages of a T_H1 orientated response, suggesting that memory cells may be produced en masse at this stage.

1.3.2. CTL Effector Mechanisms and Clonal Deletion

Once CTLp have been primed and have differentiated into mature CTL, these cells migrate to sites of inflammation. On binding pMHC, CTL affect target cell apoptosis via a cytotoxic granule exocytosis pathway involving perforin and granzyme proteases and/or via the up-regulation of FasL [258, 259].

Thus, formation of a tight junction between the effector CTL and the target cell allows the vectoral secretion of the CTL granule contents. Perforin contained within these exocytosed granules polymerises within the target cell membrane, in a manner analogous to the formation of the terminal membrane attack complex generated by the complement component C9 [260]. The resulting pores within the target cell plasma membrane allow entry of neutral serine granzyme proteases, also contained within the granular contents, which subsequently elicit target cell death by caspase-dependent and –independent mechanisms [261]. Thus, perforin-deficient mice display a 'pan-granzyme' defect whereas granzyme-$A^{-/-}$ and -$B^{-/-}$ animals reveal the contribution of these two granzymes to pathogen resistance in different models [262]. Granzyme B deficient CTL display an absolute defect in their ability to lyse allogeneic target cells within 4 hours *in vitro* [263] and it is now known that this granzyme evokes rapid apoptosis via a caspase-dependent mechanism [264] with purified granzyme B activating caspase-3, -6, -7, -8, -9 and –10 *in vitro* [265]. On the other hand, granzyme A is able to evoke target cell lysis by a caspase-independent mechanism, but this occurs with delayed kinetics in comparison to granzyme B-mediated apoptosis [266, 267]. In addition to granzymes, CTL cytotoxic granules contain granulysin that

displays direct microbicidal activity against a range of pathogens [268] and is able to enter target cells via perforin pores, thereby restricting intracellular bacterial growth [269].

CTLs display an inherent resistance to cytotoxic molecules that is mediated by serpin serine protease inhibitor-6 (SPI-6) [270] and cathepsin B (CB) [271]. CB is expressed in surface form following granule exocytosis and conveys resistance to granzyme-mediated apoptosis by cleaving an intermediate form of perforin that is formed during pore construction. Nonetheless, the transient and local expression of CB suggests that CTL may be susceptible to fratricidal attack, as can be demonstrated *in vitro* [252].

Fas-FasL interaction is additionally used by CTL, particularly $CD4^+$ CTL, to affect target cell lysis [263]. Thus, within hours of TCR engagement FasL is synthesised by CTL and is subsequently able to ligate Fas on the target cells thereby initiating caspase activation and affecting apoptosis [272]. Alternatively, CTL may secrete IFNγ and TNFα in the vicinity of target cells which are able to induce apoptosis directly in some cell types, and which activate and recruit phagocytes.

As mentioned above, as well as prolonging DC survival and increasing the T-cell priming capacity of these APC by utilising such molecules as TRANCE, T-cells are also able to induce DC apoptosis, thereby altering the dynamics of priming. Thus, systemic infection with a DC-tropic strain of LCMV results in DC depletion and concomitant immunosuppression [273]. Such T-cell-mediated DC killing may serve to down-regulate excessive CTL activation and/or allow a pre-existing CTL clonal population to inhibit the formation of another [274]. However, DCs do express SPI-6 that binds granzyme B and inhibits apoptosis induction via this pathway [275]. Interestingly, expression of SPI-6 by DCs is only induced by T_H1, but not T_H2, cells. This protective mechanism is likely to be especially important for prolonging antigen presentation during secondary CTL responses where effector CTLs may be generated rapidly (within approximately 1 hour) from the central memory pool. The effectiveness of the SPI-6-mediated inhibitory pathway is

underscored by the observation that a range of human melanoma and carcinoma cell lines express SPI-6 which conveys protection against CTL attack [276] (*c.f.*, see section 1.5.2).

During some viral infections, antigen-specific CTL may represent up to 50-70% of the $CD8^+$ T-cells [277]. Following the effector phase of the CTL response, homeostasis within the T-cell compartment is re-gained by the deletion of large numbers of antigen-specific CTL. This clonal deletion occurs via propriocidal mechanisms in both an antigen-dependent and –independent manner [278].

Upon initial antigen encounter naive CTLp secrete elevated levels of IL-2 and upregulate the high affinity component of the IL-2 receptor complex (α chain, CD25), but remain resistant to apoptosis. Nonetheless, following several rounds of division induced by IL-2, Fas and TNF are induced simultaneously with the development of an increase in susceptibility to apoptosis induced by these cytokines [279]. Antigen-induced TCR cross-linking at this stage is thus able to affect CTL apoptosis via FasL and TNF. Indeed, *gld* and *lpr* mice, which have genetic defects in FasL and Fas respectively, exhibit lymphoproliferative and autoimmune manifestations [280-282]. Similarly, patients with autoimmune lymphoproliferative syndrome have genetic defects associated with the Fas-induced death pathway. Thus, IL-2 provides positive feedback that conveys susceptibility to apoptosis, while the level of antigenic stimulation encountered by CTL dictates whether apoptosis actually occurs. This mechanism therefore accounts for immunological exhaustion observed under conditions of chronic antigen stimulation [278]. Additionally, unlike B-cells, the expression of both Fas and FasL by single T-cells, and the presence of soluble TNF and Fas, allows suicidal killing by CTL [283-285].

In contrast to activation-induced cell death (AICD) of CTL, if CTL do not receive further antigenic stimulation, passive apoptosis occurs due to 'lymphokine withdrawal', *i.e.*, diminished expression of IL-2 and down-regulation of CD25. Systemic administration of recombinant IL-2 may therefore rescue CTL from deletion following superantigen stimulation [286] and, moreover, IL-7, -15 and –4 may prevent CTL death [287-290].

Together, both forms of CTL death act to induce CTL apoptosis if too much or too little antigen and IL-2 are present. In this way, CTL expansion may be controlled within set parameters of proliferation and death in situations where environmental changes cannot be predicted.

1.4. Central & Peripheral T-cell Tolerance

1.4.1. Thymic Selection of T-lymphocytes

As neoplastic cells are derived from autologous tissue, the concept of how T-cell self tolerance is established and maintained, in the face of necessary autoreactivity, is central to our understanding of how T-cell-mediated anti-tumour immunity may be evoked without inducing autoimmunity.

T-cell tolerance to self pMHC is established centrally in the thymus, and is further re-affirmed continuously after migration to the periphery. In concordance with this, it is interesting to note that autoantigens involved in an array of autoimmune diseases (*e.g.*, myelin basic protein) are detectable at the mRNA level in the thymus and, furthermore, so-called tissue-specific transgenes (*e.g.*, rat insulin promoter-OVA) are also expressed in the thymus [291].

Thymic development ultimately results in selection of T-cells expressing a single TCR that interacts weakly with self pMHC complexes, *i.e.*, all TCRs expressed by mature T-cells are autoreactive but are of an affinity such that potentially autoimmune effector functions do not develop (see section 1.4.2). The significance of autoreactivity and our current knowledge about thymic selection are discussed below.

1.4.1.1. TCR Gene Recombination

T-lymphocytes develop from bone marrow-derived clonogenic lymphoid progenitors that have committed to the T-lineage in response to the thymic microenvironment [292]. The ensuing development may be subdivided into phases I and II. Phase I represents the

development, before CD4 and CD8 co-receptor expression, during which T-lineage-restricted precursors give rise to αβ or γδ T-lineage cells. During phase I, thymocytes display a triple negative phenotype (TN; $CD4^-8^-3^-$) which can be further subdivided based on the expression of CD25 and CD44 (H-CAM). These TN cells constitute approximately 3 % of the total thymocyte population [293].

Initial TCR gene rearrangement (β, γ or δ, not α) occurs during the TN2 to TN3 transition [294]. β gene formation requires the sequential juxta-positioning of the V, D and J TCR gene segments; initially a D segment is recombined with a J segment, followed by VDJ joining. Mice and humans carry 20-70 Vα and Vβ gene segments that encode approximately the first 90 amino acids of the TCR α and β chains respectively. Recombination is directly catalysed by the products of the recombination activating genes (RAG) -1 and -2.

A successful VDJ rearrangement at the β locus is tested by the expression and assembly of the new TCRβ chain with an invariant surrogate α chain (pre-Tα), as well as the CD3 signalling complex. Subsequent β–selection does not involve pMHC recognition, as truncated TCRβ and pre-Tα lacking an extracellular domain are able to trigger progression to the double-positive (DP) stage [295]. Thus, successful assembly of the CD3-pre-Tα-TCRβ complex is sufficient to induce thymocyte maturation to the TN4 stage. β-selection is further characterised by the silencing of the RAGs and the induction of several rounds of division [296]. Conversely, both TCRγ and δ gene rearrangement and expression are required for γδ lineage maturation at this stage. A minority of thymocytes (< 5 %) yielding productive γ- and δ-chain rearrangements enter the periphery as DN $CD3^+$ γδ T-cells. Notably, these cells have a role in DC modulation via the production of cytokines (see below).

Phase II of thymic selection involves α chain rearrangement, active TCR selection and concomitant entry to either the $CD4^+$ or $CD8^+$ T-cell lineage. Firstly, TN4 thymocytes,

having produced a functional β-chain, re-initiate RAG transcription and, thus, α chain rearrangement may begin – Vα gene segments are directly recombined with Jα segments to produce a functional α chain that can pair with the pre-existing TCRβ chain. Pairing of the immunoglobulin-like folds within the TCRα and β chains results in the formation of an antigen-binding site such that three hypervariable loops, or complementarity determining regions (CDRs), from each chain are bought into close proximity. The V gene segments directly code for CDRs 1 and 2 from both α and β chains, whereas CDR3 is created on the juxta-positioning of VJ or V(D)J segments. In this way, CDR3 from both chains provides the greatest diversity within the TCR antigen-binding site, *i.e.*, each V segment is able to pair with any (D)J segment. Moreover, joining of the coding sequences is imprecise and the enzyme terminal deoxynucleotidyl transferase randomly adds nucleotides to these junctions.

The development of thymocytes to maturation requires the appropriate interaction of TCR with pMHC complexes on thymic DCs and concomitant signalling. Indeed, only approximately 5 % of the thymocyte population expressing the TCR repertoire will migrate to the periphery, having survived either negative selection against self-pMHC and death by neglect between the TN4 and DP stages. Thus, T-cell development is blocked at the DP stage in $MHC^{-/-}$ mice, but this deficit can be overcome in foetal thymic organ culture (FTOC) by the use of anti-TCR monoclonal antibodies [297] or, alternatively, re-aggregation with wild-type thymic stromal cells [298]. Using this latter approach, it is estimated that between 5 and 20 % of the DP thymocyte population is responsive to self-MHC, thus suggesting that the germ-line TCR repertoire is skewed towards self-recognition. Indeed, certain Vα segments preferentially bind MHC class I or II, thereby resulting in the generation of $CD8^+$ and $CD4^+$ T-cells respectively (see below).

1.4.1.2. Selection of TCRs from the Germline Repertoire

Lack of interaction between the newly formed TCRs and pMHC results in death by neglect of thymocytes within approximately three days. During this time RAG transcription and α chain rearrangement continue. Engagement of pMHC via the TCR leads to the cessation

of both these processes and results in either positive or negative selection of the thymocyte. Negative selection results in thymocyte deletion and occurs when the prospective TCR engages pMHC with high affinity. In this way, negative selection establishes a high degree of self-tolerance within the T-cell compartment; approximately 50 % of thymocytes reacting with self-pMHC are eliminated during this process [299]. Unsurprisingly, thymus-resident DCs are critical in this process [300].

Whereas negative selection of thymocytes occurs on binding of high affinity TCR ligands, and results in thymocyte deletion, positive selection occurs in response to low affinity binding by the thymocyte TCR. Positive selection leads to thymocyte survival and, subsequently, CD4/8 lineage commitment.

Positive selection of thymocytes into a mature population of T-cells expressing a diverse TCR repertoire requires a diverse array of self-peptides. This requirement was first observed when mutations effecting only the peptide-binding groove of murine class I were shown to severely restrict the selected $CD8^+$ repertoire [301, 302]. Borten *et al.* demonstrated the requirement for diverse yet low abundance peptides in the positive selection of T-cells via the substitution of the class II CLIP sequence with a tight binding peptide. In these Ii-deficient mice, 95 % of the surface class II molecules are occupied by this single sequence, yet the majority of mature $CD4^+$ T-cells were selected on the remaining 5 % of molecules occupied by a diverse mixture of endogenous peptides [303]. Moreover, if these mice were backcrossed with H2-$M^{-/-}$ mice a 70 % reduction in selected $CD4^+$ T-cells was observed, while no change in class II expression was detected. Thus, not only is MHC restriction conferred during positive selection, but thymocytes are also selected on the basis on their low affinity interaction with self-peptides. This endogenous self-reactivity is essential during peripheral T-cell homeostasis and for the maintenance of self-tolerance.

1.4.1.3. CD4/8 Lineage Commitment and αβ Lineage Survival

The final stage of thymic development involves commitment of the selected DP thymocytes to either the CD4 of CD8 lineage. This lineage decision is controlled by the co-receptor-influenced duration of TCR-dependent signalling that, in turn, is thought to lead to quantitative differences in p56lck activity [293, 304]. Strong p56lck activation results in a CD4 lineage commitment, while weak activation leads to $CD8^+$ T-cell formation. Thus, transgenic mice expressing either constitutively active or dominant negative forms of p56lck induce class-I-restricted thymocytes to aberrantly differentiate into $CD4^+$ cells and class-II-restricted thymocytes to give rise to $CD8^+$ cells respectively [305]. Under normal circumstances, the degree of p56lck activation is determined by the MHC restriction of the prospective TCR; CD4-TCR aggregation is constitutively associated with high levels of p56lck activity, whereas CD8-TCR association results in low p56lck activity. In the latter case, this is due to the presence of CD8α', an isoform of CD8α which lacks a cytoplasmic tail and is thus unable to bind p56lck, thereby inhibiting CD8α-mediated p56lck activation [306]. Co-receptor binding is thought to be partly influenced by the differential binding orientation of the TCR to class I and class II MHC molecules [215].

Following selection, mature single-positive T-cells migrate from the thymus into the periphery. As these cells have been selected for low affinity interaction with self-pMHC, all are autoreactive. The level of TCR-mediated activation induced in a thymocyte does not indicate the capacity of the same recognition event to activate mature T-cells baring the same TCR. Furthermore, autoreactivity does not necessarily result in the expression of effector functions and, indeed, this property of mature naïve T-cells is now appreciated to be a prerequisite for the maintenance of self-tolerance and protective immunity. The following section aims to discuss these points.

1.4.2. Peripheral Tolerance - Dynamic Tuning of T-cell Reactivity

Mechanisms that maintain T-cell peripheral tolerance are required as the context under which antigen recognition occurs during selection and in the periphery may vary greatly, *e.g.*, differing antigen density, co-stimulatory environment, cytokine milieu and co-receptor

binding. With regard to this, it is important to note that antigen recognition does not necessarily result in the expression of effector functions and the same ligand may elicit qualitatively different responses under differing circumstances, *e.g.*, maintenance of cell viability, homeostatic division, and dynamic re-adjustment of the T-cell activation threshold. Thus, T-cell activation occurs in a hierarchical manner and autoreactive cells are therefore rarely autoimmunogenic [307, 308].

The concept of T-cell activation threshold adaptation ('tuning') is central to our understanding as to how autoreactivity is maintained without ensuing autoimmunity. Interaction of T-cells with self-ligands is thought to continuously regulate individual T-cell activation thresholds. Thus, responses can be induced, over a wide range of excitatory conditions, to relative rather than absolute changes in stimulation [308]. Indeed, freshly isolated thymocytes and mature T-cells have partially phosphorylated CD3ζ chains associated with non-phosphorylated ZAP70, a phenotype that is seen in T-cells stimulated with suboptimal ligands [309]. In this context, qualitatively different transcriptional responses ensue following TCR engagement, and this is regulated by the balance between 'excitatory' (*e.g.*, active Lck, ZAP70, Erk1) and 'de-excitatory' (*e.g.*, active SHP1) factors. Under recurrent self-ligand stimulation, excitatory factors may fluctuate but the ratio of de-excitatory-to-excitatory factors remains above that required for the expression of effector functions. Alternatively, other functional responses may take place, *e.g.*, limited proliferation. In support of this view, *in vitro* stimulation of $CD4^+$ T-cells with either antagonist or agonist ligands results in SHP1-mediated TCR desensitisation and, *in vivo*, SHP1 expression is seen to increase during development [310]. Conversely, positive tuning may occur with elevated levels of Lck - increases in peptide sensitivity and acquisition of a memory cell phenotype parallel an increase in Lck expression [311].

Peripheral tolerance is maintained at multiple levels above that of T-cell reactivity tuning, *e.g.*, DC-mediated induction of $CD4^+CD25^+$ T-regulatory cells. These mechanisms are discussed below.

1.4.3. DC-Mediated T-cell Deletion and Induction of Regulatory Function

DCs also participate in the maintenance of peripheral tolerance by deleting excessively autoreactive naïve T-cells in the absence of inflammation, *i.e.*, the absence of co-stimulation and/or appropriate cytokine milieu [312]. Furthermore, it is now appreciated that iDCs bestow regulatory functions upon naïve $CD4^+$ and $CD8^+$ T-cells [313].

Tissue-resident iDCs capture fluid-phase and cell-associated antigens from self-tissue, as well as from pathogens, in the steady state. Presentation of peptides derived from these antigens *in situ* may result in the deletion or tolerisation of the few T-cells that reach these sites under non-inflammatory conditions. Thus, iDCs are able to cross-tolerise T-cells in the steady state.

On receiving an inflammatory stimulus, DCs actively migrate to the T-cell areas of the secondary lymphoid organs. However, this process occurs continuously at low levels in non-inflammatory conditions. Langerhan cells resident in the skin survive for approximately one month before migration to the T-cell areas where their life expectancy is approximately 3 to 5 days [314]. Migratory DCs display a degree of maturation with respect to their tissue-resident counterparts – anchor receptors such as E-cadherin and α6-integrins are down-regulated, chemokine receptors such as CCR7 are induced and, moreover, MHC and co-stimulatory molecules are partially up-regulated [68]. The phenotypic changes observed in DCs during steady state migration argue against the requirement for specialised tolerising DC subsets residing within the secondary lymphoid tissue, as migratory DCs possess the ability to present stable pMHC complexes; nonetheless, limited evidence for this population does exist [87].

As anticipated, migratory DCs are loaded with antigens acquired peripherally. Notably, Huang *et al.* isolated a population of $CD4^-/OX41^-$ DCs from rat mesenteric lymph that contained remnants of intestinal epithelial cells (IEC), *i.e.*, these DCs contained IEC-specific cytokeratins and an IEC-specific isoform of non-specific esterase [315]. Additional

studies have demonstrated that DCs capture antigens from airways, blood and muscle [90].

Two separate approaches have provided evidence that migratory DCs cross-tolerise the T-cell repertoire by deletion. Firstly, mAb-antigen conjugates have been used to target antigen to DCs, *e.g.*, anti-DEC205-Hen Egg Lysozyme (HEL) conjugates specifically target HEL to draining lymph node DCs after subcutaneous injection. Subsequently, adoptively transferred transgenic HEL-specific $CD4^+$ T-cells proliferated, produced IL-2, but were rapidly deleted after approximately 7 days [316]. Conversely, co-injection of anti-DEC205-HEL and the agonistic anti-CD40 mAb FGK-45 resulted in prolonged T-cell and DC activation. Secondly, and more elegantly, transgenic mice that express either, or both, a tissue-specific peripheral neoantigen and its cognate TCR have been used in a variety of systems to study peripheral deletion. For example, HA-specific $CD8^+$ transgenic T-cells adoptively transferred into InsHA mice (that express HA under the control of the rat insulin promoter) undergo limited proliferation exclusively in the pancreatic lymph node prior to deletion [317]. In this model, the transferred T-cells display an unusual 'semi-activated' phenotype ($CD44^{hi}62L^{-}49D^{lo}25^{-}$) but, importantly, do not express IFNγ or exhibit cytolytic activity. As expected, peripheral deletion in this system was shown to be B7-independent and could be avoided by inoculation with live influenza. Interestingly, a minor population of HA-specific T-cells survive deletion due to the expression of a low affinity TCR, as assessed by K^dHA tetramer staining [318].

Together, these data suggest that, in the steady state, migratory DCs present antigen captured in peripheral tissue to naïve T-cells resulting in T-cell deletion and, moreover, that maturation stimuli are able to convert this mechanism of tolerance to active priming. Additionally, T-cells that escape deletion do so due to the expression of a low affinity TCR that is unable to detect antigen (either entirely or to the extent of eliciting effector functions) at the abundance present peripherally.

In addition to the central and peripheral deletion of self-reactive T-cells, self-tolerance is further reaffirmed by specialised subsets of T-cells, T-regulatory (Tr) cells [319]. The existence of antigen-specific Tr cells in the normal T-cell repertoire, and the control of organ-specific autoimmunity by these cells, has been demonstrated in a variety of settings. For example, neonatal thymectomy of mice (d3Tx) results in the development of organ-specific autoimmune manifestations such as gastritis and thyroiditis [320]. Additionally, Tr cells isolated from wild-type mice are able to inhibit autoimmunity in genetically susceptible strains, *e.g.*, experimental allergic encephalitis (EAE) in myelin basic protein (MBP) TCR transgenic mice [321, 322], and diabetes in a susceptible rate strain [323]. Indeed, previously unidentified mutations that convey susceptibility to autoimmunity have now been correlated with deficiencies in the Tr cell compartment [319].

Distinct subsets of Tr cells have been identified and characterised in a variety of systems (table 3). Tr1 cells may be generated *in vitro* by culturing OVA-specific $CD4^+$ transgenic T-cells with OVA in the presence of IL-10 [324]. The resulting Tr1 cells produce IL-10 and IL-5 with or without TGFβ, but with little or no IL-2, IL-4 or IFNγ, *i.e.*, a cytokine profile that is distinct from that of undifferentiated, T_H1 and T_H2 cells. *In vivo*, Tr1 clones specific for filamentous HA from *Bordetella pertussis* suppress proliferation and cytokine secretion from an unrelated influenza HA-specific T_H1 clone [325]. In both cases, suppression is mediated by IL-10.

Table 3.
Characteristics of Tr cells (adapted from [326])

	$CD4^+CD25^+$	Tr1	T_H3	$CD8^+Tr$
Surface phenotype				
CD25	+[a]	+	+	?
$CD45RB^{low}$	+	+	?	?
CD45RO	+	+	+	+
CTLA-4	+++	-	++	-
Cytokine secreted				
IL-10	+/-	+++	+	++
TGFβ	+/-	+	+++	+/-
Differentiation factors	?	IL-10, IFNα	IL-4, TGFβ	?
Suppressor Mechanism				
In vitro	Cell contact	IL-10	TGFβ	IL-10, TGFβ
In vivo	Cell contact, IL-10, TGFβ	IL-10	TGFβ	?

[a] Symbols correspond to relative surface expression or secretion by the different Tr subsets

T_H3 Tr cells were, on the other hand, first identified in studies of oral tolerance, *e.g.*, oral administration of MBP increases the frequency of MBP-specific $CD4^+$ T-cells that secrete TGFβ in multiple sclerosis patients [327]. The importance of TGFβ-mediated immunosuppression is exemplified by the fact that certain tumour lines produce TGFβ [328] and the ablation of TGFβR function in $CD4^+$ T-cells results in the spontaneous elimination of a variety of tumours with the concomitant development of inflammatory infiltrates in the colon and lung [329]. Both TGFβ and IL-10 are likely to operate at the level of both the APC and by inhibiting the proliferation of T-cells directly [319] (see section 1.5.2).

Interestingly, it has been proposed that both Tr1 and T_H3 cell are of similar origin and that these cells represent so-called 'anergic' cells that are solely generated in the periphery [330]. By contrast, accumulating evidence suggests that $CD4^+CD25^+$ Tr cells may be generated both peripherally and centrally. Indeed, grafting of xenogeneic thymic epithelium induces tolerance to a variety of peripheral tissues of donor origin and this is transferable with $CD4^+$ cells [331]. Furthermore, peripheral $CD4^+$ T-cells from athyroid rats are unable to prevent thyroiditis in TxX rats upon adoptive transfer, whereas thymocytes from these same rats contain suppressive activity [332]. Thus, Tr generation in the thymus is driven by the presence of specific autoantigens. Additionally, in double transgenic mice that express both influenza HA and the cognate class II-restricted TCR approximately 50 % of the mature HA-specific T-cells develop into $CD25^+$ Tr cells [333]. In this model, only cells expressing high avidity TCRs matured into Tr cells suggesting that a narrow affinity window enables Tr development, thereby ensuring that only a minor proportion of peripheral T-cells normally acquire regulatory capacity (5-10 % $CD4^+$ T-cells). Consistent with this hypothesis, autoimmunity may be avoided by the preferential activation of high affinity Tr cells over their pathogenic counterparts.

The exact mechanism by which $CD4^+CD25^+$ Tr cells evoke tolerance remains controversial - IL-10, CTLA-4 and both soluble and membrane-bound TGFβ have been implicated [319, 334]. Furthermore, the fact that $CD25^-$ T-cell populations also exhibit suppressor activity in

some models has led to the search for alternative markers for subset discrimination and, indeed, the precise relationship between $CD4^+CD25^+$ Tr, Tr1 and T_H3 cells at present remains ambiguous.

As well as the central generation of autoantigen-specific Tr cells, DCs activated by certain stimuli are able to induce Tr cells in the periphery [335]. Thus, *in vitro* generated DCs cultured in the presence of TNFα and PGE_2 inhibit EAE via induction of $CD4^+IL10^+$ Tr cells [336]. TNFα/PGE_2-conditioned DCs produce IL-10 in addition to expressing high levels of MHC class II and co-stimulatory molecules but, notably, these DCs do not produce IL-1β, IL-6 or IL-12. Thus, different DC stimuli evoke DC maturation within a wide spectrum of potential phenotypic and functional states, some of which are now known to induce Tr cells (figure 1.4). Indeed, the dynamic generation of Tr cells by DCs is likely to contribute to the maintenance of peripheral tolerance in the steady state.

Other examples of DC-induced Tr cells include $CD8^+$ Tr cells transiently generated in response to influenza matrix protein-pulsed immature DCs [313] and allergen-specific $CD4^+CD45RB^{low}$ Tr cells induced by killed *Mycobacterium vaccae* [326]. Thus, pathogen-specific Tr cells are transiently induced during inflammation in conjunction with CTL. These Tr cells are likely to be involved in restricting collateral immunopathology, resolving the anti-pathogen response and, thus, kerbing autoimmune responses secondary to infection. Indeed, the co-existence of tolergenic and T_H1-promoting stimuli that compete with one another for DC activation, and hence the induction of Tr cells and CTLs, is exemplified by the increased IL-10 production by murine DCs in response to yeast cells walls once TLR2 function is knocked-out [121].

Interestingly, pathogen-induced IL-10, TGFβ or IL-10 homologs encoded by some viruses could provide the appropriate cytokine milieu for the generation of Tr cells and, thus, contribute to the maintenance of persistent infection [337]. The same view could be taken to explain the existence of some tumours that express potentially immunogenic antigens (see below).

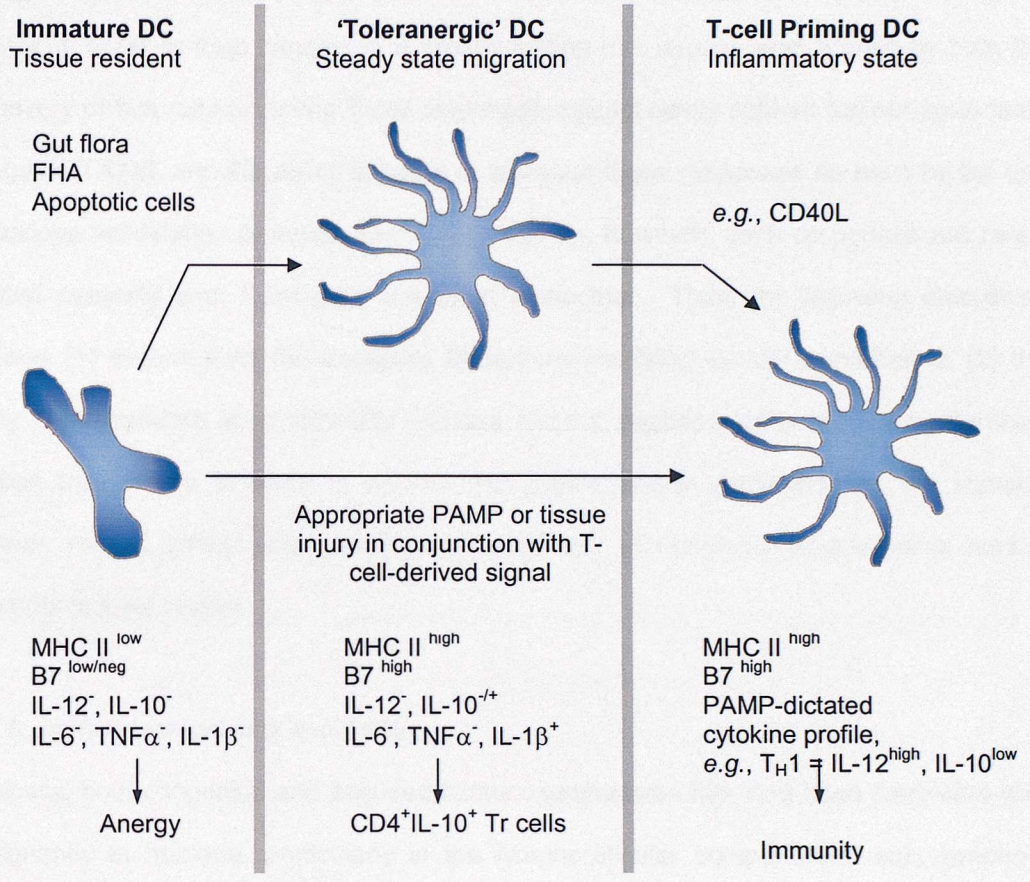


Figure 1.4. A Spectrum of DC maturation States in T-cell Tolerance & Immunity.

Immature tissue-resident DCs are characterised by their ability to capture exogenous antigen and induce T-cell anergy by inefficient presentation of this antigen, *i.e.*, lack of pro-inflammatory cytokines and co-stimulation, minimal pMHC formation. In contrast, a number of stimuli encountered in the steady state, such as apoptotic tissue and commensal bacteria, are able to induce DC migration and mature DC such that Tr cells may be induced upon antigen recognition in the absence of pro-inflammatory cytokines. Full maturation of DCs requires both PAMP recognition and T-cell derived signals such as CD40L.

Having discussed the generation and maintenance of self-tolerance, the following section aims to outline current data that indicate the existence of anti-tumour immunosurveillance mechanisms. Additionally, the means by which tumours evade detection by these systems and the potential for beneficially boosting these responses is also discussed.

1.5. Immunotherapy

In modern times, enthusiasm for the notion that anti-tumour immune responses may be elicited in order to treat cancers in a clinical setting has largely been fuelled by both the discovery of naturally-occurring T-cell responses against newly defined tumour-associated antigens (TAAs), and the ability to boost or generate these responses *de novo* by the use of various vaccination protocols [338-342]. Clearly, however, such responses are rarely elicited naturally and, if induced, are often ineffectual. Thus, the following discussion outlines (1) evidence for the existence of immune-mediated tumour surveillance, (2) the likely characteristics of a clinically relevant tumour rejection antigen, (3) mechanisms utilised by tumours in order to prevent recognition and/or eradication by the immune system, and (4) current prophylactic and 'therapeutic' vaccination protocols being used in attempts to treat cancer.

1.5.1. Immunosurveillance and TAAs

Clinically, both congenital and acquired immunosuppression has long been correlated with malignancy in humans, particularly in the lymphoreticular compartment, *e.g.*, lymphoid neoplasia resulting from drug-induced immunosuppression following stem cell transplantation [343]. However, a large number of these tumours have a strong association with viral infection, *e.g.*, Epstein-Barr virus and human herpes virus. Nonetheless, current data point to the fact that spontaneous tumours are likely to be controlled in different tissues by appropriate arms of the immune system. For example, IFNγ receptor-deficient mice are more susceptible to carcinogen-induced sarcomas and spontaneous sarcoma/lymphoma development after the loss of alleles encoding the intrinsic tumour suppressor p53 [344]. Furthermore, mice selectively deficient in NKT or NK cells display an increased susceptibility to carcinogen-induced sarcomas [344]. Indeed, cellular transformation is likely to be accompanied by the expression of stress-induced ligands (*e.g.*, MICA) which may act as receptors for NK cells, macrophages and $\gamma\delta$ T-cells thereby potentially resulting in tumour eradication at the early stages of transformation [345, 346]. Better documented however is the role of the adaptive immune response in controlling neoplasms, *e.g.*, elevated spontaneous lymphomagenesis is

observed in p53/perforin$^{-/-}$ mice with respect to p53$^{-/-}$ counterparts, and lymphomas arising in this environment may be eradicated if transplanted into wild-type mice [347]. Moreover, nude mice lacking thymus-derived T-cells have a low, but significantly higher, incidence of lymphoma than wild-type littermates, and this incidence may be further increased by stimulation with anti-μ [348]. Thus, T-cell-derived perforin, as in the T-cell compartment, contributes to the re-establishment of homeostasis following B-cell expansion, and the absence of this pathway increases susceptibility to lymphoma development [349].

In addition to the above findings, evidence for the immunosurveillance of non-haemopoietic tissue by the innate and adaptive arms of the immune response now exists [345]. Notably, mice that lack lymphocytes due to a RAG-2 deficiency all possess intestinal neoplastic lesions at 18 months of age, with 50 % of these being cancerous adenocarcinomas, this is in contrast to wild-type littermates that do not develop these lesions [344, 350]. Moreover, if RAG-2$^{-/-}$ mice are crossed with STAT-1$^{-/-}$ (STAT-1 is a transcription factor dedicated to mediating signal transduction from the IFN receptors) mice, the resulting animals develop earlier and more invasive lesions as well as mammary cancers [350]. Thus, innate and adaptive immunity are able to suppress tumours at distinct sites, thereby acting as an extrinsic tumour suppressor. Nonetheless, the late on-set of these lesions suggests that intrinsic tumour suppression is the dominant mechanism that prevents tumour development.

Indirect evidence of immunosurveillance has been corroborated by the discovery of TAAs and, moreover, the observation that expanded pools of TAA-specific T-cells exist in patients with advanced disease [351-354]. Indeed, anecdotally, some tumours occasionally undergo spontaneous regression and malignancies may be accompanied by paraneoplastic autoimmunity, which may be a good prognostic indicator. For example, cdr-2-specific CTL are associated with cerebellar degradation in seropositive breast/ovarian cancer patients [355]. Furthermore, recoverin-specific CTL generated against lung carcinomas are associated with positive prognosis, but recoverin-specific autoantibody simultaneously produced causes retinopathy [356].

Epitopes from TAAs discovered thus far arise from normal differentiation antigens that have highly restricted tissue distribution; antigens that are usually restricted to 'immunoprivileged' sites such as the testes and placenta, but are re-activated during oncogenesis; antigens with elevated expression within tumours; mutated self-antigens resulting from single-base mutations and chromosomal rearrangements; and neoantigens generated from, for example, intronic sequences or reverse-strand transcription [341, 354]. Examples of human class-I and class-II-restricted TAAs are given in table 4.

Table 4.
Examples of Tumour Cancer Antigens

Antigen Class	Example
Class-I-restricted	
Melanoma-melanocyte differentiation antigens	Melan-A (MART-1), gp100, tyrosinase, tyrosinase related protein-1 (TRP-1), TRP-2, melanocyte-stimulating hormone receptor
Cancer-testis antigens	MAGE family (1-3, & 12), BAGE, GAGE, NY-ESO-1
Mutated antigens	β-catenin, MUM-1, CDK-4, caspase-8, KIA 0205, HLA-A2-R1701
Non-mutated over-expressed shared antigens	α-fetoprotein, telomerase catalytic protein, G-250, MUC-1, carcinoembryonic antigen, p53, Her-2/neu
Class-II-restricted	
Non-mutated	gp100, MAGE-1, -3, tyrosinase, NY-ESO-1
Mutated	CDC-27, LDLR-FUT, triosephosphate isomerase

Class-I-restricted tumour antigen epitopes (TAEs) may be identified either by utilising T-cells isolated from endogenous or induced anti-tumour responses to ascertain the nature of the antigens presented or, alternatively, by using 'reverse immunology' techniques, *i.e.*, predicting epitopes from predefined tumour antigens based on consensus class-I binding motifs [357]. The former approach is usually achieved by transfecting a cDNA library derived from the tumour, which is sub-divided into cDNA pools, into APCs expressing the appropriate class-I haplotype and, subsequently, screening these pools for reactivity using isolated tumour-reactive CTL or hybridomas. The majority of class-I-restricted TAEs have been identified this way including the first, MAGE-1 [358]. Acid elution of peptides from tumour cells has also been successful, but, due to the relative insensitivity of this technique, is likely to be less useful when limited material is available [357]. The alternative approach involving epitope prediction may be utilised in conjunction with

proteasomal cleavage site prediction in order to generate candidate peptides from TAAs [359, 360]; synthesised peptides may then be used to immunise transgenic mice *in vivo* [361], or to stimulate T-cells *in vitro* that have been harvested from healthy donors or tumour patients [362] in order to ascertain the cytotoxicity of the generated CTL towards the original tumour. This technique has identified potential epitopes from pre-defined TAAs such as MAGE-3 [363], p53 [364], mucin-1 [362] and, notably, the catalytic subunit of human reverse transcriptase telomerase (hTERT) [365]. hTERT may be of great clinical relevance as it is expressed in greater than 85 % of tumours [366], is silent in a lot of tissues, and is associated with oncogenic transformation [367]. Furthermore, hTERT-transfected DCs are able to elicit anti-tumour CTL that display cytotoxicity against murine melanoma and thymoma cells of the same and different MHC backgrounds [359, 368-370].

Reverse immunology therefore relies on the identification of candidate antigens prior to epitope prediction. Antigens that are over-expressed in neoplastic tissue may be selected as candidate TAAs by differential gene expression analysis such as microarrays, RT-PCR, subtractive hybridisation and serial analysis of gene expression (SAGE) [359]. These techniques are further complemented by proteomic analysis of the tumour tissue, or SEREX (serological analysis of recombinant cDNA expression libraries) which utilises patient serum to probe cDNA libraries constructed from the tumour – TRP-1 and NY-ESO-1 were both identified using this technique [371, 372]. These techniques have been used in combination to identify new candidate TAAs [373]. However, it should be noted that mRNA and protein expression may not be suitable criteria for identifying all candidate TAAs as, for example, p53-specific CTL may be generated in tumour-bearing or healthy individuals, but cytotoxicity is restricted to neoplastic tissue even though p53 is uniformly expressed [374].

It is becoming increasingly evident that effective vaccination protocols are likely to have to include T-helper epitopes [375]. These epitopes have recently been identified using a genetic targeting expression (GTE) system. This approach uses a vector containing the Ii targeting sequence up-stream of the multi-cloning site to target tumour-derived cDNA to

class-II vesicles. In this way, transfection of vector-bound tumour cDNA into HLA-DM/DR$^+$ APCs allows the screening for class-II-restricted epitopes as for class-I epitopes [375]. This technique has been used to identify the class-II-restricted epitopes listed in table 4, which includes epitopes within previously identified TAAs that also contain class-I-restricted epitopes (*e.g.*, gp100), as well as previously unidentified TAAs. Interestingly, a mutation in CDC-27 (a component of the anaphase-promoting complex that regulates cell cycle transition) generates a class-II epitope but this is not encoded within the mutated region but, rather, is generated because the mutation allows localisation to the cytoplasm rather than to the nucleus, *i.e.*, mutated CDC-27 may access the class-II pathway [376].

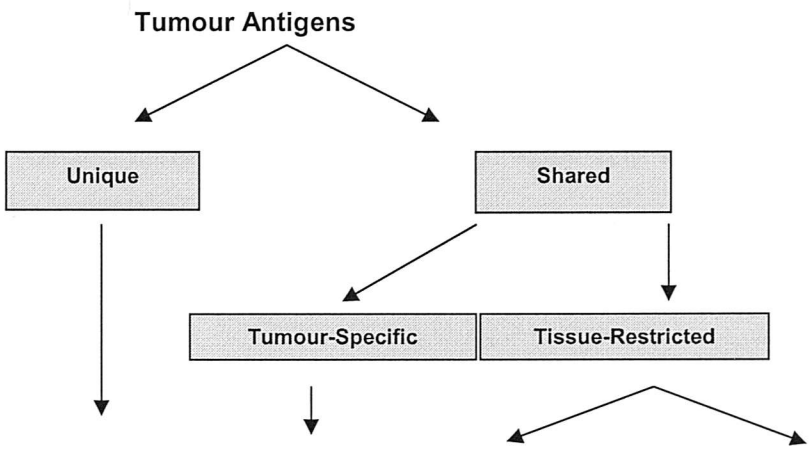
What is the evidence that expanded TAA-specific T-cells exist in cancer patients? The majority of this data stems from melanoma patients. Initial studies using fluorescenated pMHC tetrameric complexes demonstrated that both MART-1_{26-35} and tyrosinase$_{368-376}$-specific CD8$^+$ T-cells are detectable in peripheral blood of untreated melanoma patients [351, 352]. Importantly, tyrosinase-specific CD8$^+$ T-cells had a perforin$^+$/CD45RA$^+$/57$^+$/28$^{+/-}$ phenotype, which is known to correlate with cytotoxic function [253], but were unable to display ex-vivo cytotoxicity directly or after a 48 hour exposure to recombinant IL-2 [352]. Furthermore, tyrosinase-specific T-cells from this patient were unable to produce IFNγ or TNFα after *in vitro* stimulation with PMA/ionomycin, whereas CMV-specific T-cells from the same patient had intact responses [352]. Together, these studies suggested that even though TAA-specific T-cells may be detected in melanoma patients they are, most likely, anergic. Moreover, peptide vaccination of patients with MART-1_{26-35} in adjuvant (Montamide) predominantly results in the transition of MART-1-specific T-cells towards a memory phenotype (CD45RA$^-$, CCR7$^-$, CD28/27$^+$) without the generation of CD28/27$^-$ effector cells [377]. Other functional deficits have been observed using different antigens, *e.g.*, CD27$^-$ CTL elicited by gp100 vaccination largely lack perforin [378].

Recently, however, peripheral blood mononuclear cells (PBMCs) isolated from melanoma patients have been shown to produce IFNγ in response to autologous tumour in four out of seven patients tested [379]. One of these reactivities was shown to be directed against a

HLA-A2-restricted tyrosinase epitope and, indeed, CTL isolated using tetramers containing the relevant tyrosinase peptide had a $CD45RA^{+}$ $CCR7^{-}$ phenotype and displayed direct ex-vivo cytotoxicity. $IFN\gamma^{+}$ PBMCs from the other three tumour-reactive individuals displayed the same surface phenotype. Furthermore, Vβ gene expression analysis of tyrosinase-specific CTL revealed restricted usage, suggesting that these cells represent a single clonal population. Thus, even though T-cell anergy may occur [352], this event may not be the norm, at least in melanoma patients.

The lack of spontaneous regression in the above study suggests a degree of functional incompetence within the T-cell compartment; thus, either these CTL are unable to migrate to the tumour site or the tumour may have elicited some escape mechanism(s). Indeed, tyrosinase-reactive CTL did not express the skin-homing receptor CLA that is associated with the development of vitiligo [380]. Also, the previously identified anergic tyrosinase-specific T-cells lacked CD44 expression, as do naïve cells, suggesting that these cells were defective in extravasation into inflammatory sites [352, 381]. Notably, in the study by Valmori *et al.*, intracellular staining for cytokines could not identify reactivity towards class-II-restricted antigens, and this may have contributed to the blunted effectiveness of these tyrosinase-specific CTL. Furthermore, these responses are only likely to occur in late stage disease where antigen and/or 'danger' is relatively abundant, but when escape mechanisms may be established (these are discussed below) suggesting that such responses are unlikely to result in spontaneous regression. Thus, a TAA may not necessarily act as a tumour rejection antigen (TRA). Strong rejection antigens are likely to be defined by the characteristics of the T-cell pool, *i.e.*, the degree of tolerance already established against a candidate TRA as well as the avidity, diversity and precursor frequency of the cognate T-cells [354]. In this regard, it is likely that both shared and unique 'non-self' TAAs, such as mutations that are either incidental or related to the oncogenic process and viral antigens, will represent the strongest TRAs as these antigens are likely to have escaped tolerance mechanisms and high avidity cognate T-cells will be present in the periphery [354]. Alternatively, over-expressed, cancer-testis and melanoma-melanocyte differentiation antigen may possess cognate T-cells that have escaped

tolerance induction either due to 'ignorance' or by possessing a low avidity TCR; these cells may be elicited in order to effect tumour rejection but are likely to be less potent than high avidity CTL. These premises are summarised below:



	Group 1	Group 2	Group 3	Group 4
	Mutations Incidental to the Oncogenic Process	Mutations related to the Oncogenic Process or Viral Antigens	Highly Restricted	Other
Tolerance	Non	Non	Non-Partial	Partial
T-Cell Affinity	High	High	High-Interm.	Interm.-Low
Tumor Rejection Antigen	Strong	Strong	Strong-Interm.	Interm.-Poor
Example	Myosin gene 1	p53, ras, HPV	Cancer-testis antigens	Differentiation antigens

The groupings in this schematic will be used in the following discussion to refer to this classification of antigens.

It is interesting that the majority of the TAAs identified so far are of groups 3 and 4; this may reflect the fact that CTL clones generated *in vitro*, in order to screen cDNA libraries, may not contain high avidity CTL due to their susceptibility to AICD [382]. In support of this, Zorn and Hercend isolated two CTL clones expanded in situ in a case of spontaneously regressing melanoma using Vβ-specific mAbs - the predominant clone expressed Vβ16, represented 84 % of the $V\beta16^+$ T-cells in the regressing lesion, and was subsequently shown to be reactive against a myosin class I gene product containing a single point mutation [353, 383]. Notably, these CTL were shown to be cytotoxic against autologous, but not allogeneic, melanoma cells directly ex-vivo [353]. The second CTL

clone analysed was less expanded at the tumour site and was a $V\beta13^+$ MAGE-6-reactive clone [384]. Thus, both patient-specific mutated antigens and differentiation antigens may have contributed to tumour regression in this case, but the predominant reactivity was directed against a group 1 or 2 antigen. These findings have far-reaching implications for TRA identification. Firstly, most TAAs identified so far have been found by using T-cells that have been both selected *in vitro* and that have been derived from patients not undergoing regression. Secondly, 'reverse immunology' techniques rely largely on expression data for the identification of new candidate TAAs and are therefore unlikely to identify antigens in groups 1 and 2.

1.5.2. Tumour Escape Mechanisms

The above body of evidence suggests that immunosurveillance of tumours does indeed occur. Hence, in this paradigm, tumours that are able to grow successfully must be viewed as having escaped immunological detection via the development of clone phenotypes that have been dictated by the immune system, *i.e.*, immunoeditting [385]. The development of tumour varients that are able to grow within an immunocompetent host is likely to occur due a passive process of clonal selection within a heterogenous population, which itself has been produced due to the inherent genomic instability of the neoplastic cells. Conceivably, the outcome of this process is determined not only by the presence of a functional immune system, but also by other factors within the tumour environment, *e.g.*, growth factor and nutrient availability [386].

During initial growth, a population of neoplastic cells is likely to be immunologically indistinct from normal tissue in that these cells do not produce or elicit 'danger' signals; this characteristic may be viewed as the primary reason as to why tumours do not elicit an immunological response early in their development [387]. Nonetheless, it seems likely that macroscopic invasive tumours, during the process of angiogenesis and the disruption of surrounding normal tissue, elicit stress signals that are able to activate local innate cells such as DCs, *e.g.*, HSPs, necrotic cell death within solid tumour masses [386]. A number

of mechanisms may be utilised by invasive tumoour in order to invade destruction mediated by immune recognition - several lines of evidence are discussed below.

Loss of MHC expression, whether complete or allele-specific, is common in many tumours such as melanoma, colorectal carcinoma and breast carcinoma, where the frequency of MHC loss exceeds 50%; multiple mechanisms account for this, including proteosome component defects, defective TAP, point mutations in the MHC heavy chain, and loss of $\beta_2$microglobulin [388]. Additionally, decreased expression of specific antigens has been documented in correlation with both peptide vaccination and disease progression, *e.g.*, loss of gp100, tyrosinase and MART-1 during melanoma progression [389]. Thus, as MHC loss often correlates with invasiveness, and may be observed in both human and murine tumours that have recurred following successful immunotherapy, this phenomenon is assummed to aid tumour progression by preventing CTL-mediated lysis [390-392]. Nonetheless, MHC loss is likely to render tumours susceptible to NK-mediated lysis, so why do MHC-negative tumours survive in the presence of NK cells? Several possible explanations exist – down-regulation of MICA/B, lack of co-stimulation or pro-inflammatory cytokines, and/or production of immunosupressive cytokines – but these have yet to be directly linked to the suppression of NK cells by tumours [386].

Another possible mechanism that may contribute to the survival of tumours within an immunocompetent host is defective death receptor signaling. Loss of function in the Fas pathway, due to either down-modulation of Fas-mediated signaling (*e.g.*, missense mutations within the Fas gene in NHL [393]) or enhanced inhibition of this pathway (*e.g.*, elevated expression of cFLIP [394]), is likely to protect tumours against CTL-mediated lysis. In a similar fashion, the expression of SPI-6, which directly inhibits granzyme B, correlates with tumour survival, presumably as this inhibitor prevents the effectiveness of granule-mediated killing, *c.f.*, MTOC dysfunction with TILs [395]. Furthermore, apoptosis mediated via the TRAIL pathway may be suppressed as many tumours express relatively low quantities of this receptor due to, for example, dysfunction of posttranslational modification [396].

Conversely, a number of tumours, including lung, hepatocellular, and colon carcinomas, have been reported to express functional FasL which induces apoptosis of interacting Fas^+ cells; however, these observations are widely disputed, with some investigators reporting that FasL expression can not be detected when the appropriate controls are used. Furthermore, transfection of FasL into cell lines promotes a proinflammatory response associated with neutrophil infilitration [426-429]. Nonetheless, the expression of FasL by tumour cells may potentially promote activated T-cell apoptosis if this receptor were to be shed, *c.f.*, suicidal/fratricidal killing.

In addition to the evading of CTL recognition and apoptosis induction, tumours are able to induce functional incompetence at one or multiple stages during CTL development, thereby preventing tumour regression. Indeed, this is true even in experimental situations where a xenogenic model antigen is used [397-399]. These deficiencies are induced by mechanisms ranging from APC functional interference to blocking of granule-mediated cytotoxicity. For example, APC function may be impaired by tumour-derived factors such as IL-10, TGFβ and vascular endothelial growth factor [400-402] resulting in defective antigen presentation [403-405], lack of responsiveness to maturation or migratory signals [406], inability to up-regulate co-stimulatory molecules [395], and premature APC death [406].

Additionally, active suppression of anti-tumour responses may occur and is mediated by, for example, $CD4^+$ Tr and NKT cells (figure 1.5). Thus, experimental depletion of $CD4^+25^+$ Tr cells results in spontaneous CTL and NK cell-mediated tumour-specific cytotoxicity [408, 409] and, moreover, neutralisation of IL-10 in early B16 melanoma lesions abolishes continued tumour growth [410]. Similarly, both NKT [411] and γδ T-cell [412] depletion has been shown to facilitate CTL-mediated tumour rejection. In the case of NKT cells, this effect is mediated by IL-13 [411].

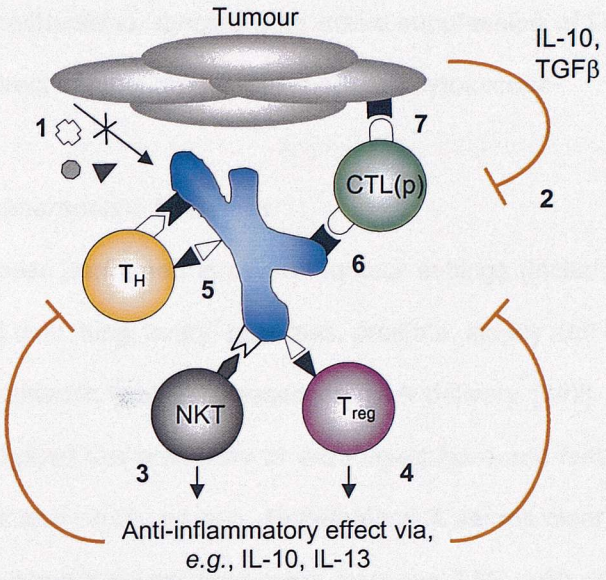


Figure 1.5. Active and Passive Mechanisms that Prevent Spontaneous Tumour Rejection

Several barriers exist that may hinder the elicitation of an effective anti-tumour T-cell response. Insufficient 'danger' signals derived from or induced by the tumour may result in ineffectual DC maturation, 1. This effect may be supplemented by active suppression of an appropriate T-cell response by the production of, for example, IL-10 and TGFβ, 2. Immunoregulatory cells such as CD1-binding NKT cells, γδ T-cells (not shown) or Tr cells may exert anti-inflammatory effects via cytokine production or cell-cell contact (not shown), 3 and 4. Absence of TAEs, weak MHC binding by TAEs or low avidity of cognate T-cells may result in blunted T-cell priming or differentiation, 5 and 6. Finally, mechanisms that directly prevent granule-mediated or FasL-mediated apoptosis protect tumour cells from CTL-mediated lysis, 6.

The development of tumour escape mechanisms following T-cell priming is exemplified by the abortive development of tumour-reactive T-cells in susceptible strains - SV-40/MUC-1 double transgenic mice, that express MUC-1 systemically and oncogenic SV40 T antigen in the pancreas under the control of an elastase promoter, develop pancreatic tumours and a concomitant MUC-1-specific ineffectual T-cell response; however, adoptive transfer of the generated MUC-1-reactive T-cell protects against tumour development in naïve transgenic mice [413], suggesting that escape mechanisms develop late in tumour growth and that prophylactic vaccination against cancer may be more effective than 'therapeutic vaccination' [414]. Indeed, if a CTL response is primed against the tumour, as detailed above, several mechanisms have been defined that may allow tumour escape.

Together, tumours remain largely unaffected by the immune system due to a wide spectrum of escape mechanisms, ranging from active suppression of DC maturation and CTL development to direct hampering of T-cell-mediated cytotoxicity.

1.5.3. Current Immunotherapeutic Strategies

Clinical studies have been performed in several tumour settings (including cancers of the breast, gastrointestinal duct, lung, ovary, pancreas, prostate, kidney and medullary thyroid) using a variety of vaccination protocols based on TAA delivery [342]. The variation in protocols used has negated the possibility of drawing-up hard-and-fast rules about what strategies are effective and which are not. Nonetheless, it seems clear that a successful vaccination is likely to have to incorporate more than one TAE, with differing restrictions being represented, in order to prevent antigen loss variants, and T-helper epitopes will have to be included. Indeed, despite the fact that most tumours do not express MHC class-II, T-help may be elicited via DCs and, thus, aid the generation and maintenance of anti-tumour CTL [375, 419].

Evidence in support of the clinical relevance of active immunotherapy comes from the fact that some conventional therapies are now known to elicit TAA-specific CTL and that patient T-cells may be re-activated *in vitro* and subsequently used to treat autologous tumours, *e.g.*, IFNα or allogeneic bone-marrow transplantation used to treat chronic mylogenous leukaemia elicits proteinase 3-specific CTL which are cytotoxic ex-vivo and which correlate with a positive clinical response [420]. Recently, Dudley *et al.* used a combination of non-myeloablative chemotherapy, high-dose IL-2 therapy and adoptive transfer of *ex-vivo*-expanded TILs to treat metastatic melanoma patients – 6 out of 13 patients showed objective clinical responses, with 4 others displaying mixed responses [421]. Vitiligo was observed in 4 cases and uveitis in one. The purpose of non-myeloablative chemotherapy in this study was presumably to abrogate pre-existing tolerance mechanisms.

Thus, several experimental approaches are now being explored to further advance the field of active immunotherapy. These approaches focus on elevating the growth and

differentiation of DCs, *in vivo* and *in vitro* loading of DCs with TAAs, and enhancing T-cell activation by increasing pMHC-TCR interaction or by eliciting co-stimulation [342, 422].

Increasing the growth and differentiation of DCs from progenitor cells has mainly been successful by the use of GM-CSF [423], although FLT3L also shows some promise [424]. GM-CSF co-transfected into the murine colon carcinoma line CT26 along with CD40L promoted the interaction of transplanted tumours with DCs and elicited regression in some cases [425]. Clinical trials of GM-CSF-expressing tumour vaccines are now underway for renal-cell cancer, melanoma and pancreatic cancer [426-428]. GM-CSF-TAA fusion proteins have also been used to target lymphoma idiotype to DCs *in vivo* [429] and, more recently, both DEC-205- and HSP70-containing conjugates have been used for the same purpose [430].

Naked DNA, recombinant replication-deficient viruses and bacteria (with and without MHC targeting sequences) have also been used to deliver TAA to DCs (see summary in [342]). Recombinant vaccinia virus encoding murine TRP-1, for example, elicits protective immunity against melanoma in both a prophylactic and 'therapeutic' setting [431, 432]. Furthermore, Xiang *et al.* used an attenuated strain of *Salmonella typhimurium* that contained a plasmid encoding ubiquitin fused to gp100 and TRP-2 epitopes to elicit protective immunity against melanoma [433].

Of particular relevance is the deliver of TAAs as naked DNA contained within a plasmid juxtaposed by a viral promter/enhancer and polyA sequence; this approach has been used to vaccinate against a number of TAAs such a mutated 53, HPV, and tumour-specific idiotypic determinants. Typically, sequences encoding idiotypic determinants derived from the amplification of V_H and V_L sequences obtained from tumour biopsies are expressed as a chimeric single-chain Fv Ig molecule fused to an immunostimulatory sequence, such as the non-toxic fragment of tetanus toxin. This latter feature, in combination with the inherent CpG motifs contained within the plasmid backbone, provides an innate danger signal and cognate help for the induction of an anti-idiotypic response. Indeed, prophylactic

intramuscular DNA vaccination of animals is able to protect recipients against surface-idiotype positive lymphomas and surface-idiotype negative myelomas via the production of anti-idiotypic antibodies and induction of idiotype-specific $CD4^+$ T-cells respectively. Furthermore, phase I/II clinical trials are underway in order to assess the efficacy of DNA vaccination against follicular centre lymphoma [for a review see 433a].

In vitro loading of DCs obtained from G-CSF-mobilised $CD34^+$ peripheral blood precursors or monocytes may be used to evoke anti-tumour responses [434]. Antigen loading is achieved by pulsing DCs with peptides, proteins, tumour lysates, apoptotic corpses or tumour-derived exosomes [32, 435, 436]. Furthermore, viral-, electroporation- or liposome-mediate delivery may be used to load DCs with the appropriate DNA or RNA [368, 437-439]. Alternatively, DC-tumour cell fusion vaccines may prove to be, in the future, clinically useful [440, 441]. Monocyte-derived peptide-pulsed mature DCs have been reported to induce clinical remission in melanoma and renal cancer patients [442, 443], but by-and-large success has been limited [444]. Possible limitations within this approach include the induction of autoimmunity and the appropriate control of DC maturation status – overly mature DC may not migrate efficiently to T-cell zones, whereas immature DCs may not prime T-cells adequately.

An alternative approach may be to enhance DC-T-cell interaction directly. In this regard, as discussed, a large number of the TAAs identified thus far only contain weak epitopes, and this is not only because high affinity cognate T-cells are absent but also because these peptides have low affinity for MHC [339]. Several groups have now demonstrated that altering the MHC anchor residues within candidate rejection epitopes may increase the binding affinity of the epitope towards MHC and, thus, T-cells may be induced that are reactive against both the altered and the original epitope. Such heteroclitic responses were shown to be effective in eradicating experimental tumours [445, 446]. Furthermore, by altering residues that interact with the TCR, similar responses may be achieved without altering MHC binding, *e.g.*, Slansky *et al.* demonstrated that by altering a TCR-interacting

residue within a peptide derived from murine leukemia virus gp70, anti-tumour immunity may be raised against CT26 which expresses this epitope [447].

A final approach that may be used in order to elicit anti-tumour CTL is elevating positive co-stimulation. This approach often takes the form of transducing B7 molecules into tumour cells [448, 449] or including B7 in DNA vaccines [450, 451]. As our knowledge of co-stimulatory ligand pairs increases, so the number of possible targets will increase. Indeed, both 4-1BB and OX40-specific mAbs are now known to exert therapeutic activity in experimental systems [4, 452].

Ultimately, ideal vaccines should be off-the-shelf reagents that allow low-cost manufacture in large scale. Despite the current lack of correlation between CTL response and clinical regression observed, the above advances might help in achieving this goal.

1.6. The Current Study

As detailed in section 1.1.3.2, CD40L has a prominent role in the licensing of DCs to prime CTL precursors, and agonistic mAbs directed against CD40 have been used in a variety of experimental settings in order to replace endogenous CD40L, thereby overcoming T-cell tolerance and affecting tumour rejection.

Until recently, these observations were being translated into the clinic using human recombinant CD40L (manufactured by Immunex, USA) in phase one and two trials [453]. Nonetheless, recombinant proteins are often rapidly eliminated from the circulation and, indeed, various recombinant CD40L constructs are experimentally less effective than anti-CD40 mAb at eradicating established lymphoma, presumably for this reason (L Haswell, unpublished observations). If successful, however, reagents that replace the requirement for endogenous CD40L may form the basis of off-the-shelf therapeutic vaccines that do not require patient HLA typing or knowledge of reactive tumour antigens prior to treatment. For these reasons, we aimed to further characterise the mechanism by which anti-CD40 elicits its therapeutic activity in two murine models of B-cell lymphoma based on the

hypothesis that this mAb either elicits a de novo, or boosts/accelerates a pre-existing, anti-tumour CTL response by enhancing the maturation of professional APCs such as DCs. This was achieved by examining DC and T-cell responses following anti-CD40, as well as the endogenous responses to these tumours. Additionally, we hypothesised that anti-CD40 may act, in part, by providing co-stimulatory signals via 4-1BB to anti-tumour CTL, thereby promoting the expansion and/or survival of these cells. This premise was based on the observation that anti-4-1BB mAb is also therapeutic in the the two tumour models used in this study [5], as well as the fact that stimulation of DCs via CD40 enhances the expression of 4-1BBL on DCs [108].

Furthermore, we aimed to establish tumour-reactive T-cell lines from animals in remission following anti-CD40 therapy, a method that previously had not successfully been implemented in this laboratory. If successful, our subsequent objective was to raise T-cell hybridomas from these animals in order to screen a lymphoma-derived cDNA library for the TRA(s) mediating tumour elimination following anti-CD40 administration. In this case, TRA identification may act to further justify clinical trials of anti-CD40 mAb, *i.e.*, if a bone fide tumour rejection antigen is identified that is relevant to human neoplasia.

2.1. Mice and Tumour Passáge

BALB/c, C57BL/6 and CBA/H mice were supplied by Harlan (Blackthorn, Oxon) and bred in a local facility. All animals used for experimentation were both sex- and age-matched (between 8 and 10 weeks of age).

The tumour models predominantly used in this study were the B-cell lymphomas A31 and BCL1 (of CBA/H and BALB/c origin respectively), as well as the T-cell lymphoma TEN-1 (BALB/c origin). A31 and BCL1 are lymphobalstic lymphomas, analogous to human chronic lymphocytic leukeamia, which both display a terminal leukaemia [454, 455]. Whereas BCL1 arose spontaneously, A31 is likely to have been induced by irradiation as this tumour was originally isolated from animals 19 months following injection of ^{90}Sr; for this reason, the BCL1 model is utilised in the majority of experiments, *i.e.*, as we believe BCL1 provides a more accurate model of naturally-occurring human disease than A31.

BCL1 and A31 lymphomas lines were maintained by *in vivo* intraperitoneal (i.p.) passáge in BALB/c and CBA mice respectively. Spleens were removed at the terminal stage of disease and single cell suspensions made as described below. piBCL1 is a subline of BCL1 that grows in culture [456]. A20, EL4 and YAC cell lines (all American Type Culture Collection) were maintained in complete media (CM; see below). X63-mIL-2 transfectant was a kind gift from Dr. Karasuyama. This cell line was routinely grown in CM supplemented with 1 mg/ml G429 (Sigma Aldrich) and was transferred to G429-free CM 24 hours prior to harvesting the supernatant; the concentration of IL-2 was subsequently estimated by ELISA (see section 2.9). The BWZ.36 CD8α fusion partner [457, 458] was a kind gift from Dr. David Woodland, and was routinely grown in ICTM containing 1 mg/ml G429 and 0.36 mg/ml hygromycin B (Sigma) (see section 2.8).

2.2. Cell Preparation, Quantitation and Culture

2.2.1. Culture Materials and Cell Quantitation

All cell lines were cultured in RPMI 1640 medium (Invitrogen), supplemented with 100 U/ml penicillin (Glaxo), 100 µg/ml streptomycin (Evans), 50 U/ml amphotericin B (Fungizone; Squibb and Sons), 2 mM L-glutamine (Invitrogen), 1 mM pyruvate (Invitrogen), 50 µM 2-mercapto-ethanol (2-ME; Sigma) and 10% Myoclone Plus Foetal Calf Serum (FCS) (Invitrogen) (CM). Cell concentrations were determined using a Coulter Industrial D Cell counter (Beckman Coulter)

2.2.2. Splenocyte Preparation, Collagenase Digestion, and DC Isolation

Single cell suspensions were routinely prepared by sieving whole spleens in 20 ml of phosphate buffered saline (PBS; 1.37 mM NaCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4) through a cell mesh (Becton Dickinson (BD) Falcon). For tumour passage, splenocyte suspensions were layered onto Lymphoprep (Apogent Discoveries Ltd.), subjected to centrifugation for 20 minutes at 2500 rpm, the interface collected, and, subsequently, $5x10^7$ cells injected i.p.

For DC isolation, whole spleens were coarsely chopped with a razor, placed in 2.5 ml of digestion mix (1 mg/ml collagenase D (Roche) and 0.05 mg/ml DNaseI (Sigma) freshly dissolved in CM) and gently agitated for 20 minutes at 37 °C. Subsequently, another 2.5 ml of digestion mix was added and the incubation continued for a further 20 minutes. The digestion was slowed by the addition of 20 ml of CM, a single cell suspension prepared by sieving through a cell nesh and the cells washed once in CM.

2.3. Antibodies

The majority of antibodies used were prepared and fluorescenated in house. Hybridoma cells were maintained in stationary culture using DMEM supplemented with 5 % FCS, supernatant protein precipitated using saturated ammonium sulphate, and mAb subsequently separated by protein A or G fractionation (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Antibodies directed against CD4, CD8, BCL1 idiotype and MHC class II were prepared by ion-excahnge chromatography on DEAE (Whatman). The purity of mAb preparations was checked by electrophoresis (Beckman

EP system; Beckman Coulter) and HPLC using a Zorbax GF250 Bio series column (Jones Chromatography).

Table 5. Monoclonal Antibody Providers

Specificity	Clone Name	Provider
B7-1, B7-2, MHC Class II, LFA1, CD11c, FcRII/III, ICAM-1, CTLA-4, IFNγ, 4-1BB	1610A1, GL-1, M5/114, TIB237, N429, 2.4G2, YN1.4.7, UC10-4F10-11, HB170, 2A1	American Type Culture Collection
CD4, CD8	YTA 3.1.2, YTS169	S. Cobbold, Sir William Dune Schoold of Pathology, Oxford
CD40	3/23	G. Klaus, National Institute of Medical Research, London
CD19	1D3	D. Fearson, University of Cambridge
Kd, Dd	K9-18, 19-191	D. Kioussis, National Institute of Medical Research, London
4-1BBL	3H3	R. Mittler
BCL1 idiotype, A31 idiotype, 41BB	MC106A5, MC39-16, LOB12	Tenovus
5-Bromo-2-deoxyuridine, IFNγ, CD8α, CD62L, CD49d	3D4, XMG1.2, 53-6.7, MEL-14, R1-2	BD Pharmingen
CD11b	M1/70.15	Serotec
ASGM1	?	Wako

In all the experiments contained within this thesis the anti-CD40 monoclonal antibody used was 3/23 rather than, for example, FGK-45; this does not reflect a functional difference, as these two antibodies perform with equal effectiveness against the model lymphomas used (Tutt, A., unpublished observations), but rather the convenience of production. For fluorescentation protocols see [2].

2.4. Measurement of Surface and Intracellular Antigens by Immunofluorescence

2.4.1. Surface Antigens - General Protocol

Each, sample containing $<10^6$ cells, was incubated at 4°C for 15 minutes with 10 µg/ml of labelled (direct; fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerthyrin (PE)) or unlabelled antibody (indirect). Cells were then washed once (direct) or twice (indirect) in PBS-1% Bovine Serum Albumin fraction V (BSA; Wilfred Smith Ltd)- 20mM sodium azide (PBS-BSA-Azide) and re-suspended at approximately $1x10^7$/ml. For indirect immunofluorescence, cells were further incubated for 30 minutes at 4°C with 10 µg/ml of a fluorescenated secondary antibody directed against the first antibody, and washed once in PBS-BSA-Azide before resuspension and subsequent analysis. If required, 7-

aminoactinomycin-D (7AAD, Sigma) at a final concentration of 2 µg/ml was added to the sample to be examined 15 minutes before acquisition. Similarly to propridium iodide, 7AAD is a reagent that flouresceses once intercalated into the DNA helix but, unlike propridium iodide, 7ADD allows the simulatenous resolution of PE-conjugated reagents.

Analysis was performed on a FACScan or FACSCalibur flow cytometer (BD Pharmingen). Unless otherwise stated, 10,000 total events were collected per sample for analysis. Cell debris was excluded by adjustment of the forward scatter (FSC) threshold parameter. Samples were analysed using either CellQuest software (Becton Dickinson) or WinMDI.

2.4.2. Intracellular Antigens

2 x10^6 Splenocytes or *in vitro* generated CTL were stimulated as described in experiments or, as a positive control, with 50 ng/ml phorbol 12-myristidate 13-acetate (PMA) and 1 µM ionomycin (Sigma). All stimulations and cell manipulations prior to fixation were performed in the presence of 10 µg/ml brefeldin A (Sigma). After the desired time (no greater than 18 hours), cells were harvested, placed in a 96-well U-bottomed plate and stained with antibodies to surface antigens as described above. This primary staining was performed in the presence of 10 µg/ml unlabelled anti-FcRII/III (2.4G2). Samples were subsequently washed twice in 200 µl of PBS and fixed for 20 minutes in 200 µl 1 % formaldehyde at room temperature. After fixation, samples were washed twice in 200 µl of PBS/0.5% BSA and re-suspended in PBS/BSA/0.5 % saponin (Sigma). After 10 minutes at room temperature, 0.25 µg of PE-labelled anti-IFNγ or irrelevant antibody in 100 µl of PBS/BSA/saponin was added to the required wells, mixed, and incubated for 30 minutes at 4 °C. After this time, samples were washed into PBS/BSA and analysed by FACS.

2.4.3. In Vivo 5-Bromo-2-deoxyuridine Administration and Ex-vivo Detection

Animals received 2 mg of BrdU (dissolved in water; Sigma) i.p. 1 hour before harvesting the required tissue. Single cell suspensions were prepared and 1x10^6 cells per sample stained for extracellular antigens, followed by fixation overnight in 1 ml of 1 % formaldehyde/PBS. Splenocytes were subsequently washed once in PBS and then

resuspended in 0.5 ml of DNase buffer (5 mM $MgCl_2$, 5mM $CaCl_2$ in PBS) containing 50 Kunitz units of DNase I (Sigma) per sample. After 1 hour at 37 °C, the samples were washed in PBS and then into 0.2 % Tween-20 (PBS). Each sample was then re-suspended in a residual volume (approximately 100 µl) and 20 µl of PE-labelled anti-BrdU or irrelevant PE-labelled antibody (provided in kit from BD Pharmingen) added for 45 minutes.

2.5. Carboxy Fluorescein Succinimidyl Ester (CFSE) labelling

Cells to be labelled were washed once in serum free media and re-suspended at $2x10^7$ /ml in the same media. CFSE was added to a final concentration of 2 µM and the cells then incubated at 37 °C for 10 minutes. The labelled cells were then washed twice in cold (4 °C) CM before re-suspension in warm CM (37 °C) at the required density.

2.6. Magnetically Assisted Cell Separation- MACS

Each sample was separated by MACS (Miltenyi Biotech) as per the manufacturers instructions – briefly, the desired number of cells was labelled with PE- or FITC-conugated mAbs as for FACS analysis and washed twice in CM at 37 °C before being re-suspended in 90 µl of CM per 10^7 cells. Subsequently, 10 µl of anti-FITC microbeads was added per 10^7 cells and the mixture incubated at 4 °C for 15 minutes. Subsequently, the bead-coated cells were washed in PBS/0.5 % BSA/2 mM EDTA and LS columns (Miltenyi Biotec; capacity to positively select 10^8 cells) pre-equilibrated on the MACS magnet (Miltenyl Biotech) with 3 ml of the same buffer. After pelleting, cells were re-suspended in 5 ml of PBS/BSA/EDTA and passed through LS columns that were subsequently washed three times with 3 ml of PBS/BSA/EDTA. The columns were then removed from the magnet, and positive fractions collected by adding 5 ml of PBS/BSA/EDTA to the columns and, subsequently, pushing the solution through using the column plunger provided.

2.7.

cDNA Preparation and Reverse Transcript-Polymerase Chain Reaction (RT-PCR)

cDNA was prepared from $1x10^6$-$1x10^7$ cells using Quickprep micro mRNA purification kit and first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) as per manufacture's instructions. Unless otherwise stated, a standard amplification involved 1 µl of each primer (100 ng/ml) being added to 1 µl of cDNA with 2.5 µl of reaction buffer (Promega) and 0.5 µl of dNTP mix (10 mM each dNTP/100 µl) in a total volume of 24.5 µl. This mixture was then pulse centrifuged and 0.5 µl of Taq polymerase (5 U/µl) added. DNA was then denatured at 94 °C for 5 minutes and PCR performed using annealing reactions of 55-62 °C for 1 minute (see table 5), an elongation temperature of 72°C for 2 minutes, and melting reaction of 94 °C for 30 seconds. Routinely, 25-30 cycles were performed in total, with a final elongation reaction of 10 minutes to ensure formation of full-length transcripts and the presence of polyA tails. All PCR reactions were carried out using a PTC-100 thermal cycler (MJ Technologies Inc.). PCR products were then loaded into 0.5-1% agarose gels (re-constituted with running buffer – 40 mM Tris-acetate, 1 mM EDTA, 0.6 mg/l ethidium bromide) and separated at 100 V, along with known standards to allow estimation of fragment size. DNA was visualised under UV light and a photographic record taken.

Table 6. RT-PCR Primer Details

Transcript Product	Annealing Temperature, °C	Product Size, base pairs (bp)	Forward; Reverse Primer (5'-3')
β-Actin	55.0	340	TGGAATCCTGTGGCATCCAT; TAAAACGCAGCTCAGTAACA
IFNγ	56.0	488	GGCCATGGTCTGAGACAATGAACG; CCTCTAGAGAATCAGCAGCGACTC
T-bet	55.0	136	GCCAGGGAACCGCTTATATG; GACGATCATCTGGGTCACATTGT
Perforin	56.6	597	AARGCYTGTGAGGAGAAGAAGA; CCCCTGTGGMCAGGAGCACATY*
IL-10	54.0	414	GACCAGCTGGACAACATACTG; TCCTGGAGTCCAGCAGACTCA
IL-12 receptor β1			CAAGCACAGGAACCACACA; CAGAGACGCGAAAATGATG
IL-12 receptor β2			AATTCAGTACCGACGCTCTCA; ATCAGGGGCTCAGGCTCTTCA
Mouse mammary tumour virus (MMTV) *env*	57.5	545	GCCGCCGCGAGATGAGA; GGCCCCTGAGTTCCCCAAAGTAT
Murine leukaemia virus (MLV) gp70	61.1	450	CTCCCCCGCCTTCTCAACAACC; GGCCAGAGCTCAACCAGGACACA

* r= purine; y = pyrlmidine

2.8. Chromium Release Assay

Target cells were washed twice in supplement-free media and labelled with 100 µl $Na_2{}^{51}CrO_4$ (Amersham) in for 60 minutes at 37°C. Excess chromium was then removed by washing 3 times with media (supplement free) and the cells resuspended in CM at $1x10^5$/ml following counting using a conventional haemocytometer. 50 µl of labelled cells was then added to 100 µl of effector cells and 50 µl of either media or blocking antibody (final concentration 50 µg/ml) in 96-well U-bottom plates. Plates were then centrifuged at 700 rpm for 3 minutes and incubated at 37 °C for 4 hours. Cells were sedimented at 1500 rpm for 5 minutes and 100 µl aliquots of supernatant removed for counting on a gamma scintillation counter (Rackgamma II, LKB). A 50 µl sample of the labelled cells was treated with 150 µl of 1% Nonidet P40 (NP40; BDH) to determine maximal release under detergent lysis. Samples were prepared in triplicate, the mean values determined and expressed in terms of percent chromium release (taking NP-40 lysis as 100%) using the equation:

Chromium Release, % = Sample - Background (cpm) / NP40 - Background (cpm) x 100

Background was determined as the amount of spontaneous chromium release from target cells alone.

2.9. T-cell Lines and Hybrids

2.9.1. Generation of anti-Tumour CTL In Vitro

CTL lines were grown from anti-CD40-treated animals 20-60 days after initial tumour challenge, i.e., 1-5x10^7 tumour cells i.v., followed four days later by 1 mg of 3/23 i.v.. Splenocytes were harvested into CM containing 10 % batch-tested (see below) FCS, and 2x10^6 cells seeded per well in 24-well plates. During the first week, cultures were supplemented with 10 ng/ml IL-7 (PeproTech), 10 µg/ml anti-CD4 and 5x10^5 irradiated (25 Grays) *in vitro* tumour line. On subsequent weeks, cultures were harvested, washed in CM and re-seeded at 1x10^6/well with 20 U/ml mIL-2 (X63-mIL-2 transfectant supernatent), 10 µg/ml anti-CD4 and 5x10^5 irradiated tumour. FCS batch testing was performed initially by assessing the proliferation of OT-1 transgenic T-cells and, subsequently, by assessing the proliferation of anti-piBCL1 CTL lines in different batches of serum. A single batch of FCS was used throughout experiments shown in Chapter 6.

2.9.2. Production of anti-piBCL1 T-cell Hybrids

Splenocytes were harvested and seeded into 24-well plates as described above for generation of CTL on the first week of activation, but the media was additionally supplemented with 20 U/ml mIL-2. After 48 hours, 10^7 cells were harvested from this culture, mixed with 10^7 BWZCD8α (which were in exponential growth at the time of fusion), and pelleted by centrifugation at 1000 rpm for 10 minutes. The cells were then washed in Iscove's modified Dulbecco's media (IMDM; Gibco) by centrifugation at 1000 rpm for 10 minutes. Subsequently, all media was aspirated off the cell pellet, the pellet loosened, and the sample placed in a sterile water bath. 1 ml of sterile 50 % polyethylene glycol 1500 (Roche) was then added over 45 seconds, followed by 10 ml of IMDM over a total of 2 minutes; 40 ml of IMDM was then added and the fusion placed in a 37 °C water bath for 5 minutes. Subsequently, the resulting cells were pelleted by centrifugation at 1000 rpm for 10 minutes, the media removed and the pellet washed in IMDM without disrupting the cells. After centrifugation at 1000 rpm for 10 minutes, the pelleted cells were re-suspended very gently in 40 ml of 'Iscove's complete tumour media' (ICTM; IMDM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% Myoclone FCS, 50 µM 2-ME, and 50 ml

'tumour cocktail', *i.e.*, 1.32 ml 45 % dextrose (Sigma), 59.5 ml 50x essential amino acids (Invitrogen), 111 ml 100x non-essential amino acids (Invitrogen), 79.3 ml pyruvate, and 8.5 g sodium bicarbonate per 500 ml of IMDM (pH 7.0)).

The fused cells were plated out in 96-well U-bottom plates at 100 µl per well by dropping from a 10 ml pipette tip. After 24 hours, 50 µl of ICTM containing 3x HAT (Invitrogen), 1 mg/ml G429 (Sigma), and 0.36 mg/ml hygromycin B (Sigma; only used 1x antibiotics at this stage) was added to each culture well and the hybrids checked everyday thereafter for signs of growth. After removing 100 µl of supernatant, 100 µl of fresh ICTM containing 1x HAT and 1x G429/hygromycin B (as detailed above) was added to the cultures every 5 days. Once single colonies were visible, they were transferred to 48-well plates and subsequently into 24-well plates, followed by flasks. During this period, HAT-containing media was slowly replaced with ICTM containing 1x HT and 1x G429/hygromycin B. Once clones were established in flasks, they were screened for reactivity against piBCL1 using a single-cell detection assay as described below. Sub-cloning was performed simply by diluting the hybrid in ICTM containing 1x HT and 1x G429/hygromycin B to a concentration of 1 cell/well in 96 U-bottom plates.

2.9.3. Measurement of Hybrid Activation (adapted from [457, 459])

Following fusion of the BWZ.36 CD8α partner with a T-cell, TCR engagement results in the expression of β-galactosidase which may be detected microscopically at the single cell level, or macroscopically after lysis of the hybridomas, following exposure to the appropriate colorimetric substrate. For the bulk assay following hybridoma lysis, T-cell hybrids were stimulated overnight with piBCL1 as described in experimental detail, then pelleted by centrifugation at 1300 rpm for 3 minutes and washed with 200 µl of PBS. After washing, 50 µl of CPRG substrate (0.15 M Chlorophenol Red β-galactoside (Roche) /0.5 % NP-40/PBS) was added to the separate samples and the reaction allowed to develop for 4-18 hours at 37 ^{o}C. Alternatively, for the single-cell assay, cells were stimulated as described, washed once in PBS, and fixed in cold 2 % formaldehyde/0.2 % glutaraldehyde for 5 minutes. Subsequently, the fixative was removed and the cells washed once in PBS

before adding 50 µl of X-Gal substrate (5 mM potassium ferrocyanide, 5mM potassium ferricyanide, 2 mM magnesium chloride, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Sigma)). The reaction was allowed to develop overnight and the cells examined microscopically the next day.

2.10. Enzyme-Linked Immunsorbant Assay (ELISA)

Primary antibody (*e.g.*, anti-IFNγ, HB170; anti-IL-2, JE56-1A12 (BD Pharmingen)) was diluted in coating buffer (15 mM Na_2C0_3, 28.5 mM $NaHC0_3$, pH 9.6) to a final concentration of 4 µg/ml, and 100 µl/well added to 'absorbant' 96 well plates (Maxisorb, Nunc) either for 1 hour at 37°C or overnight at 4°C. Unbound antibody was removed and non-specific binding sites blocked by the addition of 150 µl blocking solution (1% (w/v) BSA in PBS) for 1 hour at 37°C. The plate was then washed five times with PBS/0.05% Tween-20 and 100 µl/well of the secondary biotinylated antibody (*e.g.*, anti-IFNγ-biotin, XMG1.2, (Serotec); anti-IL-2-biotin, JES56-5H4 (BD Pharmingen)) added at 0.5 µg/ml. All dilutions were made in blocking solution. Following incubation for 90 minutes at 37°C, plates were washed as above.

HRP-Avidin (100 µl/well; BD Pharmingen) was then added to the plates at a final dilution of 1:1000 and incubated for 30 minutes. Following washing (x5), HRP substrate (20 mg o-Phenylenediamine free base (Sigma), 100 ml phospho-buffered citrate pH 5.0 + 20 µl (60% w/v) H_20_2) was added and incubated in the dark for 30 miuntes (or until developed) at room temperature. The reaction was terminated by the addition of 50 µl/well 5 M H_2S0_4 and the colour change quantified by measurement of absorbance at 495 nm on an automatic spectrophotometer (Dynatec 412, Dynatec). Unknowns were determined using standard calibration curves of known concentration on the day of each ELISA.

2.11. Transmission Electron Microscopy

Cells were fixed in the presence of their incubation media by the addition of an equal volume of glutaraldehyde (3% w/v) in 0.1 M sodium cacodylate buffer pH 7.4 for 2 hours at room temperature. Subsequently, samples were processed on-site by the Biomedical

Imaging Unit.

2.12. Purification of 4-1BB-hFc Fusion Protein

CHO-4-1BB-hFc cells were grown in GMEMS containing 25 μM methionone sulfoximine, supernatants collected and run through a 10 ml protein A column. Columns were subsequently washed in approximately 2 litres of 40 μM Tris.HCl/ 2mM EDTA (pH 8.0), prior to eluting the fusion protein using 1 ml 0.1 M glycine/2mM EDTA (pH 3) and protein estimation by ELISA against hFc (see standard protocol above).

2.13. Statistical Analysis

In many of the *in vivo* experiments performed a single animal has been used per day in each treatment group tested, hence negating the possibily of accurate quantitative analysis and restricting the analysis of the data to qualitative changes over time. In the case of figure 3.18, the x^2 test was applied to the therapy data using the programme Peto [459a].

3.1. Introduction.

Agonist mAbs directed against CD40 (*e.g.*, 3/23, FGK-45) have been shown experimentally to be particularly effective in eliciting tumour regression when compared to a range of other mAbs such as anti-CD22, anti-CD19 and anti-Id (figure 3.1., next page) [2]. Anti-CD40-mediated immunotherapy has largely been studied in models of $CD40^+$ lymphomas (*e.g.*, A31, BCL1; see below) [2, 460, 461], but is also effective in eradicating EL4 thymoma [2] and carcinoma (CMT93) [462], as well as other CD40-negative tumours [110, 463]. Unusually, the efficacy of CD40-targeted immunotherapy is best observed when administering mAb to animals bearing relatively high tumour loads such that, in the case of lymphoma, approximately 10 % of splenic lymphocytes constitute tumour cells; this may be achieved either by inoculating animals with a small number of tumour cells and delaying mAb treatment (*e.g.*, $1x10^5$ lymphoma i.v., 0.25 mg anti-CD40 days 14-17 following tumour injection), or treating early after inoculating with a relatively large number of tumour cells (*e.g.*, $5x10^7$ lymphoma i.v., 0.25 mg anti-CD40 2-5 days thereafter). Thus, immunotherapy of tumours achieved by treatment with anti-CD40 contrasts with prophylactic vaccination or vaccination in situations were only minimal tumour is present.

The observation that anti-CD40-mediated immunotherapy is dependent upon the presence of a substantial tumour burden at the time of treatment is consistent with a requirement for an active immune response against weak or limited TAA(s). Indeed, CD40-targeted immunotherapy of lymphoma is ineffectual in immunocompromised SCID mice unless these animals are reconstituted with lymphocytes from wild-type littermates [2]. Additionally, animals that have gone into remission following immunotherapy with anti-CD40 are resistant to subsequent challenges with the original tumour line, *i.e.*, immunological memory directed against TAA(s) has been established. Again, this mechanism of action is in contrast to other mAbs such as anti-idiotype and anti-CD19 which operate by signalling growth arrest and by the recruitment of effector systems respectively. Recently however, other mAbs such as anti-OX40 and anti-4-1BB have been

shown to affect tumour regression via the initiation or bolstering of an anti-tumour immune response [3, 4, 164-167, 422, 452] (see Table 7).

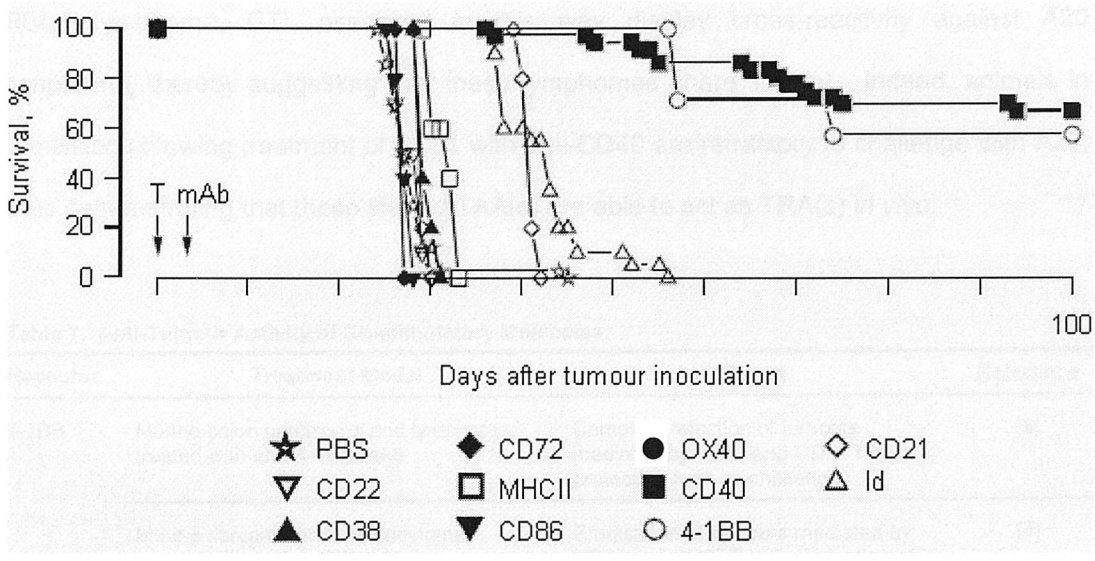


Figure 3.1. Immunotherapy of BCL1-Bearing Mice with Rat mAbs.

Groups of five age-matched BALB/c mice received $5x10^7$ BCL1 cells by i.v. injection on day 0 and, 4 days later, 1mg of rat mAb directed against the antigens shown. This figure does not form part of the work conducted for this thesis, but is adapted from [461].

Consistent with the notion that anti-CD40 operates by actively evoking an immunological response, administration of blocking mAbs directed against IFNγ, LFA-1, B7.1, or B7.2 eliminates therapeutic activity [460] and, furthermore, both IL-12 p40 and IFNγ transcripts are detectable within splenocytes harvested from tumour-bearing mice following anti-CD40 injection [2]. Additionally, mAb-mediated depletion studies have demonstrated that CD40-directed immunotherapy of A31 and BCL1 lymphomas is dependent on $CD8^+$ cells, presumably CTL, but is independent of $CD4^+$ helper T-cells [2]. These findings are consistent with the studies of Diehl *et al.* [110, 419] who demonstrated that anti-CD40 can replace the requirement for $CD4^+$ help (either in CD4-depleted or class-II knock-out animals) during the cross-priming of Ad5E1-specific CTL against Ad5E1-expressing transformed mouse embryo cells via the activation of DCs (see Chapter 1). Indeed, anti-CD40 induces a rapid expansion of $CD8^+$ lymphocytes (beginning typically within 3 days

following injection) in tumour bearing mice and this expansion is associated with the development of cytotoxic activity directed against the tumour that is detectable directly *ex-vivo* by chromium release assay [2]. Interestingly, during anti-CD40-induced eradication of BCL1 lymphoma, CTL assessed in this way display cross-reactivity against A20 lymphoma, thereby suggesting that these lymphomas share TAA(s). Indeed, animals in remission following treatment of BCL1 with anti-CD40 are refractory to challenge with A20, thus demonstrating that these shared TAA(s) are able to act as TRA(s) *in vivo*.

Table 7. Anti-Tumour Activity of Co-stimulatory Molecules.

Receptor	Treatment Model	Result	Reference
4-1BB	Murine colon carcinoma and lymphoma, treated with anti-4-1BB mAb	Complete rejection of tumours mediated by $CD4^+$ and $CD8^+$ T-cells, protection from re-challenge	[5]
	Murine sarcoma and mastocytoma, treated with anti-4-1BB mAb	Eradication of tumours mediated by $CD4^+$ and $CD8^+$ T-cells, induction of immunity	[4]
	Peptide and anti-4-1BB mAb vaccination against transformed epithelial line, melanoma, and lung carcinoma	Regression of tumours due to breaking immunological ignorance	[167]
OX-40	Murine melanoma, sarcoma, breast cancer, and colon carcinoma; treatment with OX40 ligand fusion protein or anti-OX-40 mAb.	T-helper independent $CD8^+$ CTL response, which eradicates lymphoma and provides protection from re-challenge.	[452]
CD28	Murine melanoma, transfection with B7	Rejection of transfected melanoma mediated by $CD8^+$ T-cells and protection against re-challenge with parental strain	[464]
	Murine colon carcinoma, blockade of CTLA-4 using mAbs	Rejection of B7-negative tumours resulting in immunological memory	[465]
CD27	Murine fibrosarcoma and mammary adenocarcinoma, vaccination using irradiated tumours transfected with CD27 ligand (CD70)	Promotion of anti-tumour immunity; synergisitic with B7.1 transfection	[466]
HVEM	Murine mastocytoma, intratumoural LIGHT-encoding DNA vaccination.	Tumour rejection accompanied by tumour-specific CTL activity, dependent on $CD4^+$ and $CD8^+$ T-cells; protection against re-challenge	[467]

In order to further elucidate the mechanism by which anti-CD40 induces tumour regression, we have chosen here to broaden the characterisation of anti-tumour CTL generated *in vivo* during immunotherapy induced by this mAb. In this regard, our aim was to demonstrate that anti-CD40 evokes CTL proliferation at the site of tumour and that these CTL are of a classical T_H1 phenotype displaying competence for migration into sites of

inflammation and cytotoxicity directed against the tumour present. Additionally, the dependency of this response on co-stimulation via 4-1BB/4-1BBL interactions was specifically assessed.

3.2. Materials and Methods.

Unless otherwise indicated, BCL1 or A31 lymphomas were isolated from splenocytes of animals at the terminal stage of disease as described in Chapter 2. $5x10^7$ lymphoma cells were injected i.v. followed by 1 mg of anti-CD40 when tumour represented between 2 and 10 % of the splenic lymphocytes; due to slight differences in the growth rate of these two lymphomas this was typically day 3 following tumour inoculation in the case of A31, while BCL1-bearing animals were treated 4 days after primary tumour challenge.

Surface expression of antigens by lymphocytes during immunotherapy was assessed by FACS analysis - $1x10^6$ splenocytes were incubated with 1 µg of fluorescenated mAb at 4°C for 15 minutes, followed by washing in PBS-BSA-Azide prior to acquisition on a FACScan or FACSCalibur. 5-Bromo-2-deoxyuridine incorporation was used to assess CTL proliferation *in vivo* by administering 2 mg of BrdU i.p. 1 hour before cell preparation and surface antigen staining. $1x10^6$ stained cells were then fixed overnight in 1 ml of 1 % formaldehyde and subsequently exposed to 50 Kunitz units of DNAse I for 1 hour at 37 °C before being washed and re-suspended in 0.2 % Tween-20. Each sample was then stained (4°C for 30 minutes) for BrdU incorporation using a PE-conjugated anti-BrdU mAb, washed in PBS-BSA-Azide and analysed by FACS. Where analysis of DNA content was required, 7AAD at a final concentration of 2 µg/ml was added to the sample to be examined 15 minutes before acquisition.

Typically, CTL activity was assessed prior to tumour eradication as anti-tumour CTL are rapidly eliminated following tumour regression. Thus, in order to prevent *in vitro* quenching of CTL activity, removal of residual tumour from within splenocytes preparations was performed using MACS - samples were labelled with FITC-conjugated anti-idiotype as for FACS, washed in CM at 37 °C and conjugated to anti-FITC microbeads as per

manufacturer's instructions. Samples were then washed in PBS/BSA/EDTA and separated on pre-equilibriated LS columns. Negative fractions were assessed for cytotoxic activity using a standard chromium release assay (see below). $CD8^+$ lymphocytes for cDNA preparation and subsequent RT-PCR, as well as 4-1BB-positive and –negative splenocytes for cytotoxic assays, were purified using FITC-conjugated anti-CD8 and anti-4-1BB (LOB12) respectively.

Briefly, standard chromium release assays involved $1x10^5$ ^{51}Cr-labelled piBCL1, A20 or YAC cells in 100 µl being plated in U-bottomed 96-well plates against 50 µl of the indicated effector cells at the required density and 50 µl of media contained blocking mAb to a final concentration of 50 µg/ml (if required). After brief centrifugation at 700 rpm for 3 minutes, samples were incubated at 37 °C for 4 hours, cells pelleted, and 100 µl of supernatant assessed for ^{51}Cr content on a γ scintillation counter. Maximal ^{51}Cr release was assessed under detergent lysis and the percent target cell lysis induced by each test sample calculated using the equation detailed in Chapter 2.

In order to visualise mRNA transcripts within CTL during immunotherapy, cDNA was prepared from MACS-purified $CD8^+$ lymphocytes and RT-PCR performed as detailed in Chapter 2. Similarly, ELISA was used to visualise IFNγ secretion as detailed in Chapter 2.

A 4-1BB-hFc construct was used to inhibit 4-1BB-4-1BBL interaction *in vivo* and was isolated from CHO transfectant supernatant – briefly, CHO-4-1BB-hFc cells were grown in GMEMS containing 25 µM methionone sulfoximine and supernatants run through a 10 ml protein A column. Subsequently, columns were washed in approximately 2 litres of 40 µM Tris.HCl/ 2mM EDTA (pH 8.0), prior to eluting the fusion protein using 1 ml 0.1 M glycine/2mM EDTA (pH 3) and protein estimation by ELISA. Anti-4-1BBL (3H3) was a kind gift from Dr. Robert Mittler.

3.3. Results.

3.3.1. Kinetics of Tumour Eradication and CTL Expansion.

As an introduction, figure 3.2 depicts the typical kinetics of lymphoma eradication and CTL expansion following administration of anti-CD40; CBA/H mice were inoculated with $5x10^7$ A31 i.v. and treated 3 days later with 1 mg of 3/23 i.v.. Control animals received identical doses of either A31 or anti-CD40 alone. Individual animals were culled on the days indicated and splenocytes assessed for Id or CD8 expression by FACS.

Figure 3.2 (a) shows the total number of splenocytes expressing either Id or CD8 from each group, while figure 3.2 (b) shows the percentage of Id^+ splenocytes from animals having received either A31 alone or both A31 and anti-CD40. As can be seen in panel (a), following an initial lag phase after anti-CD40 administration between days 4 and 6, during which the lymphoma continues to grow normally, tumour cells were abruptly eradicated between days 6 and 8. Similarly, following an initial lag phase between days 4 and 5, $CD8^+$ lymphocytes expanded profoundly in tumour-bearing animals in response to anti-CD40 administration, thus explaining the difference in the percentage of Id^+ cells seen between anti-CD40-treated and untreated animals on day 6 (panel (b)). This accumulation of $CD8^+$ lymphocytes rapidly dissipated following tumour regression between days 8 and 9. During this time, only minor increases in the total number of $CD8^+$ lymphocytes taken from animals that have received either tumour or anti-CD40 alone were observed.

These data bolster the notion that anti-CD40 induces tumour eradication by initiating or boosting an existing anti-tumour $CD8^+$ T-cell response resulting in the generation of class-I-restricted anti-tumour CTL that are subsequently removed following tumour eradication presumably because of antigen withdrawal (Chapter 1). Indeed, these data are in contrast to the kinetics of tumour growth arrest observed after treatment of A31 with anti-Id – this mAb induces growth arrest rapidly following injection, but is unable to eliminate the tumour over a period of 5 to 6 days [461].

3.3.2. Lymphocyte Population Kinetics During Immunotherapy.

We wished to observe the changes in the total number of $CD8^+$, $CD4^+$, and Id^- $CD19^+$ lymphocytes during anti-CD40-induced immunotherapy of both A31 and BCL1 in order to ascertain differences in these populations when administering anti-CD40 and lymphoma separately or in combination. CBA/H or BALB/c mice were inoculated i.v. with $5x10^7$ A31 or BCL1 respectively and subsequently treated 4 days later with 1 mg of 3/23 i.v.. Control animals received identical doses of either lymphoma or anti-CD40 alone. Individual animals were culled on the indicated days and splenocytes assessed for Id, CD19, CD4 or CD8 expression by FACS.

Figure 3.3 shows the total number and percent representation of $CD8^+$, $CD4^+$ and $CD19^+Id^-$ splenocytes during immunotherapy of A31 and the appropriate control groups. As can be seen, the percentage of splenocytes representing each of these populations diminished steadily in animals that received A31 alone, thereby reflecting the predominance of lymphoma growth in untreated animals. Nonetheless, as can be seen in panel (a) and (b), the total number of $CD4^+$ and $CD8^+$ lymphocytes increased in response to both A31 inoculation and anti-CD40 administration alone, notably when splenocytes were examined after day 9. In the case of $CD4^+$ lymphocytes (panel (b)), this elevation in total numbers was largely uneffected by the presence of both A31 and anti-CD40 when compared with the presence of tumour alone. Conversely, the expansion of $CD8^+$ lymphocytes in animals undergoing immunotherapy occurred with accelerated kinetics when compared with this event in animals that had received tumour alone (panel (a)).

In the case of $CD19^+Id^-$ lymphocytes (panle (c)), as expected, anti-CD40 induced proliferation of these B-cells in the absence of tumour, but markedly less so in the presence of tumour; this effect may be due to, for example, homeostatic mechanisms limiting the number of endogenous B-cells in the presence of a B-cell lymphoma, inhibitory factors secreted or induced by the tumour, preferential binding of mAb to the tumour and/or limited availability of mAb in the presence of a $CD40^+$ lymphoma. Notably, no B-cell proliferation was observed in the presence of tumour alone.

Figure 3.4 shows the $CD8^+$, $CD4^+$ and $CD19^+Id^-$ lymphocyte population variations during immunotherapy of BCL1. Again, in animals that received tumour alone, the proportion of splenocytes representing these endogenous lymphocyte populations decreased over time as the lymphoma grew (left hand panels). As was the case with A31 in CBA/H mice, anti-CD40 induced up to a 10-fold expansion of $CD8^+$ lymphocytes in tumour-bearing animals. However, in contrast to the administration of A31 or anti-CD40 alone to CBA/H mice, administration of either BCL1 or anti-CD40 to BALB/c animals resulted in only a minor accumulation of $CD8^+$ lymphocytes; nonetheless, up to a 2-fold expansion was observed in this model, but this is overshadowed by the expansion of $CD8^+$ lymphocytes when both BCL1 and anti-CD40 were administered (panel (a)). Also in contrast to the lymphocyte population changes observed in the A31 model, $CD4^+$ lymphocytes predominantly failed to increase in number in response to BCL1 alone, but appeared to do so after day 7 when animals received both tumour and anti-CD40 (panel (b)). Furthermore, endogenous B-cell proliferation was observed in response to anti-CD40 in both tumour-bearing and naïve animals, suggesting that, in this case, the dose of anti-CD40 is not limiting. Nonetheless, as with A31, no proliferation of B-cells was observed in response to BCL1 alone (panel (c)).

Together, these data regarding $CD8^+$ and $CD4^+$ lymphocyte numbers during immunotherapy of A31 and BCL1 suggest that, consistently, the combination of both lymphoma inoculation and anti-CD40 administration results in the expansion of $CD8^+$ lymphocytes which, as shown in figure 3.2, occurs concurrently with tumour eradication. Furthermore, the increase in both $CD8^+$ and $CD4^+$ lymphocyte numbers in response to A31, and to a lesser extent BCL1, alone suggests that these tumours may evoke an immune response, but this is ineffectual or inadequate for controlling neoplastic growth. This point is examined in Appendix 1.

The expansion of $CD4^+$ and $CD8^+$ lymphocytes observed predominantly in CBA/H mice in response to anti-CD40 alone may be due to the production of IFNα, and subsequently IL-

15, which drives antigen-independent proliferation of memory T-cells [252]. Alternatively, T-cell expansion in response to anti-CD40 alone may be directed against self-antigens as a result of the expansion and activation of B-cells. Indeed, figure 3.3 shows that, upon anti-CD40 administration to naïve CBA/H mice, B-cell expansion (predominating at day 8 and 9) proceeds T-cell expansion (peaking at days 10 and 11). Additionally, during pre-clinical dose-escalation studies using 3/23 both a temporary nephritis and hepatitis have been observed (T. Geldart, NAC Proposal). See Chapter 6 for discussion of this point. Another possible explanation as to why T-cells expand in response to anti-CD40 alone in CBA/H mice is that this mAb binds directly to a subset of $CD40^+$ T-cells; however, the ligation of CD40 on T-cells has been reported to enhance the survival of previously activated CTLs, thereby augmenting the generation of antigen-specific memory, but has not been shown to induce de novo proliferation of memory or naive T-cells [252].

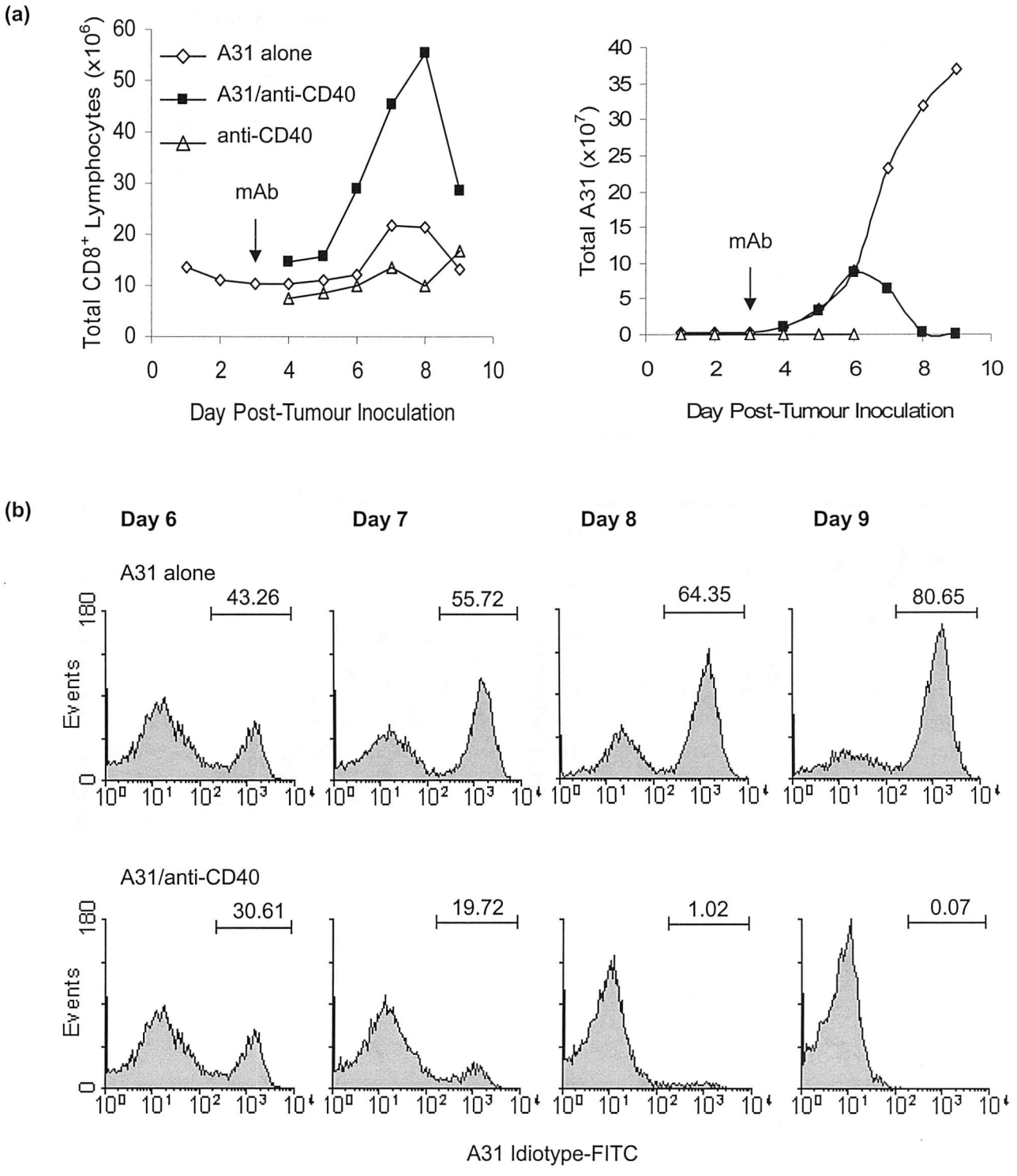


Figure 3.2. CTL Expansion and Eradication of A31 B-cell Lymphoma during Immunotherapy. CBA/H mice received $5x10^7$ A31 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) mAb i.v. on day 3. On days 1 to 9 following tumour inoculation, splenocytes were harvested from single animals in each treatment group and stained with FITC-labelled anti-A31 idiotype or FITC-labelled anti-CD8; the total number of $CD8^+$ lymphocytes and A31 lymphoma in each of the treatment groups is shown in (a). Marker values represent the percentage of gated histogram events. The percentage of A31 lymphoma cells per total splenocyte population is summaries in (b). Arrows represent the day of 3/23 administration as in the following figures. These data are representative of more than 3 separate experiments.

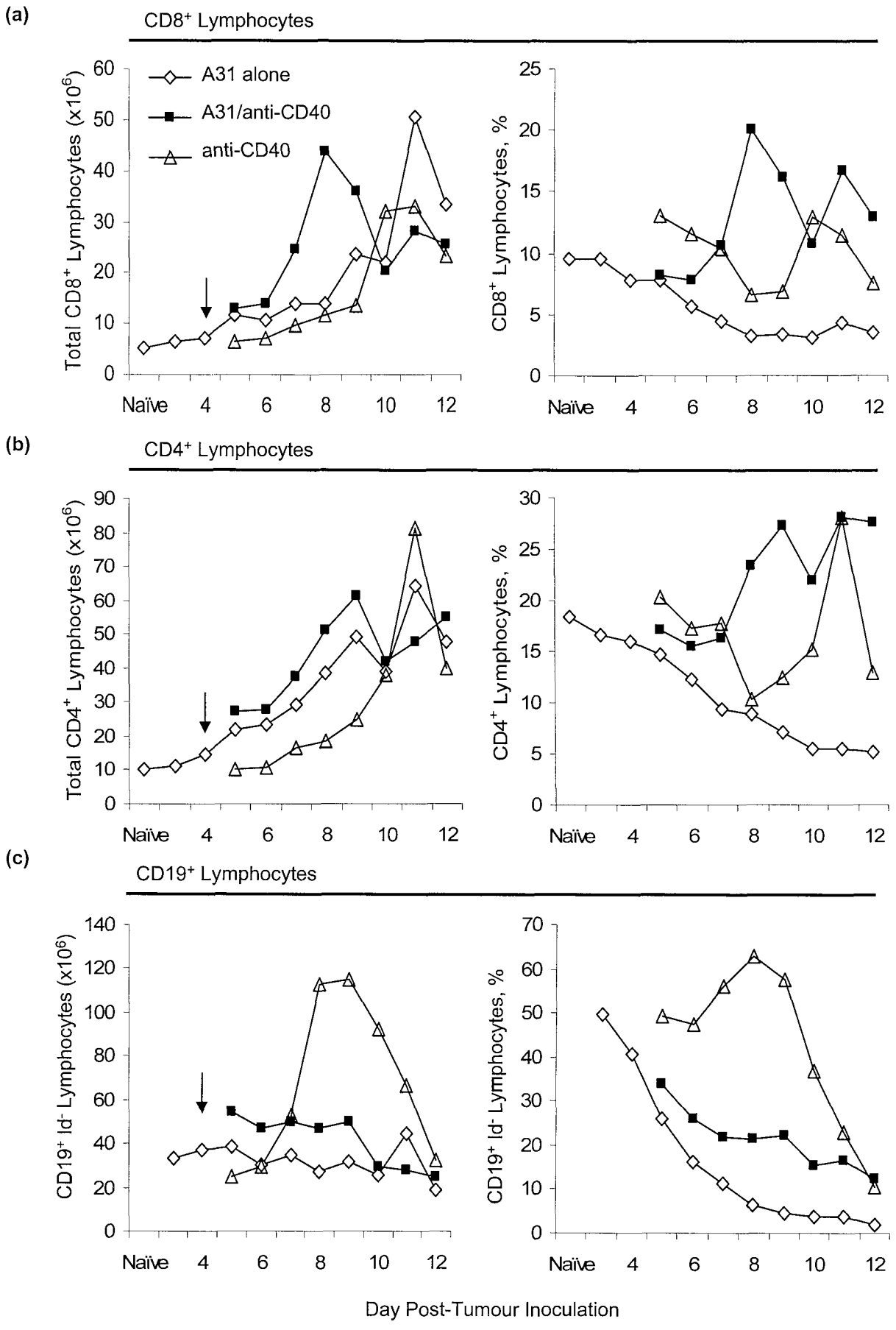


Figure 3.3. Lymphocyte Population Kinetics during Immunotherapy of A31 Lymphoma. CBA/H mice received $5x10^7$ A31 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Splenocytes were harvested on the indicated days from single animals in each treatment group and stained with FITC-conjugated anti-CD8 (a), anti-CD4 (b), or anti-CD19 (c). Each panel (a, b, or c) shows either total cell number (left) or percentage representation (right). These data are representative of 2 separate experiments. Arrows represent the day of 3/23 administration.

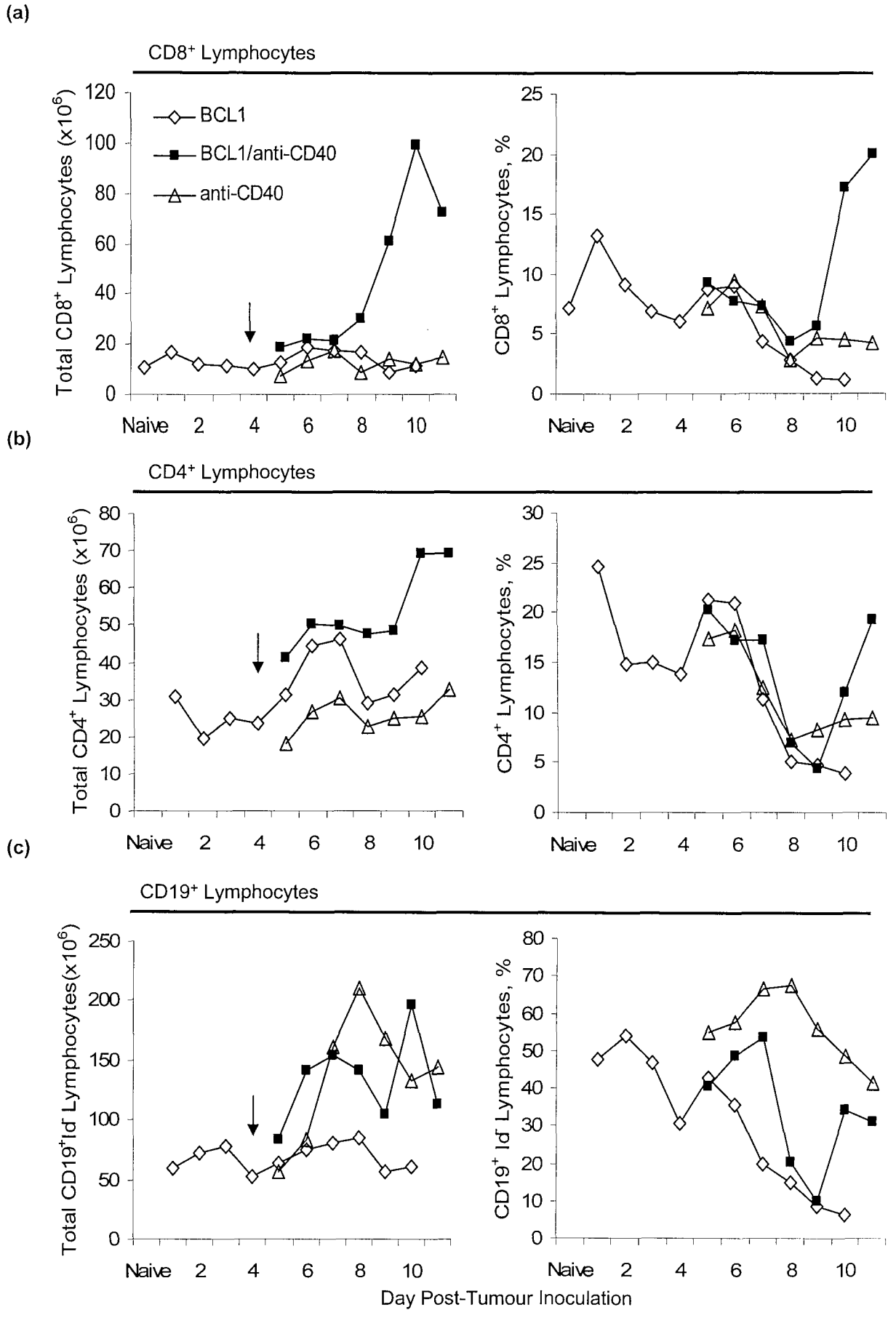


Figure 3.4. Lymphocyte Population Kinetics during Immunotherapy of BCL1 Lymphoma. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Splenocytes were harvested from single animals in each treatment group on the indicated days and stained with FITC-conjugated anti-CD8 (a), anti-CD4 (b), or anti-CD19 (c). Each panel (a, b, or c) shows either total cell number (left) or percentage representation (right). These data are representative of several separate experiments. Arrows represent the day of 3/23 administration.

3.3.3. Proliferation of $CD8^+$ Lymphocytes at the Site of Tumour in Response to anti-CD40

Next, in order to ascertain whether the increase in $CD8^+$ lymphocytes induced by anti-CD40 in the presence of tumour occurs at the tumour site within the spleen, or whether this is due to migration of lymphocytes, *in vivo* BrdU incorporation was used to visualise the proportion of $CD8^+$ lymphocytes in S phase during CD40-targeted immunotherapy of BCL1. 5-Bromo-2-deoxyuridine is an altered nucleotide that, when administered systemically, is incorporated into cells that are actively synthesising DNA; hence, upon isolation of these cells ex-vivo proliferating cells may be observed by FACS following membrane permeablisation and detection of BrdU by a specific fluorescenated mAb.

Duplicate BALB/c mice were inoculated with $5x10^7$ BCL1 i.v. and were treated 4 days later with 1 mg of 3/23 i.v.. Single control animals received identical doses of either BCL1 or anti-CD40 alone. Animals were sacrificed, 1 hour after receiving BrdU, the equivalent of 24, 48 and 72 hours following anti-CD40 administration.

As can be seen in figure 3.5, only a minimal proportion (0.1 %) of $CD8^+$ lymphocytes were in S phase under steady-state conditions (approximately 1 in 1000 cells, panel (a)), whereas following tumour inoculation this was consistently elevated to approximately 4 % of $CD8^+$ lymphocytes within 5 days. Nonetheless, after anti-CD40 administration, the proportion of $CD8^+$ lymphocytes present within tumour-bearing animals that are in S phase was rapidly increased, reaching 12 % after 72 hours in this experiment. These data are summarised in figure 3.6 (a). Additional experiments at the single time point of 72 hours following anti-CD40 administration have shown that the proportion of expanding $CD8^+$ lymphocytes may reach up to 20 to 30 % in animals undergoing immunotherapy of BCL1 (*c.f.*, figure 3.6 (c)).

As shown in figure 3.7 (a), BrdU incorporation was associated with the synthesis of DNA, as visualised by the increased incorporation of 7AAD following BrdU uptake. Indeed, $CD8^+$ lymphocytes taken from animals that had received both BCL1 and anti-CD40 demonstrated far greater DNA synthesis 72 hours following mAb administration (figure 3.7

(b)) than $CD8^+$ lymphocytes taken from animals having received either anti-CD40 or BCL1 alone.

Figure 3.6 (b) shows the forward scatter of $CD8^+$ lymphocytes (taken from animals undergoing immunotherapy of BCL1 72 hours after anti-CD40 administration) that have either incorporated BrdU or not. $BrdU^+$ $CD8^+$ lymphocytes were clearly larger (as shown by an increase in forward scatter) with respect to the $BrdU^-$ counterparts, confirming that these cells were actively dividing and were likely to be differentiating into effector cells.

Figure 3.6 (c) summarises the proportion of $CD8^+$ lymphocytes in the liver, spleen and mesenteric lymph nodes that were in S phase the equivalent of 72 hours following anti-CD40 injection in the treatment groups indicated. As can be seen, $CD8^+$ lymphocytes within the liver and the spleen were induced to proliferate in response to anti-CD40 only in the presence of tumour. Nonetheless, no proliferation was observed in the lymph nodes under these conditions.

These data therefore indicate that $CD8^+$ lymphocytes do indeed proliferate in response to the combination of BCL1 and anti-CD40. To a lesser extent, both BCL1 and anti-CD40 alone are able to induce $CD8^+$ lymphocyte proliferation in BALB/c animals despite the observation that only minor changes occur in total lymphocyte numbers in response to these two stimuli (Figure 3.4 (a)). These observations are therefore consistent with the hypothesis that BCL1 initiates an ineffectual T-cell response and that this develops into an anti-tumour response following administration of anti-CD40.

Additionally, $CD8^+$ lymphocyte proliferation occurs (at least predominantly) at the site of tumour residency, as both the liver and spleen harbour BCL1 within days of inoculation as detected by FACS, whereas BCL1 does not spread to the mesenteric lymph nodes until the terminal stages of disease (approximately 12 days following i.v. inoculation with $5x10^7$ cells; data not shown). Thus, TAA presentation is likely to occur locally, orchestrated either by the tumour itself or by local APCs.

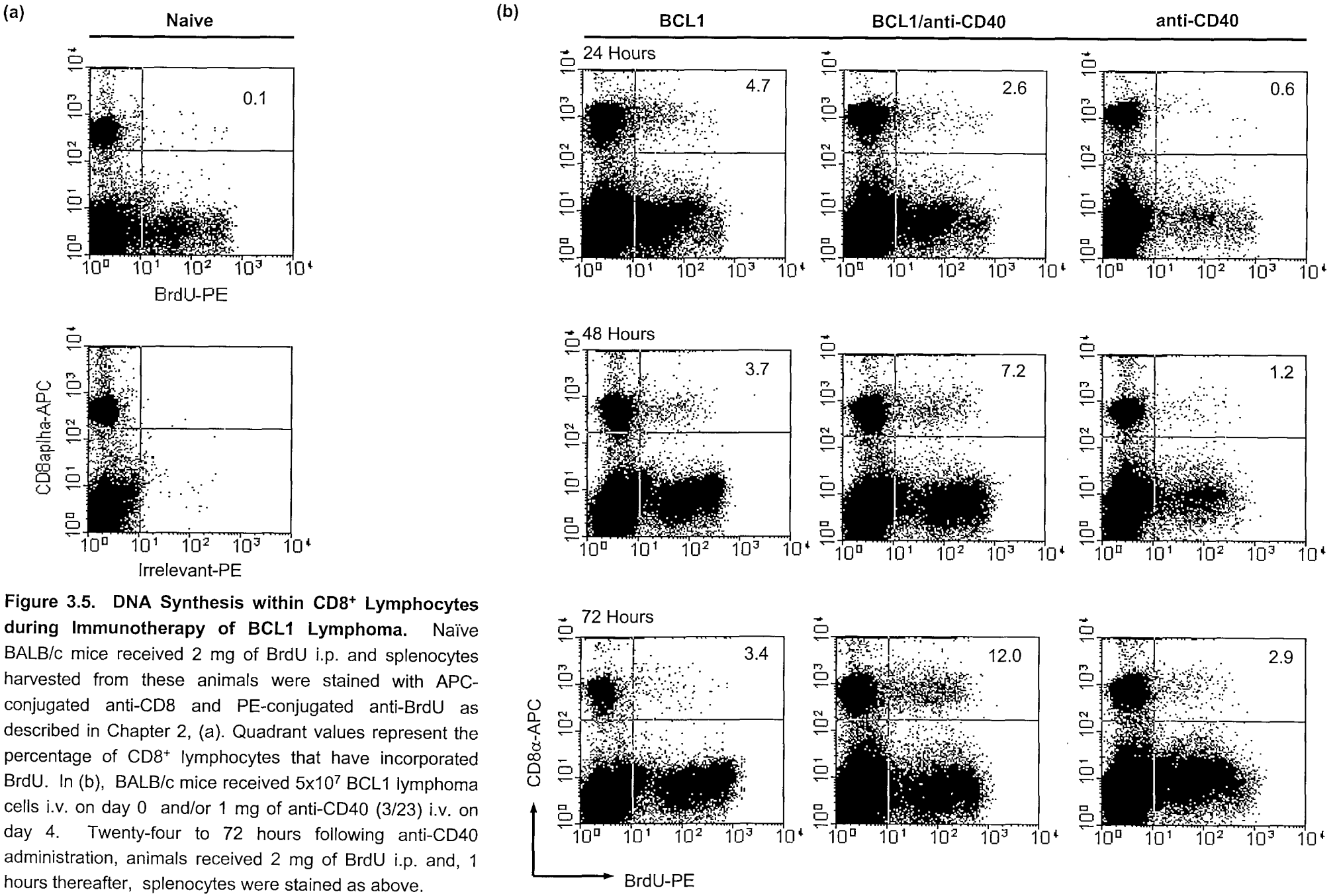


Figure 3.5. DNA Synthesis within CD8⁺ Lymphocytes during Immunotherapy of BCL1 Lymphoma. Naïve BALB/c mice received 2 mg of BrdU i.p. and splenocytes harvested from these animals were stained with APC-conjugated anti-CD8 and PE-conjugated anti-BrdU as described in Chapter 2, (a). Quadrant values represent the percentage of $CD8^+$ lymphocytes that have incorporated BrdU. In (b), BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Twenty-four to 72 hours following anti-CD40 administration, animals received 2 mg of BrdU i.p. and, 1 hours thereafter, splenocytes were stained as above.

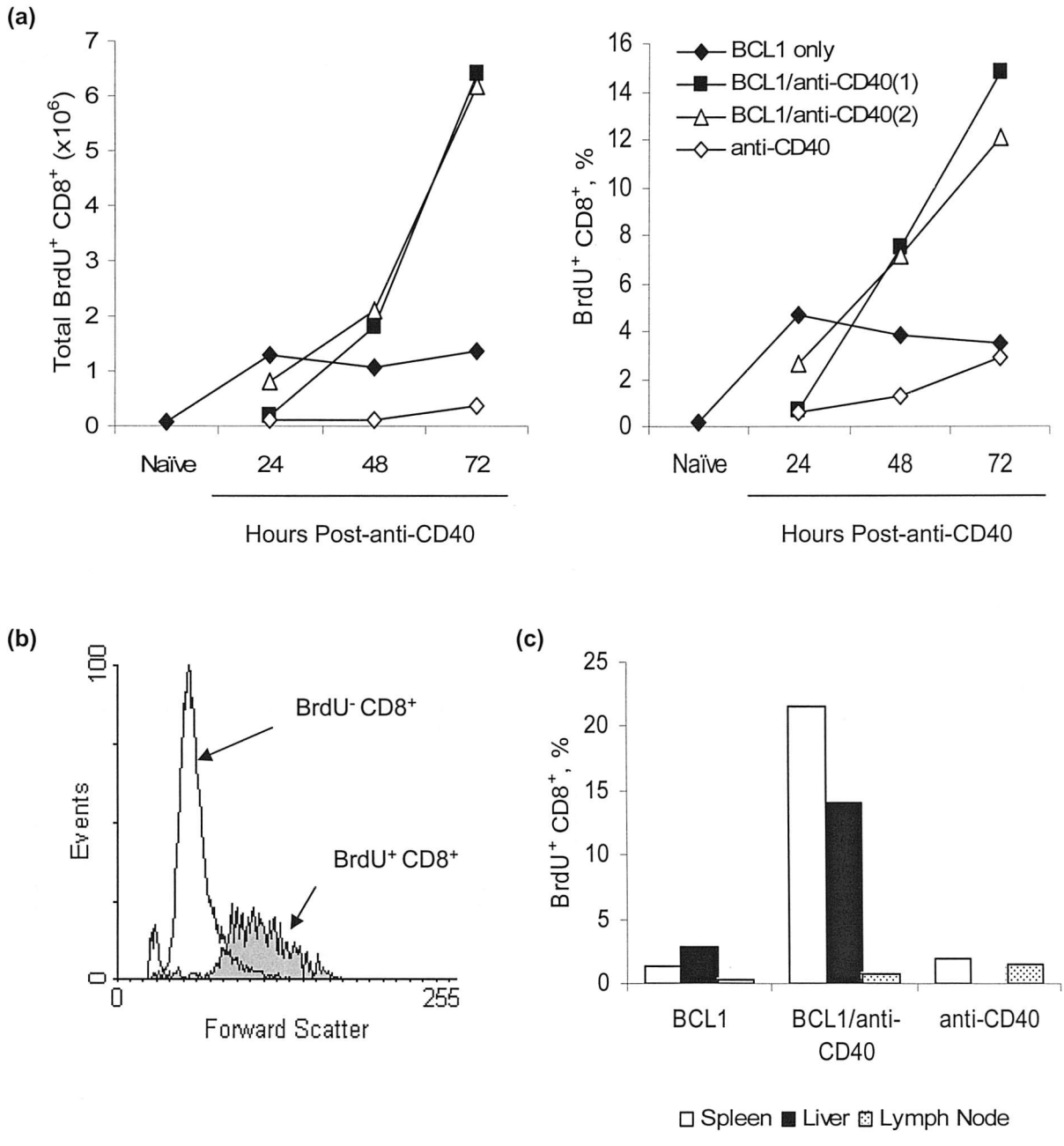


Figure 3.6. Expansion of CD8⁺ Lymphocytes at the Site of Lymphoma during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4, followed by 2 mg of BrdU i.p. 24, 48 or 72 hours thereafter. Splenocytes were harvested 1 hour following BrdU administration, stained as in figure 3.6, and the number of $CD8^+$ lymphocytes incorporating BrdU calculated, (a). Treatment groups ending in '(1)' or '(2)' represent duplicate animals within the BCL1/anti-CD40-treated group. These data are representative of two separate experiments. The forward scatter of $BrdU^+$ $CD8^+$ lymphocytes taken from tumour-bearing mice 72 hours after anti-CD40 administration is shown in (b). Similarly, in (c), at the equivalent of 72 hours following anti-CD40 administration, mesenteric lymph nodes, splenocytes and hepatocytes were harvested from duplicate animals treated as in (a), and the proportion of $CD8^+$ lymphocytes incorporating BrdU determined within these different tissues. Values represent the average of between two animals.

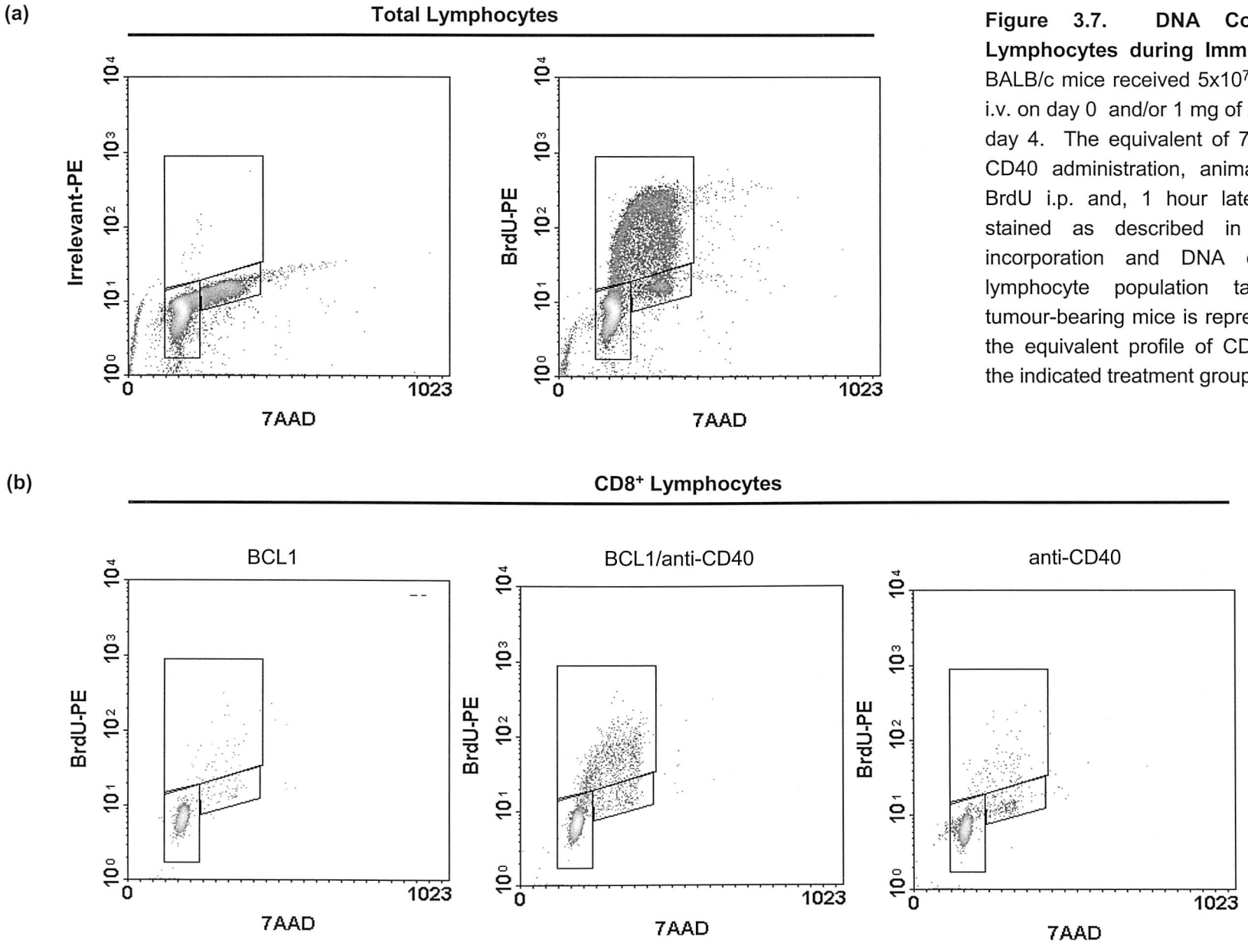


Figure 3.7. DNA Content within CD8⁺ Lymphocytes during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. The equivalent of 72 hours following anti-CD40 administration, animals received 2 mg of BrdU i.p. and, 1 hour later, splenocytes were stained as described in Chapter 2. BrdU incorporation and DNA content of the total lymphocyte population taken from untreated tumour-bearing mice is represented in (a), whereas the equivalent profile of CD8⁺ lymphocytes within the indicated treatment groups is shown in (b).

3.3.4. Phenotypic Differentiation of $CD8^+$ Lymphocytes into T_H1 Effector Cells

As $CD8^+$ lymphocytes are the primary candidates for the anti-tumour effector cells inducing lymphoma regression during anti-CD40-mediated immunotherapy, we assessed the surface phenotype of $CD8^+$ lymphocytes during immunotherapy of both A31 and BCL1 in order to confirm that anti-CD40 induces $CD8^+$ lymphocyte differentiation in tumour-bearing animals. We chose to assess CD62L (L-selectin), CD49d (α4 integrin), and 4-1BB expression on $CD8^+$ lymphocytes during immunotherapy as the expression of these antigens varies during cellular differentiation and they are involved in different aspects of lymphocyte function, *i.e.*, migration and proliferation/survival.

Both CD62L and CD49d are cell adhesion molecules involved in lymphocyte extravasation. CD62L is a member of the selectin family of cell adhesion molecules that is able to bind with low affinity to anionic oligosaccharide sequences related to sialylated Lewis x via a C-type lectin domain. CD62L is responsible for the homing of naïve lymphocytes to lymph nodes and Peyer's patches via high endothelial venules; thus, upon lymphocyte activation, CD62L is down-regulated thereby inhibiting lymphocyte re-circulation and promoting the retention of lymphocytes at sites of inflammation. CD49d is a constitutively expressed integrin that is able to complex with CD29 and β7 subunits to form VLA-4 ($\alpha4\beta1$) and $\alpha4\beta7$ integrins respectively. By binding VCAM-1 and CS-1-containing fibronectin, these CD49d-containing integrins are involved in all stages of lymphocyte-endothelium interaction - initial tethering, rolling, and firm adhesion of lymphocytes. Thus, upon lymphocyte differentiation towards terminal effector cells, CD49d is up-regulated.

CBA/H or BALB/c mice were inoculated intravenously with $5x10^7$ A31 or BCL1 respectively and treated 4 days later with 1 mg of 3/23 i.v.. Control animals received identical doses of either lymphoma or anti-CD40 alone. Individual animals were culled on the indicated days and splenocytes assessed for CD8 as well as CD49d and CD62L expression by FACS. Data regarding 4-1BB expression by $CD8^+$ lymphocytes during immunotherapy is discussed in section 3.2.7.

Figure 3.8 shows the expression of CD49d (panel (a)) and CD62L (panel (b)) on $CD8^+$ lymphocytes on the equivalent of day 8 and 12 following tumour challenge during immunotherapy of A31, while figure 3.9 summaries these data until day 12 following tumour inoculation. Similarly, figure 3.10 shows the equivalent summary of data obtained during immunotherapy of BCL1; panel (a) and (b) of this figure represent data from different therapies.

As can be seen in figure 3.8 (a), on the equivalent of day 8 following initial tumour challenge, CD49d expression was up-regulated on $CD8^+$ lymphocytes in response to both A31 and anti-CD40 alone with respect to the naïve control. This effect was augmented on day 8 in animals that have received both tumour and anti-CD40, but this was only on day 8 and was not so on days 5 to 7 (figure 3.9 (a)) – the percentage of $CD49d^{hi}$ $CD8^+$ lymphocytes was identical on days 5 to 7 in animals that have either received A31 alone or both A31 and anti-CD40. Similarly, the percentage of $CD62L^{lo}$ $CD8^+$ lymphocytes was comparable in these two treatment groups (figure 3.9 (b)). Notably, following tumour eradication in animals that had received A31 and anti-CD40, the percentage of both $CD49d^{hi}$ and $CD62L^{lo}$ $CD8^+$ lymphocytes diminished, whereas in untreated tumour-bearing animals the proportion of $CD49d^{hi}$ and $CD62L^{lo}$ $CD8^+$ lymphocytes continued to increase (figure 3.8 (a) and (b), day 12; figure 3.9 (a) and (b), days 10 to 12). Furthermore, the proportion of both $CD49d^{hi}$ and $CD62L^{lo}$ $CD8^+$ lymphocytes in animals that had received anti-CD40 alone continued to increase until day 10 (figure 3.9).

Similarly, as can be seen in figure 3.10, both BCL1 and anti-CD40 alone were able to induce the up-regulation of CD49d and the down-regulation of CD62L on $CD8^+$ lymphocytes, albeit to a lesser extent than in the A31 model. However, in contrast to the immunotherapy of A31, the presence of both BCL1 and anti-CD40 augmented the proportion of $CD8^+$ lymphocytes displaying a $CD49d^{hi}/62L^{lo}$ phenotype. Similarly, however, following lymphoma regression, the percentage of $CD49d^{hi}$ $CD8^+$ lymphocytes decreased towards naïve levels (figure 3.10 (a)), but continued to increase in untreated tumour recipients.

As can be seen in figure 3.9, the total number of both $CD49d^{hi}$ and $CD62L^{lo}$ $CD8^+$ lymphocytes was elevated in animals undergoing immunotherapy of A31 prior to a similar response in animals that had received A31 or anti-CD40 alone. In contrast, in the BCL1 model, maximal accumulation of either $CD49d^{hi}$ or $CD62L^{lo}$ $CD8^+$ lymphocytes only occurred in animals that had received both tumour and anti-CD40 (figure 3.10).

These data suggest that both BCL1 and A31 lymphomas alone are able to initiate $CD8^+$ T-cell differentiation, at least as regards competence for migration towards sites of inflammation, and that this is only augmented by anti-CD40 in the case of BCL1, but not A31. Interestingly, Hernandez *et al.* demonstrated that $CD8^+$ lymphocytes undergoing peripheral deletion do not up-regulate CD49d and are deficient in their ability to down-regulate CD62L ([317]; see discussion, section 3.3). Thus, these data are consistent with the hypothesis that the $CD8^+$ lymphocyte differentiation observed in response A31 or BCL1 does not represent differentiation in response to self-antigen prior to peripheral deletion. In addition to this, it is clear (especially in the A31 model) that the administration of anti-CD40 to tumour-bearing animals accelerates this endogenous response, perhaps preventing 'exhaustion' of CTL. The differentiation of $CD8^+$ lymphocytes in response to anti-CD40 alone may be directed against self-antigen, as will be discussed in Chapter 6.

As concluded in section 3.2.1, these data show further that A31 is able to induce $CD8^+$ lymphocyte proliferation to a greater extent than BCL1, but that this occurs more rapidly when anti-CD40 is administered. In contrast, the combination of BCL1 and anti-CD40 consistently results in a far greater expansion of 'activated' $CD8^+$ lymphocytes than is observed when either anti-CD40 or tumour is present. These data are therefore consistent with the hypothesis that anti-CD40 augments a pre-existing immunological response largely by providing proliferative and/or survival signals to $CD8^+$ lymphocytes, possibly by inducing co-stimulatory ligands (*e.g.*, B7, 4-1BBL), co-stimulatory receptors (4-1BB, CD27) and/or cytokines (IL-12, -2, -15), and that this results in either a greater or quicker accumulation of anti-tumour CTL than in untreated tumour recipients.

3.3.5. $CD8^+$ Lymphocyte Responsiveness to Tumour Alone

As an aside, we wished to assess the specificity of the $CD8^+$ lymphocyte expansion and differentiation observed in response to A31 and BCL1 alone, as well as to establish whether this occurs in response to the T-lymphoma TEN-1 which does not respond to anti-CD40 *in vivo*. Thus, CBA/H mice received either $5x10^7$ A31 or naïve CBA splenocytes i.v., whereas BALB/c mice received $5x10^7$ BCL1, TEN-1, or naïve BALB/c splenocytes i.v. Eight days following this injection, splenocytes were harvested from recipient animals, or naïve littermates. The time-point of 8 days following inoculation was chosen as this generally corresponds to the time at which maximal $CD8^+$ lymphocyte expansion is seen if anti-CD40 is administered to tumour-bearing animals, thereby providing a comparison.

As can be seen in figure 3.11 (a), $CD8^+$ lymphocyte expansion was observed in response to A31, BCL1 and TEN-1, but did not occur when naïve splenocytes were administered to either CBA/H or BALB/c mice. The expansion on $CD8^+$ lymphocytes in response to A31, BCL1 and TEN-1 was 4.6-, 2.1- and 2.7-fold respectively, as compared with the number of $CD8^+$ lymphocytes in naïve littermates; these data were confirmed by the incorporation of BrdU into $CD8^+$ lymphocytes as early as days 2 to 4 following inoculation with either A31, BCL1, or TEN-1 (figure 3.12). Interestingly, little accumulation of $CD8^+$ lymphocytes was observed in to A31 within the first 4 days after inoculation (figure 3.12), thereby suggesting that, in this model, proliferative signals precede the up-regulation of anti-apoptotic molecules in order to effect $CD8^+$ lymphocyte accumulation.

$CD8^+$ lymphocytes additionally expressed 4-1BB in response to A31 and BCL1 (see section 3.3.8/.9), but not in response to TEN-1 or administration of naïve splenocytes; this pattern was reflected in the expression of CD49d, which was effectively up-regulated in response to A31 and BCL1, less so in response to TEN-1, and not at all in response to an injection of splenocytes. The expression of CD25 acted as a negative control in these experiments, as no expression was observed in response to any of these stimuli. These data were confirmed by the assessment of CD49d and CD62L expression on $CD8^+$ lymphocytes in animals that were at the terminal stage of disease, as induced by

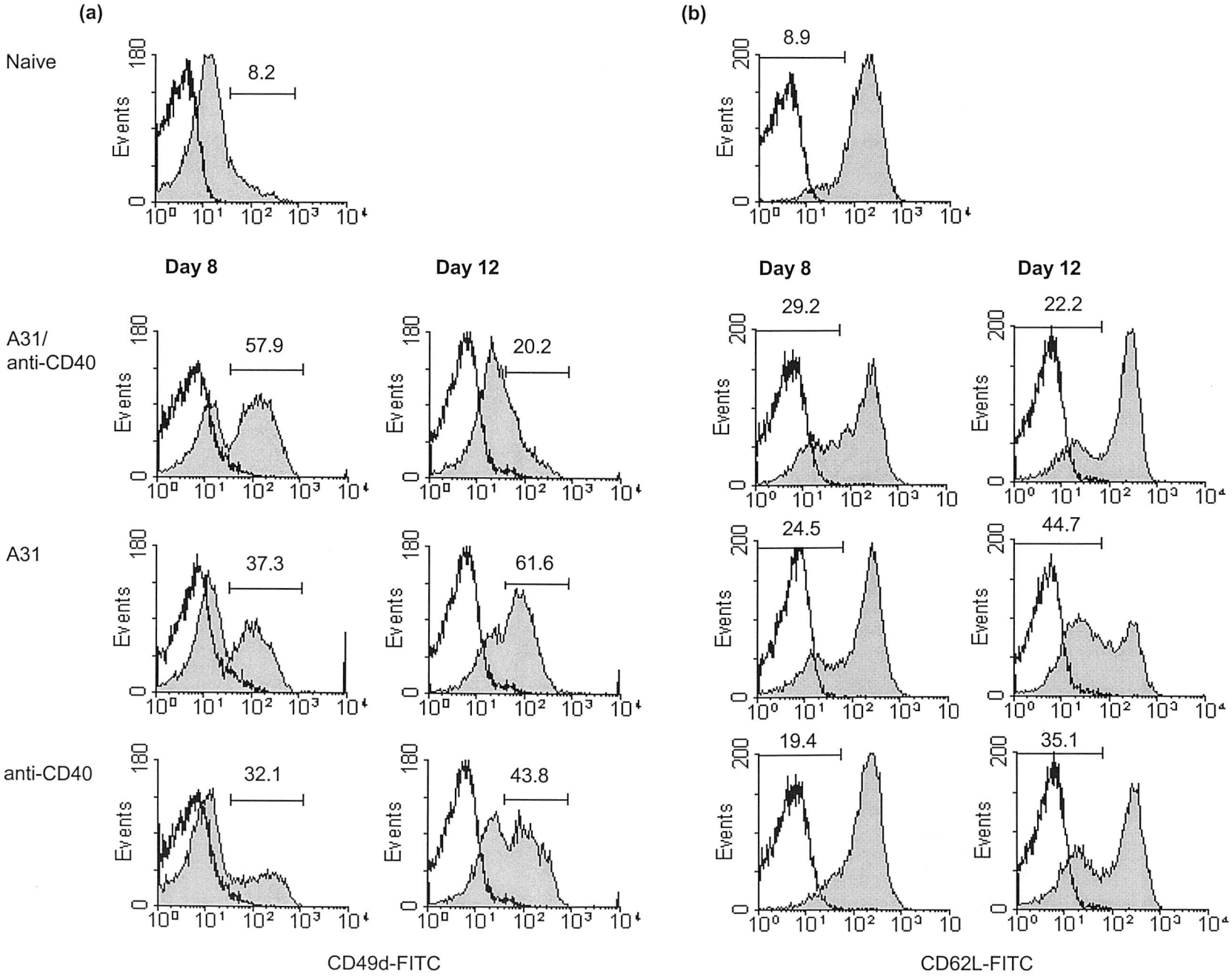


Figure 3.8. CD8+ Lymphocyte Activation as assessed by CD49d and CD62L Expression during Immunotherapy of A31. CBA/H/H mice received $5x10^7$ A31 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Splenocytes were harvested on the indicated days and stained with PE-conjugated anti-CD8 and either FITC-conjugated anti-CD49d, -CD62L (solid grey histograms, panels (a) and (b) respectively), or irrelevant mAb (open histograms).

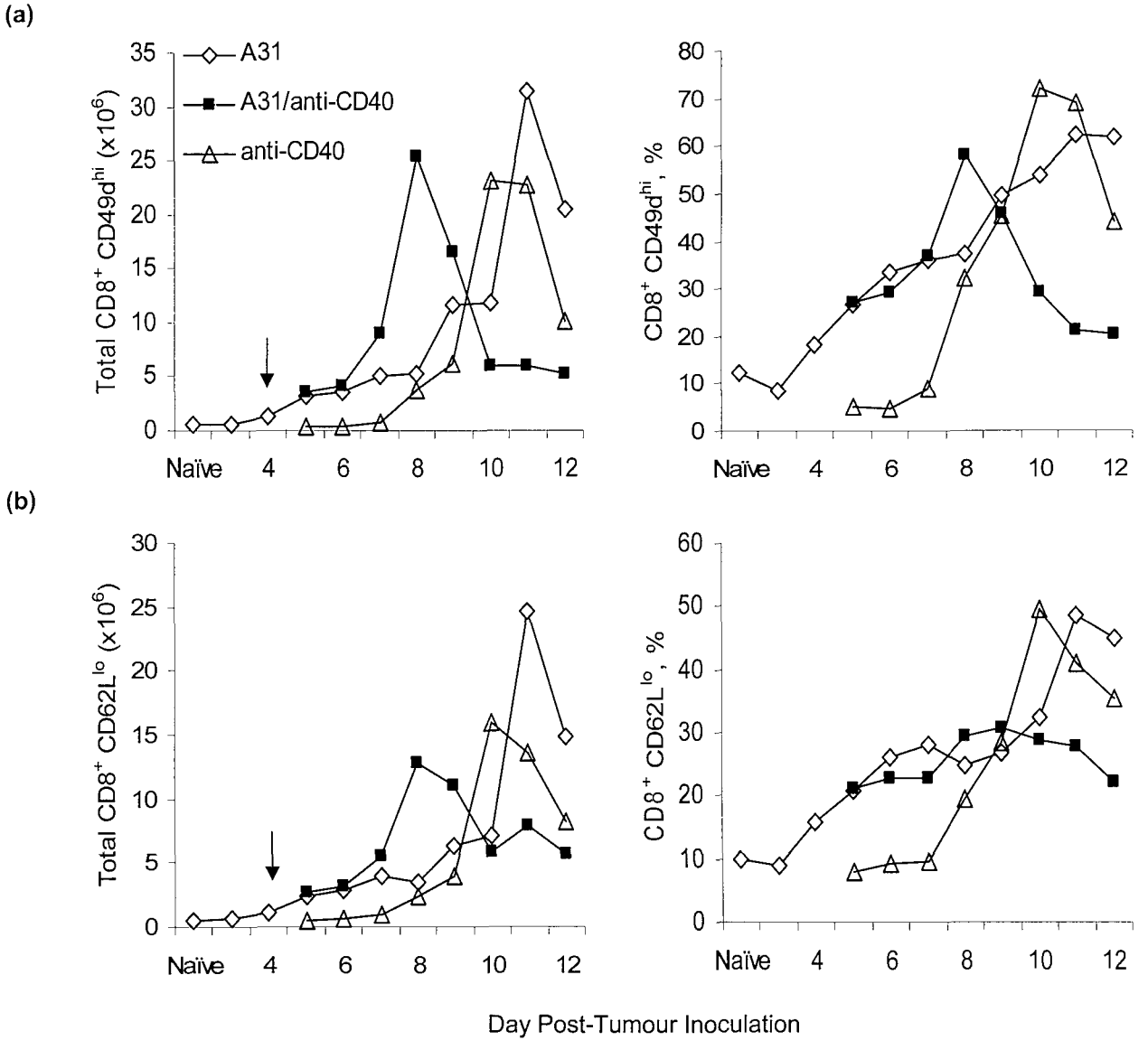


Figure 3.9. Kinetics of CD8⁺ Lymphocyte Activation as assessed by CD49d and CD62L Expression during Immunotherapy of A31. CBA/H/H mice received $5x10^7$ A31 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. As shown in figure 3.8, splenocytes were harvested on the indicated days from single animals in each treatment group and stained with PE-conjugated anti-CD8 and either FITC-conjugated anti-CD49d (a) or FITC-conjugated anti-CD62L (b). These data are representative of two separate experiments. Arrows represent the day of 3/23 administration.

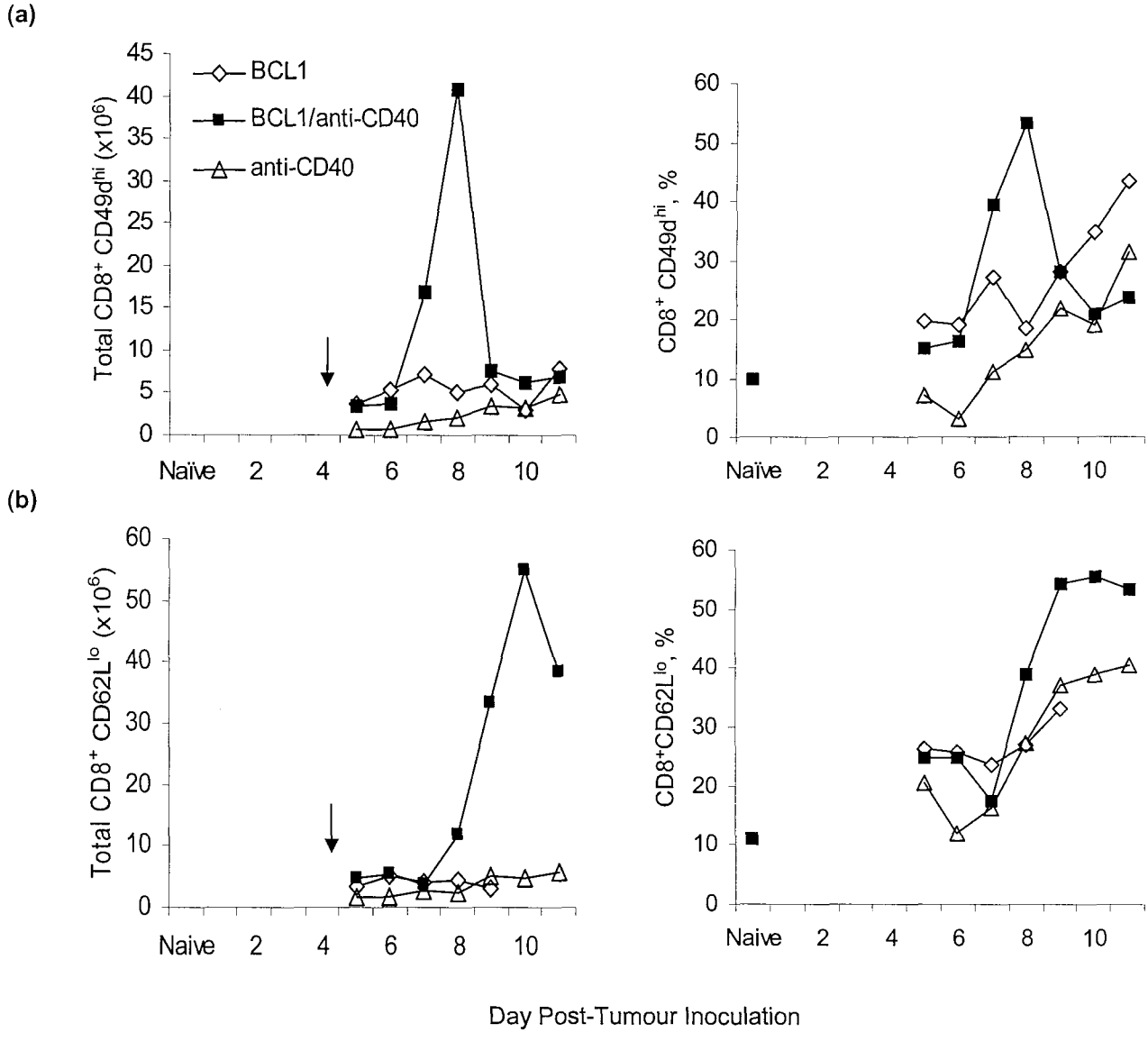


Figure 3.10. Kinetics of CD8⁺ Lymphocyte Activation as assessed by CD49d and CD62L Expression during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Splenocytes were harvested on the indicated days from single animals in each treatment group and stained with PE-conjugated anti-CD8 and either FITC-conjugated anti-CD49d (a) or FITC-conjugated anti-CD62L (b). These data are representative of two separate experiments. Arrows represent the day of 3/23 administration.

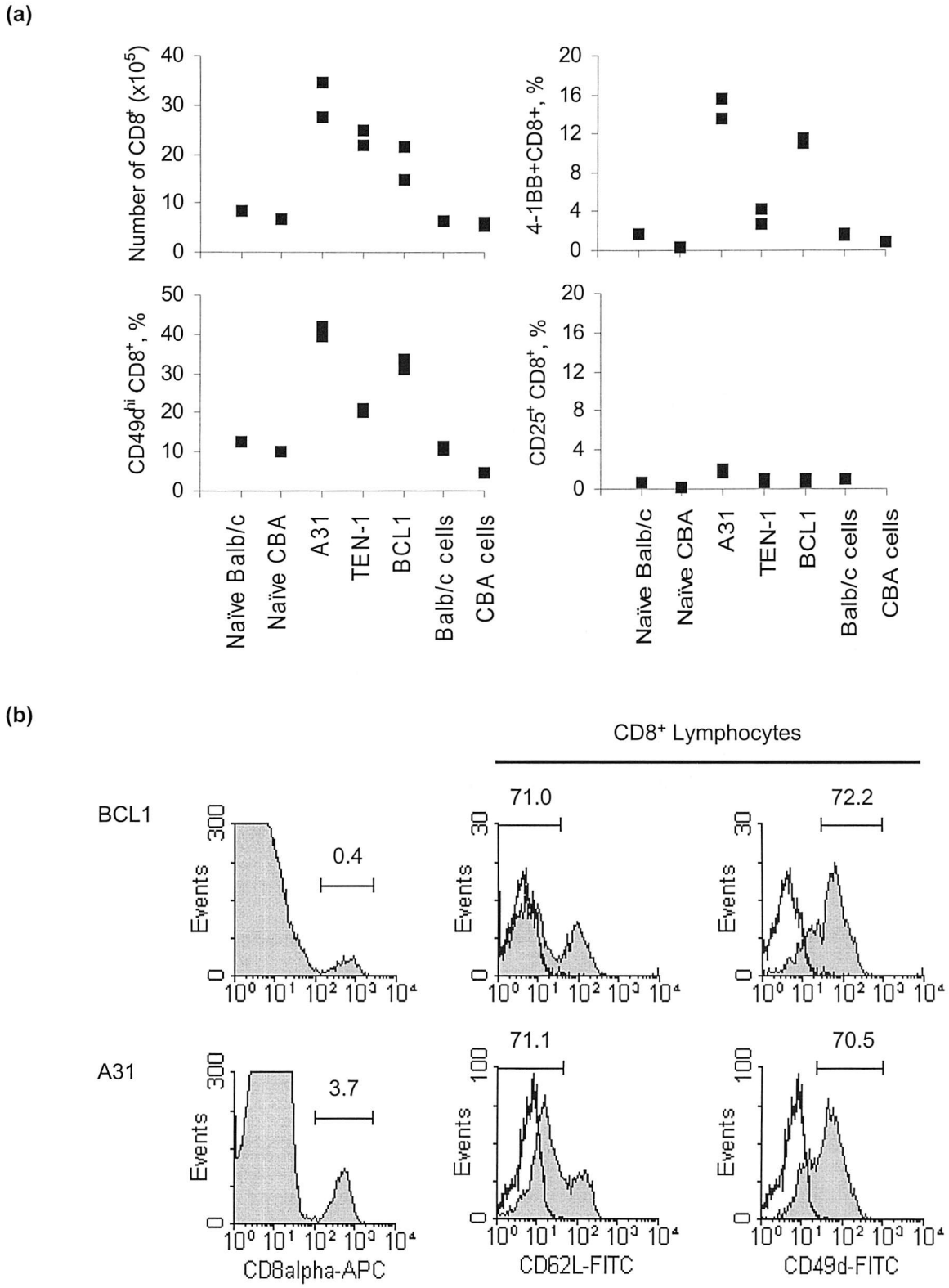


Figure 3.11. Endogenous CD8⁺ Lymphocyte Responsiveness to A31, BCL1 and TEN-1. Duplicate naive BALB/c mice received either $5x10^7$ TEN-1, BCL1, or BALB/c splenocytes, whereas naive CBA/H mice received either $5x10^7$ A31 or CBA/H splenocytes i.v. on day 0; splenocytes were harvested from recipient animals and naive littermates on day 8. Subsequently, splenocytes were stained with FITC-conjugated anti-4-1BB (LOB12), -CD49d, or -CD25, and PE-conjugated anti-CD8, and the percentage of CD8⁺ lymphocytes determined (a). Naive BALB/c or CBA/H mice received $5x10^7$ BCL1 or A31 i.v. respectively. At the terminal stage of disease (approximately 12 days following tumour inoculation), splenocytes were harvested and stained with APC-conjugated anti-CD8α as well as FITC-conjugated anti-CD49d, -CD62L (solid grey histograms), or an irrelevant mAb (open histograms); CD8⁺ lympocytes were gated and CD49d and CD62L expression assessed, (b).

administration of A31 or BCL1 (figure 3.11 (b)) – approximately 71 % of $CD8^+$ lymphocytes in such animals demonstrated an 'activated' $CD49d^{hi}$ $CD62L^{lo}$ phenotype.

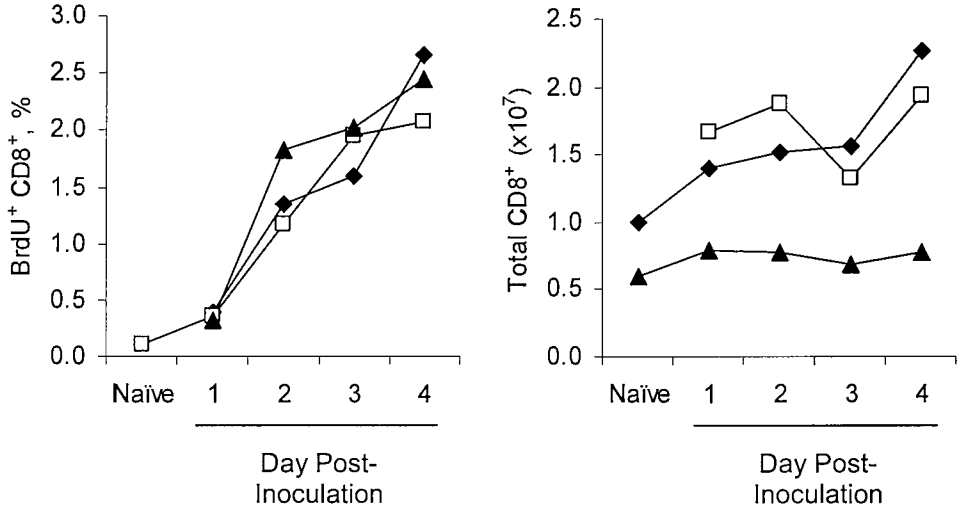


Figure 3.12.

Proliferation of $CD8^+$ Lymphocytes in Response to A31, BCL1 and TEN-1.

CBA/H mice received $5x10^7$ A31 i.v., while BALB/c mice received $5x10^7$ TEN-1 or BCL1 i.v. on day 0. On days 1 to 4 following tumour inoculation, animals received 2 mg BrdU i.p. 1 hour prior to the harvesting of splenocytes. Subsequently, splenocytes were stained with PE-conjugated anti-BrdU and APC-conjugated anti-CD8α. Values shown represent the mean calculated between duplicate animals.

Together, these data demonstrate that $CD8^+$ lymphocytes accumulate and differentiate, importantly, in a tumour-specific fashion and, therefore, that these tumours present antigens that are either unique or expressed at elevated levels with respect to normal tissue. Furthermore, this effect differs in magnitude in response to the different tumours studied here, with A31 inducing the greatest accumulation of $CD8^+$ lymphocytes, and TEN-1 inducing the least.

3.3.6. Anti-Tumour Cytotoxicity and T_H1 Phenotype of CTL

In order to confirm that anti-CD40 does indeed promote the production of T_H1-like class-I-restricted CTL in tumour-bearing animals, and to examine these cells further, we assessed

the cytotoxic capacity of these cells during immunotherapy using a standard chromium release assay in the presence or absence of blocking antibodies directed against, for example, class-I, CD8 and LFA-1. In addition, $CD8^+$ lymphocytes were removed from animals during immunotherapy and, subsequently, transcripts encoding proteins classically involved in T_H1 effector function (IFNγ and perforin) and differentiation (IL-12 receptor and T-bet) visualised. The production of T-bet (T box expressed in T-cells) transcripts was investigated as this transcription factor is induced by IFNγ and is responsible for mediating IFNγ expression by T-cells [468]. Furthermore, T-bet induces the expression of the IL-12 receptor β2 chain, thus promoting the differentiation of uncommitted naïve T-cells towards a T_H1 phenotype [469]. Indeed, ectopic expression of T-bet in $CD4^+$ T_H2 cells permits IFNγ expression following PMA-iononmycin stimulation simultaneously with T_H2 cytokine production (IL-4, -5, and -10) [469].

For the cytotoxicity assays, duplicate BALB/c mice were inoculated with $5x10^7$ BCL1 i.v. and treated 4 days later with 1 mg of 3/23 i.v.. Splenocytes were harvested on the indicated days following anti-CD40 administration, residual tumour removed by MACS with anti-Id-coated microbeads, and the cytotoxicity of the residual splenocytes assessed against piBCL1, A20 and YAC cells. When assessing the production of mRNA transcripts by $CD8^+$ lymphocytes during immunotherapy of BCL1, animals received lymphoma and anti-CD40 as above and, additionally, control animals received identical doses of either BCL1 or anti-CD40 alone. Individual animals were culled on the days indicated, $CD8^+$ splenocytes purified by MACS with anti-CD8-coated microbeads, and cDNA prepared from $1x10^5$ of these cells. As a positive control, cDNA was prepared from an anti-piBCL1 CTL line (Line A) 1 day following re-stimulation with tumour and IL-2 (see Chapter 5).

As can be seen in figure 3.13, only splenocytes taken from animals that had received both BCL1 and anti-CD40 developed noticeable cytotoxic activity directed against BCL1 after 120 hours following anti-CD40 administration, *i.e.*, at the maxima of the $CD8^+$ lymphocyte response in this experiment. Furthermore, this activity was completely blocked by anti-CD8, confirming the presence of $CD8^+$ CTL. As expected, anti-CD40 increased NK cell

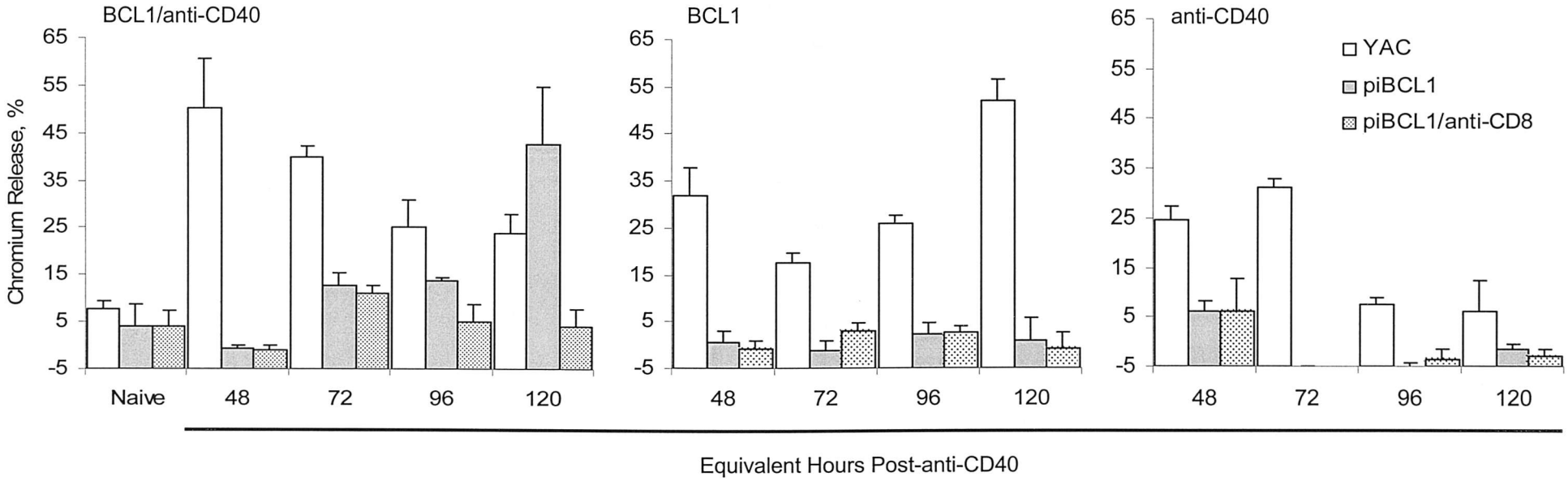


Figure 3.13. Acquisition of Anti-Tumour Cytotoxic Activity Over Time Following anti-CD40 Administration. Duplicate BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. On the equivalent of 48 to 120 hours following anti-CD40 administration, splenocytes were harvested, residual tumour removed by MACS directed against BCL1 idiotype, and the remaining splenocytes assayed for cytotoxic activity against piBCL1 (in the presence or absence of 50 µg/ml anti-CD8) and YAC cells at an effector-to-target ratio of 100:1. As a control, naïve splenocytes were also assayed for cytotoxic activity against the same targets after the equivalent manipulation. These data are represent one of two separate experiments. Values represent means from duplicate mice assayed in triplicate, while error bars represent standard deviation in these data.

activity as seen when comparing YAC cytolysis at 48 and 72 hours following anti-CD40 administration with cytolysis of YAC cells achieved when using splenocytes from naïve animals [470]. Interestingly, YAC cytolysis was also consistently elevated by the presence of tumour, as compared to naïve controls.

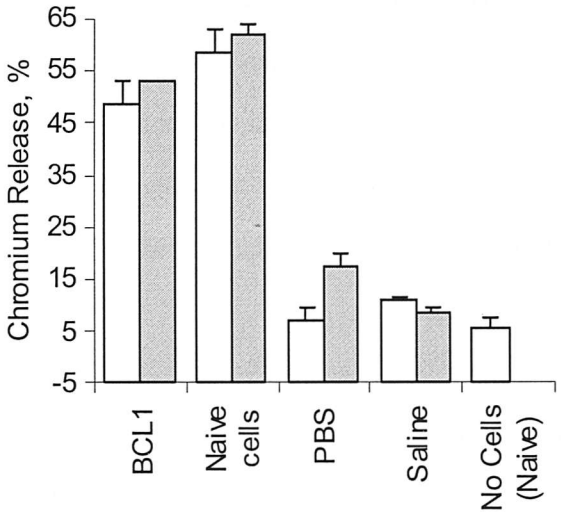


Figure 3.14. NK-mediated anti-BCL1 Cytotoxicity in Response to Cell Injection. Naive BALB/c mice received $5x10^7$ BCL1, $5x10^7$ naive BALB/c splenocytes, 200 μl PBS, or 200 μl sterile saLine i.v. on day 0. Splenocytes were harvested from recipient or naive animals on day 3 following inoculation and, subsequently, tumour cells removed using anti-BCL1-FITC MACS beads. The residual cells were assayed for cytotoxicity against YAC and piBCL1 cells by standard chromium release assay at an effector-to-target ratio of 100:1.

In order to assess whether the elevated cytolysis of YAC by NK cells resulted from the administration of cells (*i.e.*, tumour or splenocytes), and not from the injection of contaminants, duplicate BALB/c mice were injected with BCL1 or naïve splenocytes as above, or with PBS or sterile saline. As can be seen in figure 3.14, the injection of either naïve splenocytes or BCL1 resulted in the elevation of YAC cytolysis by NK cells as before, but this effect was not observed with PBS or sterile saline. These data therfore demonstrate that murine NK cells develop cytotoxic activity following intravenous injection of syngeneic cells, and not solely in response to tumour injection. As changes in the DC

(see Chapter 4) and $CD8^+$ lymphocyte compartments occur specifically in response to tumour administration, it seems unlikely therefore, that the activation of NK cells affects these responses.

Figure 3.15 (a) depicts the cytotoxic activity of splenocytes, following tumour removal, harvested from tumour-bearing animals 3 days after anti-CD40 administration (at the peak of the $CD8^+$ lymphocyte response in this experiment). Again, splenocytes assessed at this time-point following anti-CD40 injection displayed cytotoxic activity against piBCL1 and YAC, as well as A20 cells. The cytotoxic activity directed against piBCL1 was inhibited by the addition of blocking mAbs directed against Kd, ICAM-1, LFA-1, B7.1, and CD8 (figure 3.15 (b)).

These data therefore confirm that class-I-restricted $CD8^+$ lymphocytes develop anti-tumour CTL activity following exposure to both BCL1 and anti-CD40, and that this is directly detectable ex-vivo at the maxima of the CTL response. The elevated NK cell activity observed in response to anti-CD40 has been attributed to both IL-12 and IFNγ production from APCs following systemic administration of this mAb [470].

Panel (c) of figure 3.15 shows the expression of the mRNA species induced within $CD8^+$ lymphocytes in the treatment groups shown. Both $CD8^+$ lymphocytes extracted from animals undergoing immunotherapy with anti-CD40 (lane 2) and Line A CTL (lane 4; 'Line A' is an *in vitro* generated anti-piBCL1 $CD8^+$ T-cell line, see Chapter 5) expressed transcripts encoding IFNγ, and this was reflected in the elevated expression of mRNA encoding T-bet and both chains of the IL-12 receptor complex in these cells. Similarly, $CD8^+$ lymphocytes from animals undergoing immunotherapy expressed the highest number of transcripts encoding perforin, even when compared to *in vitro* generated CTL. Furthermore, $CD8^+$ lymphocytes from animals having received only BCL1 did not express transcripts encoding perforin, IFNγ, or either of the IL-12 receptor chains, but did contain mRNA encoding T-bet. Notably, transcripts encoding IL-10 were not detectable in any of the tested samples. Unexpectedly, $CD8^+$ lymphocytes from animals having received just

anti-CD40 appeared to express IFNγ, as well as low levels of mRNA encoding T-bet, perforin and the IL-12 receptor subunits, as compared to $CD8^+$ lymphocytes from animals undergoing immunotherapy. These data contained in figure 3.15 (c) do however require an addition control of mRNA extracted from naive animals before any firm conclusions can be drawn.

These data confirm that $CD8^+$ lymphocytes are induced to differentiate towards a T_H1 phenotype in the presence of tumour and anti-CD40. Furthermore, the acquisition of a $CD49d^{hi}CD62L^{lo}$ phenotype and limited proliferation of $CD8^+$ lymphocytes in response to BCL1 alone is not associated with the marked expression of mRNA encoding perforin or IFNγ (despite the expression of T-bet), and this may offer an explanation for the ineffectiveness of this response. Interestingly, this may not be the case in animals that have received anti-CD40 alone, but the finding that $CD8^+$ lymphocytes from these animals express IFNγ may be attributed to contamination by $CD8^-$ cells (see figure legend for percent purity). Notably, the lack of mRNA encoding IL-10 in lanes 1 and 2 suggests that no contaminating tumour was present at the time of cDNA preparation, as BCL1 is known to produce IL-10 both *in vivo* and *in vitro* ([2] and unpublished observations).

3.3.7. Generation of Tumour-specific T-cell Memory

In order to confirm that the CTL response evoked in response to anti-CD40 in the presence of tumour generates T-cell memory that protects against future tumour inoculation, we harvested splenocytes from animals in remission following immunotherapy of either A31 or BCL1 and, subsequently, assessed the production of IFNγ from these cells following co-culture with the appropriate tumour.

As can be seen in figure 3.16 (a) and (b), splenocytes taken from animals 20 to 60 days following immunotherapy produced IFNγ in response to the original tumour in a titratable fashion. Tumour-independent IFNγ production was observed from splenocytes taken from CBA/H mice having received A31 and anti-CD40 (panel (a)); this is shown by the production of approximately 1 ng/ml IFNγ in the absence of A31. Interestingly, far less

IFNγ production was observed from splenocytes taken from animals having undergone immunotherapy of BCL1 when these cells were co-cultured with A20, rather than piBCL1 (note different axis).

These data therefore confirm that anti-CD40 generates an anti-tumour T-cell response that results in the establishment of protective T-cell memory. Importantly, intracellular staining has confirmed that $CD8^+$ lymphocytes are responsible for IFNγ production in this system (French RR, unpublished observations). Interestingly, despite the fact that animals having undergone immunotherapy of BCL1 are refractory to challenge with A20 [460], these data suggest that only a minor proportion of BCL1-reactive T-cells are cross-reactive with A20, but this is sufficient to convey cross-protection *in vivo*.

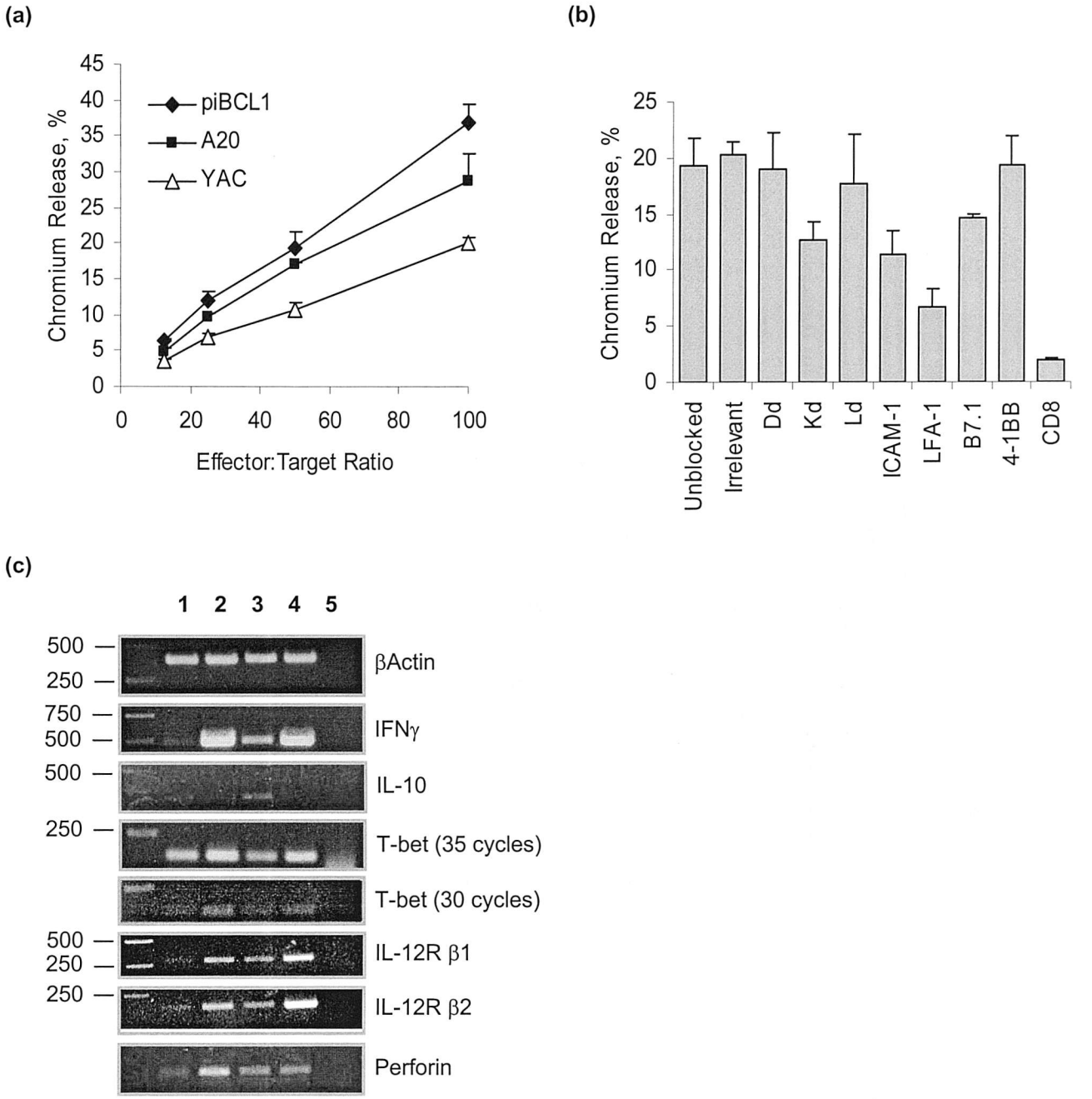


Figure 3.15. Acquisition of anti-Tumour Cytotoxic Activity and T_H1 Phenotype by $CD8^+$ Lymphocytes during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and 1 mg of anti-CD40 (3/23) i.v. on day 4. Three days following anti-CD40 administration, splenocytes were harvested from a single animal, residual tumour removed by MACS, and the remaining splenocytes assayed for cytotoxic activity against piBCL1, A20 and YAC cells, (a). Cytotoxicity (effector:target ratio = 50:1) directed against piBCL1 was assessed in the presence of mAbs (to a final concentration of 50 μg/ml) directed against the antigens shown, (b). Mean and standard deviation are based on triplicate assay measurements. These data are representative of three separate experiments. In (c), BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. Subsequently, $CD8^+$ lymphocytes were removed from splenocytes the equivalent of 3 days following anti-CD40 administration by MACS, cDNA prepared from $1x10^5$ of these cells and RT-PCR performed as described in Chapter 2. $CD8^+$ lymphocyte purity was 94.6, 95.8, and 77.2 % for BCL1-, BCL1/anti-CD40-, and anti-CD40-treated mice respectively. Lanes: 1, BCL1 alone; 2, BCL1/anti-CD40; 3, anti-CD40; 4, CTL Line A (an anti-piBCL1 CTL Line generated *in vitro*; day 1 post-restimulation – see Chapter 6); 5, no cDNA.

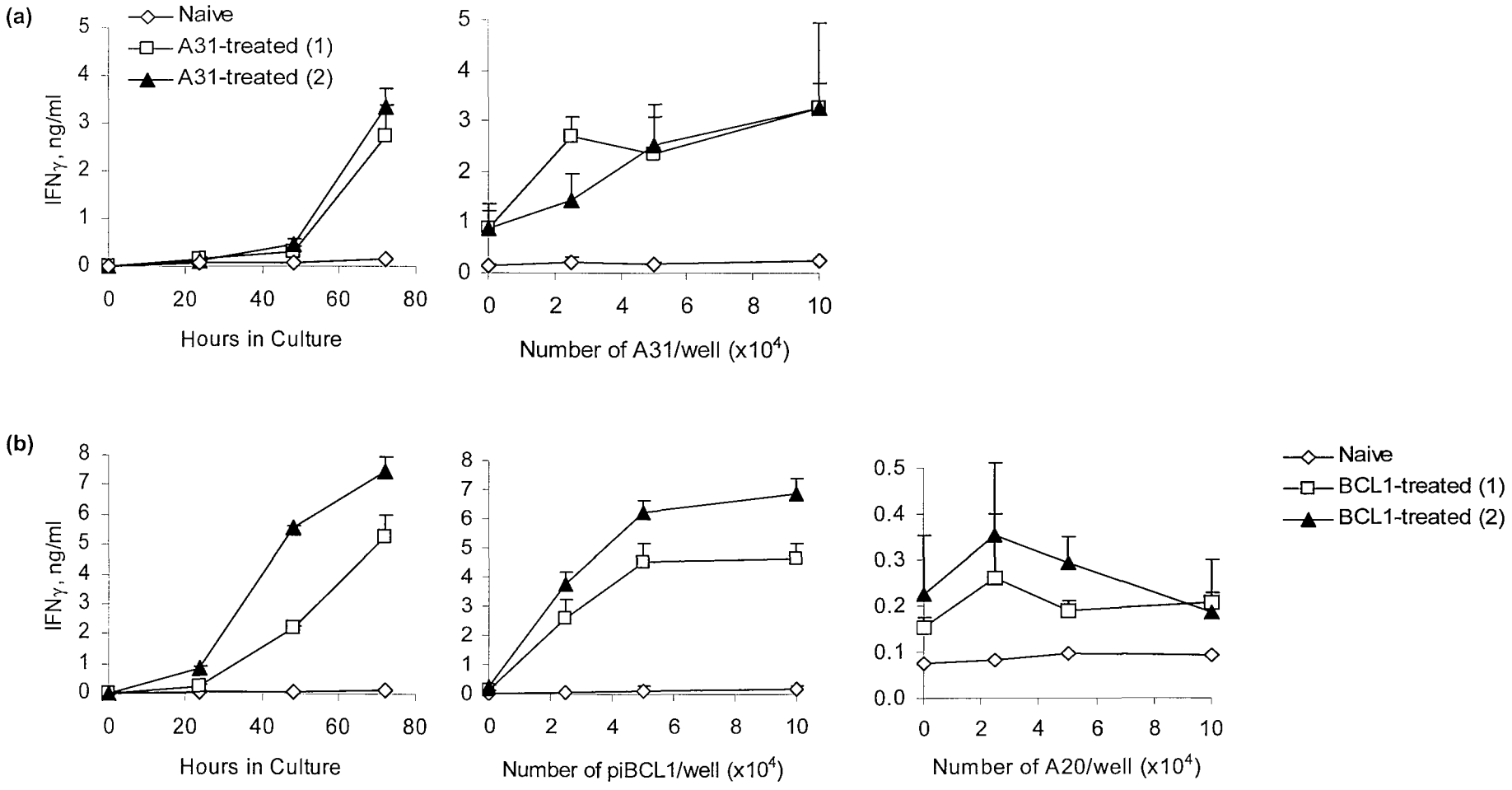


Figure 3.16. Anti-CD40 Generates Tumour-specific CTL Memory. CBA/H or BALB/c mice received $5x10^7$ A31 (a) or BCL1 (b) lymphoma cells i.v. on day 0 respectively, followed by 1 mg of anti-CD40 (3/23) i.v. on day 4. Between days 20 and 60, splenocytes were harvested from single animals in each treatment group, 10^6 co-cultured overnight with the indicated number of irradiated tumour cells, and IFNγ production estimated by standard ELISA of culture supernatents. Splenocytes from naïve and anti-CD40-treated animals were used as controls where shown. The kinetics of IFNγ production was assayed by using 10^5 irradiated tumour cells, whereas the dose response was assayed after 72 hours in culture. These data are representative of numerous separate experiments performed in this laboratory. (1) and (2) represent duplicate animals in the indicated groups. Error bars represent triplicate cultures and assay measurements.

3.3.8. Expression of 4-1BB on anti-Tumour CTL During Immunotherapy

In order to address the mechanism by which anti-CD40 provides proliferative and/or survival signals to anti-tumour $CD8^+$ lymphocytes, we assessed the expression of 4-1BB on $CD8^+$ lymphocytes during immunotherapy of BCL1 and A31. We considered 4-1BB to be potentially important in generating anti-tumour CTL responses during anti-CD40-mediated immunotherapy as, firstly, 4-1BB has been reported to preferentially provide co-stimulatory signals to $CD8^+$ lymphocytes (consistent with the more rapid and elevated expression of 4-1BB on $CD8^+$ lymphocytes over $CD4^+$ lymphocytes) [5, 471], elevating proliferation and survival by positively regulating IL-2 and Bcl-xL expression respectively [118]. Secondly, the expression of 4-1BBL, as well as the B7 family of co-stimulatory ligands, is elevated on DCs following stimulation via CD40 [108, 117]. Thirdly, 4-1BB expression on $CD8^+$ lymphocytes is enhanced by CD28-mediated co-stimulation under conditions of sub-optimal anti-CD3 stimulation, such as that provided by a tumour [108, 166]. Finally, agonist mAbs directed against 4-1BB have been shown in this laboratory to induce regression of both BCL1 and A31 [5].

As shown in figure 3.17 (a), 4-1BB is not expressed on $CD8^+$ lymphocytes taken from naïve animals, whereas, interestingly, BCL1 alone induced 4-1BB expression on a small proportion of $CD8^+$ lymphocytes; panel (b) shows that approximately 10 % of $CD8^+$ lymphocytes expressed 4-1BB by the equivalent of 4 days following anti-CD40 (*i.e.*, day 8 following tumour inoculation), and that these cells accumulated slightly over time. Nonetheless, administration of anti-CD40 to tumour-bearing mice increased the proportion of $CD8^+$ lymphocytes expressing 4-1BB, and this occurred simultaneously with the usual profound $CD8^+$ lymphocyte expansion. Importantly, administration of anti-CD40 alone did not induce 4-1BB expression on $CD8^+$ lymphocytes and was associated with only a minor $CD8^+$ lymphocyte expansion. These data were confirmed in the A31 lymphoma model - figure 3.18 (a) shows data demonstrating that 4-1BB is expressed on $CD8^+$ lymphocytes during immunotherapy of A31 as well as BCL1.

In order to demonstrate that the 4-1BB$^+$ CD8$^+$ lymphocytes observed in the above experiments were also the CD8$^+$ lymphocytes induced to proliferate in animals undergoing immunotherapy, BrdU incorporation was used (as in section 3.3.3) to visualise proliferative CD8$^+$ lymphocytes ex-vivo, and these cells were co-stained for 4-1BB expression. As shown in figure 3.18 (b), the majority (63 %) of 4-1BB$^+$ CD8$^+$ lymphocytes in animals having received both BCL1 and anti-CD40 were BrdU$^+$, whereas only a minority of 4-1BB-negative CD8$^+$ lymphocytes were shown to have incorporated BrdU (21 %). It is likely that all these BrdU$^+$ T-cells are 4-1BB$^+$, but 4-1BB is mildly soluble in Tween-20 (used during staining for incorporated BrdU), thus only allowing the accurate gating of cells expressing very high quantities of 4-1BB. This difficulty could not be overcome by cross-linking 4-1BB following staining with the primary mAb (data not shown). An alternative explanation for this data may be that several rounds of lymphocyte division could be required in order to allow 4-1BB expression (*c.f.*, CD49d, [317]).

Together, these data indicate that BCL1 is able to induce the expression of 4-1BB by CD8$^+$ lymphocytes, but the proportion of CD8$^+$ lymphocytes expressing this molecule is increased following administration of anti-CD40. This increase in the proportion of CD8$^+$ lymphocytes expressing 4-1BB following administration of anti-CD40 is also observed in the A31 lymphoma model (figure 3.18 (a), and data not shown) and may be due to increased B7 expression on, for example, DCs following anti-CD40 administration that, subsequently, results in the increased expression of 4-1BB on minimally stimulated tumour-specific CD8$^+$ lymphocytes (see discussion, section 3.4.). The expression of 4-1BB on proliferative CD8$^+$ lymphocytes during immunotherapy suggests that expression of this molecule may be a pre-requisite for, or at least contribute to, the clonal expansion of CD8$^+$ lymphocytes that then develop into anti-tumour CTL during anti-CD40-mediated immunotherapy.

Thus, in order to demonstrate that the observed 4-1BB$^+$ CD8$^+$ lymphocytes do indeed develop into anti-tumour CTL, we separated splenocytes from animals undergoing immunotherapy into 4-1BB$^+$ and 4-1BB$^-$ fractions using MACS (figure 3.19 (a)) and

assessed the anti-tumour cytotoxicity of each fraction. As can be seen in figure 3.19 (b), as previously demonstrated, unfractionated splenocytes from animals undergoing immunotherapy of BCL1 (3 days following anti-CD40 administration) displayed cytotoxic activity against piBCL1 and, to a lesser extent, YAC cells. However, when 4-1BB$^+$ cells were removed from these splenocytes, the anti-piBCL1 activity was completely abolished. Furthermore, the 4-1BB$^+$ splenocytes lysed piBCL1 with greater efficiency than the unfractionated population, presumably because of an enrichment of anti-tumour CTL. These data therefore conclusively demonstrate that anti-tumour CTL elicited by anti-CD40 in BCL1-bearing animals express 4-1BB.

3.3.9. Expression of 4-1BB on CD8$^+$ lymphocytes in Response to Tumour Alone

As 4-1BB expression on CD8$^+$ lymphocytes following administration of anti-CD40 to tumour-bearing animals is associated with the acquisition of anti-tumour cytotoxicity, and interactions via CD28 act to amplify 4-1BB expression induced by TCR-mediated signalling [5, 108], we chose to assess whether or not the expression of 4-1BB on CD8$^+$ lymphocytes following exposure to BCL1 or A31 was inhibited by the presence of blocking mAbs to B7.1 or B7.2. As shown in figure 3.20 (a), 4-1BB expression is induced on approximately 10 % of CD8$^+$ lymphocytes 8 days following inoculation with BCL1, and this expression is completely abrogated by the presence of mAbs directed against B7.1 and B7.2 (panel (a) and (b)). Nonetheless, CD8$^+$ lymphocyte expansion in response to BCL1 alone was only partly reduced in the presence of blocking mAbs to B7.1 and B7.2 (figure 3.20 (b)). In a similar fashion, 4-1BB expression was induced upon CD8$^+$ lymphocytes following inoculation of CBA/H mice with A31, with this effect being inhibited in the presence of anti-B7 mAb (figure A1.3. (b)). In this case, the expansion of CD8$^+$ lymphocytes in response to A31 was effectively inhibited by anti-B7 mAb (figure 3.20. (b)).

Thus, the expression of 4-1BB on CD8$^+$ lymphocytes following exposure to BCL1 and A31 is dependent on interactions via CD28, thereby suggesting that TAA(s) present on these tumours act as weak agonists that rely on amplification of TCR-mediated signals to induce T-cell differentiation. Hence, the elevation of B7 expression by DCs following

administration of anti-CD40 is likely to elevate the proportion of $CD8^+$ T-cells expressing 4-1BB and aid in the generation of an anti-tumour CTL population.

3.3.10. 4-1BB-4-1BBL Interactions during CTL Expansion and Tumour Regression

Because of the expression of 4-1BB on anti-tumour CTL generated during anti-CD40-induced immunotherapy, and because of the therapeutic activity of anti-4-1BB mAbs for the treatment of BCL1 and A31, we wished to assess the requirement for co-stimulation via 4-1BB during CTL expansion and, ultimately, tumour eradication induced by anti-CD40. A construct consisting of murine 4-1BB extracellular domain and the constant region of human IgG (4-1BB-hFc; see Chapter 2) or the anti-4-1BBL mAb 3H3 were used *in vivo* to inhibit the interaction of 4-1BB with its endogenous ligand.

Firstly, duplicate BALB/c mice received $5x10^7$ BCL1 i.v. followed 4 days later, where appropriate, by 1 mg anti-CD40 i.v.. Animals having anti-CD40 also received either 200 µg of 4-1BB-hFc fusion protein i.p on days 5, 6 and 7 following tumour inoculation, or the equivalent dosage of human IgG as a control. BrdU was administered as in section 3.3.

As shown in figure 3.21 (a), as compared to animals that had received tumour alone, the total number of $4\text{-}1BB^+$ $CD8^+$ lymphocytes (*i.e.*, the population of lymphocytes containing anti-tumour CTL) was increased in animals undergoing immunotherapy by 3 days following anti-CD40 administration whether 4-1BB-hFc was present or not. However, by day 4 following anti-CD40 the number of $4\text{-}1BB^+$ $CD8^+$ lymphocytes was almost four-fold higher in animals that had received hIgG compared to those that had received the 4-1BB-hFc construct. These data were reflected in the number of $CD8^+$ lymphocytes in S phase in each of the treatment groups (panel (b)), but less so in the percentage of proliferative $CD8^+$ lymphocytes (panel (c)).

These data therefore suggest that, on the proviso that the 4-1BB-hFc construct completely abolishes the interaction of 4-1BB with 4-1BBL, anti-CD40 is able to induce the proliferation and accumulation of $4\text{-}1BB^+$ $CD8^+$ lymphocytes firstly in a 4-1BB-independent

manner, but only maximally if 4-1BB-4-1BBL interactions are intact. These data are therefore consistent with the observation that CD28- and 4-1BB-mediated co-stimulation operate during temporally distinct phases of T-cell differentiation, with CD28 initiating T-cell expansion prior to 4-1BB expression [108, 169]. Moreover, the fact that this effect is predominantly observed in the number of $CD8^+$ lymphocytes accumulated, rather than the percentage of proliferative cells, suggest that this may be achieved by enhancing survival.

In order to assess how these observations impact upon tumour eradication induced by anti-CD40, we administered either the 4-1BB-hFc construct or an anti-4-1BBL mAb to animals during immunotherapy of BCL1. As shown in figure 3.22, our initial experiment demonstrated that two tumour-bearing animals having received both anti-CD40 and anti-4-1BBL where unable to overcome the lymphoma and were culled on day 18 along with animals that had received tumour alone; the remaining animals in this group succumbed to the lymphoma over the course of the next 40 days, while animals that had received anti-CD40, but not anti-4-1BBL, survived past 100 days ($p<0.01$ BCL1/anti-CD40/anti-4-1BBL versus BCL1/anti-CD40). In our second experiment, similarly to the first, 3 out of 5 tumour-bearing animals that received anti-CD40 and, either, anti-4-1BBL or 4-1BB-hFc, had to be culled alongside animals that had received tumour alone. However, unlike our preliminary experiment, the surviving animals from these groups continued past 90 days, with the final 2 animals that had received anti-CD40 and anti-4-1BBL being culled on days 92 and 105 ($p<0.01$ BCL1/anti-CD40/anti-4-1BBL versus BCL1/anti-CD40).

These data therefore support the hypothesis that anti-CD40 is able to promote tumour-specific $CD8^+$ lymphocyte expansion in, firstly, a 4-1BB-independent manner (likely to be mediated by CD28) and, secondly, a 4-1BB-dependent manner [108, 169]; and that blocking 4-1BB-4-1BBL interactions prevents the profound $CD8^+$ lymphocyte expansion that is required for clearance of a large quantity of lymphoma. Furthermore, inhibiting interactions between 4-1BB and its ligand appears to hinder the generation of T-cell memory in this system, thereby accounting for the resurgence of tumour in these animals over time; this point is discussed below.

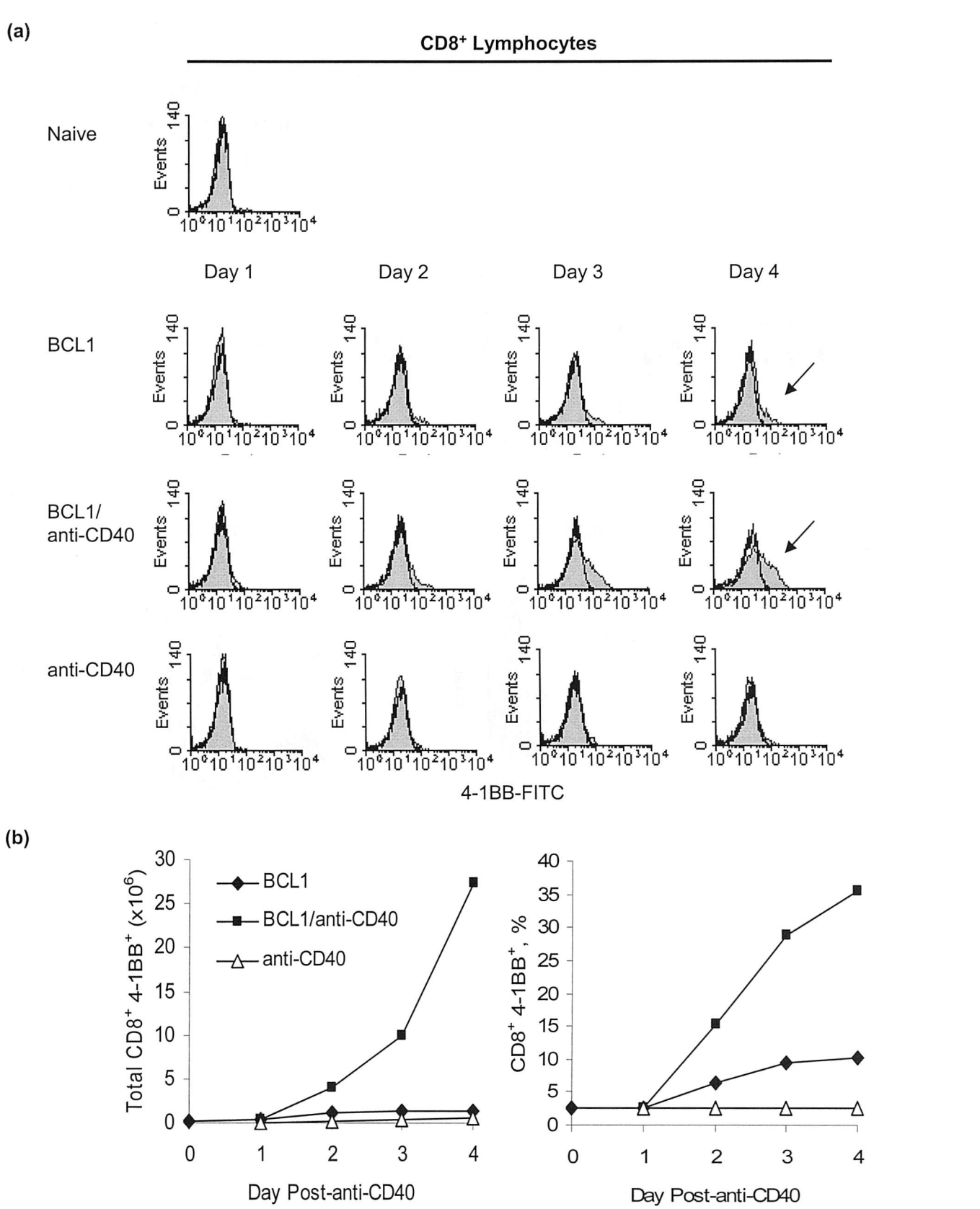


Figure 3.17. Elevated Expression of 4-1BB on CD8⁺ Lymphoyctes following anti-CD40 Administration. BALB/c mice received 5x10^7 BCL1 lymphoma cells i.v. on day 0 and/or 1 mg of anti-CD40 (3/23) i.v. on day 4. On days 1 to 4 following anti-CD40 administration, splenocytes were harvested from single animals in each treatment group and stained with FITC-conjugated anti-4-1BB (LOB12; solid grey histograms) or an irrelevant mAb (open histograms) and PE-conjugated anti-CD8, (a). Arrows highlight 4-1BB expression. These data are summarised in (b). These data are representative at two separate experiments.

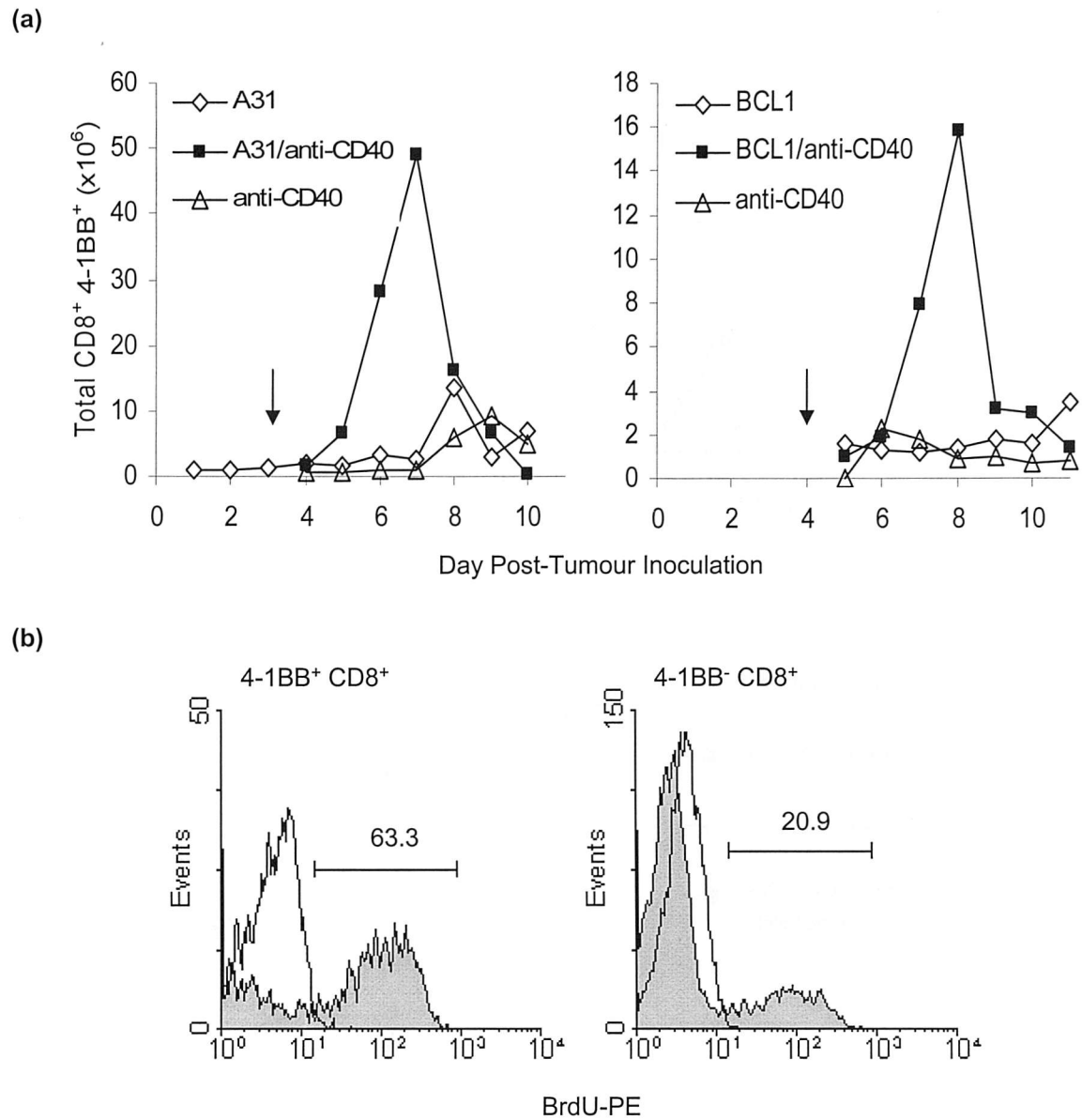


Figure 3.18. Expansion of 4-1BB$^+$ CTL During Immunotherapy of A31 and BCL1. CBA/H or BALB/c mice received 5x10^7 A31 or BCL1 lymphoma cells i.v. on day 0 respectively, and/or 1 mg of anti-CD40 (3/23) on the days shown by arrows. On the indicated days following tumour inoculation, splenocytes were harvested from single animals in each treatment group and stained with FITC-conjugated anti-4-1BB (LOB12 for BCL1, 2A1 for A31) and PE-conjugated anti-CD8, (a). In (b), BALB/c mice received BCL1 and anti-CD40 as above; three days following anti-CD40 administration, animals received BrdU and, one hour later, splenocytes were harvested and CD8$^+$ lymphocytes stained for incorporated BrdU as described in Chapter 2. Additionally, splenocytes were stained with FITC-conjugated anti-4-1BB. Solid grey histograms represent staining with PE-conjugated anti-BrdU, whereas open histograms are irrelevant binding controls. Marker values represent the percentage of either 4-1BB-positive or –negative CD8$^+$ lymphocytes having incorporated BrdU. These data are representative of separate experiments performed three times.

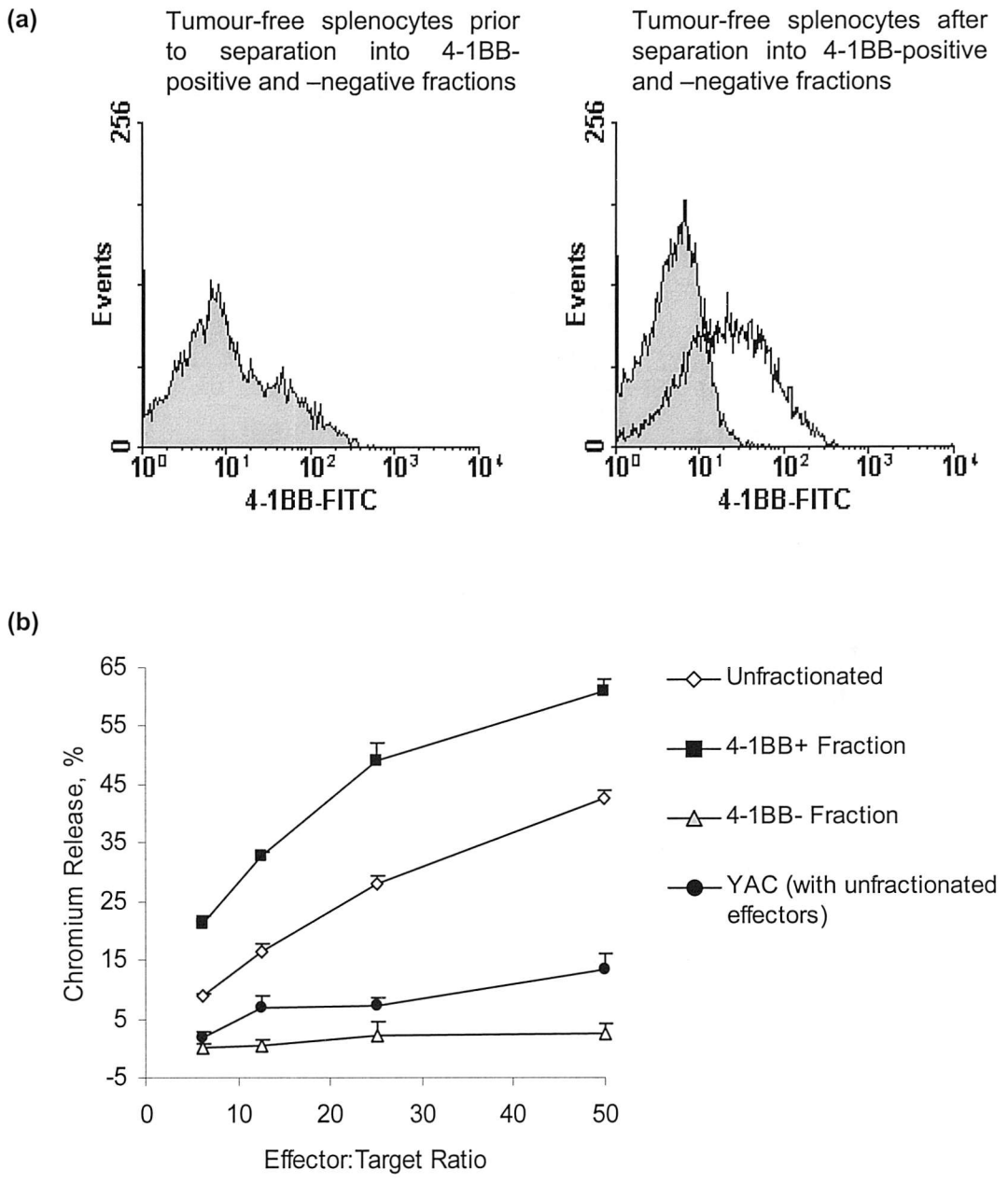


Figure 3.19. 4-1BB$^+$ CD8$^+$ Lymphocytes Contain the anti-Tumour CTL Generated during Immunotherapy BCL1. BALB/c mice received 5x10^7 BCL1 lymphoma cells i.v. on day 0 and 1 mg of anti-CD40 (3/23) on day 4. Three days following anti-CD40 administration, splenocytes were harvested from a single animal, residual tumour removed by MACS directed against BCL1 idiotype using a MACS depletion column, and the remaining splenocytes separated by further MACS directed against 4-1BB (again, using a depletion column), (a). The separated tumour-free 4-1BB$^+$ and 4-1BB$^-$ fractions were assayed for cytotoxic activity against piBCL1, with unfractionated tumour-free splenocytes being additionally assayed against YAC cells, (b). Mean and standard deviation are representative of triplicate assay measurements. These data are representative of two separate experiments.

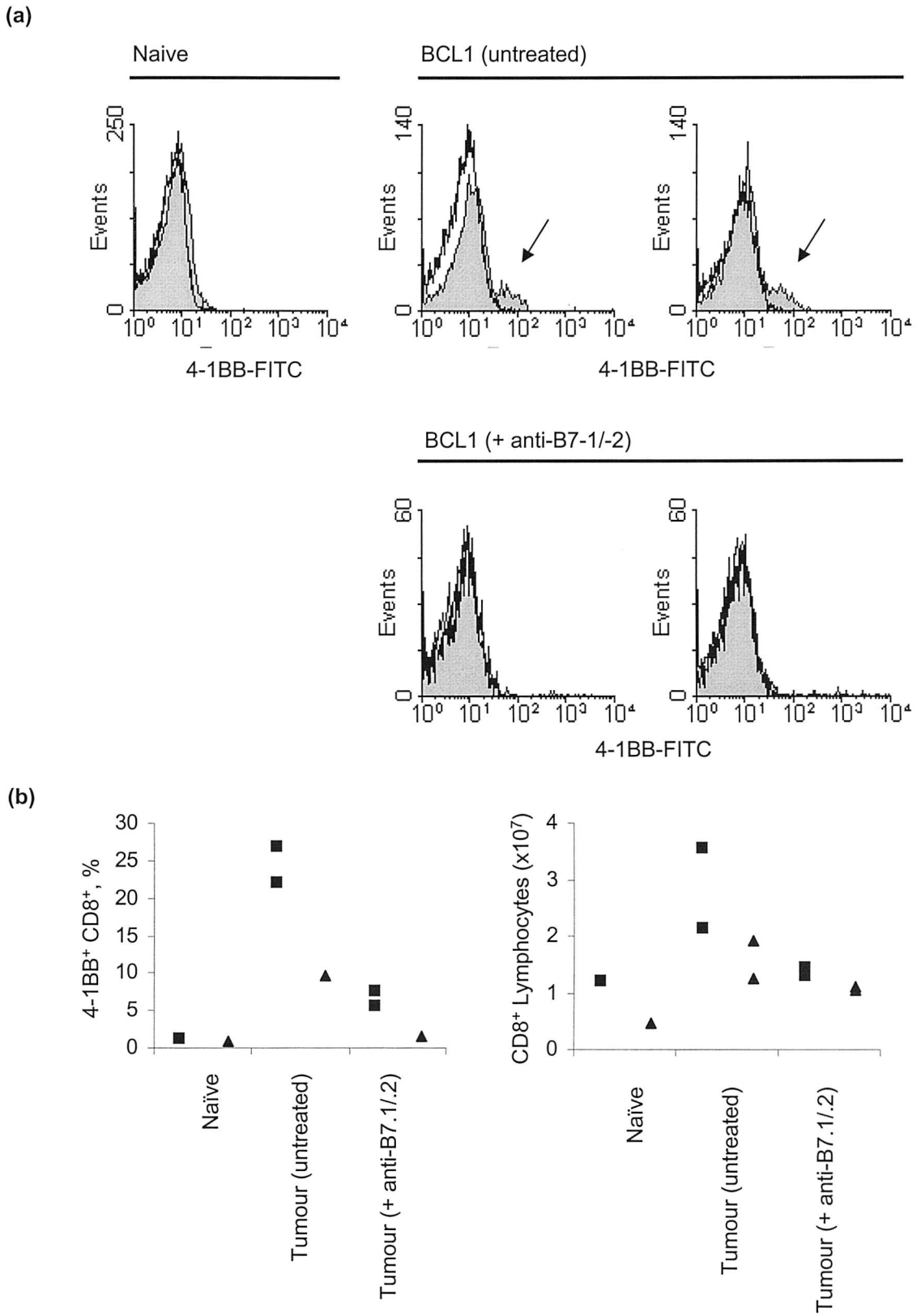


Figure 3.20. Expression of 4-1BB on CD8⁺ Lymphocytes in Response to Lymphoma Alone is Blocked by anti-B7. Duplicate BALB/c mice received $5x10^7$ BCL1 on day 0 and splenocytes were subsequently harvested from recipient animals and naive littermates on day 8. Animals receiving anti-B7.1 and anti-B7.2 had 0.5 mg of each mAb i.p. daily, until day 8. Splenocytes were stained with FITC-conjugated anti-4-1BB (LOB12; solid grey histograms) or an irrelevant mAb (open histograms) and PE-conjugated anti-CD8; histograms represent the expression of 4-1BB on CD8⁺ lymphocytes, (a). Similarly, in (b), either BALB/c or CBA/H mice received $5x10^7$ BCL1 or A31 i.v. respectively; this was followed by administration of anti-B7.1/anti-B7.2 and sample processing as in (a). The total number of CD8⁺ lymphocytes and the percentage of CD8⁺ lymphocytes expressing 4-1BB in each group is shown.

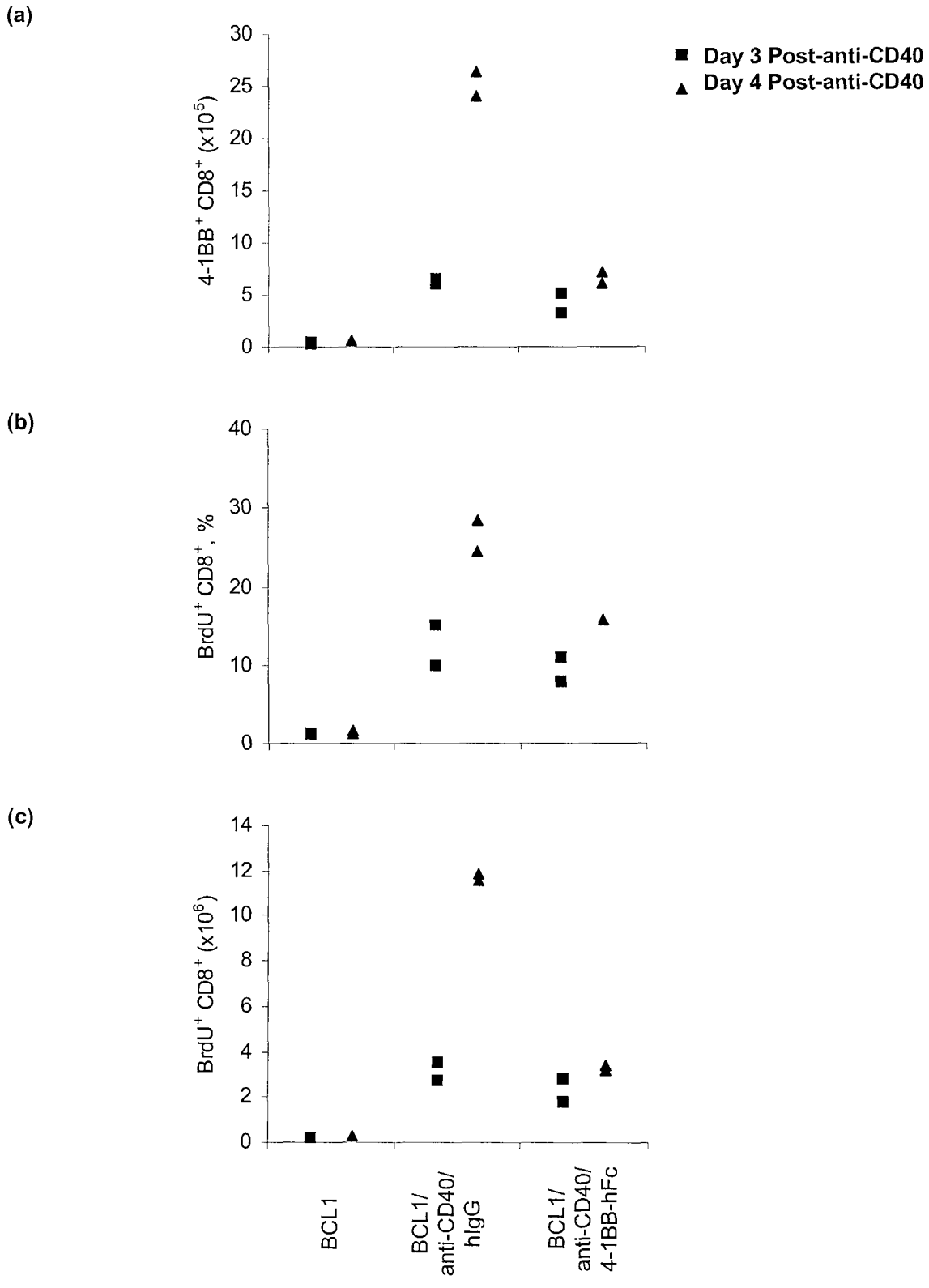


Figure 3.21. Blocking 4-1BB-4-1BBL Interactions Reduces CTL Expansion during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and, where appropriate, 1 mg of anti-CD40 (3/23) on day 4. Additionally, where indicated, animals received 200 μg of 4-1BB-hFc construct or human IgG i.p. on days 5, 6 and 7. The equivalent of three or four days following anti-CD40 administration (days 7 and 8 following tumour inoculation), duplicate animals received 2 mg of BrdU i.p. one hour before splenocytes were harvested and stained for BrdU incorporation as described in Chapter 2. These data are based on two animals per group and are representative of two separate experiments.

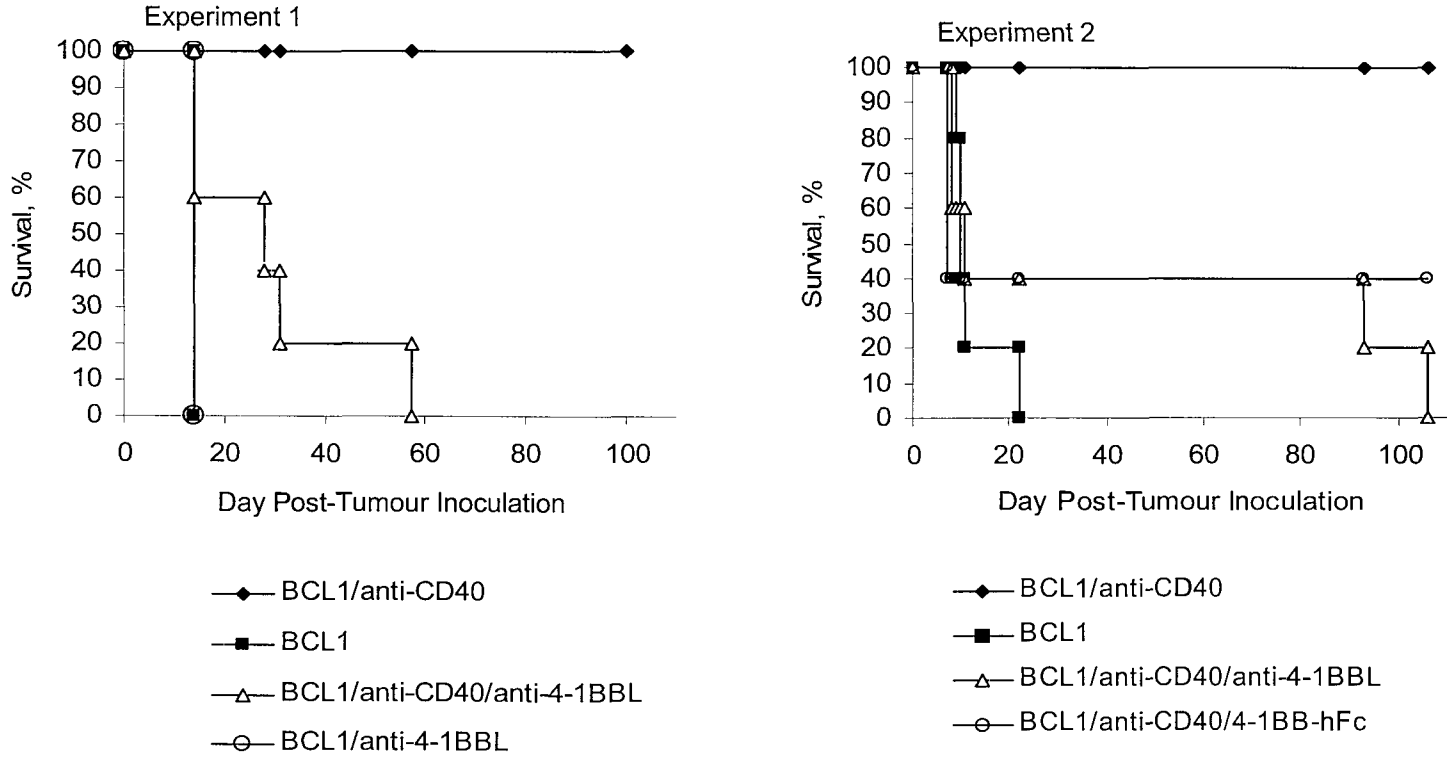


Figure 3.22. Blocking 4-1BB-4-1BBL Interactions Blocks the Efficacy of anti-CD40-mediated Immunotherapy of BCL1. In experiment 1, groups of 5 BALB/c mice received $5x10^7$ BCL1 lymphoma cells i.v. on day 0 and, where appropriate, 1 mg of anti-CD40 (3/23) on day 4. Additionally, where indicated, animals received 200 µg of anti-4-1BBL i.p. on day 4. In experiment 2, animals received tumour and anti-CD40 as in experiment 1, but only 160 µg of anti-4-1BBL was used on day 4. Additionally, where indicated, animals received 200 µg of 4-1BB-hFc i.p. on days 5, 6 and 7.

3.4. Discussion

Together, these data build on published work and demonstrate that anti-CD40 bolsters a pre-existing T-cell response directed against these model lymphomas, thereby resulting in the generation of class-I-restricted $CD8^+$ anti-tumour CTL that affect tumour eradication and, subsequently, provide protection against potential endogenous or exogenous tumour challenge.

Anti-tumour CTL develop typically within 3 days of anti-CD40 administration and are of a T_H1 phenotype as denoted by the production of mRNA transcripts encoding IFNγ, T-bet, perforin and the IL-12 receptor. $CD8^+$ lymphocytes clearly proliferate in response to BCL1, and to a greater extent A31, but these data suggest that only limited differentiation of these lymphocytes occurs as competence for migration towards sites of inflammation is achieved without, at least in the case of BCL1-responsive $CD8^+$ lymphocytes, the expression of effector molecules such as IFNγ. Thus, these data are consistent with the hypothesis that $CD8^+$ lymphocytes divide in response to these lymphomas, but daughter cells do not survive effectively and, hence, effector functions can not be evolved. Hypothetically, effector function may be generated at the late stages of tumour development, but at this stage CTL may be prone to AICD due to the prevalence of antigen, or, tumour escape mechanisms may have evolved.

As is discussed primarily in Chapter 6, these data additionally support the hypothesis that co-stimulation of $CD8^+$ lymphocytes via 4-1BB plays a role in allowing increased proliferation and survival of anti-tumour $CD8^+$ lymphocytes following anti-CD40 administration, *i.e.*, via the induction of 4-1BBL on, for example, DCs or the lymphoma itself. Nonetheless, this role is dispensable in some cases, probably when tumour eradication occurs at relatively low tumour burdens and, thus, when less profound CTL expansion (mediated by CD28) is required in order to overcome the tumour, *c.f.*, production of neutralizing antibody against LCMV is independent of 4-1BBL, but becomes so at low viral titers [159, 163].

Notably, even if animals were able to clear the primary tumour under conditions were 4-1BB-4-1BBL interactions were inhibited, often the animals relapsed some days later. As stated in Chapter 1, Bertram *et al.* demonstrated that interactions via 4-1BB sustain influenza-specific CTL late in the primary response, and that 4-1BB-mediated co-stimulation is required for the optimal generation of a memory pool of $CD8^+$ lymphocytes in this system; either by providing an elevated primary clonal burst size and by effecting survival within the memory pool [169]. Thus, even though CD28-mediated clonal expansion of $CD8^+$ lymphocytes may affect primary lymphoma eradication in some cases, co-stimulation via 4-1BB is required for the generation of optimal tumour-specific T-cell memory directed against BCL1.

The expression of 4-1BB on $CD8^+$ lymphocytes in response to BCL1 and A31 (figure 3.19) alone supports the notion that anti-4-1BB mAb may operate by providing direct co-stimulation to antigen-experienced $CD8^+$ lymphocytes. Indeed, $CD4^+$ lymphocytes also express 4-1BB in response to these lymphomas in the absence of anti-CD40 (Patrick C, unpublished observations), and protection against A31 mediated by anti-4-1BB is dependent on both $CD4^+$ and $CD8^+$ cells [5]. Nonetheless, this does not negate the possibility that anti-4-1BB acts to stimulate DCs which subsequently boost the pre-existing anti-tumour T-cell response by providing, for example, IL-6, IL-12 and B7-mediated co-stimulation [116, 117].

4.1. Introduction.

Dendritic cells are critical in the initiation of both T_H1 and T_H2 responses [12]. Direct binding of PAMPs or the indirect sensing of 'danger' signals evoked by pathogens (such as IFNα) initiates a terminal process of DC maturation that is characterised by the production of cytokines and co-stimulatory molecules, and the migration of DCs to the T-cell zones of the secondary lymphoid organs [199, 208]. In the spleen, this involves the migration of resident DCs from the marginal zone into the PALS, and occurs simultaneously with the acquisition of a $CD8\alpha^+$ $DEC205^+$ phenotype [199]. This observation accounts for why $CD8\alpha^+$ DCs are found to produce more IFNγ and IL-12 than their $CD8\alpha^-$ counterparts, as well as being the DCs that are often shown to have endocytosed and cross-presented antigen [57, 203, 472].

The ligation of CD40 on DCs was initially thought to activate DC maturation in a de novo fashion, but it is now appreciated that signals via CD40 on DCs act to amplify pre-existing responses initated by PAMPs or endogenous danger signals [120]. Indeed, CD40 ligation on splenic DCs *in vitro* can lead to the release of classically pro-inflammatory or anti-inflammatory cytokines, depending upon the PAMP administered, *e.g.*, CD40 ligation preceded by exposure to yeast extract results in IL-10 production, whereas IL-12 is produced if DCs are exposed to STAg and functional CD40L [121]. In this way, DCs act to translate the pathogen threat encountered into an appropriate T-cell response.

We thus postulated that anti-CD40 may be acting to amplify DC maturation following exposure to lymphoma-derived/evoked signals, thereby replacing the need for endogenous CD40L (typically provided by antigen-experienced $CD4^+$ lymphocytes) and boosting the observed endogenous T-cell response. This hypothesis was strengthened by the finding that anti-CD40 is able to replace the need for T-help during the cross-priming of CTL in B-cell deficient animals [419]. Thus, in order to test this hypothesis, we assessed the surface

phenotype of DCs during immunotherapy of BCL1 with anti-CD40 mAb, and used these data as an indication of the maturation state of the DCs.

4.2. Materials and Methods.

Unless otherwise indicated, BCL1 lymphoma was isolated from splenocytes of animals at the terminal stage of disease as described in Chapter 2. $5x10^7$ lymphoma cells were injected i.v. followed by 1 mg of anti-CD40 when tumour represented between 2 and 10 % of the splenic lymphocytes; typically, on day 4 after primary tumour challenge.

Prior to detection of surface antigens by fluorescenated mAbs, splenic fragments were digested in CM containing 1mg/ml collagenase D and 0.05 mg/ml DNAse I for two rounds of digestion, each of 20 minutes at 37 °C with gently shaking. This protocol was adapted from published work [120] and was used in order to strip DCs from collagen filaments, hence, allowing their detection by FACS. Surface expression of antigens was assessed by FACS analysis – briefly, $1x10^6$ splenocytes were incubated with 1 µg of fluorescenated mAb at 4°C for 15 minutes, followed by washing in PBS-BSA-Azide prior to acquisition on a FACScan or FACSCalibur. In order to allow live/dead cell discrimination without forward/side scatter gating (DCs and macrophages are too amorphous to allow this), 7AAD at a final concentration of 2 µg/ml was added each sample 15 minutes before acquisition.

Purification of $CD11c^+$ cells was performed by MACS - samples were labelled with PE-conjugated anti-CD11c as for FACS, washed in CM at 37 °C and bound to anti-PE microbeads as described in Chapter 2. Samples were then washed in PBS/BSA/EDTA and separated on pre-equilibriated LS columns. Subsequently, these columns were extensively washed on the magnet with PBS/BSA/EDTA in order to remove any non-specifically bound cells, and the $CD11c^+$ cells removed by forcing PBS/BSA/EDTA through the column once off the magnet. $CD11c^+$ fractions were typically > 70% pure and were subsequently assessed for surface antigen expression by using FITC-conjugated mAbs as described above.

4.3. Results.

4.3.1. Identification of Splenic DCs by FACS

Firstly we wished to develop a reliable protocol in order to assess the surface phenotype of $CD11c^+$ splenic DCs, as we had concluded that T-cell induction is likely to occur locally during immunotherapy (see Chapter 3). CD11c is the αX subunit of the p150/95 integrin (CD11c/CD18) which is know to bind ICAM-1, fibrinogen, and iC3b and is expressed on marcophages, activated T-cells (such as intraepithelial lymphocytes) [473], activated B-cells, murine plasmocytoid DCs, and at relatively high levels by murine splenic DCs. Thus, following collagen digestion, we used a PE-labelled mAb (N429) directed against CD11c to distinguish splenic DCs.

As can be seen in figure 4.1 (a), following collagen digestion a proportion of the splenocytes possessed autofluorescent properties as, without any fluorescenated mAb bound, these cells fluoresced into FL1 and FL2 channels equally (top left). The addition of PE-conjugated anti-CD11c allowed the distinction of $CD11c^+$ cells expressing this integrin over a range of approximately two logs, and this included the autofluorescent cells. Furthermore, this staining was ablated by binding unfluorescenated anti-CD11c to splenocytes prior to addition of the fluorescenated mAb. By gating $7AAD^+$ dead cells the forward/side scatter properties of the autofluorescent cells and the $CD11c^{hi}$ cells were assessed (figure 4.1 (b)).

As shown in figure 4.2, once autofluorescent cells are removed from the gate, splenocytes expressing high levels (MFI > 300) of CD11c were seen to be distinct from cells expressing lower levels (MFI < 300), as $CD11c^{hi}$ cells constitutively expressed class-II, B7.1 and CD40. Furthermore, $CD11c^{hi}$ cells could be divided into CD8α-positive and –negative fractions (figure 4.3 (a)), with these two populations displaying distinct phenotypes – notably, $CD8\alpha^+$ $CD11c^{hi}$ cells expressed higher levels of ICAM-1 and LFA-1 than their $CD8\alpha^-$ counterparts (figure 4.3 (b)). Furthermore, $CD8\alpha^+$ $CD11c^{hi}$ cells expressed DEC-205, whereas the $CD8\alpha^-$ $CD11c^{hi}$ cells did not (figure 4.3 (b)). As expected, a proportion of the $CD8\alpha^-$ DCs also expressed CD4.

Data shown in figures 4.1-4.3 are therefore consistent with published work demonstrating that murine splenic DCs express high levels of CD11c and that this marker may therefore be used to distinguish these cells [57, 120, 203]. Notably, injection of cholera toxin into transgenic animals expressing human cholera toxin receptor under the control of the murine CD11c promoter results in the selective ablation of $CD11c^{hi}$ DCs and a concomitant loss of T-cell responses to cell-associated antigen, *e.g.*, osmotically-loaded OVA [15]. The autoflourescent cells observed here (figure 4.1 (a)) have previously been defined as macrophages and, indeed, these cells constitutively express F4-80 (which suggests that these cells are of a macrophage lineage; data not shown) and have a classically monocytic morphology (figure 4.1. (b)). It should be noted however that, consistent with published data, a sub-population of murine splenic DCs also express this marker (figure 4.3) [474, 475].

4.3.2. Assessment of Surface Antigen Expression on DCs During Immunotherapy

Following the development of a procedure to discriminate DCs by FACS, we next wished to assess the maturation state of DCs during the immunotherapy of BCL1 in order to see if anti-CD40 augments DC maturation in the presence of lymphoma and, thus, to observe if anti-CD40 may be acting in this way to affect anti-tumour CTL expansion. BALB/c mice received $5x10^7$ BCL1 i.v. and/or 1 mg anti-CD40 i.v. 4 days later. Spleen were subsequently harvested the equivalent of 24 to 96 hours following anti-CD40, collagen digested, and DCs visualised by FACS as described.

As shown in figure 4.4 (a), DCs taken from naïve animals expressed ICAM-1 at a MFI of 42 and this expression was approximately doubled when either BCL1 or anti-CD40 were present alone. However, at 96 hours following anti-CD40, DCs isolated from animals undergoing immunotherapy expressed approximately 5-fold more ICAM-1 than those extracted from naïve animals (MFIs of 42 versus 238). The kinetics of these changes in ICAM-1, as well as B7.1, B7.2, and LFA-1 expression by DCs is shown in panel (b). B7.1, ICAM-1 and B7.2 displayed a similar pattern of expression, when compared with each

other, in response to BCL1 and/or anti-CD40 - the expression of all these molecules was elevated (approximately 2-fold) on DCs in response to the lymphoma, as compared to naïve controls, but this expression was elevated in the presence of both BCL1 and anti-CD40, most notably 72 and 96 hours following mAb administration. In contrast, injection of anti-CD40 into naïve animals resulted in the elevated expression of B7.1, B7.2, and ICAM-1 that reached a maxima more rapidly than when anti-CD40 was administered to tumour-bearing animals, *i.e.*, at 48 hours following administration. LFA-1 displayed a distinict pattern of expression when compared with the expression of ICAM-1, B7.1 and B7.2 as tumour inoculation resulted in an approximately 3-fold induction of LFA-1 on DCs, but this expression was unaltered following anti-CD40 administration. Nonetheless, administration of anti-CD40 to naïve animals resulted in the elevated expression of LFA-1 on DCs which reached a maxima 72 hours following mAb administration.

In addition to these changes in the surface expression of B7.1, B7.2, ICAM-1 and LFA-1, the total number of DCs was consistently elevated in animals that were inoculated with BCL1 (approximately $3x10^6$ DCs/spleen in animals that had received BCL1 versus $5x10^5$ DCs/spleen in naïve animals) and, again, this effect was profoundly augmented in animals that were undergoing immunotherapy (figure 4.4 (c)). Anti-CD40 alone also induced the accumulation of DCs (maximally at 72 hours following injection), but to a lesser extent than BCL1.

Together, these data are therefore consistent with the hypothesis that BCL1 lymphoma is able to induce DC maturation and accumulation such that an ineffectual T-cell response is elicited, *i.e.*, consisting of limited T-cell accumulation and differentiation without the generation of effector function. Following this, maturation of DC in tumour-bearing animals is enhanced by anti-CD40, thereby either directly affecting, or at least contributing to, the production of anti-tumour CTL and tumour eradication. Nonetheless, the fact that the expression of B7.1, ICAM-1, and, to a lesser extent, B7.2 was elevated on the lymphoma following anti-CD40 administration (figure 4.5 (a)) suggests that enhanced recognition of

antigen on the lymphoma following exposure to anti-CD40 may also contribute to the generation of an anti-tumour CTL response.

In order to investigate the possibility that the presence of a $CD40^+$ lymphoma in animals that are subsequently injected with anti-CD40 may delay enhanced DC maturation in comparison to that observed in the absence of tumour (as shown in figure 4.4 (b)) due to limited mAb availablility *in vivo*, we injected naïve BALB/c mice intravenously with either 50 µg or 1000 µg of anti-CD40 and assessed the binding of this mAb to DCs 1 day thereafter. As can be seen in figure 4.5 (b), the quantity of anti-CD40 bound to DCs, as detected by a fluorescenated anti-rat-Fc mAb, when administering either 50 µg or 1000 µg of anti-CD40 is comparible. Thus, even if the lymphoma were to bind 95 % of the injected mAb, this mechanism can not account for the different expression kinetics of the antigens detailed in figure 4.4 when anti-CD40 is administered in the presence or absence of tumour.

4.3.3. The Binding of anti-CD40 to DCs

On the assumption that enhanced DC maturation induced by anti-CD40 is a critical component of the therapeutic activity of this mAb, we next wished to demonstrate that DC maturation was not being induced by the binding of this mAb to Fc receptors on the splenic DCs. Thus, naïve BALB/c mice received either 1 mg of anti-CD40, 1mg of anti-CD19, or 200 µl of PBS i.v. and, subsequently, splenic DCs were assessed for the binding of Rat Fc. Anti-CD19 is of the same isotype (Rat γ2a) as 3/23 and, even though this mAb has mild therapeutic activity against BCL1 if a low tumour inoculum is used (*i.e.*, 10^5), it has no therapeutic activity against BCL1 if a greater number of tumour cells are administered, *i.e.*, $5x10^7$, as has been used here [461]. Importantly, both CD40 and CD19 are expressed to a similar degree by BCL1 (data not shown), but only CD40 is expressed by murine DCs (figure 4.3).

As shown in figure 4.6 (a), splenic DCs taken from animals having received PBS did indeed express CD40 and Fc receptors γRII/III, but not CD19. As expected, following administration of anti-CD19, the expression of CD40 was maintained, while when anti-

CD40 was administered *in vivo* the binding of anti-CD40 was blocked *in vitro*. Nonetheless, as can be seen in panel (b), the injection of either anti-CD19 or anti-CD40 resulted in the association of DCs with Rat-Fc 24 hours following mAb injection.

These data are therefore consistent with the hypothesis that both anti-CD19 and anti-CD40 are able to bind murine splenic DCs following intravenous injection of 1 mg of mAb, but that this occurs exclusively by Fc receptor binding and predominantly by antigen binding respectively (anti-CD40 may also bind Fc receptors, but the proportion of anti-CD40 bound to DCs in this way could not be assessed in this experiment). Thus, if enhanced DC activation is related to the therapeutic activity of anti-CD40, this effect is mediated by specific binding of CD40 to splenic DCs; this hypothesis will have to be further tested by assessing DC activation following administration of anti-CD19 and, ideally, by selectively ablating the DC compartment during immunotherapy.

4.3.4. Dendritic Cell Responsiveness to tumour Alone

Next, we wished to assess the response of DCs to these same tumours (A31, BCL1, and TEN-1), so as so determine whether the endogenous $CD8^+$ lymphocyte response observed may have been affected by these APCs. Thus, CBA/H or BALB/c mice received tumour as detailed in section 3.3.5 and DC phenotypes were assessed following collagen digestion.

As shown in figure 4.7 (a), the total yield of DCs from animals having received either A31, BCL1, or TEN-1 was greatly (4-12-fold) elevated when compared to that obtained from naïve animals or those having received an injection of naïve splenocytes. Furthermore, DCs from tumour-bearing animals expressed higher levels of ICAM-1 and CD40, whereas the expression of B7.1 by DCs was only markedly elevated in response to A31 and TEN-1. Interestingly, the expression of B7.2 was greatly elevated on DCs following administration of TEN-1, but only fractionally after injection of A31 and BCL1.

The expression of CD40 by DCs following exposure to tumour was of particular importance to us as elevated expression of CD40 by DCs has been shown to occur in response to

PAMP-mediated signals and, thus, may enhance the sensitivity of DCs to CD40 ligation [120]. We therefore chose to assess this effect more closely, specifically asking the question: is CD40 expression on DCs elevated in response to A31, BCL1 or TEN-1 by the time anti-CD40 would be administered in order to affect therapy? Thus, BALB/c mice received $5x10^7$ BCL1 alone, while CBA/H mice received the same number of A31 i.v.; splenocytes were harvested from duplicate tumour-bearing animals (and naïve littermates) on days 4 and 3 following inoculation respectively.

As shown in figure 4.7 (b), the expression of CD40 on DCs was elevated approximately 2-fold in response to both A31 and BCL1 by day 3 or 4 following inoculation respectively; furthermore, this increase in CD40 expression was found to continue if A31 was left untreated (figure 4.7 (c)).

Together, these data demonstrate that DCs receive a signal analogous to a PAMP-derived signal following tumour inoculation, but not following an injection of splenocytes. It is less clear from this data that B7.1 expression is elevated following BCL1 inoculation, but this does indeed occur as shown in figure 4.4 (b), albeit to a very slight degree (< 2-fold). More profound changes are seen in the expression of ICAM-1 and LFA-1 (figures 4.4 (b) and 4.7 (a)). Additionally, the approximately 4-fold increase in the expression of B7.2 following inoculation with TEN-1 suggests that this molecule does not contribute to 4-1BB expression in this system as 4-1BB is not expressed on $CD8^+$ lymphocytes following TEN-1 incoulation (figure 3.11).

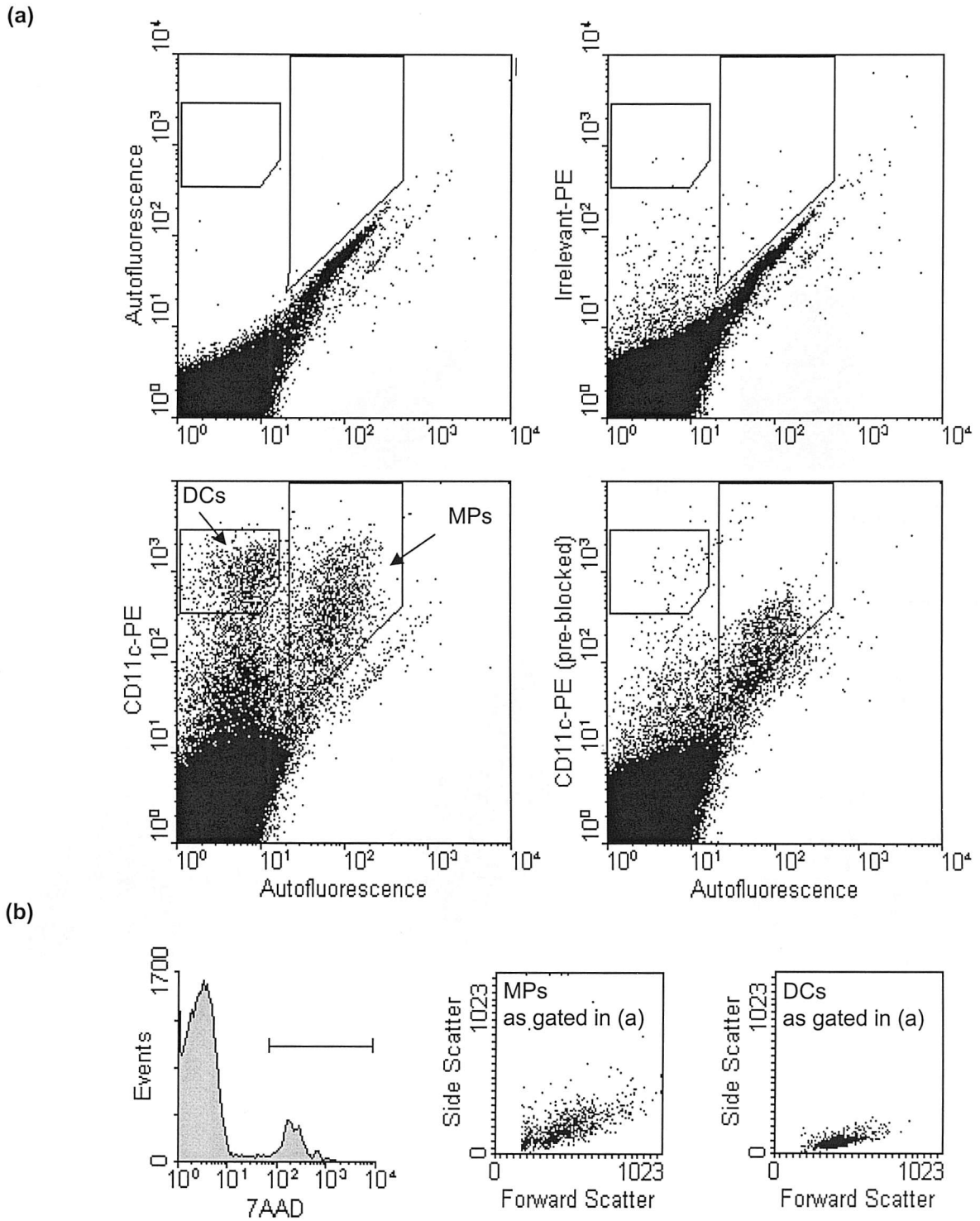


Figure 4.1. Identification of Splenic Macrophages and DCs. Spleen fragments from naïve BALB/c mice were digested with collagenase D as described in Chapter 2 and the recovered splenocytes stained with PE-conjugated anti-CD11c or an isotype-matched PE-conjugated irrelevant mAb. The specificity of this staining was confirmed by pre-incubating the splenocytes with 20 µg/ml unconjugated anti-CD11c, (a). Dead cells were excluded using 7AAD, and macrophages or dendritic cells assessed by gating as shown in (a), (b).

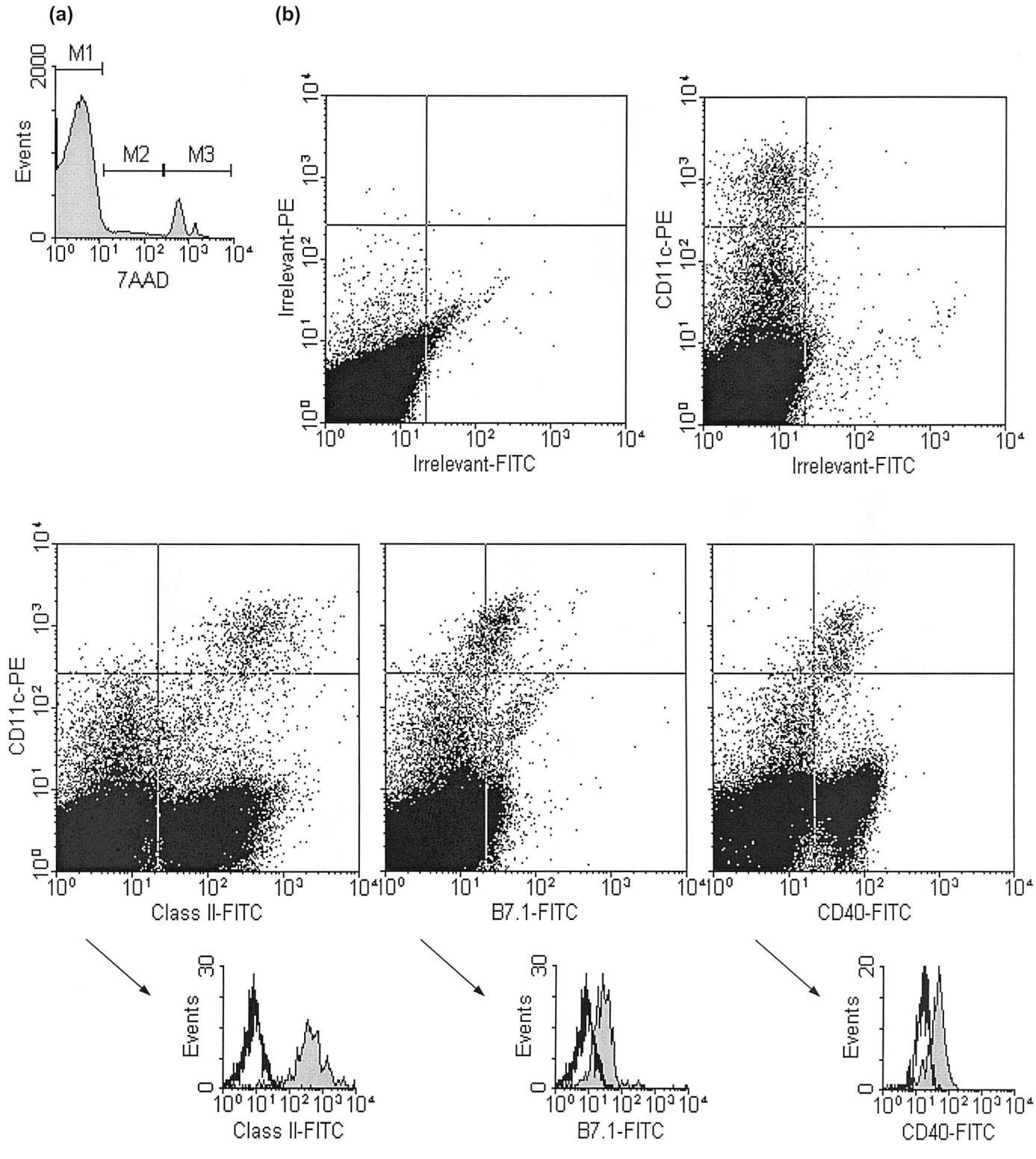


Figure 4.2. Phenotypic Analysis of Splenic DCs. Spleen fragments from naïve BALB/c mice were digested with collagenase D as described in Chapter 2 and the recovered splenocytes stained with PE-conjugated anti-CD11c or an isotype-matched PE-conjugated irrelevant mAb. Dead cells (M3) and apoptotic cells, as well as macrophages (M2), were excluded using 7AAD and autofluorescent properties, (a). Subsequently, the phenotype of dendritic cells was assessed by staining the splenocytes with FITC-conjugated mAbs, (b). Open histograms depict the binding of irrelevant mAb.

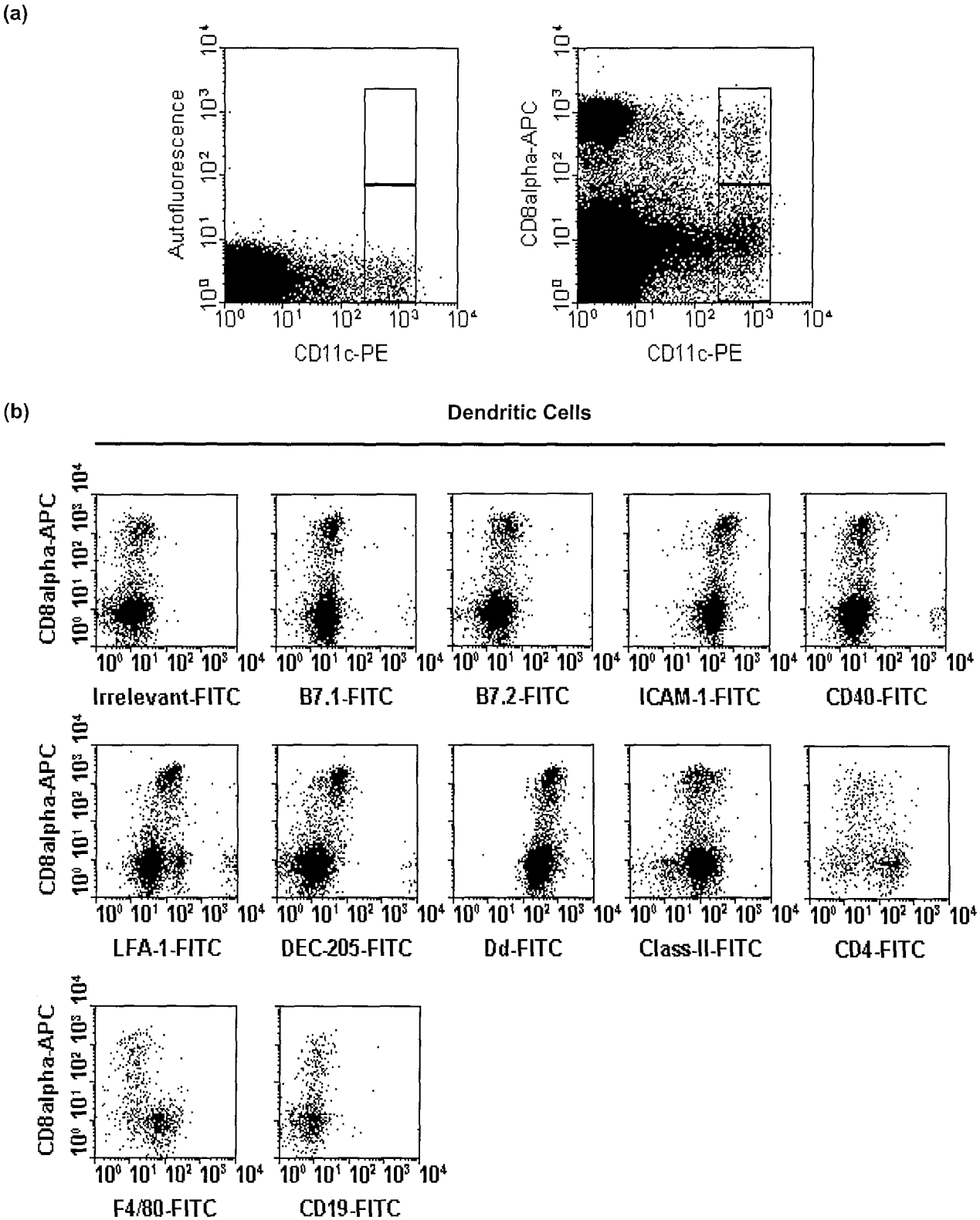


Figure 4.3. Differential Phenotypic Analysis of CD8α⁺ and CD8α⁻ Splenic DCs. Spleen fragments from naïve BALB/c mice were digested with collagenase D as described in Chapter 2 and the recovered splenocytes stained with PE-conjugated anti-CD11c. Dead cells and apoptotic cells, as well as macrophages, were excluded using 7AAD and autofluorescent properties. If required, CD8α-positve or -negative splenic DCs were distinguished using APC-conjugated anti-CD8α, (a). The phenotype of these two DC subsets was assessed simultaneously by staining with FITC-conjugated mAbs, (b).

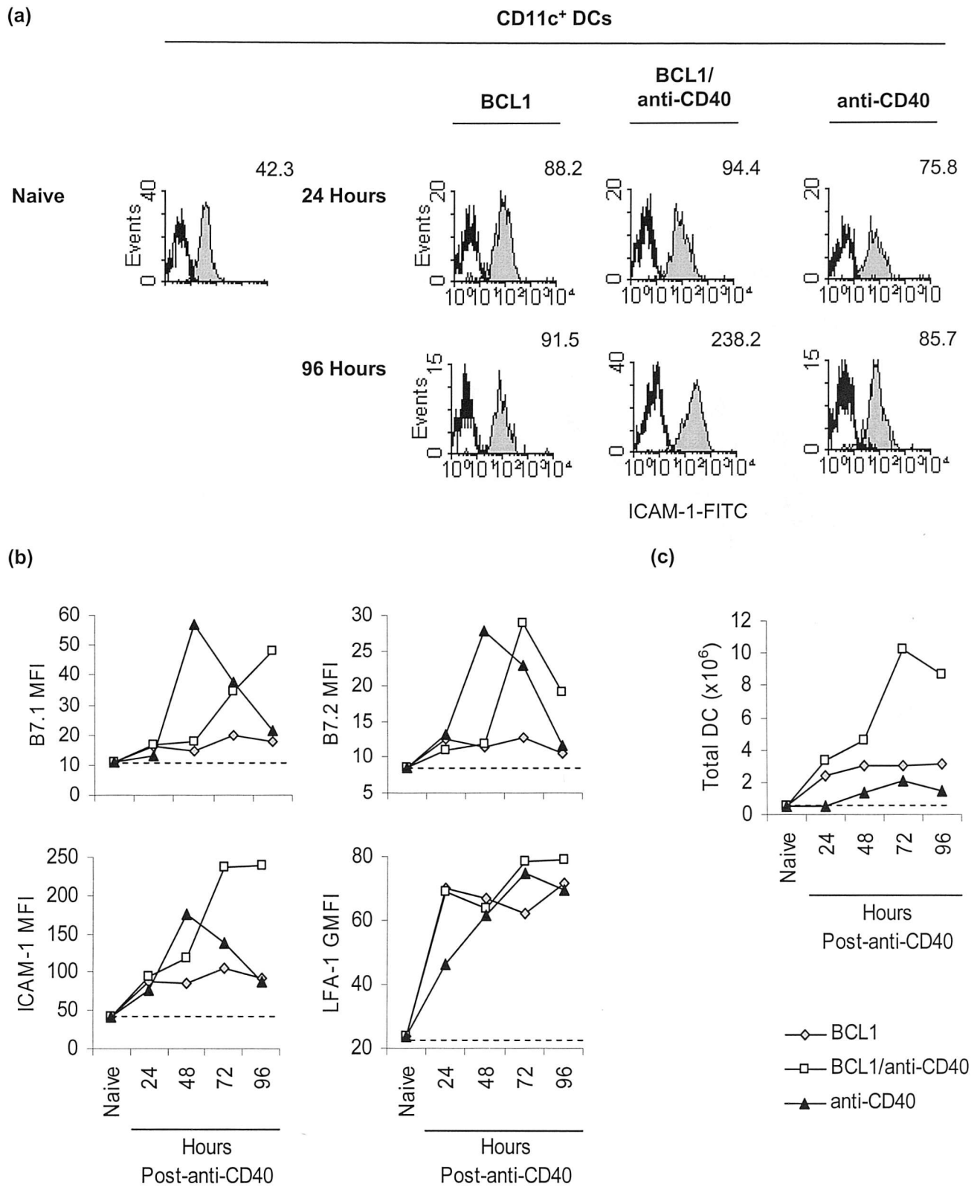


Figure 4.4. Dendritic Cell Phenotype During Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 i.v. on day 0 and/or 1 mg anti-CD40 (3/23) i.v. on day 4. Subsequently, spleen fragments from single animals taken the equivalent of 24 to 96 hours following anti-CD40 administration were digested with collagenase D as described in Chapter 2, and the recovered splenocytes stained with PE-conjugated anti-CD11c, 7AAD and the FITC-conjugated mAbs directed against the antigens indicated (solid grey histograms) or against an irrelevant antigen (open histograms). Dead cells, apoptotic cells and macrophages were excluded, and DCs gated as in figure 4.2. The expression of ICAM-1 on DCs at the equivalent of 24 and 96 hours after anti-CD40 administration is shown in (a), whereas the data for all antigens assessed over time is summarised in (b). Numbers associated with the hisotgrams in (a) represent the MFI of ICAM-1 expression by DCs. Total DC numbers were calculated over time, (c). Dashed Lines represent the antigen expression on naïve DCs. These data are representative of two separate experiments.

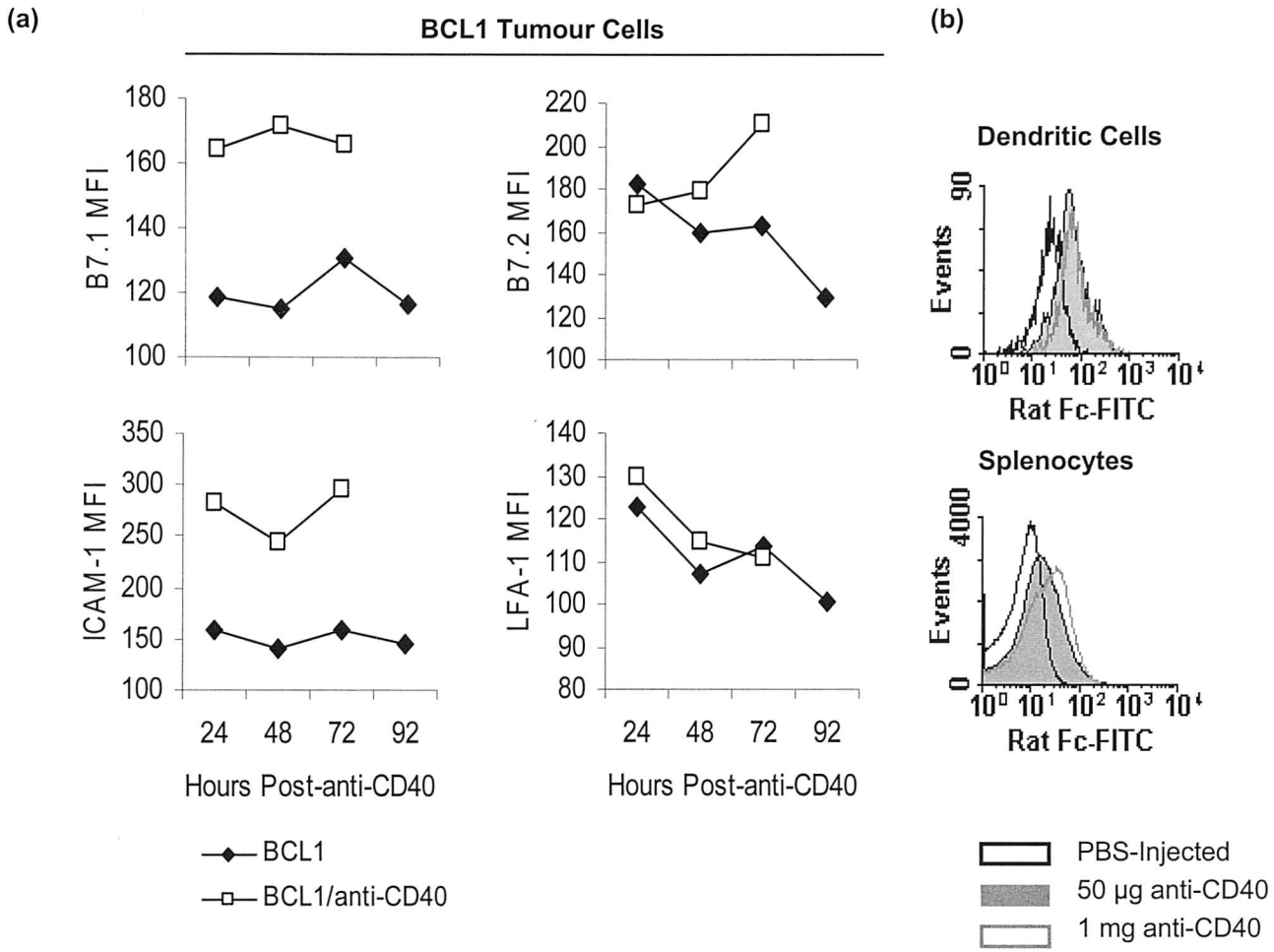


Figure 4.5. Tumour Phenotype during Immunotherapy of BCL1 and anti-CD40 Binding to DCs. BALB/c mice received $5x10^7$ BCL1 i.v. on day 0 and/or 1 mg anti-CD40 (3/23) i.v. on day 4. Subsequently, spleen fragments from single animals taken at the equivalent of 24 to 96 hours following anti-CD40 administration were digested with collagenase D as described in Chapter 2, and the recovered splenocytes stained with PE-conjugated anti-BCL1 idiotype and FITC-conjugated mAbs directed against the antigens shown, (a). In (b), naïve BALB/c mice received either 1 mg or 50 µg of anti-CD40 i.v. 1 day prior to the harvesting of splenocytes by collagenase D digestion and the staining of DCs with FITC-conjugated anti-Rat-Fc. PBS-injected animals were used as a negative control.

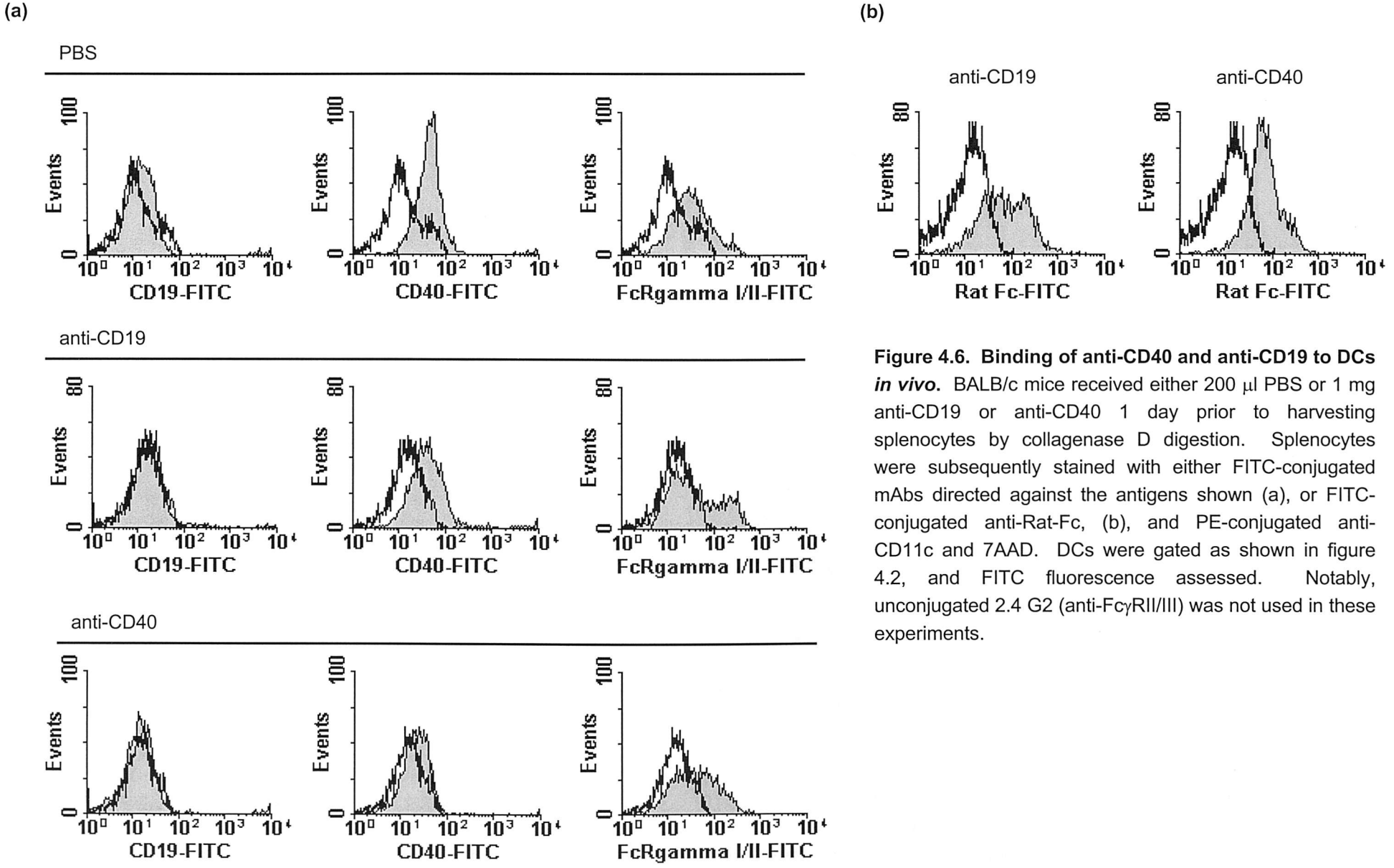


Figure 4.6. Binding of anti-CD40 and anti-CD19 to DCs *in vivo*. BALB/c mice received either 200 μl PBS or 1 mg anti-CD19 or anti-CD40 1 day prior to harvesting splenocytes by collagenase D digestion. Splenocytes were subsequently stained with either FITC-conjugated mAbs directed against the antigens shown (a), or FITC-conjugated anti-Rat-Fc, (b), and PE-conjugated anti-CD11c and 7AAD. DCs were gated as shown in figure 4.2, and FITC fluorescence assessed. Notably, unconjugated 2.4 G2 (anti-FcγRII/III) was not used in these experiments.

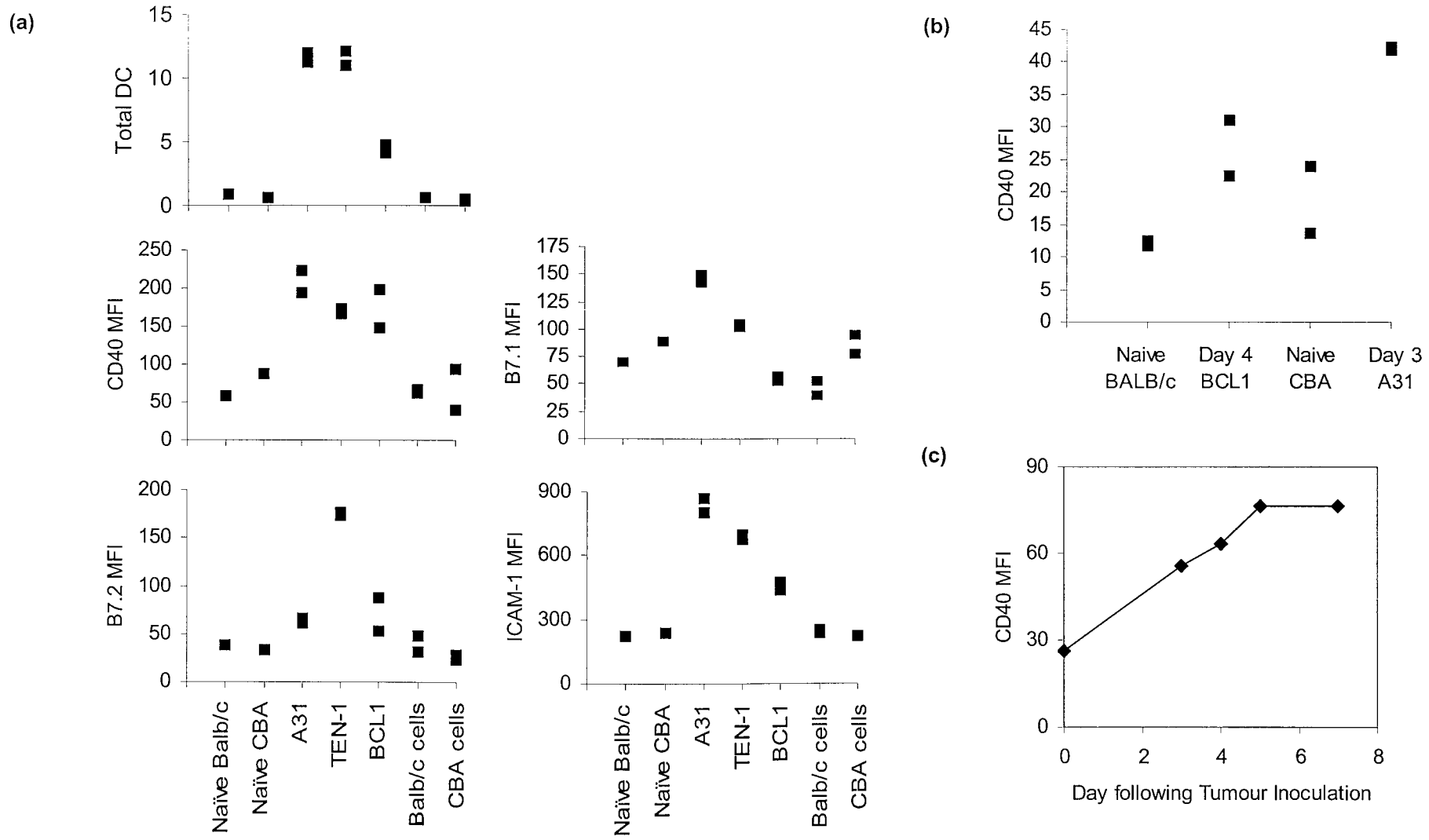


Figure 4.7. Partial DC Maturation in Response to A31, BCL1, and TEN-1. In (a), duplicate naive BALB/c mice received either $5x10^7$ TEN-1, BCL1, or BALB/c splenocytes, whereas naive CBA/H mice received either $5x10^7$ A31 or CBA/H splenocytes i.v. on day 0. On day 8, spleen fragments from animals were digested with collagenase D as described in Chapter 2, and the recovered splenoctyes stained with PE-conjugated anti-CD11c, 7AAD and the FITC-conjugated mAbs directed against the antigens indicated. Dead cells, apoptotic cells and macrophages were excluded, and DCs gated as in figure 4.2. In (b), duplicate naive BALB/c mice received $5x10^7$ BCL1 while naive CBA/H mice received $5x10^7$ A31 i.v.; 3 or 4 days (as indicated) following tumour inoculation, CD40 expression on splenic DCs was assessed as in (a). Similarly, in (c), naive CBA/H mice received $5x10^7$ A31 i.v. and the expression of CD40 on splenic DCs was assessed as in (a) on days 4 to 8 following tumour inoculation.

4.3.5.

Isolation of DCs and Macrophages During Immunotherapy & Determination of Phenotype

In order to assess whether or not the enhanced DC maturation observed during anti-CD40-mediated immunotherapy is a consistent phenomenon, and in order to acquire more accurate data of these observations (by allowing the assessment of a greater number of $CD11c^+$ cells), we chose to purify $CD11c^+$ cells from animals after having received both BCL1 and anti-CD40.

Duplicate BALB/c mice received $5x10^7$ BCL1 i.v. and/or 1 mg anti-CD40 i.v. 4 days later. Splenocytes were subsequently harvested, following collagen digestion, the equivalent of 72 hours following anti-CD40, $CD11c^+$ cells separated from the splenocytes by MACS and these cells stained with FITC-conjugated mAbs. The time-point of 72 hours following anti-CD40 injection was chosen as previous experiments demonstrated that at this time splenic DCs taken from animals undergoing immunotherapy consistently displayed a distinct phenotype from DCs taken from the other treatment groups, and, moreover, this precedes tumour eradication.

As can be seen in figure 4.8 (a), MACS purification of $CD11c^+$ cells allowed the distinction of macrophages and DCs from $CD11c^-$ cells with ease. As shown in panel (b), as previously observed, DCs taken from animals undergoing immunotherapy expressed higher levels of B7.1, B7.2, and ICAM-1, when compared to DCs from animals having received either BCL1 or anti-CD40 alone. Again, the expression of LFA-1 by DCs was unaffected in tumour-bearing animals whether anti-CD40 was administered or not. These data, taken from duplicate animals, are summarised in figure 4.9 (a), whereas the equivalent data for the expression of these antigens by macrophages is shown in panel (b). As can be seen, the pattern of B7.1/.2 expression by DCs in each treatment group is broadly reflected by macrophages; the combination of BCL1 and anti-CD40 did however induce ICAM-1 expression by macrophages to a far greater extent than on DCs (*i.e.*, off the scale when measured side-by-side with DCs due to their autofluorescent properties) and LFA-1 expression was slightly elevated under the same conditions.

As for DCs (figure 4.10 (a)), the number of macrophages per spleen in animals undergoing immunotherapy was greater than in those animals that had received BCL1 or anti-CD40 alone, but, in comparison to DCs the total number of cells is less.

Together, these data for DCs are consistent with that obtained prior to purification (*i.e.*, figure 4.4) and therefore confirm that a process of DC maturation/accumulation is initiated following injection of BCL1 lymphoma, and that this process is enhanced by anti-CD40 administration. Moreover, this effect is also observed in the macrophage compartment.

In order to assess the phenotype of $CD8\alpha^+$ and $CD8\alpha^-$ DC during immunotherapy, these populations were detected using an APC-conjugated mAb to stain MACS-purified $CD11c^+$ cells. As is shown in figure 4.10 (b), 72 hours following anti-CD40 administration, compared to DCs from animals that received tumour alone, both CD8α–positive and –negative DCs taken from animals during immunotherapy had an elevated expression of ICAM-1, whereas B7.1 was only noticeable elevated on $CD8\alpha^-$ DCs.

These data appear inconsistent with the notion that $CD8\alpha^-$ DCs migrate from the marginal zone of the spleen to the PALS with the concomitant acquisition of a $CD8\alpha^+$ phenotype [199], as the elevated expression of B7.1 on $CD8\alpha^-$ DCs is not reflected in the $CD8\alpha^+$ population. The inability to correlate the maturation of $CD8\alpha^-$ DCs with similar changes in the $CD8\alpha^+$ population may be due to the preferential binding of anti-CD40 to $CD8\alpha^-$ DCs in the marginal zone adjacent to the circulation and/or the possibility that T-cell priming may have already occurred, *i.e.*, anti-tumour T-cells may be present outside of the PALS. Nonetheless, systemic administration of anti-CD40 to tumour-bearing animals clearly enhances the maturation of $CD8\alpha^+$ DCs as reflected by the elevated ICAM-1 expression in this population.

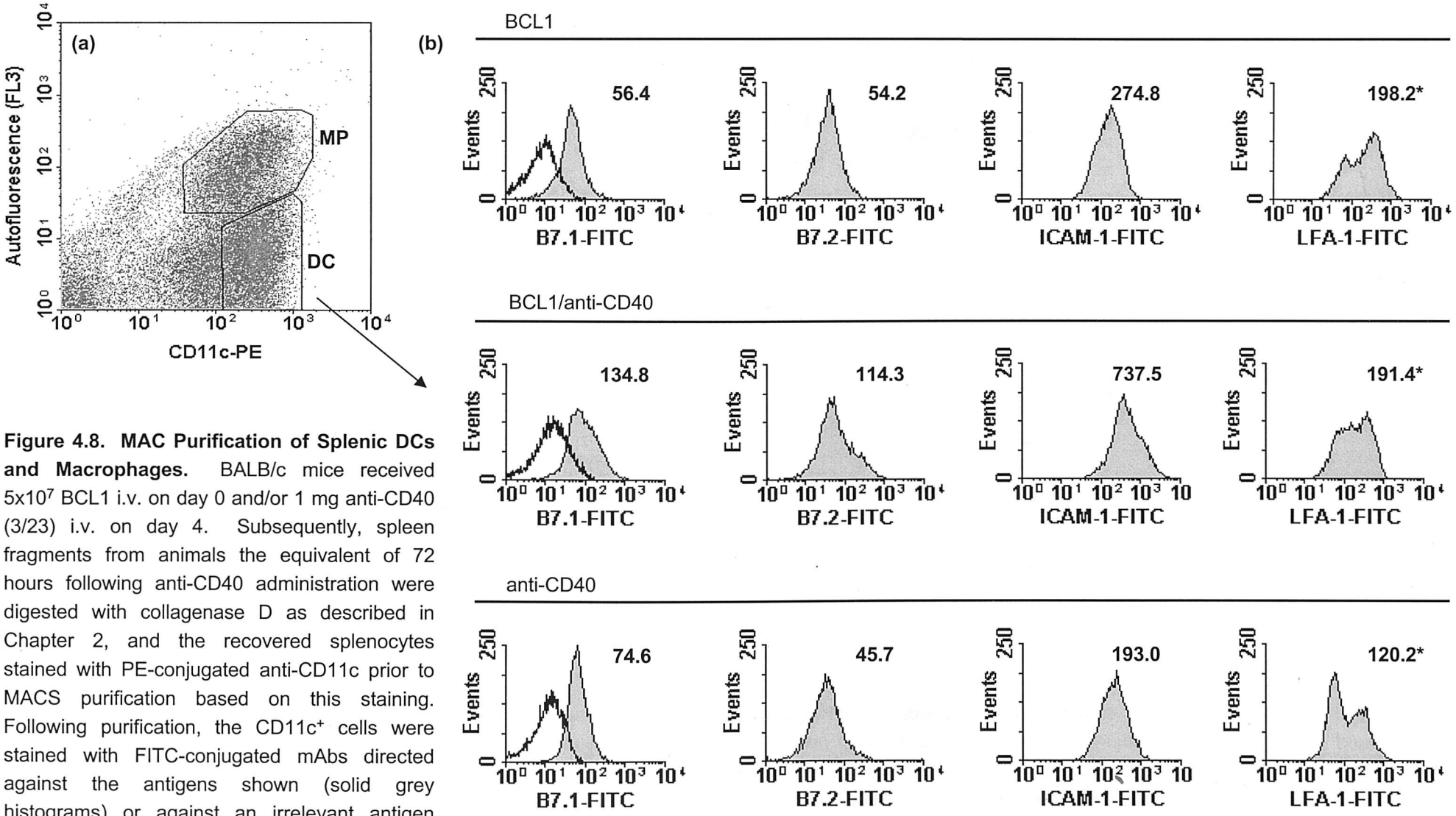


Figure 4.8. MAC Purification of Splenic DCs and Macrophages. BALB/c mice received $5x10^7$ BCL1 i.v. on day 0 and/or 1 mg anti-CD40 (3/23) i.v. on day 4. Subsequently, spleen fragments from animals the equivalent of 72 hours following anti-CD40 administration were digested with collagenase D as described in Chapter 2, and the recovered splenocytes stained with PE-conjugated anti-CD11c prior to MACS purification based on this staining. Following purification, the $CD11c^+$ cells were stained with FITC-conjugated mAbs directed against the antigens shown (solid grey histograms) or against an irrelevant antigen (open histograms). The expression of these antigens by DCs is shown in (b) based on the gating indicated in (a).

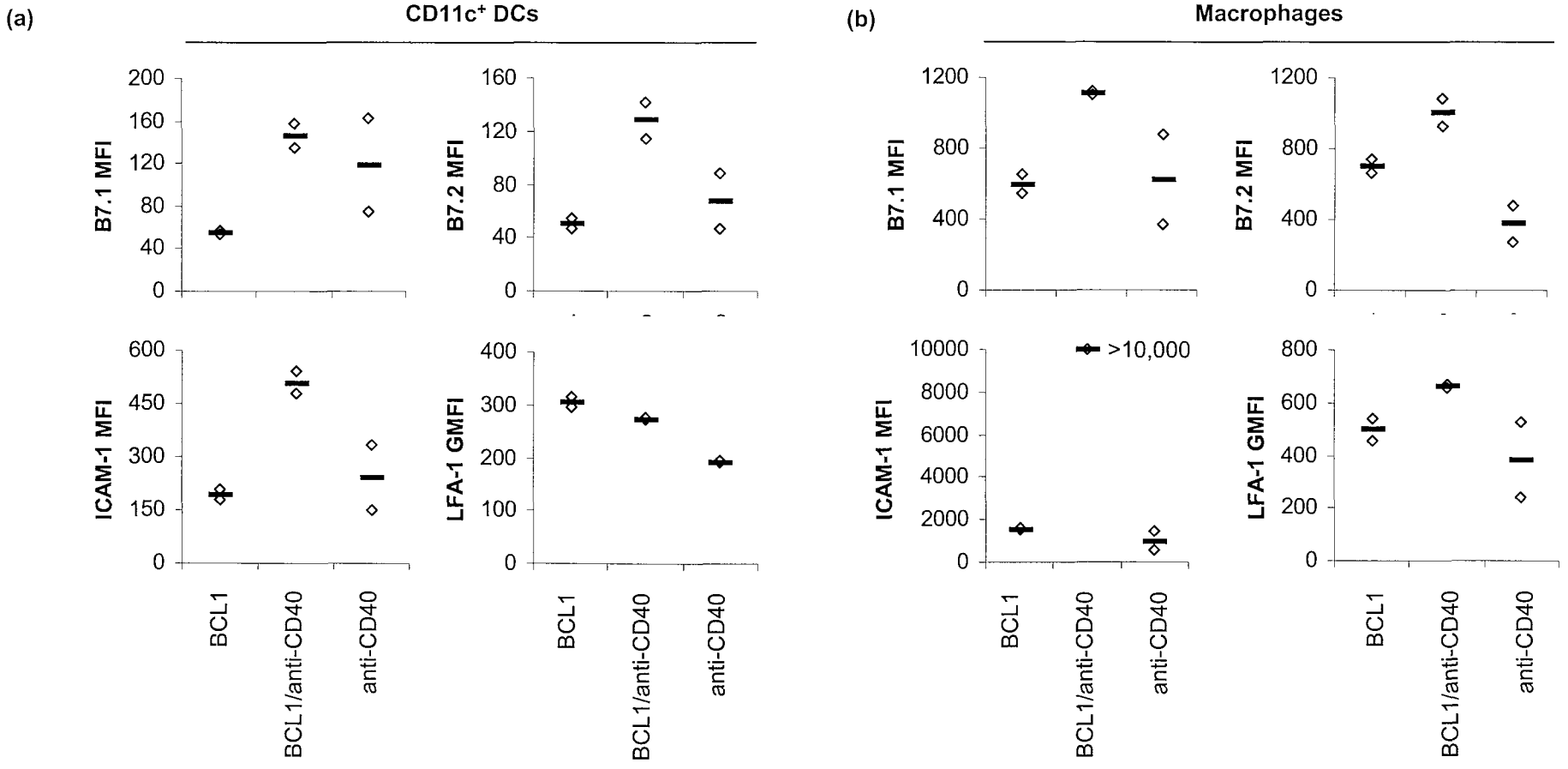


Figure 4.9. Phenotypic Analysis of DCs and Macrophages during Immunotherapy of BCL1 after Isolation by MACS. BALB/c mice received $5x10^7$ BCL1 i.v. on day 0 and/or 1 mg anti-CD40 (3/23) i.v. on day 4. Subsequently, spleen fragments from duplicate animals the equivalent of 72 hours following anti-CD40 administration were digested with collagenase D as described in Chapter 2, and the recovered splenocytes stained with PE-conjugated anti-CD11c prior to MACS purification based on this staining. Following purification, the $CD11c^+$ cells were stained with FITC-conjugated mAbs directed against the antigens shown. The expression of these antigens by DCs (a) and MPs (b) is shown based on the gating indicated in figure 4.7. MFI values obtained from individual animals are shown as grey diamonds and the mean between these indicated by a horizontal Line. These data are representative of three separate experiments.

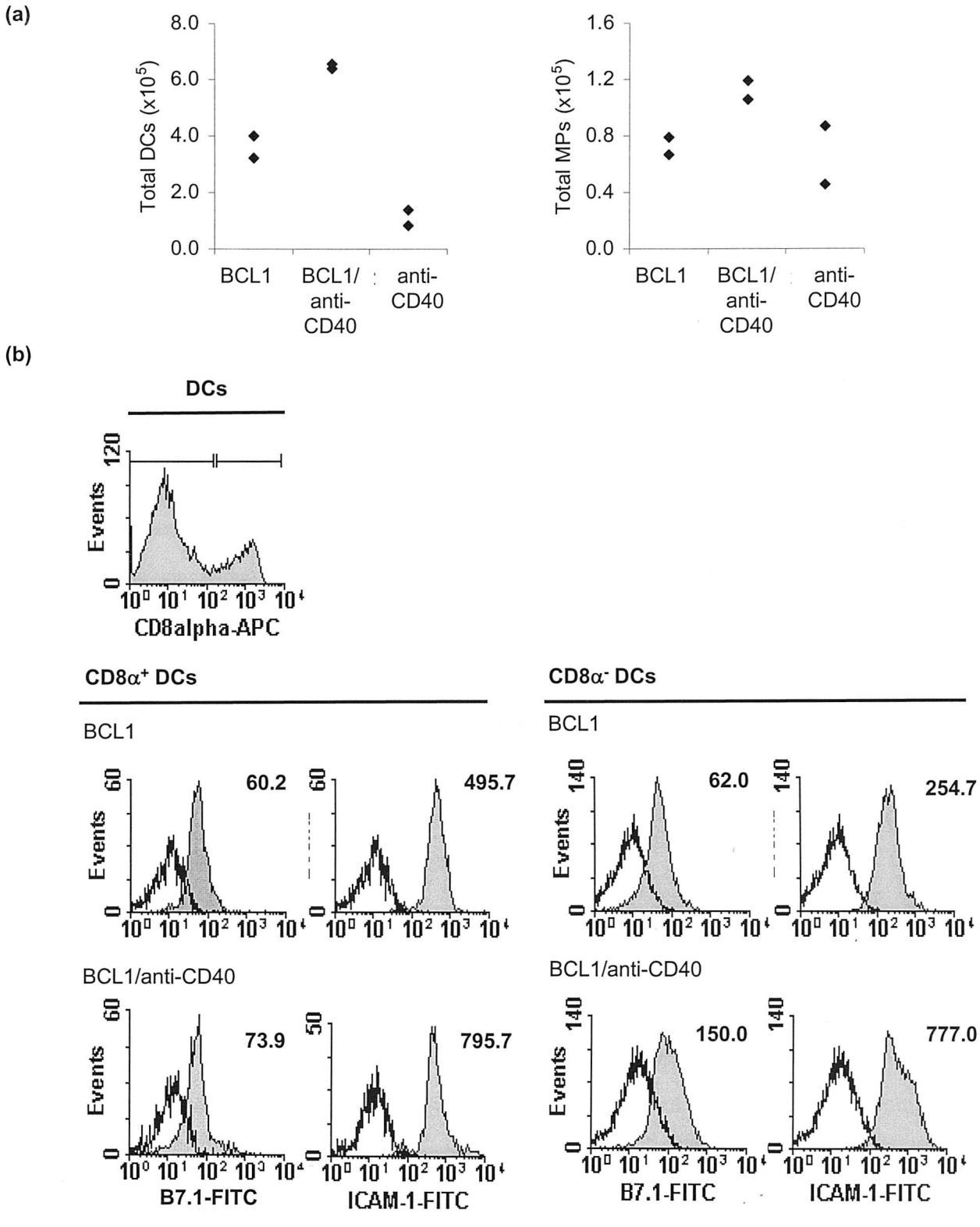


Figure 4.10. Enumeration of APC Populations and Phenotypic Analysis of CD8α⁺ and CD8α⁻ DCs during Immunotherapy of BCL1. BALB/c mice received $5x10^7$ BCL1 i.v. on day 0 and/or 1 mg anti-CD40 (3/23) i.v. on day 4. Subsequently, spleen fragments from duplicate animals the equivalent of 72 hours following anti-CD40 administration were digested with collagenase D as described in Chapter 2, and the recovered splenocytes stained with PE-conjugated anti-CD11c prior to MACS purification and FACS analysis; the total number of DCs and macrophages was then calculated (a). In (b), CD11c⁺ cells were stained with APC-conjugated anti-CD8α and FITC-conjugated mAbs directed against the indicated antigens (solid grey histograms) or towards an irrelevant antigen (open histograms). CD8α⁺ and CD8α⁻ DCs were gated as shown, and the expression of the indicated antigens assessed, (b). B7.2 and LFA-1 expression was also assessed (data not shown). These data are representative of two separate experiments. Numbers associated with each hisotgram represent the MFI of B7.1 or ICAM-1 expression by DCs.

4.4. Discussion

We have demonstrated here that anti-CD40 is able to enhance a pre-existing process of DC maturation that is likely to contribute to the generation of a functional anti-tumour CTL response from an ineffectual T-cell response. The characteristics, and possible initiating factors, of the endogenous T-cell and DC responses are discussed in Appendix 1.

Here, DC maturation in the presence of BCL1 was associated with the up-regulation of B7.1, B7.2, ICAM-1, and LFA-1, all of which, except LFA-1, were boosted by the administration of anti-CD40, and all of which are required for tumour eradication during immunotherapy [460]. It seems likely therefore that anti-CD40 replaces the need for signalling via endogenous CD40L, thereby providing enhanced stimulation of antigen-specific T-cells and enhanced DC-T-cell interaction via these and other, unexamined, molecules, *e.g.*, 4-1BBL, IL-12, mature IL-18 [117, 476]. Furthermore, the enhanced accumulation of splenic DCs in tumour-bearing animals following anti-CD40 administration is likely to favour the generation of a CTL response as do high DC:T-cell ratios [213]; this accumulation of DCs may be due to increased proliferation (in situ or at a distinct site, *e.g.*, the BM), survival, and/or migration into the spleen.

In addition to the enhanced maturation of DC, the expression of both B7.1 and ICAM-1 was also up-regulated upon BCL1 cells following anti-CD40 administration, suggesting that enhanced cross-presentation of antigen to tumour-specific T-cells by the tumour itself may also contribute to the therapeutic activity of this mAb. Indeed, CTL lines raised from animals in remission following immunotherapy of BCL1 rely on interactions via B7 and LFA-1 for target cell recognition preceding IFNγ production (see Chapter 6). However, T-cell responses may only be initiated in the absence of BM-derived APCs when the avidity of the interaction between T-cell and cognate antigen is high, *e.g.*, during LCMV infection, thereby suggesting that even though enhanced presentation of antigen by the lymphoma following anti-CD40 administration may occur, this is unlikely to be an effective route for the priming of an anti-tumour T-cell response. Furthermore, the effectiveness of anti-CD40

against $CD40^-$ tumours additionally argues that cross-priming of tumour-specific T-cells by DCs is an effective route via which tumour rejection can be elicited.

Finally, the DC maturation observed in the presence of anti-CD40 alone may be initiated by contaminating LPS and, indeed preparations of 3/23 contain >250 ng/ml of LPS, as estimated by limulus amebocyte lysate assay (Tutt, A, unpublished observations). Nonetheless, anti-CD40 that has been passed through a polymixin column is able to mediate immunotherapy of BCL1, suggesting that the lymphoma is indeed able to provide 'danger' stimuli (Tutt, A, unpublished observations), or that LPS contamination remains at a sufficient level to provide this stimulus. Moreover, the accelerated DC maturation observed upon injection of anti-CD40 into naïve animals, when compared to the kinetic of DC maturation during immunotherapy, suggests that DC maturation is inhibited in tumour-bearing animals, especially as this observation can not be accounted for by the quantity of mAb binding DCs; this point is discussed further in Chapter 6.

5.1. Introduction.

In addition to intrinsic mechanisms of tumour suppression (*e.g.*, p53), both innate and adaptive arms of the immune system act as extrinsic suppressors of neoplasia, *e.g.*, RAG-$2^{-/-}$ animals have a higher incidence of microscopic tumours than do wild-type littermates, and the severity of these lesions is increased if these animals are crossed with STAT-$1^{-/-}$ animals [350]. Indeed, extensive evidence now exists demonstrating that the adaptive immune system is able to respond to TAAs resulting in, either the ineffectual expansion and/or differentiation of TAA-specific T-cells (*e.g.*, [344, 351, 352, 356, 478]), or, infrequently, the spontaneous regression of some tumours, notably melanomas [353]. Thus, current therapeutic strategies aim to generate or boost such responses in order to affect tumour eradication.

Animals in remission following immunotherapy of either A31 or BCL1 are refractory to challenge with these tumours and this protective effect is mediated, in the case of BCL1, solely by $CD8^+$ cells [460], whereas both $CD4^+$ and $CD8^+$ cells are required in order to provide a secondary response against A31 (Tutt, A, unpublished observation); currently, the identity of the antigens that act to mediate tumour rejection in this system is unknown. Thus, in order to allow further research into the mechanism by which this mAb exerts its therapeutic activity (for example, by allowing the generation of MHC class-I tetrameric complexes), and in order to assess if a bona fide TRA(s) exists in this murine model, we aimed to identify the TRA(s) in this system by: (1) generating both anti-tumour CTL lines and T-cell hybridomas from animals in remission following immunotherapy of BCL1, a procedure that was not established in this laboratory, and (2) generating and, subsequently, screening a cDNA library from BCL1 using these CTL lines and/or hybridomas. The system to be used during the screening phase of this procedure is to involve the transient transfection of COS-7 cells that had previously been stably transfected with the appropriate murine class-I haplotype [480, 481].

5.2. Materials and Methods.

Unless otherwise indicated, BCL1 lymphoma cells were isolated from spleens of animals at the terminal stage of disease as described in Chapter 2. $5x10^7$ lymphoma cells were injected i.v. followed by 1 mg of anti-CD40 when tumour represented between 2 and 10 % of the splenic lymphocytes; typically, 4 days following tumour inoculation. In the case of EL4, $1x10^7$ *in vitro* cultured cells were injected i.v. followed, 4 days later, with 1 mg of anti-CD40 i.v..

CTL lines were grown from anti-CD40-treated animals 20-60 days after initial tumour challenge. Splenocytes were harvested into CM containing 10 % batch-tested FCS, and $2x10^6$ cells seeded per well in 24-well plates. During the first week, cultures were supplemented with 10 ng/ml IL-7, 10 µg/ml anti-CD4 and $5x10^5$ irradiated *in vitro* tumour line (such as piBCL1 or EL4). On subsequent weeks, cultures were harvested, washed in CM and re-seeded at $1x10^6$/well with 20 U/ml mIL-2, 10 µg/ml anti-CD4 and $5x10^5$ irradiated tumour. CTL activity was assessed by standard chromium release assay – $1x10^5$ ^{51}Cr-labelled piBCL1, A20, P815, CT26 or YAC cells in 100 µl of CM were plated in U-bottomed 96-well plates against 50 µl of the indicated effector cells at the required density and 50 µl of CM contained blocking mAb to a final concentration of 50 µg/ml (if required). After brief centrifugation at 700 rpm for 3 minutes, samples were incubated at 37 °C for 4 hours, cells pelleted and 100 µl of supernatant assessed for ^{51}Cr content on a γ scintillation counter. Maximal ^{51}Cr release was assessed under detergent lysis and the percent target cell lysis induced by each test sample calculated using the equation detailed in Chapter 2. Alternatively, IFNγ release was used to assess CTL activity as described in Chapter 2.

In order to generate T-cell hybridomas from animals in remission following immunotherapy of BCL1, splenocytes were harvested from these animals 20 days following initial tumour challenge and seeded into 24-well plates as if to generate CTL on the first week of activation (media was additionally supplemented with 20 U/ml mIL-2). After 48 hours, 10^7 cells were harvested from this culture, mixed with 10^7 BWZCD8α, and fused using PEG-

1000 as described in Chapter 2. The resultant hybridomas were progressively grown into HT-containing ICTM from HAT-containing ICTM and assessed for reactivity against piBCL1 either by a single cell β-galactosidase detection assay (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) substrate), or by lysing the hybridomas following exposure to tumour and detecting released β-galactosidase (Chlorophenol Red β-galactoside substrate).

Surface expression of antigens by lymphocytes was assessed by FACS analysis - $1x10^6$ cells were incubated with 1 μg of fluorescenated mAb at 4°C for 15 minutes, followed by washing in PBS-BSA-Azide prior to acquisition on a FACScan or FACSCalibur. Cells to be labelled with CFSE were washed once in serum free media and re-suspended at $2x10^7$ /ml in the same media. CFSE was added to a final concentration of 2 μM and the cells then incubated at 37 °C for 10 minutes. The labelled cells were then washed twice in cold (4 °C) CM before re-suspension in warm CM (37 °C) at the required density.

5.3. Results.

5.3.1. The Establishment of anti-Tumour CTL lines

Initially we wished to determine the most favourable cytokine conditions under which to establish anti-tumour CTL lines from animals in remission; such animals were used based on the hypothesis that anti-CD40-induced tumour regression generates tumour-specific T-cell memory. Thus, animals were inoculated with $5x10^7$ BCL1 i.v. followed by 1 mg of anti-CD40 i.v. 4 days later; subsequently, 20 days after the initial tumour challenge, splenocytes were cultured with anti-CD4 and either IL-7 or murine IL-2 in the presence of irradiated piBCL1. Preliminary experiments indicated that anti-CD4 had to be used to prevent the outgrowth of $CD4^+$ lymphocytes in these cultures (data not shown).

As shown in figure 5.1 (a), the presence of lymphoma with anti-CD4 alone did not allow the outgrowth of cytotoxic tumour-reactive T-cells, but this was achieved by the addition of IL-7 (on day 0) or IL-2, either on day 0 or day 4 of culture. Nonetheless, the most efficient outgrowth of tumour-reactive T-cells was achieved in the presence of IL-7 and anti-CD4, as

cytotoxicity directed against piBCL1 obtained under these conditions was greatest and equalled that achieved when an [anti-CD3 x anti-BCL1 idiotype] bispecific antibody was present in the chromium release assay, *i.e.*, all the surviving cytotoxic T-cells were specific for the lymphoma. Importantly, even though mildly enhanced when compared to splenocytes cultured in the presence of anti-CD4 alone, cytotoxicity directed against NK-sensitive YAC leukaemia cells was only moderate under these conditions

Cytotoxicity directed against piBCL1 was assessed at the end of weeks 2 and 4, and was found to increase with culture period (figure 5.1 (b)). Furthermore, anti-tumour cytotoxicity was blocked by the presence of mAbs directed against CD8 and, in this experiment, the BALB/c class-I haplotype D^d (figure 5.1 (c)).

Using this procedure, two new CTL lines reactive against piBCL1 (Line A and B) were established using the combination of IL-7 and anti-CD4 during the first week of culture and, thereafter, IL-2 and anti-CD4. Cytotoxicity was measured each week and lines were considered to be established when the level of cytotoxicity no longer increased with culture period. As shown in figure 5.2 (a), Line A demonstrated cytotoxic activity against both piBCL1 and A20, but not YAC, P815 (a murine mastocytoma cell line) or CT26 (a murine carcinoma cell line), all if which are derived from BALB/c mice. Interestingly, the cytolysis of both A20 and piBCL1 by Line A negates the possibility that these cells are reactive against idiotypic determinants. Line B also displayed cytotoxic activity against piBCL1, although to a lesser extent than Line A, but, was not reactive against A20 or any of the other cell lines tested.

In order to demonstrate that tumour-reactive CTL can be raised from animals having undergone immunotherapy for a different tumour (specifically a CD40-negative tumour other than a lymphoma), duplicate C57BL/6 mice were inoculated with $1x10^7$ EL4 thymoma cells i.v. and, 4 days later, with 1 mg of anti-CD40 i.v.; subsequently, 20 days after the initial tumour challenge, splenocytes were cultured as for piBCL1-reactive CTL and their reactivity against EL4 assessed after two weeks in culture, *i.e.*, two rounds of stimulation.

As can be seen in figure 5.2 (b), these cultures demonstrated cytotoxic activity against EL4, but not YAC cells.

In order to assess the clonal diversity of the generated piBCL1-specific CTL, we visualised the Vβ gene useage in Line A and B by using a panel of fluorescenated anti-V-region mAbs. Line A predominantly consisted of T-cells using the Vβ8 gene segment, whereas approximately 55 % of Line B consisted of T-cells using either the Vβ10 or Vβ8 gene segment (Figure 5.3).

These data therefore indicate that $CD8^+$ class-I-restricted anti-tumour CTL can be raised *in vitro* from animals in remission following immunotherapy of BCL1 or EL4. The fact that the combination of IL-7 and anti-CD4 results in the generation of CTL that are all tumour-specific within one week of culture (figure 5.1 (a)), but that anti-tumour cytotoxicity is increased (at a set effector-to-target ratio) over the course of approximately the next four weeks, suggests that high avidity clones may grow to predominate these cultures. This conclusion is supported by the predominance of $CD8^+$ lymphocytes utilising the same Vβ gene segment within established lines (figure 5.3). Which clones grow to predominate is likely to be largely influenced by the conditions under which immunotherapy took place *in vivo* (*e.g.*, the tumour burden present during the primary response) and the survival of these clones within the memory pool, *i.e.*, the frequency of these clones within the $CD8^+$ lymphocyte compartment when starting each culture *in vitro*.

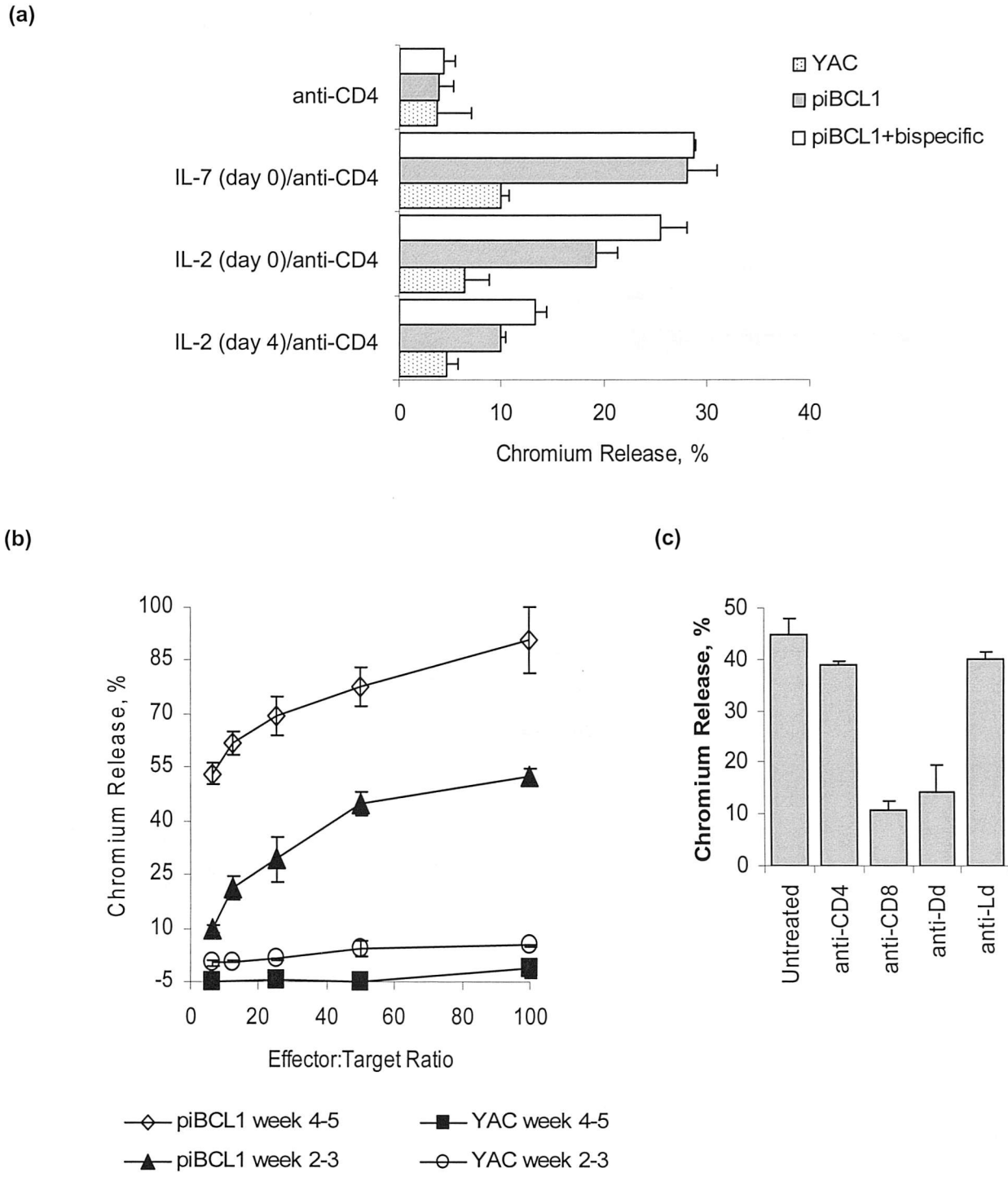
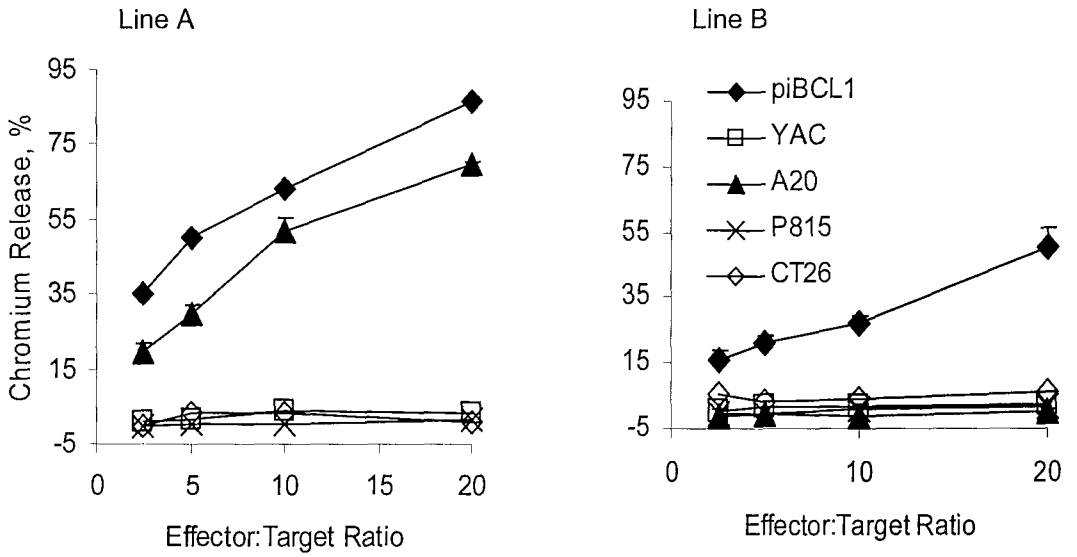


Figure 5.1. Generation of piBCL1-specific CTL from Animals Following Immunotherapy for BCL1. BALB/c mice received $5x10^7$ BCL1 lymphoma cells on day 0 followed by 1 mg of anti-CD40 on day 4. Once animals were in remission (typically days 20-60), splenocytes were harvested and $2x10^6$ co-cultured with $5x10^5$ irradiated piBCL1 as described in Chapter 2. In (a), splenocytes were co-cultured with irradiated piBCL1 under a variety of conditions (anti-CD4, 10 µg/ml;, IL-7, 10 ng/ml; IL-2, 20 U/ml) for 7 days and a standard chromium release assay performed at an effector:target ratio of 100:1. Where indicated [anti-CD3 x anti-BCL1 idiotype] bispecific antibody was used at a final concentration of 1 µg/ml. Cultures given IL-7/anti-CD4 were re-seeded and re-stimulated weekly by plating $1x10^6$ responders with $5x10^5$ irradiated piBCL1 in the presence of mIL-2 and anti-CD4; cytotoxic activity was assessed after 2 weeks and 4 weeks in culture by standard chromium release assay, (b). After two weeks in culture, responding cells were also assayed at an effector:target ratio of 50:1 for cytotoxicity against piBCL1 in the presence of 50 µg/ml of the indicated mAbs, (c).

(a)



(b)

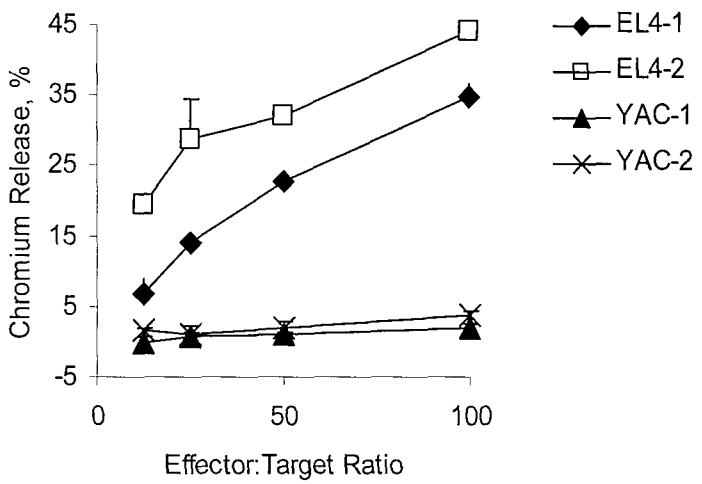


Figure 5.2. Cytotoxicity of Established anti-BCL1 and anti-EL4 CTL Lines. In (a), BALB/c mice received $5x10^7$ BCL1 lymphoma cells on day 0 followed by 1 mg of anti-CD40 on day 4. Once animals were in remission (typically days 20-60), splenocytes were harvested and $2x10^6$ co-cultured with $5x10^5$ irradiated piBCL1 as described in Chapter 2. Cultures having had IL-7/anti-CD4 were re-seeded and re-stimulated weekly by plating $1x10^6$ responders with $5x10^5$ irradiated piBCL1 in the presence of mIL-2 and anti-CD4. Cultures were tested for cytotoxicity, directed against the targets indicated, 7 days after the last re-stimulation. Lines were considered to be established when cytotoxicity stopped increasing after rounds of stimulation. In (b), C57BL/6 mice received $1x10^7$ EL4 thymoma cells i.v. on day 0 followed by 1 mg of anti-CD40 on day 4. Once animals were in remission, splenocytes were harvested and $2x10^6$ co-cultured with $5x10^5$ irradiated EL4 as described in Chapter 2. Cultures having had IL-7/anti-CD4 were re-seeded and re-stimulated weekly by plating $1x10^6$ responders against $5x10^5$ irradiated EL4 in the presence of mIL-2 and anti-CD4. Cultures were tested for cytotoxicity, directed against the indicated targets, 7 days after prior re-stimulation. Means and standard deviation represent the mean values from triplicate assay measurements.

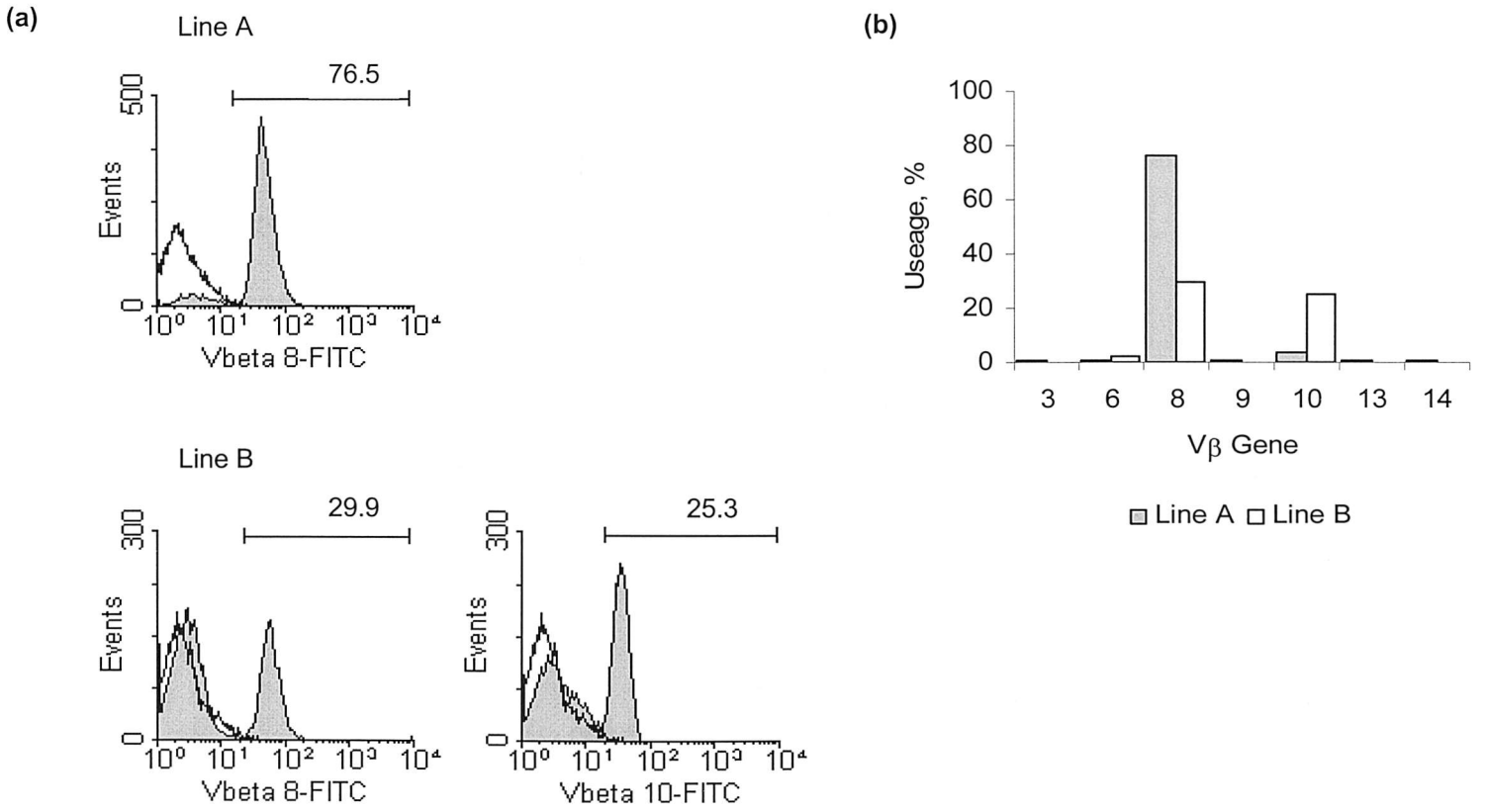


Figure 5.3. Vβ Gene Useage by Established anti-piBCL1 CTL Lines. BALB/c mice received $5x10^7$ BCL1 lymphoma cells on day 0 followed by 1 mg of anti-CD40 on day 4. Once animals were in remission, splenocytes were harvested and $2x10^6$ co-cultured with $5x10^5$ irradiated piBCL1 as described in Chapter 2. Cultures having had IL-7/anti-CD4 were re-seeded and re-stimulated weekly by plating $1x10^6$ responders against $5x10^5$ irradiated piBCL1 in the presence of mIL-2 and anti-CD4. Lines were considered to be established when cytotoxicity stopped increasing after rounds of stimulation. Anti-piBCL1 Line A and B were both stained with PE-conjugated anti-CD8 and FITC-conjugated mAbs directed against the Vβ region indicated (solid grey histograms) or an irrelevant antigen (open histograms) 7 days after the last re-stimulation. Representative data are shown in (a), and data from all Vβ regions tested are shown in (b).

5.3.2. Characterisation of anti-piBCL1 CTL Lines

Next, we chose to characterise Line A and Line B in order to demonstrate that these CTL reflected the characteristics of the responding $CD8^+$ lymphocytes seen *in vivo* during immunotherapy and, furthermore, to see if these lines were suitable for the screening of a cDNA library.

As shown in figure 5.4 (a) and (b), cytolysis of piBCL1 by Line A was blocked by mAbs directed against D^d and LFA-1. Furthermore, pre-incubation of Line A with concanomycin A, a reagent which prevents lytic granule acidification via inhibition of vacuolar H^+ ATPase and, hence, granule-mediated cytotoxicity [482], abolished the cytotoxic activity of Line A (figure 5.4 (c)). Conversely, the pre-incubation of Line A in 10 μM brefeldin A, conditions under which Fas-mediated cytotoxicity is prevented, failed to inhibit cytolysis of piBCL1.

Proliferation of Line A in response to piBCL1 was assessed by CFSE staining of these cells and detecting cell division by FACS on days 1 to 6 following stimulation – as can be seen in figure 5.5 (a), Line A proliferated in response to piBCL1 and IL-2, with the greatest period of growth being between days 2 and 3. Additionally, both Line A and B expressed 4-1BB under these conditions 24 hours following stimulation (figure 5.5 (a)), but this expression was greatly reduced at 72 hours or if IL-2 was omitted (figure 5.5 (b)).

Figure 5.6 (a) shows that stimulation of both Line A and B with piBCL1 induced IFNγ production from these CTL, and that this was elevated with the number of tumour cells present with respect to the number of CTL (*i.e.*, at lower effector-to-target ratios). At effector-to-target ratios higher than 2:1, IFNγ production was maximal 24 hours following stimulaton, but, notably with Line A, at 2:1 IFNγ accumulation continued from 24 to 72 hours. Furthermore, as with cytotoxic activity (figure 5.2 (a)), Line A produced IFNγ in response to A20, though this was to a lesser extent than in response to piBCL1, whereas Line B did not (figure 5.6 (b)).

IFNγ production by Line A and B in response to piBCL1 was inhibited in the presence of mAbs directed against CD8, LFA-1, B7.1, B7.2, and, to a much lesser extent, ICAM-1 (figure 5.6 (c)). However, mAbs directed against 4-1BB failed to prevent IFNγ production from both lines in response to piBCL1.

Together, these data demonstrate that CTL raised *in vitro* from animals in remission share the broad characteristics of the $CD8^+$ lymphocytes known to respond to BCL1 *in vivo* during anti-CD40-mediated immunotherapy, *i.e.*, proliferation, cytotoxic activity, 4-1BB expression, and IFNγ production in response to tumour. Furthermore, both IFNγ production and anti-tumour cytotoxicity are dependent upon interactions via LFA-1, as is $CD8^+$ lymphocyte expansion following administration of anti-CD40 [460] and anti-tumour cytotoxicity when assessed directly ex-vivo during immunotherapy (figure 3.13).

Interestingly, Line A mediated cytolysis of piBCL1 via the granule exocytosis pathway, thereby arguing against the possibility that anti-CD40 may be operating by the up-regulation of Fas expression on the lymphoma and, hence, rendering neoplastic cells susceptible to anti-tumour CTL. However, this single piece of data is by no means conclusive, and the possibility that other changes in the tumour, such as altered TAP expression, may contribute to the generation of effective anti-tumour CTL can not be excluded. Nonetheless, such effects are unlikely to constitute the predominant mechanisms by which anti-CD40 exerts its therapeutic effect (see Chapter 6).

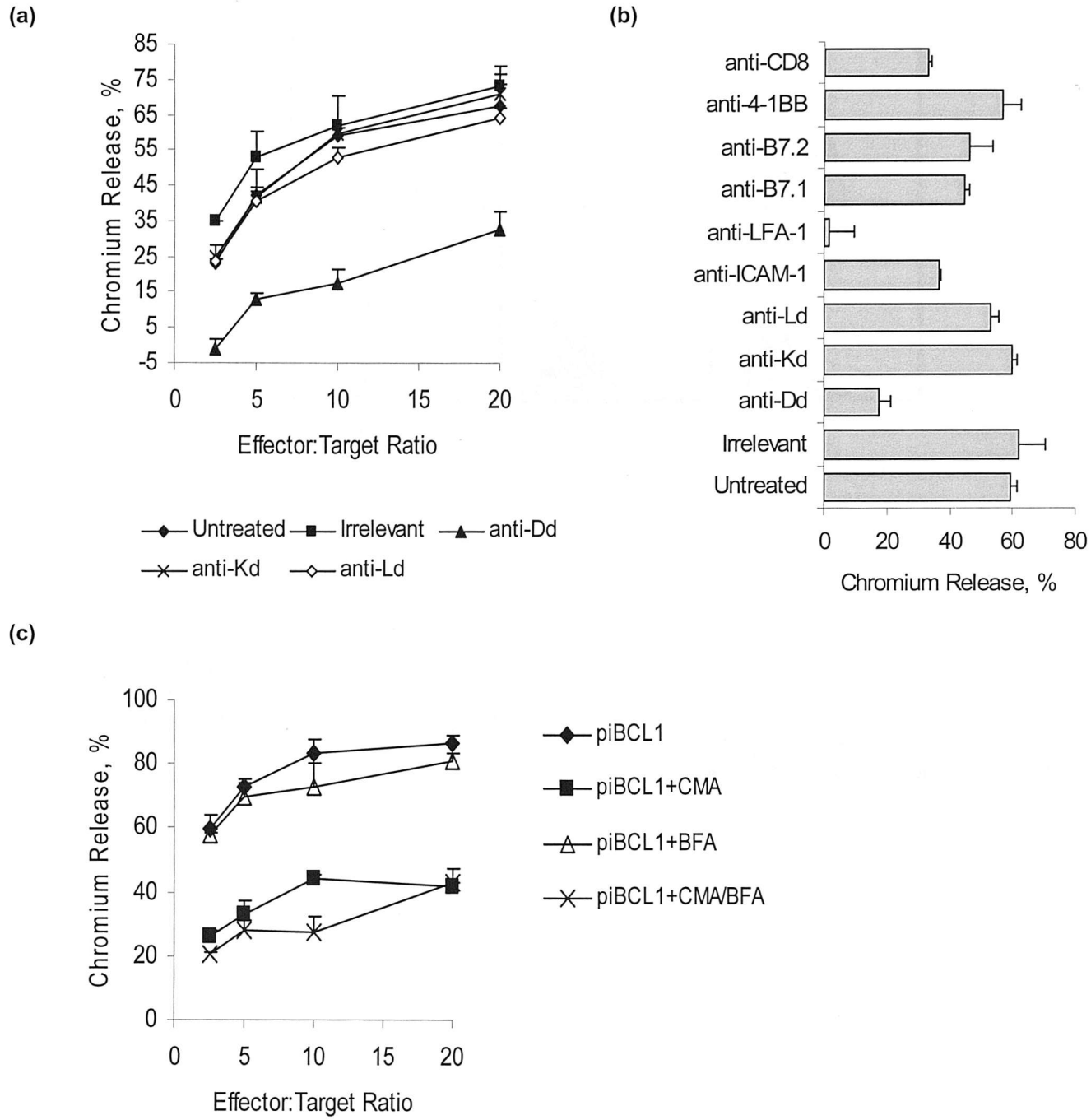


Figure 5.4. Target Recognition and Cytotoxicity Mechanism Utilised by Established anti-piBCL1 CTL Lines. BALB/c mice received $5x10^7$ BCL1 lymphoma cells on day 0 followed by 1 mg of anti-CD40 on day 4. Once animals were in remission (typically days 20-60), splenocytes were harvested and $2x10^6$ co-cultured with $5x10^5$ irradiated piBCL1 as described in Chapter 2. Cultures having had IL-7/anti-CD4 were re-seeded and re-stimulated weekly by plating $1x10^6$ responders against $5x10^5$ irradiated piBCL1 in the presence of mIL-2 and anti-CD4. Lines were considered to be established when cytotoxicity stopped increasing after rounds of stimulation. Cultures were tested for cytotoxicity, directed against the targets indicated, 7 days after the last re-stimulation. In (a), cytotoxicity was assessed by standard chromium release assay at the effector:target ratio shown in the presence of 50 µg/ml of mAbs directed against the indicated targets. Alternatively, cytotoxicity was assessed by standard chromium release assay at an effector:target ratio of 10:1 in the presence of 50 µg/ml of mAbs directed against the indicated targets, (b). In (c), effector cells were pre-incubated with 30 nM of concanamycin A (CMA) or 10 µM of brefeldin A (BFA) 2 hours prior to assessing cytotoxicity in the presence of the same concentration of CMA or BFA.

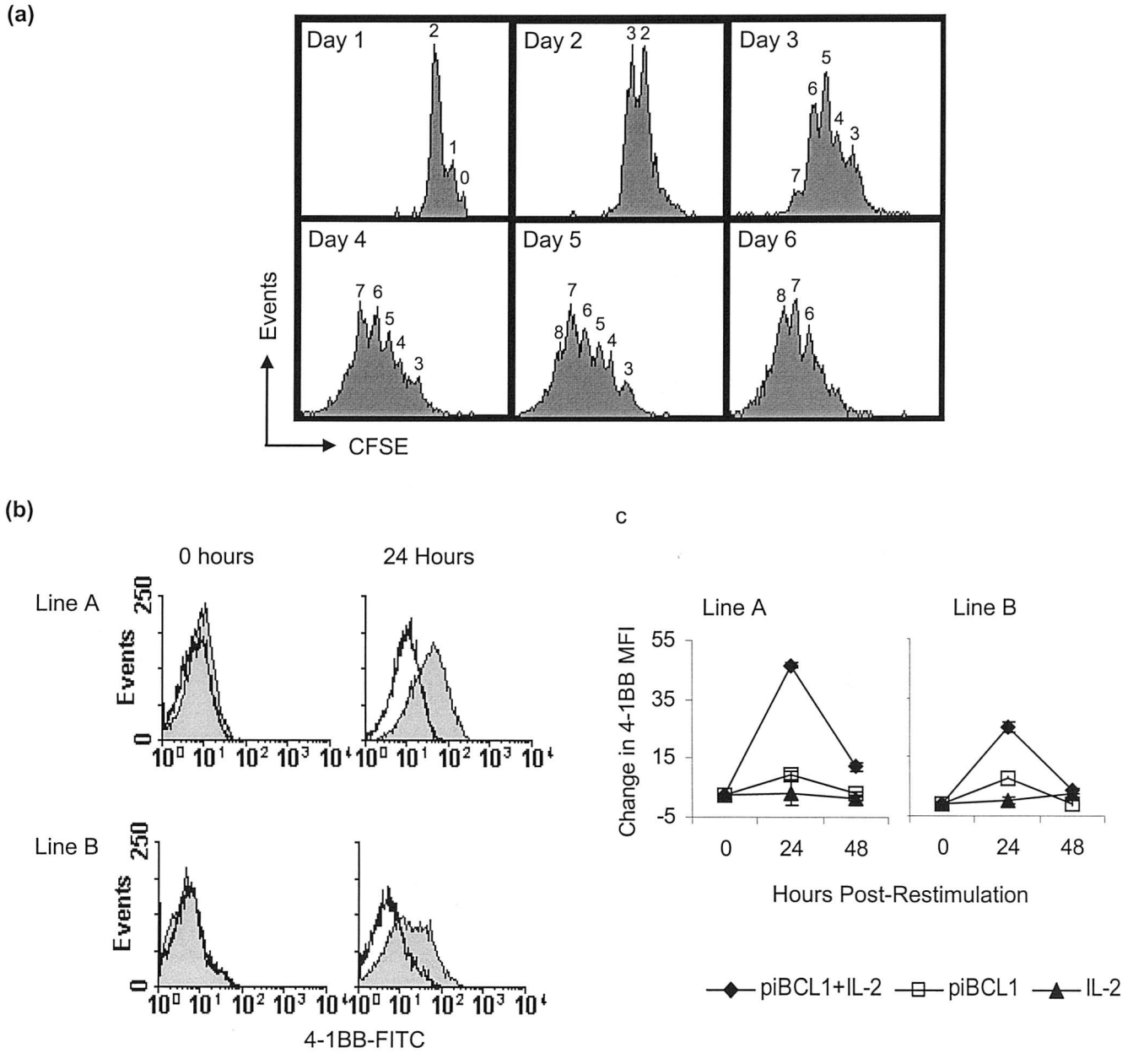


Figure 5.5. Proliferation and 4-1BB Expression by Established anti-piBCL1 CTL Lines. Anti-piBCL1 Line A was labelled with CFSE as detailed in Chapter 2 and re-stimulated under standard conditions. On days 1-6 following re-stimulation, individual cultures of Line A were harvested and CFSE fluorescence assessed by FACS, (a). The number of divisions is indicated above each peak. In (b), both Line A and B were re-stimulated under conditions used for weekly stimulation and, after 0, 24 and 48 hours, cells stained with PE-conjugated anti-CD8 and FITC-conjugated anti-4-1BB (solid grey histograms) or an irrelevant mAb (open histograms). Changes in 4-1BB expression over time (0, 24 and 48 hours) following re-stimulation under the conditions shown are summarised in (c). Error bars represent the maximum and minimum values obtained in each group.

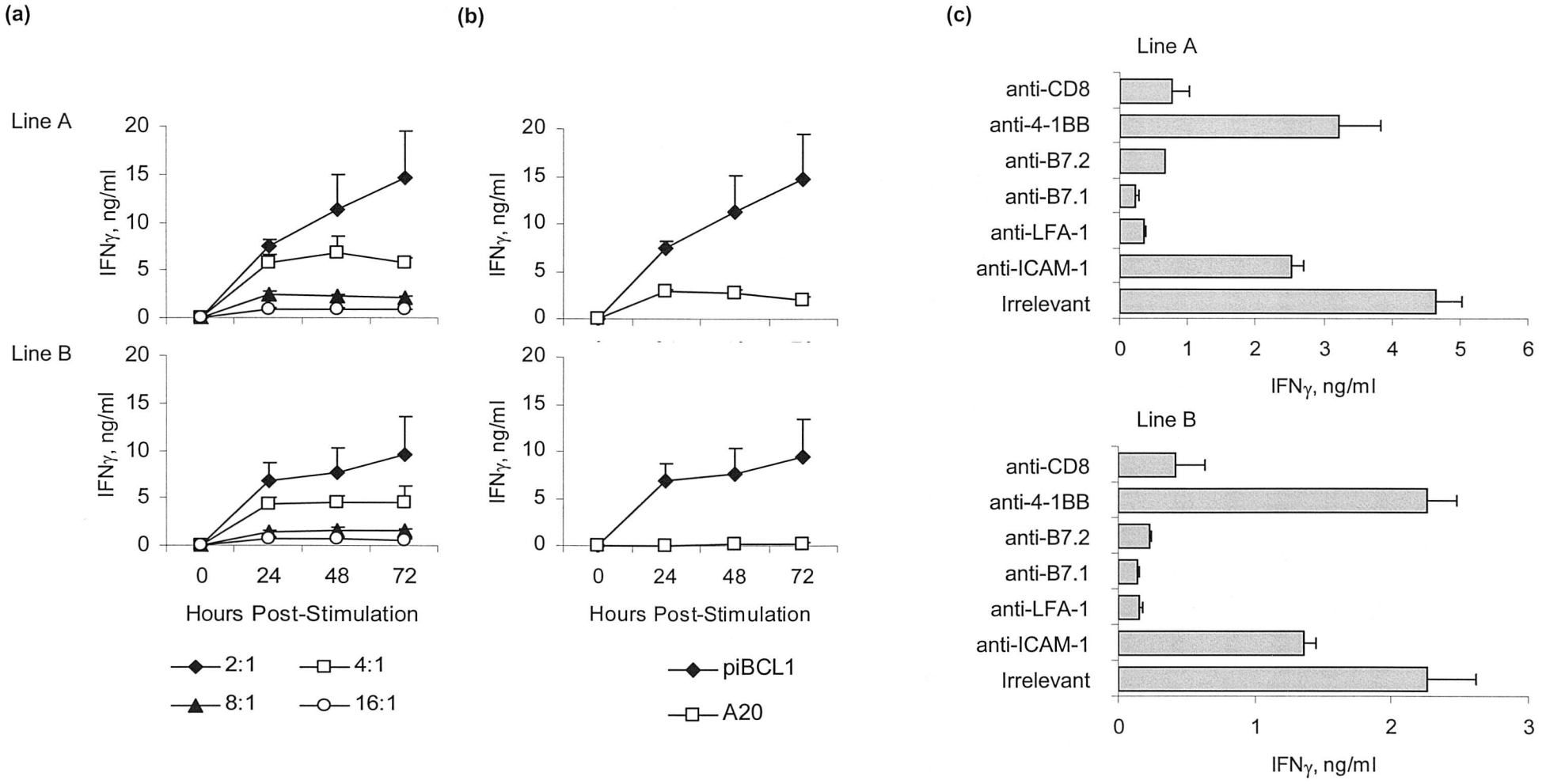


Figure 5.6. IFNγ Secretion by Established anti-piBCL1 CTL Lines. 1x10^5 responder cells from established anti-piBCL1 CTL Lines A and B were restimulated with irradiated piBCL1 at the effector:target ratio shown, and IFNγ secretion assayed by standard ELISA after 0, 24, 48, and 72 hours, (a). In (b), responder cells were re-stimulated with irradiated A20 or piBCL1 at an effector:target ratio of 2:1 and IFNγ secretion assayed as in (a). Alternatively, IFNγ secretion was estimated by standard ELISA after culturing 1x10^5 of Line A or B in the presence of 5x10^4 irradiated piBCL1 for 24 hours in the presence of 50 µg/ml mAb of specificity shown, (c).

5.3.3. Generation and Characterisation of anti-piBCL1 Hybridomas

In addition to the generation of class-I-restricted CTL lines, we wished to raise T-cell hybridomas from animals in remission following immunotherapy as such hybrids are classically used to screen cDNA libraries since large numbers of cells can be rapidly accumulated without the need for re-stimulation, and hybrid fusion partners often contain constructs that allow rapid assessment of activation. Indeed, we chose to use the BWZ.36 CD8α fusion partner which is derived from the αβBW5147 cell line by stably transfection with both the CD8α gene and an inducible NFAT-LacZ construct (Nuclear factor of activated T-cell; NFAT, enhancer element of the IL-2 gene) [457]. Thus, following fusion of this partner with a T-cell, TCR engagement results in the expression of β-galactosidase which can be detected microscopically at the single cell level, or macroscopically after lysis of the hybridomas, following exposure to the appropriate colorimetric substrate (see Chapter 2). In this way, such hybridomas provide a convenient and sensitive assay of TCR engagement.

In order to generate piBCL1-reactive hybrids, animals were inoculated with $5x10^7$ BCL1 i.v. followed by 1 mg of anti-CD40 i.v. 4 days later; subsequently, 20 days after the initial tumour challenge, splenocytes were cultured with anti-CD4, IL-7, IL-2, and irradiated piBCL1 prior to fusion as described in Chapter 2.

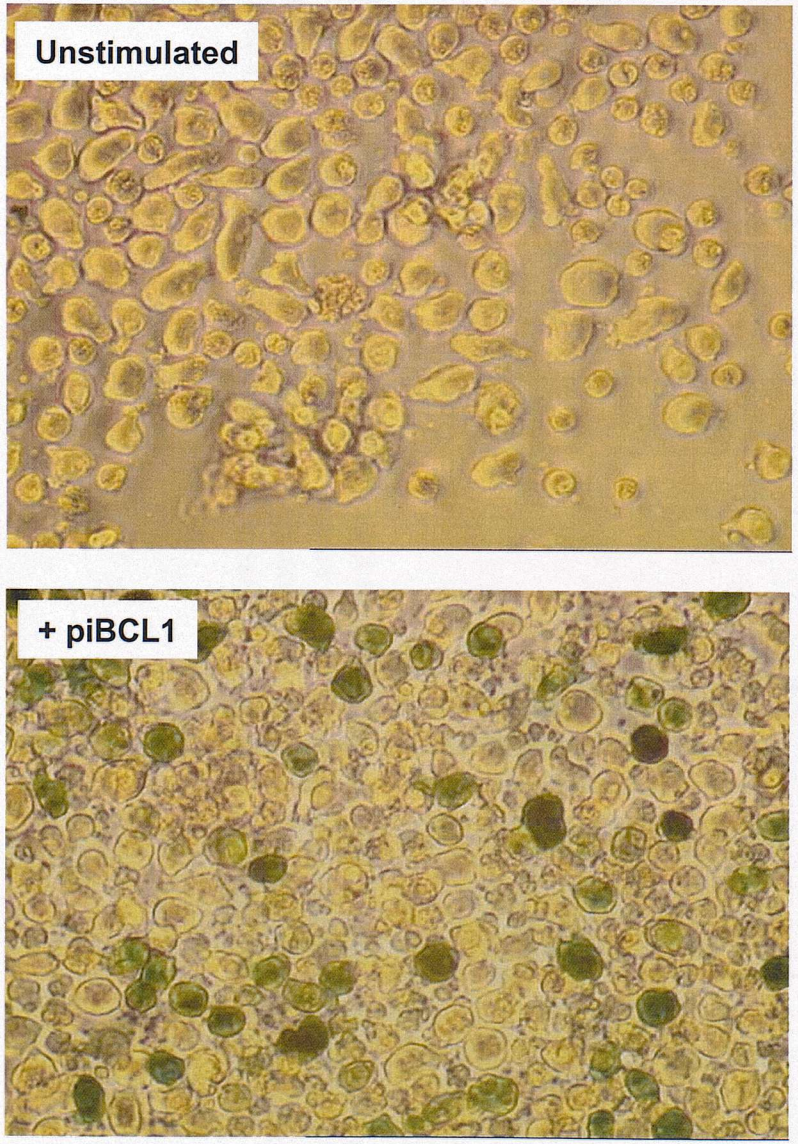
Three tumour-reactive hybrids were generated from a single fusion, Hybrids A, B and C. As shown in figure 5.7, these hybrids demonstrated β-galactosidase expression following exposure to piBCL1, as assessed both at the single cell level (panel (a)) and after lysis of the hybrids (panel (b)). Importantly, in the absence of tumour, little or no β-galactosidase was detectable (figure 5.7 (a)). Hybrids A and B expressed CD8 and, to a lesser extent CD3, but not CD4 (figure 5.8 (a)); sub-cloning of these two hybrids allowed the segregation of hybridomas expressing high or low quantities of CD3 (as shown in figure 5.8 (b)). In contrast to Hybrids A and B, Hyrbid C seemed to be losing CD8 expression, but expressed CD4 and higher levels of CD3 than Hybrids A and B (figure 5.8 (a)). Accordingly, the expression of β-galactosidase by Hybrid C in response to piBCL1 was abolished in the

presence of anti-class II mAb (figure 5.7 (b)), while anti-class I mAb inhibited the activation of Hybrids A and B (figure 5.9 (a)). The anti-class-I mAb used in this experiment was HB79 as this mAb binds murine class-I in a haplotype-independent manner; haplotype-specific mAbs were unable to inhibit β-galactosidase expression by Hybrids A and B (data not shown); this point will be discussed in section 5.4 below.

Next, we wished to characterise the $CD8^+$ class-I-restricted Hybrids (A and B) so as to assess if, as with piBCL1-reactive CTL, activation of these hybrids was dependent upon LFA-1, B7.1, or B7.2. As shown in figure 5.9 (a), mAbs directed against ICAM-1 or LFA-1 only slightly inhibited β-galactosidase expression from Hybrid A following exposure to piBCL1, while activation of Hybrid B was similarly effected by mAbs directed against ICAM-1, B7.1, and B7.2; however, anti-LFA-1 did reduce β-galactosidase expression from Hybrid B by approximately 30 % (estimated using β-galactosidase expression in the presence of anti-class-I as 100 % inhibition). Additionally, Hybrid A demonstrated reactivity towards both piBCL1 and CT26, while Hybrid B was only reactive against piBCL1 (figure 5.9 (b)). Neither of these two hybrids demonstrated reactivity against A20, P815, or WEHI.

These data within figures 5.7 - 5.9 therefore demonstrate for the first time that both class-I- and class-II-restricted T-cell hybridomas can be generated from animals in remission following immunotherapy with anti-CD40; the implications of this are discussed below.

(a)



(b)

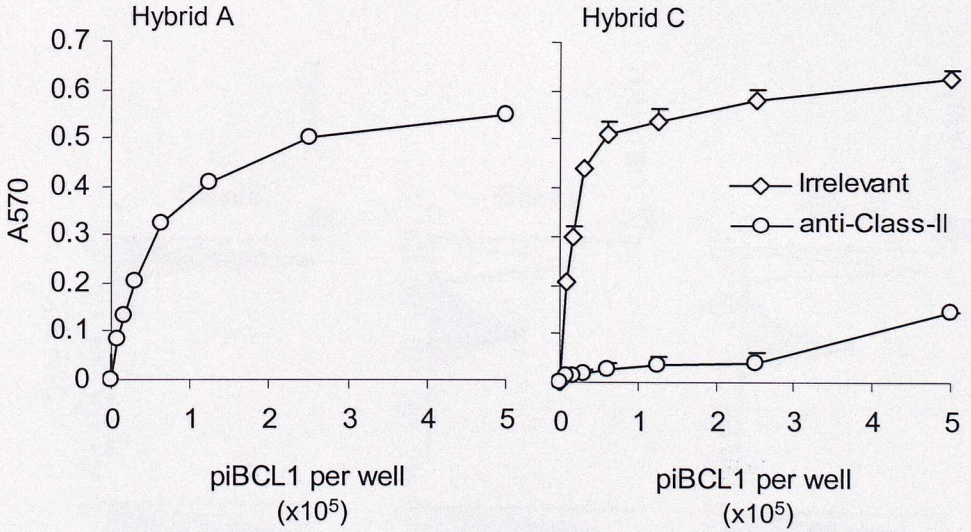
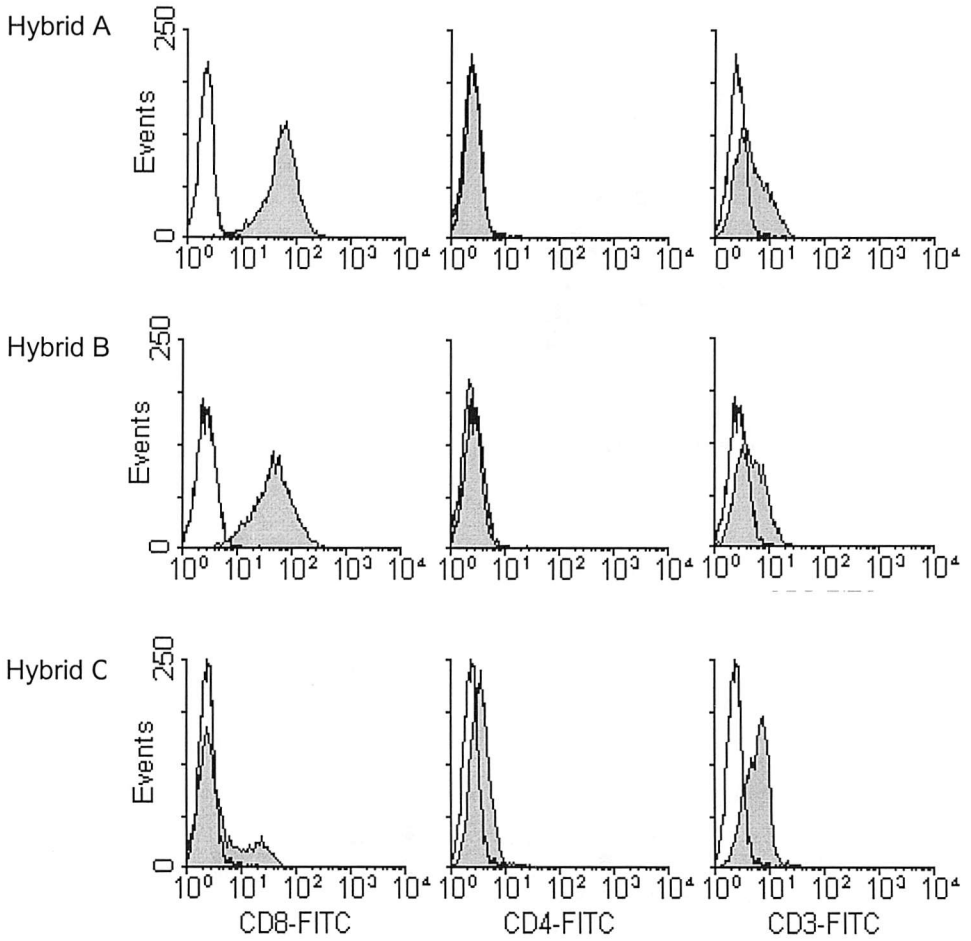


Figure 5.7. Generation of piBCL1-specific Hybridomas from Animals following Immunotherapy of BCL1. $1x10^5$ Hybrid A cells were cultured with $5x10^5$ piBCL1 overnight, a single cell β-galatosidase detection assay performed (Chapter 2), and reactive cells visualised microscopically, (a). Similarly, in (b), Hybrid A or Hybrid C cells were cultured overnight with the indicated number of piBCL1 and, subsequently, a bulk β-galatosidase detection assay performed as detailed in Chapter 2. In the case of Hybrid C, blocking mAb was used at a final concentration of 50 μg/ml.

(a)



(b)

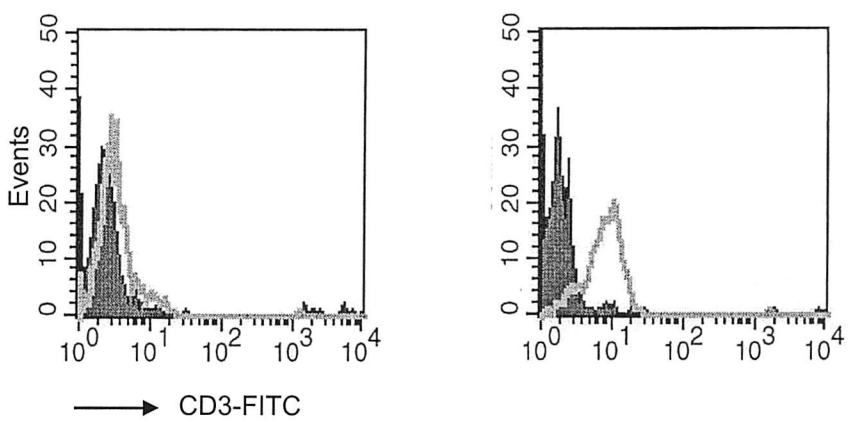


Figure 5.8. Phenotype and Cloning of piBCL1-specific Hybridomas. Once grown into small flasks, piBCL1-reactive hybrids A, B and C were stained with FITC-conjugated anti-CD8, -CD3, -CD4 (solid grey histograms) , or an irrelevant mAb (open histograms), (a). $CD8^+$ Hybrids A and B were then sub-cloned as detailed in Chapter 2 and individual clones stained with FITC-conjugated anti-CD3, (b).

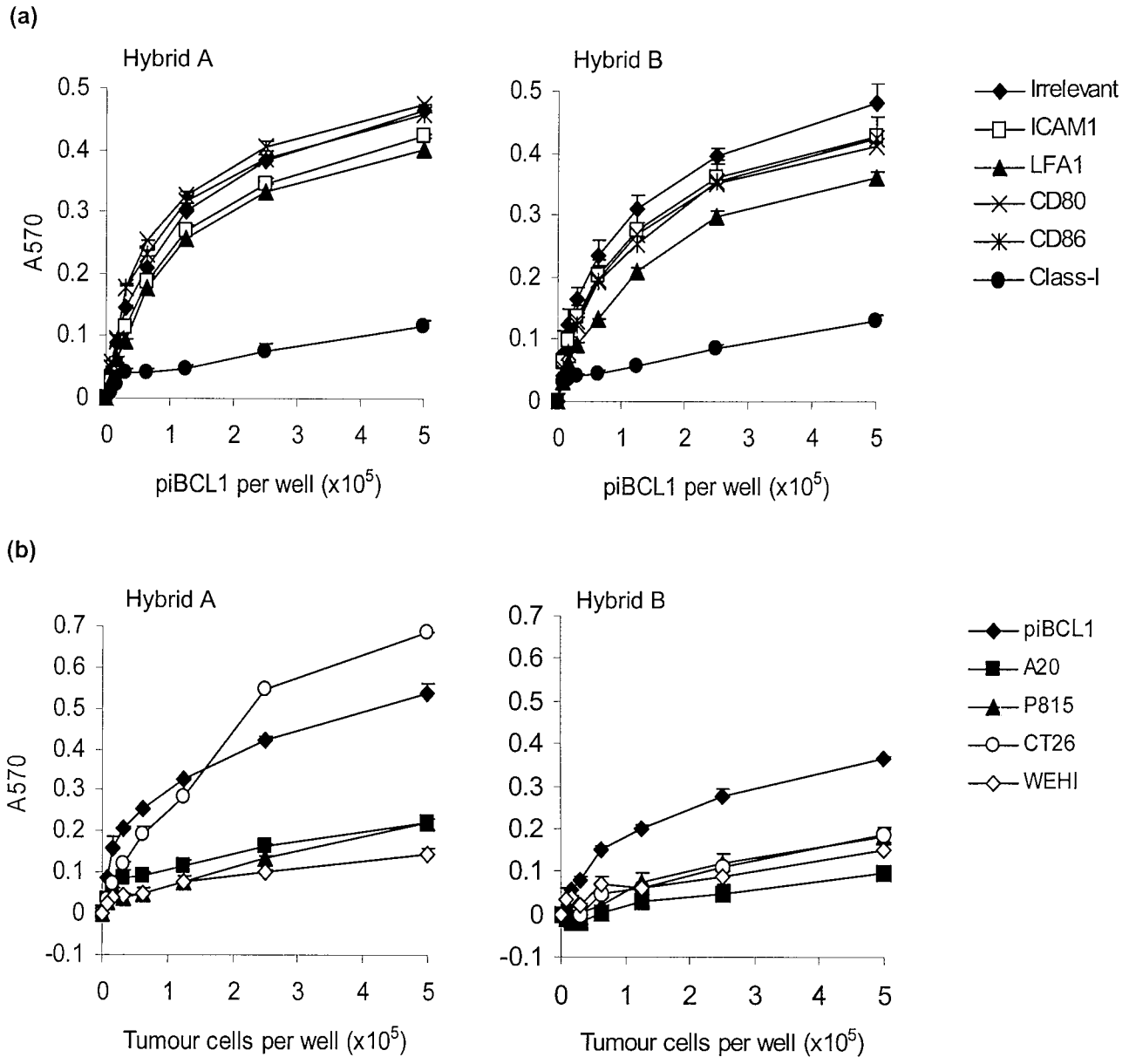


Figure 5.9. Reactivity of piBCL1-specific Hybridomas A and B. $1x10^5$ Hybrid A or B cells were cultured with the indicated number of piBCL1 overnight in the presence of 50 µg/ml mAbs specific for the antigens shown. Subsequently, a bulk β-galatosidase detection assay was performed as detailed in Chapter 2, (a). In (b), $1x10^5$ Hybrid A or B cells were cultured overnight with the indicated number of with P815, A20, CT26, WEHI, or piBCL1. Again, anti-tumour reactivity was assessed by using a bulk β-galactosidase detection assay.

5.4. Discussion

These data demonstrate for the first time that tumour-reactive CTL may be raised from splenocytes taken from animals in remission following immunotherapy with anti-CD40, and thereby confirm that a proportion of the responding $CD8^+$ lymphocytes seen during immunotherapy develop into either central or effector memory cells which may be reactivated on exposure to tumour.

Line A demonstrated cytotoxicity and IFNγ production in response to both piBCL1 and A20 (figures 5.2 and 5.6), while Line B only demonstrated responsiveness to piBCL1. Assuming that the same $V\beta8^+$ CTL clone within Line A is responsible for both A20 and piBCL1 responsiveness, these data suggest two points. Firstly, A20 and piBCL1 display the same epitope that is either produced as a result of a common event related to the oncogenic process (such as the expression of a developmental protein or the generation of a neoantigen), or as a result of a viral infection (see Chapter 1). The comparable cytolysis of A20 and piBCL1 by Line A, but the differing IFNγ production in response to these tumours, suggests that A20 and piBCL1 are similarly susceptible to granule-mediated cytotoxicity, and that this shared epitope is expressed at sufficiently similar levels in order to mediated analogous cytotoxicity, but not IFNγ production. Indeed, IFNγ production by Line A in response to piBCL1 was dependent upon interactions via B7.1 and B7.2 (figure 5.6), as is the *in vivo* expansion of $CD8^+$ lymphocytes during immunotherapy of BCL1, [460] whereas cytolysis of piBCL1 by Line A was not (figure 5.4). Secondly, in addition to data contained within section 5.3.2, the fact that Line A responded to both piBCL1 and A20 reinforces the relationship between these CTL lines and those CTL that provide protection against secondary *in vivo* challenge with tumour, as animals that are in remission following immunotherapy of BCL1 are refractory to challenge with A20; this shared epitope is therefore able to act as a bona fide TRA. Indeed, co-injection of piBCL1-reactive CTL lines with BCL1 protects against tumour outgrowth (Patrick, CC, unpublished observations).

Tumour-reactice CTL lines, even if cloned, are unlikely to be useful in the screening of a cDNA library generated from the lymphomas. Firstly, the large number of cells required for

rounds of screening would be difficult to obtain as, even though these CTL rapidly divide on re-stimulation, only low yields are achieved, presumably because of AICD. Secondly, IFNγ production (which is the most likely assay to be used in the screening process) from these CTL is dependent upon interactions via B7 and LFA-1, implying that stable COS-7 transfectants expressing the appropriate murine class-I, B7.1, B7.2, and LFA-1 would have to be generated in order to detect weakly agonistic epitopes; this also applies if chromium release assay were to be used during screening, as interactions via LFA-1 are required for cytolysis of piBCL1 by Line A (figure 5.4. (b)). On the other hand, class-I-restricted T-cell hybridomas, such as those described here, overcome these two problems as large numbers can be easily obtained and, at least in the case of Hybrids A and B, activation of these hybrids is far less dependent on interactions via B7.1, B7.2, and LFA-1. It is hard to validate the relevance of these hybrids as many $CD8^+$ lymphocytes may be reactive against piBCL1 following immunotherapy with BCL1, but which antigens act as the dominant TRAs is unknown, *i.e.*, epitopes may exist that result in T-cell activation, but not tumour eradication *in vivo*. Hence, it may be pertinent in the future to raise such hybrids from CTL clones that, firstly, have been cloned from CTL lines early after initiating *in vitro* culture (so as to maintain diversity) and, secondly, that are known to mediate tumour eradication *in vivo* on adoptive transfer. Moreover, sub-cloning of these hybrids prior to screening a cDNA library would be essential, firstly, in order to obtain a genetically stable population and, secondly, in order to isolate hybrids that express high levels of CD3 and, hence, are likely to express more β-galactosidase upon TCR engagement than clones that express less CD3 (*c.f.*, heterogenous responders seen in figure 5.7 (a)).

Our cumulative data suggest that anti-CD40 mAb operates via the bolstering of a pre-existing anti-tumour T_H1 response, at least in part, by the amplification of tumour-initiated DC licensing which subsequently provides enhanced co-stimulatory signals to tumour-specific CTL. In this way, the differentiation and/or accumulation of anti-tumour CTL is enhanced sufficiently in order to affect tumour eradication. Moreover, anti-tumour CTL may be isolated, either as primary lines or immortalised hybridomas, from animals in remission following immunotherapy therefore, potentially, allowing the identification of the class-I-restricted epitopes acting as TRAs during anti-CD40-mediated immunotherapy.

The expression of 4-1BB on $CD8^+$ splenic lymphocytes following exposure to A31 and BCL1 (figure 3.14 and figure A1.1), and the increase in the proportion of these cells expressing 4-1BB following administration of anti-CD40 (figure 3.14), suggests that interactions via this receptor are likely to deliver survival and/or proliferative signals to anti-tumour CTL, as has been documented in other *in vivo* systems [108, 118, 149, 160, 162, 483]. Indeed, during immunotherapy, all BCL1-specific $CD8^+$ lymphocytes generated that have differentiated to possess cytotoxic activity express this receptor (figure 3.16), and inhibition of the interactions via this receptor prevents primary tumor eradication in some cases (figure 3.18).

Importantly, on the assumption that the 4-1BB-hFc construct used in these experiments provides efficient inhibition of interacts via 4-1BB, our data demonstrate limited tumour-specific CTL accumulation may occur in a 4-1BB-independent manner following administration of anti-CD40 to tumour-bearing animals as some $CD8^+$ lymphocytes differentiate to express 4-1BB in the presence of the 4-1BB-hFc fusion protein (figure 3.17). Furthermore, tumour eradication may also occur independently of interactions via 4-1BB, but this is clearly not always the case (figure 3.18). The generation of anti-tumour CTL in the absence of 4-1BB-mediated co-stimulation, and the eradication of tumour in these cases, is likely to be mediated via CD28 as administration of anti-B7 mAbs completely abolishes both $CD8^+$ lymphocytes accumulation and immunotherapy [460].

Indeed, 4-1BB-mediated co-stimulation of antigen-specific CTL has been shown to be temporally segregated from CD28-mediated co-stimulation, with interactions via CD28 being required to induce 4-1BB expression by $CD8^+$ lymphocytes in situations of sub-optimal antigen stimulation, such as those induced upon tumour inoculation [108, 169]. Specifically, Diehl *et al.* demonstrated that cross-priming of adenovirus-specific CTL in animals depleted of $CD4^+$ lymphocytes could be restored by the administration of anti-4-1BB mAb, and that this effect was abrogated if interactions via B7 were inhibited by pre-exposing animals to CTLA-4Ig fusion protein [108]. Similarly, the lack of expression of 4-1BB by $CD8^+$ lymphocytes following exposure to BCL1 and A31 in the presence of anti-B7 mAbs suggests that this response is indeed occurring under conditions of sub-optimal TCR engagement (figure A1.3), *i.e.*, B7-mediated co-stimulation is required in order to amplify TCR-mediated signaling such that 4-1BB expression may be induced. Indeed, 4-1BB-mediated co-stimulation of T-cells has only been demonstrated to be independent of CD28 under situations of strong TCR signaling, such as plate-bound anti-CD3 or allogeneic APC [154, 158]. Similarly, the efficacy of anti-4-1BB mAb against sarcoma may be improved by co-administration of FLT3 which promotes the generation of DCs from haematopoietic stem cells, *i.e.*, by enhancing the antigen presentation capacity of the host [168].

It has previously been demonstrated that interactions via 4-1BB enhance the accumulation of CTL predominantly by enhancing the survival of these cells following exposure to antigen, *e.g.*, following peptide vaccination, the proliferation of CFSE-labeled adenovirus-specific CTL was shown to be uneffected by the presence of anti-4-1BB but, rather, the accumulation of CTL was greatly elevated in animals that had received this mAb in comparison to animals that had received control mAb [108]. Indeed, this is also the case for anti-CD3-stimulated $CD8^+$ lymphocytes *in vitro*, as well as for superantigen-stimulated T-cells *in vivo* [149, 150]. In agreement with these data, the lack of correlation between the proportion of $CD8^+$ lymphocytes entering S-phase and the accumulation of these cells 4 days following anti-CD40 administration suggests that 4-1BB-mediated co-stimulation may act in a similar fashion in this model (figure 3.17), but this possibility needs confirming by, for example, co-staining 4-1BB^+ $CD8^+$ lymphocytes for intracellular BcL-xL [161].

Despite the possibility that anti-CD40 may act to enhance the antigen presentation capacity of CD40-positive lymphomas (figure 4.6), the fact that anti-CD40 is effective against CD40-negative tumours [460] suggests that the enhanced co-stimulation of tumour-specific CTL following administration of this mAb is likely to occur via the up-regulation of, for example, B7.1, B7.2 and 4-1BBL, as well as other B7-like molecules and TNF family members (*e.g.*, CD70), on DCs (figure 4.4). The contribution of DC-derived cytokines such as IL-12 p75 and mature IL-18 has yet to be fully assessed in this system, but preliminary data demonstrate that anti-CD40 is able to affect eradication of BCL1 in IL-12 $p40^{-/-}$ animals (G. Crowther, unpublished data).

The kinetics of DC maturation observed during immunotherapy, as visualised in figure 4.4, are consistent with the dynamic boosting of a pre-existing response, rather than the de novo priming of an anti-tumour response, as no lag exists between DC 'activation' and $CD8^+$ lymphocytes expansion or 4-1BB expression by these cells. Furthermore, the fact that anti-CD40 mAb induces DC maturation rapidly in the absence of tumour, whereas, in comparison, DC maturation is slow in the presence of both tumour and anti-CD40, suggests that active suppression of this response occurs; these points are drawn upon below.

A mechanism in explanation of the therapeutic activity of agonist anti-CD40 mAb based on the enhanced licensing of DCs presenting tumour-derived CTL epitopes is in contrast to the findings that systemic administration of anti-CD40 may alleviant arthritis that has been induced by immunisation of susceptible DBA/1-TCRβ transgenic mice with type II collagen [484, 485]. In this model of T_H1-driven arthritis, as in experimentally-induced EAE, recovery from the autoimmune disease may be affected by adoptive transfer of autoantigen-specific IL-10-producing B-cells that require CD40 ligation in order to secrete IL-10 [486]. How can agonist anti-CD40 mAb alleviate autoimmunity in one model and enhance anti-tumour immunity in another? Timing of mAb administration could be critical in effecting outcome as, despite the fact that anti-CD40 is administered following antigen

inoculation in both models, tumour inoculation only initiates an ineffectual CTL response, whereas immunization with collagen in Freund's adjuvant induces a fully functional T-cell response, *i.e.*, anti-CD40 is acting to promote priming in these models of tumour immunotherapy, but is acting to deviate the response in models of arthritis. Hypothetically, another contributing factor that may explain why CD40 agonists induce opposite outcomes in different disease models may lie in the different sources of antigen – intravenously injected cell-associated tumour antigen may not provide an abundance of soluble antigen available for B-cell receptor ligation, whereas collagen emulsified in Freund's adjuvant is likely to provide epitopes for both B-cells and T-cells. Furthermore, even if tumour-reactive IL-10-producing B-cells existed and were to be stimulated following anti-CD40 administration to tumour-bearing animals, the IL-10 produced is unlikely to significantly effect the ensuing anti-tumour CTL response as this response occurs anyway in the presence of tumour-derived IL-10 (see below). The persistence of anti-CD40 mAb is likely to be an important factor in driving the anti-tumour CTL response in the face of tumour-derived IL-10; indeed, despite the fact that administration of 50 μg or 1000 μg of anti-CD40 results in similar quantities of this mAb binding splenic DCs (and the total splenocytes population, figure 4.5. (b)), only doses of >200 μg of anti-CD40 affect successful immunotherapy [2].

Ochsenbein *et al.* used LCMV gp33-transfected murine tumours of several different tissue origins (lung carcinoma, T-cell lymphoma, melanoma, mastocytoma, and fibrosarcoma) to demonstrate that anti-tumour CTL priming relies on the metastasis of tumours into the secondary lymphoid tissue and that, even if this occurs, CTL may remain ignorant due to the formation of 'barriers' (consisting of collagen or factor VIII-related haemostasis factors) around solid tumour masses [487]. Interestingly, in this study, 10^4-10^7 irradiated or 10^5 live EL4-gp33 cells injected intravenously were able to prime gp33-specific CTL, hence, in the case of the live tumour cells, affecting self-rejection. Nonetheless, injection of high doses (10^7-10^8) of the same live tumour resulted in specific CTL unresponsiveness as assessed by the inability to control simultaneous LCMV infection. Furthermore, exposure of animals to LCMV prior to tumour inoculation protected recipients from subsequent high-dose

tumour inoculation; thus, increased CTL precursor frequencies were concluded to have prevented unresponsiveness. In other studies, such vaccination strategies have been used to affect tumour remission, but, in these situations low CTL precursor frequency can not be used as an explanation for the success of vaccination. For example, P14/RIP(gp x Tag2) animals, that express both the oncogenic SV40 large T antigen and LCMV gp within the pancreatic islets, develop spontaneous insulinomas with the concomitant activation of high-avidity gp33-specific P14 T-cells within the local draining lymph node; this response occurs in the absence of T-cell anergy or deletion, but does not result in tumour eradication, presumably because of limited cross-presentation within the lymph node which results in incomplete CTL differentiation [488]. Thus, in this model, administration of gp33 peptide followed by anti-CD40 mAb, or vaccination of animals with LCMV, is likely to enhance host survival by promoting the differentiation of T-cells towards both central and effector memory phenotypes (see Chapter 1).

A number of factors are likely to contribute to the lack of tumour rejection observed following inoculation of animals with either high or low doses of the tumours used here; unlike EL4-gp33, these tumours remain tumourgenic even at inoculums of 10^4 i.v. [2]. Nonetheless, it seems unlikely that anti-tumour $CD8^+$ lymphocytes remain ignorant of the presence of these lymphomas, even though this may be the case before seeding of the spleen following intravenous injection - a lag exists between tumour inoculation and detection of tumour IgM idiotype within the spleen by FACS, with the duration of this lag being dependent upon the tumour dose (data not shown). However, following migration of the lymphoma to the spleen, data shown here demonstrate that these tumours initiate DC and T-cell differentiation and accumulation but, as is the case in P14/RP(gp x Tag2) animals and in the majority of melanoma cases, this effect may be incomplete or too slow to overcome tumour outgrowth. Indeed, administration of anti-CD40 to animals that bear greater than 10 % BCL1 or A31 lymphoma within the splenic lymphocyte compartment fails to affect tumour rejection (data not shown). This may be due to several factors: firstly, both BCL1 and A31 are known to produce large quantities of IL-10 at the mRNA level [2] and, thus, tumour-derived IL-10 is likely to restrict antigen presentation by splenic DCs within

the tumour microenvironment, as has been observed, for example, with MC38 colon adenocarcinoma [32, 489]. Indeed, this may account for the delayed DC maturation observed in tumour-bearing animals, in comparison to naïve animals, following anti-CD40 administration (figure 4.4); this hypothesis could be tested by comparing the rate of DC maturation following anti-CD40 administration to BCL1-bearing animals in the presence or absence of neutralizing anti-IL-10 mAb. Secondly, the T-cell epitopes presented by the tumours used here may act only as minimal agonists, thereby minimising $CD4^+$ lymphocyte activation and, subsequently, limiting the amplification of DC licensing as well as $CD8^+$ lymphocyte differentiation. The nature of the epitopes acting as TRAs for these lymphomas is discussed below.

Despite the fact that human tumours associated with viral infection only become clinically apparent under immunosuppressive states, it is becoming increasingly obvious that several murine tumour lines used to model human disease present viral epitopes [490, 491]. Specifically, EL4 thymoma presents a CTL epitope derived from the endogenous MMTV *env* gene [125], whereas CT26 colon carcinoma presents the AH-1 peptide which is derived from gp70 of the endogenous MLV [124]. In both cases, expression of these epitopes results from demethylation of DNA during the oncogenic process [492, 493]. Hence, we postulated that the induction of a T_H1 response that occurs following inoculation with A31, BCL1, and, to a lesser extent, TEN-1 (see Appendix 1), may occur as a result of either the presence of viral T-cell epitopes and/or virally-derived 'danger' signals such as double-stranded mRNA or IFNα.

As can be seen in figure 6.1 (a), BCL1, TEN-1, A31, A20, and EL4 all expressed transcripts encoding the MMTV *env* protein whereas, importantly, BALB/c splenocytes did not. Furthermore, all these cell lines, except A31, expressed mRNA transcripts encoding MLV gp70; the expression of gp70 by CT26 was detectable, but was exceptionally low in comparison to the other tumour lines. Electron microscopy was used to additionally demonstrate that BCL1 cells produce retrovirus-like particles (panel (b)). Overall then, it is

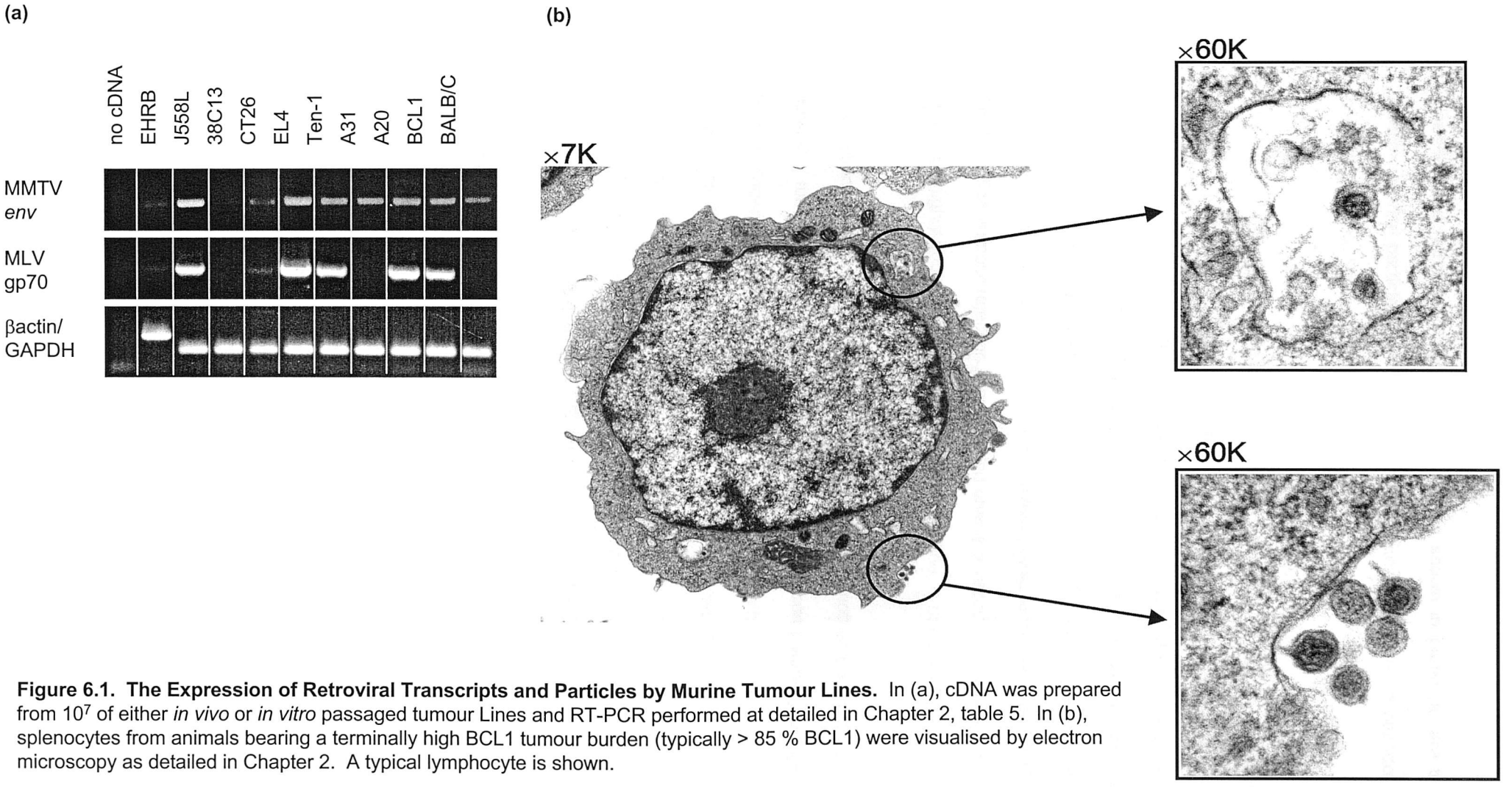


Figure 6.1. The Expression of Retroviral Transcripts and Particles by Murine Tumour Lines. In (a), cDNA was prepared from 10^7 of either *in vivo* or *in vitro* passaged tumour Lines and RT-PCR performed at detailed in Chapter 2, table 5. In (b), splenocytes from animals bearing a terminally high BCL1 tumour burden (typically > 85 % BCL1) were visualised by electron microscopy as detailed in Chapter 2. A typical lymphocyte is shown.

clear that the retroviral transcripts visualised as shown in panel (a) are translated into protein, as viral particles may be formed and, hence, virally-derived peptides are likely to become available for presentation.

Thus, the endogenous DC and T-cell response generated as a reaction to these tumours may well be directed against weakly agonist viral epitopes encoded by these viruses, but this response fails to elicit tumour eradication; this conclusion does not just apply to class-I-restricted epitopes, but also class-II epitopes, *i.e.*, both limited $CD4^+$ lymphocyte activation and, thus, DC licensing, as well as weakly agonistic $CD8^+$ lymphocyte epitopes, may curtail this anti-tumour response (*c.f.*, figure 1.5). Indeed, despite the fact that CT26 presents the AH-1 peptide, tumour rejection is not elicited unless a vaccination scheme, such as the use of a more highly agonistic AH-1-like heteroclitic peptide, is used [447].

In addition to epitopes derived from viral sequences, another possible source of weakly agonistic tumour-specific epitopes may be minor histocompatability antigens that differ from endogenous antigens due to genetic drift. Nonetheless, the possibility that the tumours studied here present viral epitopes is supported, firstly, by the fact that early studies using BCL1 lymphoma demonstrated that the surface IgM of this lymphoma binds MLV gp70 and, hence, initiates the transformation process [494] and, secondly, vaccination of animals against CT26 by the use of anti-CD25-mediated Tr cell depletion protects these animals from challenge with BCL1, *i.e.*, CT26 colon carcinoma and BCL1 lymphoma present a shared epitope(s) which must therefore be derived from either a cancer-testis antigen(s) or viral anitgen(s); notably, this epitope(s) is not derived from gp70 as animals that are vaccinated with anti-CD25 and CT26 are unable to control vaccinia virus encoding gp70, whereas animals vaccinated with a CT26-GM-CSF transfectant can [495]. Additionally, the cross-protection between BCL1 and A20, as demonstrated *in vivo* [2] and via the isolation of cross-reactive CTL (figure 5.2), may be mediated by either a shared cancer-testis antigen, viral antigen, or a tissue-differentiation antigen (see Chapter 1).

If we consider the tumour-associated epitopes presented by these lymphomas to be weakly agonistic, anti-CD40 mAb may be seen to affect tumour eradication largely by increasing the longevity and density of tumour-associated epitope presentation (via MHC up-regulation and enhancement of antigen-processing), as well as by enhancing the supply of co-stimulatory signals, thereby allowing low-affinity anti-tumour T-cells to adequately differentiate in response to these epitopes [111, 496]. Unfortunately, this premise raises the possibility that anti-CD40 may induce autoimmunity by allowing the differentiation of low-affinity autoreactive T-cell into effector cells, a prospect that other investigators have in fact hoped would affect tumour regression with an 'acceptable' concomitant damage to healthy tissue, *e.g.*, vaccination against melanoma using vaccinia-TRP-1 induces vitiligo and melanoma regression [431, 497]. Indeed, pre-clinical toxicological studies using 3/23 have demonstrated that, in animals that had received greater than 100 μg of mAb, this anti-murine CD40 mAb induces nephritis and hepatitis that is detectable histopathologically and that are both associated with lymphocytic infiltrates (T Geldart, NAC proposal). Importantly, these abnormalities are self-resolving, typically within 3 weeks of mAb administration.

The nephritis and hepatitis observed following 3/23 administration is induced by CD40-mediated signaling, as when anti-human CD40 (chimeric LOB7/4, see below) is administered to mice it does not induce these effects, suggesting that these events could potentially occur in humans following CD40 ligation. Indeed, phase I trials demonstrated a reversible elevation in serum transaminases, a phenomenon associated with hepatitis, following administration of recombinant human CD40L [453] (this effect was also observed following administration of > 100 μg of 3/23 to animals). Nonetheless, of 32 patients in this dose escalation trial, 1 complete remission (interestingly, of a carcinoma) and 1 partial remission were observed, as well as stabilisation of disease in 12 cases. Thus, phase I trials initiated here at Southampton hope to use a chimeric anti-human CD40 mAb (chimeric LOB7/4; consisting of the constant regions of human IgG_1 and the variable regions of a murine anti-human CD40 mAb) at doses that avoid toxicity associated with autoimmunity without compromising anti-tumour activity.

In regard to this, the T-cell response observed against the murine lymphomas used here, compounded by the evidence that humans tumours are able to elicit TAA-specific T-cells without affecting rejection (see Chapter 1), suggests that tumour-associated epitopes generated during the oncogenic process (whether these be due to mutation or aberrant gene expression) may act as stronger agonists than do endogenous bona fide self-ligands, thus allowing immunotherapeutic strategies to affect tumour rejection without generating adverse damage to normal tissue. Indeed, this hypothesis forms the basis of the categorisation of TAAs shown in Chapter 1, *i.e.*, vaccination using unique TAAs elicits high-avidity T-cells that affect efficient tumour rejection as no tolerance exists against these neoantigens, whereas only low-affinity T-cells may be recruited against TAAs related to self-epitopes and, thus, successful tumour regression is often associated with autoimmunity. Accordingly, identification of the epitopes present on BCL1 that allow tumour rejection during immunotherapy would allow us to assess the avidity of the generated CTL via the construction of MHC tetramers.

The use of chimeric anti-human CD40 may therefore allow the generation of anti-tumour CTL of a higher avidity that those reactive to self-ligands without requiring a foreknowledge of the TAAs being targeted; in this way, such a reagent may provide an universal off-the-shelf product that may be manufactured cheaply in bulk using hollow fibre fermenters, as have other clinically-available mAbs which have been found to be useful for the treatment of several diseases, *e.g.*, anti-TNFα (Infliximab) may be used to treat both Crohn's disease and rheumatoid arthritis. Furthermore, the use of a chimerised antibody allows relatively high tolerable doses to be used by reducing the possibility that a human anti-mouse antibody response may be induced. Additionally, the recycling of chimerised antibody via binding of the human IgG Fc regions to Fc_n receptors on endothelial cells, unlike recombinant protein, allows relatively long serum half-lives to be achieved, thus negating the need for repeated high-dose administration in order to achieve clinical efficacy [1, 498].

Overall, data shown here advance our understanding of the mechanism by with anti-CD40 mAb acts to eradicate murine lymphoma and, moreover, provide a basis for the identification of the class-I-restricted epitopes acting as TRAs during immunotherapy which may, hopefully, further validate the use of related mAbs in clinical trials to treat human lymphoma.

6.1. Further Work

In addition to assessing the efficacy of chimeric anti-CD40 mAb in clinical trials, the immediate area of most academic interest is likely to be the generation of an array of BCL-reactive hybridomas (see Chapter 5, section 5.4.). These hybridomas could be used to screen a BCL1-derived cDNA library that has already been constructed in-house using a Superscript plasmid system (Gibco). Synthetic peptides representing tumour-associated epitopes identified in this way could be used to construct MHC class-I tetrameric complexes that may be used to assess the frequency and affinity of anti-tumour T-cells induced by immunotherapy for BCL1 (both in the primary and secondary responses, as well as in the memory pool). Furthermore, such tetrameric complexes could be used in more detailed studies of the T-cell response during immunotherapy, for example, in order to assess: (1) the clonal diversity of tumour-induced T-cells *in vivo*, both before and after anti-CD40 administration, and (2) deficiencies in tumour-primed T-cells prior to anti-CD40 administration as indicated by perforin, cytokine (IFNγ, TNFα, IL-2) and surface antigen (4-1BB, CAMs, CD27) expression. Additionally, synthetic peptides could be used to assess the density and affinity for MHC of epitopes presented by the tumour (and APCs) before and after anti-CD40 administration. Thus, the identification of the tumour-associated epitopes acting to affect tumour rejection during anti-CD40-induced immunotherapy would allow more insight into the mechanism by which this mAb exerts its therapeutic activity and, ultimately, allow the generation of more rationally designed therapeutics.

Additional experiments that could provide insight into the mechanism by which anti-CD40 operates include the profiling of mRNA transcripts from splenic DCs isolated from animals undergoing immunotherapy, and from animals that have received tumour or anti-CD40

alone. Specifically, it would be of interest to see the proportion of T_H1- versus T_H2-polarising cytokine transcripts in DCs isolated from these animals, *i.e.*, are IL-10-encoding transcripts produced by DCs in tumour-bearing animals, and is IL-12 p35/40-encoding mRNA present following anti-CD40 administration? Furthermore, as IFNγ is essential for immunotherapy [460], but preliminary results suggest that IL-12 p40 plays a redundant role, it would be interesting to assess if splenic DCs produce IFNγ following tumour inoculation and/or anti-CD40 administration. Notably, the production of mature IL-18 by DCs (which may also evoke IFNγ production by T-cells) could not be assessed by mRNA profiling as mature IL-18 is produced from a consistitively expressed precursor. Thus, western blotting of splenocyte lysates would have to be performed in order to observe the production of this cytokine and, additionally, neutralizing mAbs could be used to assess if this cytokine is essential for immunotherapy.

Finally, as we have shown here that co-stimulatory signals via 4-1BB are essential for the optimal expansion of tumour-specific $CD8^+$ lymphocytes and, hence, immunotherapy (figures 3.17 and 3.18), the expression of 4-1BBL by splenic DCs is likely to be increased by administration of anti-CD40, as has already been shown in the absence of tumour [108, 117]. Preliminary experiments using a 4-1BB-hFc fusion protein to stain splenic DCs for 4-1BBL expression have failed as the binding of a control human IgG to splenic DCs following anti-CD40 administration increases, even in the presence of FcR-blocking mAb. Thus, a fluorescenated anti-4-1BBL mAb is required in order to perform this experiment accurately and, indeed, this mAb is currently being raised in-house.

1. Glennie, M.J. and P.W. Johnson, *Clinical trials of antibody therapy.* Immunol Today, 2000. **21**(8): p. 403-10.
2. French, R.R., *et al., CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help.* Nat Med, 1999. **5**(5): p. 548-53.
3. Guinn, B.A., *et al., 4-1BBL cooperates with B7-1 and B7-2 in converting a B cell lymphoma cell line into a long-lasting antitumor vaccine.* J Immunol, 1999. **162**(8): p. 5003-10.
4. Melero, I., *et al., Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors.* Nat Med, 1997. **3**(6): p. 682-5.
5. Taraban, V.Y., *et al., Expression and costimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of anti-tumor immune responses.* Eur J Immunol, 2002. **32**(12): p. 3617-27.
6. Lanzavecchia, A. and F. Sallusto, *The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics.* Curr Opin Immunol, 2001. **13**(3): p. 291-8.
7. Lanzavecchia, A. and F. Sallusto, *Regulation of T cell immunity by dendritic cells.* Cell, 2001. **106**(3): p. 263-6.
8. Larsson, M., J.F. Fonteneau, and N. Bhardwaj, *Dendritic cells resurrect antigens from dead cells.* Trends Immunol, 2001. **22**(3): p. 141-8.
9. Liu, Y.J., *et al., Dendritic cell lineage, plasticity and cross-regulation.* Nat Immunol, 2001. **2**(7): p. 585-9.
10. Liu, Y.J., *Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity.* Cell, 2001. **106**(3): p. 259-62.
11. Mellman, I. and R.M. Steinman, *Dendritic cells: specialized and regulated antigen processing machines.* Cell, 2001. **106**(3): p. 255-8.
12. Moser, M. and K.M. Murphy, *Dendritic cell regulation of TH1-TH2 development.* Nat Immunol, 2000. **1**(3): p. 199-205.
13. Reis e Sousa, C., *Dendritic cells as sensors of infection.* Immunity, 2001. **14**(5): p. 495-8.
14. Lo, D., *et al., Integrating innate and adaptive immunity in the whole animal.* Immunol Rev, 1999. **169**: p. 225-39.
15. Jung, S., *et al., In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-20.
16. Heath, W.R. and F.R. Carbone, *Cytotoxic T lymphocyte activation by cross-priming.* Curr Opin Immunol, 1999. **11**(3): p. 314-8.

17. den Haan, J.M. and M.J. Bevan, *Antigen presentation to CD8+ T cells: cross-priming in infectious diseases.* Curr Opin Immunol, 2001. **13**(4): p. 437-41.
18. Cresswell, P., *et al.*, *The nature of the MHC class I peptide loading complex.* Immunol Rev, 1999. **172**: p. 21-8.
19. Morel, S., *et al.*, *Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells.* Immunity, 2000. **12**(1): p. 107-17.
20. Bates, E.E., *et al.*, *Identification and analysis of a novel member of the ubiquitin family expressed in dendritic cells and mature B cells.* Eur J Immunol, 1997. **27**(10): p. 2471-7.
21. Bevan, M.J., *Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay.* J Exp Med, 1976. **143**(5): p. 1283-8.
22. Huang, A.Y., *et al.*, *In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter.* Immunity, 1996. **4**(4): p. 349-55.
23. Sigal, L.J., *et al.*, *Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen.* Nature, 1999. **398**(6722): p. 77-80.
24. Albert, M.L., B. Sauter, and N. Bhardwaj, *Dendritic cells acquire antigen from apoptotic cells and induce class I- restricted CTLs.* Nature, 1998. **392**(6671): p. 86-9.
25. Yrlid, U. and M.J. Wick, *Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells.* J Exp Med, 2000. **191**(4): p. 613-24.
26. Mitchell, D.A., S.K. Nair, and E. Gilboa, *Dendritic cell/macrophage precursors capture exogenous antigen for MHC class I presentation by dendritic cells.* Eur J Immunol, 1998. **28**(6): p. 1923-33.
27. Rodriguez, A., *et al.*, *Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells.* Nat Cell Biol, 1999. **1**(6): p. 362-8.
28. Bellone, M., *et al.*, *Processing of engulfed apoptotic bodies yields T cell epitopes.* J Immunol, 1997. **159**(11): p. 5391-9.
29. Regnault, A., *et al.*, *Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization.* J Exp Med, 1999. **189**(2): p. 371-80.
30. Machy, P., K. Serre, and L. Leserman, *Class I-restricted presentation of exogenous antigen acquired by Fcgamma receptor-mediated endocytosis is regulated in dendritic cells.* Eur J Immunol, 2000. **30**(3): p. 848-57.
31. Rovere, P., *et al.*, *Dendritic cell presentation of antigens from apoptotic cells in a proinflammatory context: role of opsonizing anti-beta2-glycoprotein I antibodies.* Arthritis Rheum, 1999. **42**(7): p. 1412-20.

32. Guermonprez, P., *et al.*, *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. **20**: p. 621-67.
33. Cho, B.K., *et al.*, *A proposed mechanism for the induction of cytotoxic T lymphocyte production by heat shock fusion proteins.* Immunity, 2000. **12**(3): p. 263-72.
34. Singh-Jasuja, H., *et al.*, *The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor.* Eur J Immunol, 2000. **30**(18): p. 2211-2215.
35. Basu, S., *et al.*, *Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway.* Int Immunol, 2000. **12**(11): p. 1539-46.
36. Binder, R.J., *et al.*, *Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells in vivo.* J Immunol, 2000. **165**(11): p. 6029-35.
37. Yewdell, J.W., C.C. Norbury, and J.R. Bennink, *Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines.* Adv Immunol, 1999. **73**: p. 1-77.
38. Castellino, F., *et al.*, *Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways.* J Exp Med, 2000. **191**(11): p. 1957-64.
39. Merkenschlager, M., *et al.*, *Intrathymic deletion of MHC class I-restricted cytotoxic T cell precursors by constitutive cross-presentation of exogenous antigen.* Eur J Immunol, 1999. **29**(5): p. 1477-86.
40. Watts, C., *Antigen processing in the endocytic compartment.* Curr Opin Immunol, 2001. **13**(1): p. 26-31.
41. Arndt, S.O., *et al.*, *Functional HLA-DM on the surface of B cells and immature dendritic cells.* Embo J, 2000. **19**(6): p. 1241-51.
42. Fiebiger, E., *et al.*, *Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells.* J Exp Med, 2001. **193**(8): p. 881-92.
43. Bevec, T., *et al.*, *Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L.* J Exp Med, 1996. **183**(4): p. 1331-8.
44. Inaba, K., *et al.*, *The formation of immunogenic major histocompatibility complex class II- peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli.* J Exp Med, 2000. **191**(6): p. 927-36.
45. Turley, S.J., *et al.*, *Transport of peptide-MHC class II complexes in developing dendritic cells.* Science, 2000. **288**(5469): p. 522-7.
46. Pierre, P., *et al.*, *Developmental regulation of MHC class II transport in mouse dendritic cells.* Nature, 1997. **388**(6644): p. 787-92.

47. Riese, R.J., *et al.*, *Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading.* Immunity, 1996. **4**(4): p. 357-66.
48. Fanger, N.A., *et al.*, *Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells.* J Immunol, 1996. **157**(2): p. 541-8.
49. Esposito-Farese, M.E., *et al.*, *Membrane and soluble Fc gamma RII/III modulate the antigen-presenting capacity of murine dendritic epidermal Langerhans cells for IgG-complexed antigens.* J Immunol, 1995. **155**(4): p. 1725-36.
50. Reis e Sousa, C., P.D. Stahl, and J.M. Austyn, *Phagocytosis of antigens by Langerhans cells in vitro.* J Exp Med, 1993. **178**(2): p. 509-19.
51. Basu, S., *et al.*, *CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin.* Immunity, 2001. **14**(3): p. 303-13.
52. Ohashi, K., *et al.*, *Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex.* J Immunol, 2000. **164**(2): p. 558-61.
53. Gregory, C.D., *CD14-dependent clearance of apoptotic cells: relevance to the immune system.* Curr Opin Immunol, 2000. **12**(1): p. 27-34.
54. Nouri-Shirazi, M., *et al.*, *Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses.* J Immunol, 2000. **165**(7): p. 3797-803.
55. Savill, J. and V. Fadok, *Corpse clearance defines the meaning of cell death.* Nature, 2000. **407**(6805): p. 784-8.
56. Albert, M.L., *et al.*, *Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes.* J Exp Med, 1998. **188**(7): p. 1359-68.
57. Belz, G.T., *et al.*, *CD36 is differentially expressed by CD8+ splenic dendritic cells but is not required for cross-presentation in vivo.* J Immunol, 2002. **168**(12): p. 6066-70.
58. Schulz, O., *et al.*, *CD36 or alphavbeta3 and alphavbeta5 integrins are not essential for MHC class I cross-presentation of cell-associated antigen by CD8 alpha+ murine dendritic cells.* J Immunol, 2002. **168**(12): p. 6057-65.
59. Mevorach, D., *et al.*, *Complement-dependent clearance of apoptotic cells by human macrophages.* J Exp Med, 1998. **188**(12): p. 2313-20.
60. Reddien, P.W. and H.R. Horvitz, *CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans.* Nat Cell Biol, 2000. **2**(3): p. 131-6.
61. Geijtenbeek, T.B., *et al.*, *DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking.* Nat Immunol, 2000. **1**(4): p. 353-7.
62. Geijtenbeek, T.B., *et al.*, *DC-SIGN, a dentritic cell-specific HIV-1 receptor present in placenta that infects T cells in trans-a review.* Placenta, 2001. **22 Suppl A**: p. S19-23.
63. Rescigno, M., *et al.*, *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.* Nat Immunol, 2001. **2**(4): p. 361-7.

64. Sallusto, F., *et al.*, *Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products.* J Exp Med, 1995. **182**(2): p. 389-400.
65. de Baey, A. and A. Lanzavecchia, *The role of aquaporins in dendritic cell macropinocytosis.* J Exp Med, 2000. **191**(4): p. 743-8.
66. Gunzer, M., *et al.*, *Dendritic cells and tumor immunity.* Semin Immunol, 2001. **13**(5): p. 291-302.
67. Kelsall, B.L., *et al.*, *Dendritic cells at the host-pathogen interface.* Nat Immunol, 2002. **3**(8): p. 699-702.
68. Randolph, G.J., *Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators.* Semin Immunol, 2001. **13**(5): p. 267-74.
69. Rock, F.L., *et al.*, *A family of human receptors structurally related to Drosophila Toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.
70. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.
71. Beutler, B., *Tlr4: central component of the sole mammalian LPS sensor.* Curr Opin Immunol, 2000. **12**(1): p. 20-6.
72. Lien, E., *et al.*, *Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products.* J Biol Chem, 1999. **274**(47): p. 33419-25.
73. Poltorak, A., *et al.*, *Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation.* Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2163-7.
74. Krieg, A.M., *The role of CpG motifs in innate immunity.* Curr Opin Immunol, 2000. **12**(1): p. 35-43.
75. Wagner, H., *Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity.* Curr Opin Microbiol, 2002. **5**(1): p. 62-9.
76. Bauer, S. and H. Wagner, *Bacterial CpG-DNA licenses TLR9.* Curr Top Microbiol Immunol, 2002. **270**: p. 145-54.
77. Anderson, K.V., *Toll signaling pathways in the innate immune response.* Curr Opin Immunol, 2000. **12**(1): p. 13-9.
78. Akashi, S., *et al.*, *Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages.* J Immunol, 2000. **164**(7): p. 3471-5.
79. Wright, S.D., *Toll, a new piece in the puzzle of innate immunity.* J Exp Med, 1999. **189**(4): p. 605-9.
80. Takeuchi, O., *et al.*, *Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components.* Immunity, 1999. **11**(4): p. 443-51.

81. Matzinger, P., *Tolerance, danger, and the extended family.* Annu Rev Immunol, 1994. **12**: p. 991-1045.
82. Matzinger, P., *An innate sense of danger.* Ann N Y Acad Sci, 2002. **961**: p. 341-2.
83. Matzinger, P., *An innate sense of danger.* Semin Immunol, 1998. **10**(5): p. 399-415.
84. Haziot, A., *et al.*, *Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice.* Immunity, 1996. **4**(4): p. 407-14.
85. Sauter, B., *et al.*, *Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells.* J Exp Med, 2000. **191**(3): p. 423-34.
86. Melcher, A., *et al.*, *Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression.* Nat Med, 1998. **4**(5): p. 581-7.
87. Gallucci, S., M. Lolkema, and P. Matzinger, *Natural adjuvants: endogenous activators of dendritic cells.* Nat Med, 1999. **5**(11): p. 1249-55.
88. Srivastava, P., *Roles of heat-shock proteins in innate and adaptive immunity.* Nat Rev Immunol, 2002. **2**(3): p. 185-94.
89. Wells, A.D. and M. Malkovsky, *Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view.* Immunol Today, 2000. **21**(3): p. 129-32.
90. Steinman, R.M., *et al.*, *The induction of tolerance by dendritic cells that have captured apoptotic cells.* J Exp Med, 2000. **191**(3): p. 411-6.
91. Binder, R.J., N.E. Blachere, and P.K. Srivastava, *Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules.* J Biol Chem, 2001. **276**(20): p. 17163-71.
92. Thery, C., *et al.*, *Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73.* J Cell Biol, 1999. **147**(3): p. 599-610.
93. Zitvogel, L., *et al.*, *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes.* Nat Med, 1998. **4**(5): p. 594-600.
94. Wolfers, J., *et al.*, *Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming.* Nat Med, 2001. **7**(3): p. 297-303.
95. Gallucci, S. and P. Matzinger, *Danger signals: SOS to the immune system.* Curr Opin Immunol, 2001. **13**(1): p. 114-9.
96. Sansonetti, P.J., *et al.*, *Caspase-1 activation of IL-1beta and IL-18 are essential for Shigella flexneri-induced inflammation.* Immunity, 2000. **12**(5): p. 581-90.
97. Banchereau, J., *et al.*, *The CD40 antigen and its ligand.* Annu Rev Immunol, 1994. **12**: p. 881-922.
98. van Kooten, C. and J. Banchereau, *CD40-CD40 ligand.* J Leukoc Biol, 2000. **67**(1): p. 2-17.
99. van Kooten, C. and J. Banchereau, *Functions of CD40 on B cells, dendritic cells and other cells.* Curr Opin Immunol, 1997. **9**(3): p. 330-7.

100. Grewal, I.S. and R.A. Flavell, *CD40 and CD154 in cell-mediated immunity.* Annu Rev Immunol, 1998. **16**: p. 111-35.
101. Guerder, S. and P. Matzinger, *A fail-safe mechanism for maintaining self-tolerance.* J Exp Med, 1992. **176**(2): p. 553-64.
102. Cassell, D. and J. Forman, *Linked recognition of helper and cytotoxic antigenic determinants for the generation of cytotoxic T lymphocytes.* Ann N Y Acad Sci, 1988. **532**: p. 51-60.
103. Schoenberger, S.P., *et al.*, *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-3.
104. Bennett, S.R., *et al.*, *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-80.
105. Boog, C.J., *et al.*, *Abolition of specific immune response defect by immunization with dendritic cells.* Nature, 1985. **318**(6041): p. 59-62.
106. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell.* Nature, 1998. **393**(6684): p. 474-8.
107. Toes, R.E., *et al.*, *An adenovirus type 5 early region 1B-encoded CTL epitope-mediating tumor eradication by CTL clones is down-modulated by an activated ras oncogene.* J Immunol, 1995. **154**(7): p. 3396-405.
108. Diehl, L., *et al.*, *In vivo triggering through 4-1BB enables Th-independent priming of CTL in the presence of an intact CD28 costimulatory pathway.* J Immunol, 2002. **168**(8): p. 3755-62.
109. Feltkamp, M.C., *et al.*, *Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells.* Eur J Immunol, 1993. **23**(9): p. 2242-9.
110. Diehl, L., *et al.*, *CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy.* Nat Med, 1999. **5**(7): p. 774-9.
111. Ludewig, B., *et al.*, *Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells.* J Immunol, 2001. **166**(6): p. 3678-87.
112. Heath, W.R. and F.R. Carbone, *Cross-presentation, dendritic cells, tolerance and immunity.* Annu Rev Immunol, 2001. **19**: p. 47-64.
113. Kurts, C., *et al.*, *CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity.* J Exp Med, 1997. **186**(12): p. 2057-62.
114. Josien, R., *et al.*, *TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo.* J Exp Med, 2000. **191**(3): p. 495-502.

115. Morel, Y., *et al.*, *The TNF superfamily members LIGHT and CD154 (CD40 ligand) costimulate induction of dendritic cell maturation and elicit specific CTL activity.* J Immunol, 2001. **167**(5): p. 2479-86.
116. Wilcox, R.A., *et al.*, *Cutting edge: Expression of functional CD137 receptor by dendritic cells.* J Immunol, 2002. **168**(9): p. 4262-7.
117. Futagawa, T., *et al.*, *Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells.* Int Immunol, 2002. **14**(3): p. 275-86.
118. Kwon, B., H.W. Lee, and B.S. Kwon, *New insights into the role of 4-1BB in immune responses: beyond CD8+ T cells.* Trends Immunol, 2002. **23**(8): p. 378-80.
119. Sotomayor, E.M., *et al.*, *Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40.* Nat Med, 1999. **5**(7): p. 780-7.
120. Schulz, O., *et al.*, *CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal.* Immunity, 2000. **13**(4): p. 457-62.
121. Edwards, A.D., *et al.*, *Microbial recognition via toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering.* J Immunol, 2002. **169**(7): p. 3652-60.
122. Kodaira, Y., *et al.*, *Phenotypic and functional maturation of dendritic cells mediated by heparan sulfate.* J Immunol, 2000. **165**(3): p. 1599-604.
123. Termeer, C., *et al.*, *Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4.* J Exp Med, 2002. **195**(1): p. 99-111.
124. Huang, A.Y., *et al.*, *The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9730-5.
125. Malarkannan, S., *et al.*, *The mouse mammary tumor virus env gene is the source of a CD8+ T-cell- stimulating peptide presented by a major histocompatibility complex class I molecule in a murine thymoma.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13991-6.
126. Schuurhuis, D.H., *et al.*, *Immature dendritic cells acquire CD8(+) cytotoxic T lymphocyte priming capacity upon activation by T helper cell-independent or -dependent stimuli.* J Exp Med, 2000. **192**(1): p. 145-50.
127. Maxwell, J.R., *et al.*, *Contrasting the roles of costimulation and the natural adjuvant lipopolysaccharide during the induction of T cell immunity.* J Immunol, 2002. **168**(9): p. 4372-81.
128. Carreno, B.M. and M. Collins, *The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses.* Annu Rev Immunol, 2002. **20**: p. 29-53.
129. Coyle, A.J. and J.C. Gutierrez-Ramos, *The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function.* Nat Immunol, 2001. **2**(3): p. 203-9.

130. Liang, L. and W.C. Sha, *The right place at the right time: novel B7 family members regulate effector T cell responses.* Curr Opin Immunol, 2002. **14**(3): p. 384-90.
131. van der Merwe, P.A., *et al.*, *CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics.* J Exp Med, 1997. **185**(3): p. 393-403.
132. Lanzavecchia, A., G. Lezzi, and A. Viola, *From TCR engagement to T cell activation: a kinetic view of T cell behavior.* Cell, 1999. **96**(1): p. 1-4.
133. Kuchroo, V.K., *et al.*, *B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy.* Cell, 1995. **80**(5): p. 707-18.
134. Girvin, A.M., *et al.*, *A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade.* J Immunol, 2000. **164**(1): p. 136-43.
135. Salomon, B., *et al.*, *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-40.
136. London, C.A., M.P. Lodge, and A.K. Abbas, *Functional responses and costimulator dependence of memory CD4+ T cells.* J Immunol, 2000. **164**(1): p. 265-72.
137. Chambers, C.A., *The expanding world of co-stimulation: the two-signal model revisited.* Trends Immunol, 2001. **22**(4): p. 217-23.
138. Yoshinaga, S.K., *et al.*, *Characterization of a new human B7-related protein: B7RP-1 is the ligand to the co-stimulatory protein ICOS.* Int Immunol, 2000. **12**(10): p. 1439-47.
139. Hutloff, A., *et al.*, *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.* Nature, 1999. **397**(6716): p. 263-6.
140. Coyle, A.J., *et al.*, *The CD28-related molecule ICOS is required for effective T cell-dependent immune responses.* Immunity, 2000. **13**(1): p. 95-105.
141. McAdam, A.J., *et al.*, *Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells.* J Immunol, 2000. **165**(9): p. 5035-40.
142. Khayyamian, S., *et al.*, *ICOS-ligand, expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells.* Proc Natl Acad Sci U S A, 2002. **99**(9): p. 6198-203.
143. Liu, X., *et al.*, *B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes in vivo.* J Exp Med, 2001. **194**(9): p. 1339-48.
144. Freeman, G.J., *et al.*, *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
145. Dong, H., *et al.*, *B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion.* Nat Med, 1999. **5**(12): p. 1365-9.

146. Nishimura, H., *et al., Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses.* Int Immunol, 1998. **10**(10): p. 1563-72.
147. Croft, M., *Activation of naive, memory and effector T cells.* Curr Opin Immunol, 1994. **6**(3): p. 431-7.
148. Tseng, S.Y., *et al., B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells.* J Exp Med, 2001. **193**(7): p. 839-46.
149. Hurtado, J.C., Y.J. Kim, and B.S. Kwon, *Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death.* J Immunol, 1997. **158**(6): p. 2600-9.
150. Takahashi, C., R.S. Mittler, and A.T. Vella, *Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal.* J Immunol, 1999. **162**(9): p. 5037-40.
151. Wilcox, R.A., *et al., Signaling Through NK Cell-Associated CD137 Promotes Both Helper Function for CD8(+) Cytolytic T Cells and Responsiveness to IL-2 But Not Cytolytic Activity.* J Immunol, 2002. **169**(8): p. 4230-6.
152. Alderson, M.R., *et al., Molecular and biological characterization of human 4-1BB and its ligand.* Eur J Immunol, 1994. **24**(9): p. 2219-27.
153. DeBenedette, M.A., *et al., Costimulation of CD28- T lymphocytes by 4-1BB ligand.* J Immunol, 1997. **158**(2): p. 551-9.
154. Chu, N.R., *et al., Role of IL-12 and 4-1BB ligand in cytokine production by CD28+ and CD28- T cells.* J Immunol, 1997. **158**(7): p. 3081-9.
155. Saoulli, K., *et al., CD28-independent, TRAF2-dependent costimulation of resting T cells by 4- 1BB ligand.* J Exp Med, 1998. **187**(11): p. 1849-62.
156. DeBenedette, M.A., *et al., Role of 4-1BB ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP.* J Exp Med, 1995. **181**(3): p. 985-92.
157. Hurtado, J.C., *et al., Potential role of 4-1BB in T cell activation. Comparison with the costimulatory molecule CD28.* J Immunol, 1995. **155**(7): p. 3360-7.
158. DeBenedette, M.A., *et al., Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus.* J Immunol, 1999. **163**(9): p. 4833-41.
159. Tan, J.T., *et al., 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination.* J Immunol, 2000. **164**(5): p. 2320-5.
160. Cannons, J.L., *et al., 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy.* J Immunol, 2001. **167**(3): p. 1313-24.
161. Maus, M.V., *et al., Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB.* Nat Biotechnol, 2002. **20**(2): p. 143-8.

162. Cooper, D., P. Bansal-Pakala, and M. Croft, *4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity.* Eur J Immunol, 2002. **32**(2): p. 521-9.
163. Tan, J.T., *et al.*, *4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses.* J Immunol, 1999. **163**(9): p. 4859-68.
164. Guinn, B.A., *et al.*, *4-1BBL enhances anti-tumor responses in the presence or absence of CD28 but CD28 is required for protective immunity against parental tumors.* Cell Immunol, 2001. **210**(1): p. 56-65.
165. May, K.F., Jr., *et al.*, *Anti-4-1BB monoclonal antibody enhances rejection of large tumor burden by promoting survival but not clonal expansion of tumor-specific CD8+ T cells.* Cancer Res, 2002. **62**(12): p. 3463-65.
166. Melero, I., *et al.*, *Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway.* Eur J Immunol, 1998. **28**(3): p. 1116-21.
167. Wilcox, R.A., *et al.*, *Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors.* J Clin Invest, 2002. **109**(5): p. 651-9.
168. Miller, R.E., *et al.*, *4-1BB-specific monoclonal antibody promotes the generation of tumor- specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner.* J Immunol, 2002. **169**(4): p. 1792-800.
169. Bertram, E.M., P. Lau, and T.H. Watts, *Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection.* J Immunol, 2002. **168**(8): p. 3777-85.
170. Yoshinaga, S.K., *et al.*, *T-cell co-stimulation through B7RP-1 and ICOS.* Nature, 1999. **402**(6763): p. 827-32.
171. MacPherson, G.G., *et al.*, *Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence.* J Immunol, 1995. **154**(3): p. 1317-22.
172. Yanagihara, S., *et al.*, *EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation.* J Immunol, 1998. **161**(6): p. 3096-102.
173. Sallusto, F., C.R. Mackay, and A. Lanzavecchia, *The role of chemokine receptors in primary, effector, and memory immune responses.* Annu Rev Immunol, 2000. **18**: p. 593-620.
174. Robbiani, D.F., *et al.*, *The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)- dependent mobilization of dendritic cells to lymph nodes.* Cell, 2000. **103**(5): p. 757-68.

175. Gunn, M.D., *et al.*, *A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes.* Proc Natl Acad Sci U S A, 1998. **95**(1): p. 258-63.
176. Hirao, M., *et al.*, *CC chemokine receptor-7 on dendritic cells is induced after interaction with apoptotic tumor cells: critical role in migration from the tumor site to draining lymph nodes.* Cancer Res, 2000. **60**(8): p. 2209-17.
177. Propato, A., *et al.*, *Apoptotic cells overexpress vinculin and induce vinculin-specific cytotoxic T-cell cross-priming.* Nat Med, 2001. **7**(7): p. 807-13.
178. Abdi, K., *IL-12: the role of p40 versus p75.* Scand J Immunol, 2002. **56**(1): p. 1-11.
179. Park, A.Y. and P. Scott, *Il-12: keeping cell-mediated immunity alive.* Scand J Immunol, 2001. **53**(6): p. 529-32.
180. Grumont, R., *et al.*, *c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription.* J Exp Med, 2001. **194**(8): p. 1021-32.
181. Trinchieri, G., *Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity.* Annu Rev Immunol, 1995. **13**: p. 251-76.
182. Belladonna, M.L., *et al.*, *IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells.* J Immunol, 2002. **168**(11): p. 5448-54.
183. Frucht, D.M., *et al.*, *IFN-gamma production by antigen-presenting cells: mechanisms emerge.* Trends Immunol, 2001. **22**(10): p. 556-60.
184. Konecny, P., *et al.*, *Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro.* Eur J Immunol, 1999. **29**(6): p. 1803-11.
185. Heinzel, F.P., *et al.*, *Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon.* Infect Immun, 1994. **62**(10): p. 4244-9.
186. Lehmann, J., *et al.*, *IL-12p40-dependent agonistic effects on the development of protective innate and adaptive immunity against Salmonella enteritidis.* J Immunol, 2001. **167**(9): p. 5304-15.
187. Oppmann, B., *et al.*, *Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12.* Immunity, 2000. **13**(5): p. 715-25.
188. Reis e Sousa, C., *et al.*, *In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas.* J Exp Med, 1997. **186**(11): p. 1819-29.
189. Yamane, H., T. Kato, and H. Nariuchi, *Effective stimulation for IL-12 p35 mRNA accumulation and bioactive IL- 12 production of antigen-presenting cells interacted with Th cells.* J Immunol, 1999. **162**(11): p. 6433-41.

190. Reis e Sousa, C., A. Sher, and P. Kaye, *The role of dendritic cells in the induction and regulation of immunity to microbial infection.* Curr Opin Immunol, 1999. **11**(4): p. 392-9.
191. Skok, J., J. Poudrier, and D. Gray, *Dendritic cell-derived IL-12 promotes B cell induction of Th2 differentiation: a feedback regulation of Th1 development.* J Immunol, 1999. **163**(8): p. 4284-91.
192. Park, A.Y., B.D. Hondowicz, and P. Scott, *IL-12 is required to maintain a Th1 response during Leishmania major infection.* J Immunol, 2000. **165**(2): p. 896-902.
193. Yap, G., M. Pesin, and A. Sher, *Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii.* J Immunol, 2000. **165**(2): p. 628-31.
194. Iwasaki, M., *et al.*, *A mandatory role for STAT4 in IL-12 induction of mouse T cell CCR5.* J Immunol, 2001. **167**(12): p. 6877-83.
195. Shortman, K. and L. Wu, *Parentage and heritage of dendritic cells.* Blood, 2001. **97**(11): p. 3325.
196. Shortman, K., *Burnet oration: dendritic cells: multiple subtypes, multiple origins, multiple functions.* Immunol Cell Biol, 2000. **78**(2): p. 161-5.
197. Rodewald, H.R., T. Brocker, and C. Haller, *Developmental dissociation of thymic dendritic cell and thymocyte lineages revealed in growth factor receptor mutant mice.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15068-73.
198. Brasel, K., *et al.*, *Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures.* Blood, 2000. **96**(9): p. 3029-39.
199. Martinez del Hoyo, G., *et al.*, *CD8alpha+ dendritic cells originate from the CD8alpha- dendritic cell subset by a maturation process involving CD8alpha, DEC-205, and CD24 up- regulation.* Blood, 2002. **99**(3): p. 999-1004.
200. Ruedl, C. and M.F. Bachmann, *CTL priming by CD8(+) and CD8(-) dendritic cells in vivo.* Eur J Immunol, 1999. **29**(11): p. 3762-7.
201. Maldonado-Lopez, R., *et al.*, *CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo.* J Exp Med, 1999. **189**(3): p. 587-92.
202. Pooley, J.L., W.R. Heath, and K. Shortman, *Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells.* J Immunol, 2001. **166**(9): p. 5327-30.
203. den Haan, J.M., S.M. Lehar, and M.J. Bevan, *CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo.* J Exp Med, 2000. **192**(12): p. 1685-96.
204. Reis e Sousa, C. and R.N. Germain, *Analysis of adjuvant function by direct visualization of antigen presentation in vivo: endotoxin promotes accumulation of antigen- bearing dendritic cells in the T cell areas of lymphoid tissue.* J Immunol, 1999. **162**(11): p. 6552-61.

205. Huang, L.Y., *et al., IL-12 induction by a TH1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10.* J Immunol, 2001. **167**(3): p. 1423-30.
206. Kadowaki, N., *et al., Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.* J Exp Med, 2001. **194**(6): p. 863-9.
207. d'Ostiani, C.F., *et al., Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo.* J Exp Med, 2000. **191**(10): p. 1661-74.
208. Reis e Sousa, C., *et al., Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology.* Immunity, 1999. **11**(5): p. 637-47.
209. Moron, G., *et al., CD8alpha- CD11b+ dendritic cells present exogenous virus-like particles to CD8+ T cells and subsequently express CD8alpha and CD205 molecules.* J Exp Med, 2002. **195**(10): p. 1233-45.
210. Ohteki, T., *et al., Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells.* J Exp Med, 1999. **189**(12): p. 1981-6.
211. Kalinski, P., *et al., T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal.* Immunol Today, 1999. **20**(12): p. 561-7.
212. Iwasaki, A. and B.L. Kelsall, *Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells.* J Exp Med, 1999. **190**(2): p. 229-39.
213. Tanaka, H., *et al., Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors. Role of stimulator/responder ratio.* J Exp Med, 2000. **192**(3): p. 405-12.
214. Wang, J.H. and E.L. Reinherz, *Structural basis of T cell recognition of peptides bound to MHC molecules.* Mol Immunol, 2002. **38**(14): p. 1039-49.
215. Hogquist, K.A., *Signal strength in thymic selection and lineage commitment.* Curr Opin Immunol, 2001. **13**(2): p. 225-31.
216. Konig, R., *Interactions between MHC molecules and co-receptors of the TCR.* Curr Opin Immunol, 2002. **14**(1): p. 75-83.
217. Kane, L.P., J. Lin, and A. Weiss, *Signal transduction by the TCR for antigen.* Curr Opin Immunol, 2000. **12**(3): p. 242-9.
218. Singer, A.L. and G.A. Koretzky, *Control of T cell function by positive and negative regulators.* Science, 2002. **296**(5573): p. 1639-40.
219. Joazeiro, C.A., *et al., The tyrosine kinase negative regulator c-Cbl as a RING-type, E2- dependent ubiquitin-protein ligase.* Science, 1999. **286**(5438): p. 309-12.
220. van Leeuwen, J.E., P.K. Paik, and L.E. Samelson, *Activation of nuclear factor of activated T cells-(NFAT) and activating protein 1 (AP-1) by oncogenic 70Z Cbl requires an intact phosphotyrosine binding domain but not Crk(L) or p85 phosphatidylinositol 3-kinase association.* J Bioi Chem, 1999. **274**(8): p. 5153-62.

221. Zhang, Z., *et al.*, *Dual regulation of T cell receptor-mediated signaling by oncogenic Cbl mutant 70Z.* J Biol Chem, 1999. **274**(8): p. 4883-9.
222. Krummel, M.F. and M.M. Davis, *Dynamics of the immunological synapse: finding, establishing and solidifying a connection.* Curr Opin Immunol, 2002. **14**(1): p. 66-74.
223. van der Merwe, P.A., *Formation and function of the immunological synapse.* Curr Opin Immunol, 2002. **14**(3): p. 293-8.
224. van Der Merwe, P.A. and S.J. Davis, *Immunology. The immunological synapse--a multitasking system.* Science, 2002. **295**(5559): p. 1479-80.
225. Hudrisier, D., *et al.*, *The efficiency of antigen recognition by CD8+ CTL clones is determined by the frequency of serial TCR engagement.* J Immunol, 1998. **161**(2): p. 553-62.
226. Bachmann, M.F., *et al.*, *Peptide-induced T cell receptor down-regulation on naive T cells predicts agonist/partial agonist properties and strictly correlates with T cell activation.* Eur J Immunol, 1997. **27**(9): p. 2195-203.
227. McFarland, W., *Microspikes on the lymphocyte uropod.* Science, 1969. **163**(869): p. 818-20.
228. Nieto, M., *et al.*, *Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis.* J Exp Med, 1997. **186**(1): p. 153-8.
229. Delon, J., *et al.*, *Antigen-dependent and -independent Ca2+ responses triggered in T cells by dendritic cells compared with B cells.* J Exp Med, 1998. **188**(8): p. 1473-84.
230. Dustin, M.L., *et al.*, *Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity.* J Biol Chem, 1997. **272**(49): p. 30889-98.
231. Wulfing, C., *et al.*, *Kinetics and extent of T cell activation as measured with the calcium signal.* J Exp Med, 1997. **185**(10): p. 1815-25.
232. Bachmann, M.F., *et al.*, *Four types of Ca2+ signals in naive CD8+ cytotoxic T cells after stimulation with T cell agonists, partial agonists and antagonists.* Eur J Immunol, 1997. **27**(12): p. 3414-9.
233. Viola, A., *The amplification of TCR signaling by dynamic membrane microdomains.* Trends Immunol, 2001. **22**(6): p. 322-7.
234. Wulfing, C., *et al.*, *Costimulation and endogenous MHC ligands contribute to T cell recognition.* Nat Immunol, 2002. **3**(1): p. 42-7.
235. Montixi, C., *et al.*, *Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains.* Embo J, 1998. **17**(18): p. 5334-48.
236. Xavier, R., *et al.*, *Membrane compartmentation is required for efficient T cell activation.* Immunity, 1998. **8**(6): p. 723-32.
237. Kabouridis, P.S., A.I. Magee, and S.C. Ley, *S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes.* Embo J, 1997. **16**(16): p. 4983-98.

238. Stamper, C.C., *et al.*, *Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses.* Nature, 2001. **410**(6828): p. 608-11.
239. Thomas, M.L., *The regulation of antigen-receptor signaling by protein tyrosine phosphatases: a hole in the story.* Curr Opin Immunol, 1999. **11**(3): p. 270-6.
240. Viola, A., *et al.*, *T lymphocyte costimulation mediated by reorganization of membrane microdomains.* Science, 1999. **283**(5402): p. 680-2.
241. Wulfing, C., *et al.*, *The vav exchange factor is an essential regulator in actin-dependent receptor translocation to the lymphocyte-antigen-presenting cell interface.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10150-5.
242. Bachmann, M.F., *et al.*, *Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells.* J Exp Med, 1999. **189**(10): p. 1521-30.
243. Lanzavecchia, A. and F. Sallusto, *Antigen decoding by T lymphocytes: from synapses to fate determination.* Nat Immunol, 2001. **2**(6): p. 487-92.
244. Gunzer, M., *et al.*, *Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential.* Immunity, 2000. **13**(3): p. 323-32.
245. Gretz, J.E., A.O. Anderson, and S. Shaw, *Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex.* Immunol Rev, 1997. **156**: p. 11-24.
246. Stoll, S., *et al.*, *Dynamic imaging of T cell-dendritic cell interactions in lymph nodes.* Science, 2002. **296**(5574): p. 1873-6.
247. Lee, K.H., *et al.*, *T cell receptor signaling precedes immunological synapse formation.* Science, 2002. **295**(5559): p. 1539-42.
248. McLellan, A., *et al.*, *MHC class II and CD40 play opposing roles in dendritic cell survival.* Eur J Immunol, 2000. **30**(9): p. 2612-9.
249. Anderson, D.M., *et al.*, *A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function.* Nature, 1997. **390**(6656): p. 175-9.
250. Wong, B.R., *et al.*, *TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor.* J Exp Med, 1997. **186**(12): p. 2075-80.
251. Lanzavecchia, A. and F. Sallusto, *Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells.* Science, 2000. **290**(5489): p. 92-7.
252. Sprent, J. and C.D. Surh, *T cell memory.* Annu Rev Immunol, 2002. **20**: p. 551-79.
253. Sallusto, F., *et al.*, *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
254. Kaech, S.M. and R. Ahmed, *Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells.* Nat Immunol, 2001. **2**(5): p. 415-22.

255. Foulds, K.E., *et al.*, *Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses.* J Immunol, 2002. **168**(4): p. 1528-32.
256. Zhao, K., *et al.*, *Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling.* Cell, 1998. **95**(5): p. 625-36.
257. Langenkamp, A., *et al.*, *Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells.* Nat Immunol, 2000. **1**(4): p. 311-6.
258. Henkart, P.A., *Mechanism of lymphocyte-mediated cytotoxicity.* Annu Rev Immunol, 1985. **3**: p. 31-58.
259. Rouvier, E., M.F. Luciani, and P. Golstein, *Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity.* J Exp Med, 1993. **177**(1): p. 195-200.
260. Lowrey, D.M., *et al.*, *Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9.* Proc Natl Acad Sci U S A, 1989. **86**(1): p. 247-51.
261. Sarin, A., *et al.*, *Target cell lysis by CTL granule exocytosis is independent of ICE/Ced-3 family proteases.* Immunity, 1997. **6**(2): p. 209-15.
262. Harty, J.T., A.R. Tvinnereim, and D.W. White, *CD8+ T cell effector mechanisms in resistance to infection.* Annu Rev Immunol, 2000. **18**: p. 275-308.
263. Shresta, S., *et al.*, *How do cytotoxic lymphocytes kill their targets?* Curr Opin Immunol, 1998. **10**(5): p. 581-7.
264. Heusel, J.W., *et al.*, *Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells.* Cell, 1994. **76**(6): p. 977-87.
265. Andrade, F., *et al.*, *Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis.* Immunity, 1998. **8**(4): p. 451-60.
266. Shresta, S., *et al.*, *Granzyme A initiates an alternative pathway for granule-mediated apoptosis.* Immunity, 1999. **10**(5): p. 595-605.
267. Beresford, P.J., *et al.*, *Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation.* Immunity, 1999. **10**(5): p. 585-94.
268. Cho, S., *et al.*, *Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12210-5.
269. Liu, X., *et al.*, *DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis.* Cell, 1997. **89**(2): p. 175-84.
270. Barry, M. and R.C. Bleackley, *Cytotoxic T lymphocytes: all roads lead to death.* Nat Rev Immunol, 2002. **2**(6): p. 401-9.
271. Balaji, K.N., *et al.*, *Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation.* J Exp Med, 2002. **196**(4): p. 493-503.

272. Enari, M., *et al., A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD.* Nature, 1998. **391**(6662): p. 43-50.
273. Borrow, P., C.F. Evans, and M.B. Oldstone, *Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression.* J Virol, 1995. **69**(2): p. 1059-70.
274. Ronchese, F. and I.F. Hermans, *Killing of dendritic cells: a life cut short or a purposeful death?* J Exp Med, 2001. **194**(5): p. F23-6.
275. Medema, J.P., *et al., Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells.* J Exp Med, 2001. **194**(5): p. 657-67.
276. Medema, J.P., *et al., Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11515-20.
277. Murali-Krishna, K., *et al., Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection.* Immunity, 1998. **8**(2): p. 177-87.
278. Lenardo, M., *et al., Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment.* Annu Rev Immunol, 1999. **17**: p. 221-53.
279. Zheng, L., *et al., T cell growth cytokines cause the superinduction of molecules mediating antigen-induced T lymphocyte death.* J Immunol, 1998. **160**(2): p. 763-9.
280. Cohen, P.L. and R.A. Eisenberg, *Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease.* Annu Rev Immunol, 1991. **9**: p. 243-69.
281. Watanabe-Fukunaga, R., *et al., Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis.* Nature, 1992. **356**(6367): p. 314-7.
282. Ramsdell, F., *et al., gld/gld mice are unable to express a functional ligand for Fas.* Eur J Immunol, 1994. **24**(4): p. 928-33.
283. Tanaka, M., *et al., Expression of the functional soluble form of human fas ligand in activated lymphocytes.* Embo J, 1995. **14**(6): p. 1129-35.
284. Kayagaki, N., *et al., Metalloproteinase-mediated release of human Fas ligand.* J Exp Med, 1995. **182**(6): p. 1777-83.
285. Mariani, S.M., *et al., Interleukin 1 beta-converting enzyme related proteases/caspases are involved in TRAIL-induced apoptosis of myeloma and leukemia cells.* J Cell Biol, 1997. **137**(1): p. 221-9.
286. Kuroda, K., *et al., Implantation of IL-2-containing osmotic pump prolongs the survival of superantigen-reactive T cells expanded in mice injected with bacterial superantigen.* J Immunol, 1996. **157**(4): p. 1422-31.
287. Boise, L.H., *et al., Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division.* Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5491-5.

288. Critchfield, J.M., *et al., T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis.* Science, 1994. **263**(5150): p. 1139-43.
289. Kung, J.T., D. Beller, and S.T. Ju, *Lymphokine regulation of activation-induced apoptosis in T cells of IL- 2 and IL-2R beta knockout mice.* Cell Immunol, 1998. **185**(2): p. 158-63.
290. Nakajima, H., *et al., The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis.* J Exp Med, 1997. **185**(2): p. 189-95.
291. Anderson, M.S., *et al., Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-401.
292. Shortman, K. and L. Wu, *Early T lymphocyte progenitors.* Annu Rev Immunol, 1996. **14**: p. 29-47.
293. Berg, L.J. and J. Kang, *Molecular determinants of TCR expression and selection.* Curr Opin Immunol, 2001. **13**(2): p. 232-41.
294. Capone, M., R.D. Hockett, Jr., and A. Zlotnik, *Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes.* Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12522-7.
295. Irving, B.A., F.W. Alt, and N. Killeen, *Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains.* Science, 1998. **280**(5365): p. 905-8.
296. Goldrath, A.W. and M.J. Bevan, *Selecting and maintaining a diverse T-cell repertoire.* Nature, 1999. **402**(6759): p. 255-62.
297. Zerrahn, J., W. Held, and D.H. Raulet, *The MHC reactivity of the T cell repertoire prior to positive and negative selection.* Cell, 1997. **88**(5): p. 627-36.
298. Merkenschlager, M., *et al., How many thymocytes audition for selection?* J Exp Med, 1997. **186**(7): p. 1149-58.
299. van Meerwijk, J.P., *et al., Quantitative impact of thymic clonal deletion on the T cell repertoire.* J Exp Med, 1997. **185**(3): p. 377-83.
300. Mazda, O., *et al., Requirement of dendritic cells and B cells in the clonal deletion of Mls-reactive T cells in the thymus.* J Exp Med, 1991. **173**(3): p. 539-47.
301. Nikolic-Zugic, J. and M.J. Bevan, *Role of self-peptides in positively selecting the T-cell repertoire.* Nature, 1990. **344**(6261): p. 65-7.
302. Sha, W.C., *et al., Positive selection of transgenic receptor-bearing thymocytes by Kb antigen is altered by Kb mutations that involve peptide binding.* Proc Natl Acad Sci U S A, 1990. **87**(16): p. 6186-90.
303. Barton, G.M. and A.Y. Rudensky, *Requirement for diverse, low-abundance peptides in positive selection of T cells.* Science, 1999. **283**(5398): p. 67-70.
304. Yasutomo, K., *et al., The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate.* Nature, 2000. **404**(6777): p. 506-10.

305. Hernandez-Hoyos, G., *et al.*, *Lck activity controls CD4/CD8 T cell lineage commitment.* Immunity, 2000. **12**(3): p. 313-22.
306. Salmon, P., *et al.*, *The role of CD8 alpha' in the CD4 versus CD8 lineage choice.* J Immunol, 1999. **163**(10): p. 5312-8.
307. Grossman, Z. and W.E. Paul, *Self-tolerance: context dependent tuning of T cell antigen recognition.* Semin Immunol, 2000. **12**(3): p. 197-203; discussion 257-344.
308. Grossman, Z. and W.E. Paul, *Autoreactivity, dynamic tuning and selectivity.* Curr Opin Immunol, 2001. **13**(6): p. 687-98.
309. Dorfman, J.R., *et al.*, *CD4+ T cell survival is not directly linked to self-MHC-induced TCR signaling.* Nat Immunol, 2000. **1**(4): p. 329-35.
310. Germain, R.N. and I. Stefanova, *The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation.* Annu Rev Immunol, 1999. **17**: p. 471-522.
311. Lucas, B., *et al.*, *Divergent changes in the sensitivity of maturing T cells to structurally related ligands underlies formation of a useful T cell repertoire.* Immunity, 1999. **10**(3): p. 367-76.
312. Fazekas de St Groth, B., *DCs and peripheral T cell tolerance.* Semin Immunol, 2001. **13**(5): p. 311-22.
313. Dhodapkar, M.V. and R.M. Steinman, *Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans.* Blood, 2002. **100**(1): p. 174-7.
314. Moodycliffe, A.M., *et al.*, *CD40-CD40 ligand interactions in vivo regulate migration of antigen- bearing dendritic cells from the skin to draining lymph nodes.* J Exp Med, 2000. **191**(11): p. 2011-20.
315. Huang, F.P., *et al.*, *A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes.* J Exp Med, 2000. **191**(3): p. 435-44.
316. Hawiger, D., *et al.*, *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* J Exp Med, 2001. **194**(6): p. 769-79.
317. Hernandez, J., *et al.*, *Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen.* J Exp Med, 2001. **194**(6): p. 707-17.
318. Nugent, C.T., *et al.*, *Characterization of CD8+ T lymphocytes that persist after peripheral tolerance to a self antigen expressed in the pancreas.* J Immunol, 2000. **164**(1): p. 191-200.
319. Maloy, K.J. and F. Powrie, *Regulatory T cells in the control of immune pathology.* Nat Immunol, 2001. **2**(9): p. 816-22.
320. Shevach, E.M., *Regulatory T cells in autoimmmunity*.* Annu Rev Immunol, 2000. **18**: p. 423-49.

321. Olivares-Villagomez, D., Y. Wang, and J.J. Lafaille, *Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis.* J Exp Med, 1998. **188**(10): p. 1883-94.

322. Van de Keere, F. and S. Tonegawa, *CD4(+) T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice.* J Exp Med, 1998. **188**(10): p. 1875-82.

323. Mordes, J.P., *et al.*, *Transfusions enriched for W3/25+ helper/inducer T lymphocytes prevent spontaneous diabetes in the BB/W rat.* Diabetologia, 1987. **30**(1): p. 22-6.

324. Groux, H., *et al.*, *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.

325. McGuirk, P., C. McCann, and K.H. Mills, *Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis.* J Exp Med, 2002. **195**(2): p. 221-31.

326. McGuirk, P. and K.H. Mills, *Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases.* Trends Immunol, 2002. **23**(9): p. 450-5.

327. Fukaura, H., *et al.*, *Induction of circulating myelin basic protein and proteolipid protein- specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients.* J Clin Invest, 1996. **98**(1): p. 70-7.

328. Nagy, N. and F. Vanky, *Transforming growth factor beta, (TGFbeta) secreted by immunogenic ex vivo human carcinoma cells, counteracts the activation and inhibits the function of autologous cytotoxic lymphocytes. Pretreatment with interferon gamma and tumor necrosis factor alpha reduces the production of active TGFbeta.* Cancer Immunol Immunother, 1998. **45**(6): p. 306-12.

329. Gorelik, L. and R.A. Flavell, *Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells.* Nat Med, 2001. **7**(10): p. 1118-22.

330. Seddon, B. and D. Mason, *The third function of the thymus.* Immunol Today, 2000. **21**(2): p. 95-9.

331. Modigliani, Y., A. Bandeira, and A. Coutinho, *A model for developmentally acquired thymus-dependent tolerance to central and peripheral antigens.* Immunol Rev, 1996. **149**: p. 155-20.

332. Seddon, B. and D. Mason, *Peripheral autoantigen induces regulatory T cells that prevent autoimmunity.* J Exp Med, 1999. **189**(5): p. 877-82.

333. Jordan, M.S., *et al.*, *Anergy and suppression regulate CD4(+) T cell responses to a self peptide.* Eur J Immunol, 2000. **30**(1): p. 136-44.

334. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. **194**(5): p. 629-44.
335. Lutz, M.B. and G. Schuler, *Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?* Trends Immunol, 2002. **23**(9): p. 445-9.
336. Menges, M., *et al.*, *Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity.* J Exp Med, 2002. **195**(1): p. 15-21.
337. Boussiotis, V.A., *et al.*, *IL-10-producing T cells suppress immune responses in anergic tuberculosis patients.* J Clin Invest, 2000. **105**(9): p. 1317-25.
338. Schultze, J.L., *et al.*, *Tumour immunotherapy: new tools, new treatment modalities and new T- cell antigens.* Vox Sang, 2001. **80**(2): p. 81-9.
339. Perales, M.A., *et al.*, *Strategies to overcome immune ignorance and tolerance.* Semin Cancer Biol, 2002. **12**(1): p. 63-71.
340. Yu, Z. and N.P. Restifo, *Cancer vaccines: progress reveals new complexities.* J Clin Invest, 2002. **110**(3): p. 289-94.
341. Rosenberg, S.A., *Progress in human tumour immunology and immunotherapy.* Nature, 2001. **411**(6835): p. 380-4.
342. Pardoll, D.M., *Spinning molecular immunology into successful immunotherapy.* Nat Rev Immunol, 2002. **2**(4): p. 227-38.
343. Aguilar, L.K., C.M. Rooney, and H.E. Heslop, *Lymphoproliferative disorders involving Epstein-Barr virus after hemopoietic stem cell transplantation.* Curr Opin Oncol, 1999. **11**(2): p. 96-101.
344. Kaplan, D.H., *et al.*, *Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7556-61.
345. Smyth, M.J., D.I. Godfrey, and J.A. Trapani, *A fresh look at tumor immunosurveillance and immunotherapy.* Nat Immunol, 2001. **2**(4): p. 293-9.
346. Groh, V., *et al.*, *Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6879-84.
347. Smyth, M.J., *et al.*, *Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma.* J Exp Med, 2000. **192**(5): p. 755-60.
348. Gershwin, M.E., *et al.*, *Anti-mu induces lymphoma in germfree congenitally athymic (nude) but not in heterozygous (nu/+) mice.* J Immunol, 1983. **131**(4): p. 2069-73.
349. Trapani, J.A. and M.J. Smyth, *Functional significance of the perforin/granzyme cell death pathway.* Nat Rev Immunol, 2002. **2**(10): p. 735-47.
350. Shankaran, V., *et al.*, *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.

351. Romero, P., *et al., Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen- experienced tumor-specific cytolytic T lymphocytes.* J Exp Med, 1998. **188**(9): p. 1641-50.
352. Lee, P.P., *et al., Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients.* Nat Med, 1999. **5**(6): p. 677-85.
353. Mackensen, A., *et al., Direct evidence to support the immunosurveillance concept in a human regressive melanoma.* J Clin Invest, 1994. **93**(4): p. 1397-402.
354. Gilboa, E., *The makings of a tumor rejection antigen.* Immunity, 1999. **11**(3): p. 263-70.
355. Albert, M.L., *et al., Tumor-specific killer cells in paraneoplastic cerebellar degeneration.* Nat Med, 1998. **4**(11): p. 1321-4.
356. Maeda, A., *et al., Identification of human antitumor cytotoxic T lymphocytes epitopes of recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome.* Eur J Immunol, 2001. **31**(2): p. 563-72.
357. Schultze, J.L. and R.H. Vonderheide, *From cancer genomics to cancer immunotherapy: toward second-generation tumor antigens.* Trends Immunol, 2001. **22**(9): p. 516-23.
358. van der Bruggen, P., *et al., A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.* Science, 1991. **254**(5038): p. 1643-7.
359. Kessler, J.H., *et al., Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis.* J Exp Med, 2001. **193**(1): p. 73-88.
360. Nussbaum, A.K., *et al., PAProC: a prediction algorithm for proteasomal cleavages available on the WWW.* Immunogenetics, 2001. **53**(2): p. 87-94.
361. Pascolo, S., *et al., A MAGE-A1 HLA-A A*0201 epitope identified by mass spectrometry.* Cancer Res, 2001. **61**(10): p. 4072-7.
362. Brossart, P., *et al., Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies.* Blood, 1999. **93**(12): p. 4309-17.
363. van der Bruggen, P., *et al., A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3.* Eur J Immunol, 1994. **24**(12): p. 3038-43.
364. Melief, C.J. and W.M. Kast, *T-cell immunotherapy of cancer.* Res Immunol, 1991. **142**(5-6): p. 425-9.
365. Vonderheide, R.H., *et al., The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes.* Immunity, 1999. **10**(6): p. 673-9.
366. Kim, N.W., *et al., Specific association of human telomerase activity with immortal cells and cancer.* Science, 1994. **266**(5193): p. 2011-5.

367. Greenberg, R.A., *et al.*, *Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse.* Cell, 1999. **97**(4): p. 515-25.
368. Nair, S.K., *et al.*, *Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells.* Nat Med, 2000. **6**(9): p. 1011-7.
369. Heiser, A., *et al.*, *Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA.* J Immunol, 2001. **166**(5): p. 2953-60.
370. Heiser, A., *et al.*, *Human dendritic cells transfected with renal tumor RNA stimulate polyclonal T-cell responses against antigens expressed by primary and metastatic tumors.* Cancer Res, 2001. **61**(8): p. 3388-93.
371. Cox, A.L., *et al.*, *Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines.* Science, 1994. **264**(5159): p. 716-9.
372. Falk, K., *et al.*, *Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules.* Nature, 1991. **351**(6324): p. 290-6.
373. Klade, C.S., *et al.*, *Identification of tumor antigens in renal cell carcinoma by serological proteome analysis.* Proteomics, 2001. **1**(7): p. 890-8.
374. Lim, S.H., *et al.*, *Defining tumor antigens: mRNA, protein or cytotoxicity?* Trends Immunol, 2002. **23**(5): p. 236-7; author reply 237-8.
375. Wang, R.F., *The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity.* Trends Immunol, 2001. **22**(5): p. 269-76.
376. Wang, R.F., *et al.*, *Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen.* Science, 1999. **284**(5418): p. 1351-4.
377. Speiser, D.E., *et al.*, *In vivo activation of melanoma-specific CD8(+) T cells by endogenous tumor antigen and peptide vaccines. A comparison to virus-specific T cells.* Eur J Immunol, 2002. **32**(3): p. 731-41.
378. Monsurro, V., *et al.*, *Functional heterogeneity of vaccine-induced CD8(+) T cells.* J Immunol, 2002. **168**(11): p. 5933-42.
379. Valmori, D., *et al.*, *Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(-) effector subset exerting ex vivo tumor-specific cytolytic activity.* Cancer Res, 2002. **62**(6): p. 1743-50.
380. Ogg, G.S., *et al.*, *High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo.* J Exp Med, 1998. **188**(6): p. 1203-8.
381. DeGrendele, H.C., P. Estess, and M.H. Siegelman, *Requirement for CD44 in activated T cell extravasation into an inflammatory site.* Science, 1997. **278**(5338): p. 672-5.
382. Busch, D.H. and E.G. Pamer, *MHC class I/peptide stability: implications for immunodominance, in vitro proliferation, and diversity of responding CTL.* J Immunol, 1998. **160**(9): p. 4441-8.

383. Zorn, E. and T. Hercend, *A natural cytotoxic T cell response in a spontaneously regressing human melanoma targets a neoantigen resulting from a somatic point mutation.* Eur J Immunol, 1999. **29**(2): p. 592-601.

384. Zorn, E. and T. Hercend, *A MAGE-6-encoded peptide is recognized by expanded lymphocytes infiltrating a spontaneously regressing human primary melanoma lesion.* Eur J Immunol, 1999. **29**(2): p. 602-7.

385. Gavin, P.D., *et.al., Cancer immunoediting: from immunosurveillance to tumor escape.* Nat.Immunol. 2002 **3**(11):p. 991-998

386. Khong, H.T. & Restifo, N.P., *Natural selection of tumor varients in the generation of "tumor escape" phenotypes.* Nat.Immunol. 2002 **3**(11): p.999-1005

387. Restifo, N.P., *et.al. Assumptions of the tumor 'escape' hypothesis.* Semin.Cancer Biol. 2002 **12**: p.81-86

388. Algarra, I., *et al. MHC class I antigens in tumours.* Int.J.Clin.Lab.Res. 1997 **27**:95-102

389. de Varies, T.J., *et al., Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1 and tyrosinase in human melanoma cell lines and human melonocytic lesions.* Can. Res. 1997 **57**:3223-3229

390. Cabrera, T. *et al. High frequency of altered HLA I phenotypes in invasive breast carcinomas.* 1996 **50**:127-134

391. Restifo, N.P. *et.al. Loss of functional β2-microglobulin in metastatic melanoma from five patients receiving immunotherapy.* J. Natl.Can.Inst. 1996 **88:**100-108

392. Zheng, P., Sarma, S., Guo, J. & Liu, Y., *Two mechanisms of tumour evasion of preexisting cytotoxic T-cell responses: lessons from recurring tumours* Can.Res. 1999 **59:**3461-3467

393. Gronbaek, K., *et.al., Somatic Fas mutations in NHL:association with extranodal disease and autoimmunity.* Blood 1998 **92:**3018-3024.

394. Medema, J.P., *et.al. Immune escape of tumour in vivo by expression of cellular FLICE-inhibitory protein.* J.Exp.Med. 1999 **190:**1033-1038.

395. Radoja, S., *et al., CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis.* J Immunol, 2001. **167**(9): p. 5042-51.

396 Hersey, P. & Zhang, X.D. *How melonoma cells evade TRAIL-mediated apoptosis* Nat.Rev.Can. 2001 **1**:142-150

397. Ohlen, C., *et al., CD8(+) T cell tolerance to a tumor-associated antigen is maintained at the level of expansion rather than effector function.* J Exp Med, 2002. **195**(11): p. 1407-18.

398. Nelson, D.J., *et al., Tumor progression despite efficient tumor antigen cross-presentation and effective "arming" of tumor antigen-specific CTL.* J Immunol, 2001. **166**(9): p. 5557-66.

399. Sarma, S., *et al.*, *Cytotoxic T lymphocytes to an unmutated tumor rejection antigen P1A: normal development but restrained effector function in vivo.* J Exp Med, 1999. **189**(5): p. 811-20.
400. Grabbe, S., S. Bruvers, and R.D. Granstein, *Effects of immunomodulatory cytokines on the presentation of tumor- associated antigens by epidermal Langerhans cells.* J Invest Dermatol, 1992. **99**(5): p. 66S-68S.
401. Beissert, S., *et al.*, *IL-10 inhibits tumor antigen presentation by epidermal antigen-presenting cells.* J Immunol, 1995. **154**(3): p. 1280-6.
402. Gabrilovich, D.I., *et al.*, *Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells.* Nat Med, 1996. **2**(10): p. 1096-103.
403. Hiltbold, E.M., *et al.*, *The mechanism of unresponsiveness to circulating tumor antigen MUC1 is a block in intracellular sorting and processing by dendritic cells.* J Immunol, 2000. **165**(7): p. 3730-41.
404. Johnsen, A.K., *et al.*, *Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis.* J Immunol, 1999. **163**(8): p. 4224-31.
405. Gabrilovich, D.I., I.F. Ciernik, and D.P. Carbone, *Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts.* Cell Immunol, 1996. **170**(1): p. 101-10.
406. Kiertscher, S.M., *et al.*, *Tumors promote altered maturation and early apoptosis of monocyte- derived dendritic cells.* J Immunol, 2000. **164**(3): p. 1269-76.
407. Chaux, P., *et al.*, *Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression. A role in the immune tolerance to antigenic tumors.* Adv Exp Med Biol, 1997. **417**: p. 525-8.
408. Onizuka, S., *et al.*, *Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody.* Cancer Res, 1999. **59**(13): p. 3128-33.
409. Shimizu, J., S. Yamazaki, and S. Sakaguchi, *Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity.* J Immunol, 1999. **163**(10): p. 5211-8.
410. Seo, N., S. Hayakawa, and Y. Tokura, *Mechanisms of immune privilege for tumor cells by regulatory cytokines produced by innate and acquired immune cells.* Semin Cancer Biol, 2002. **12**(4): p. 291-300.
411. Terabe, M., *et al.*, *NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway.* Nat Immunol, 2000. **1**(6): p. 515-20.
412. Seo, N., *et al.*, *Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells.* J Immunol, 1999. **163**(1): p. 242-9.

413. Mukherjee, P., *et al.*, *Mice with spontaneous pancreatic cancer naturally develop MUC-1- specific CTLs that eradicate tumors when adoptively transferred.* J Immunol, 2000. **165**(6): p. 3451-60.
414. Finn, O.J. and G. Forni, *Prophylactic cancer vaccines.* Curr Opin Immunol, 2002. **14**(2): p. 172-7.
415. Restifo, N.P., *Countering the 'counterattack' hypothesis.* Nat Med, 2001. **7**(3): p. 259.
416. Restifo, N.P., *Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape.* Nat Med, 2000. **6**(5): p. 493-5.
417. Restifo, N.P., *Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity.* Curr Opin Immunol, 2000. **12**(5): p. 597-603.
418. Chappell, D.B., *et al.*, *Human melanoma cells do not express Fas (Apo-1/CD95) ligand.* Cancer Res, 1999. **59**(1): p. 59-62.
419. Diehl, L., *et al.*, *The role of CD40 in peripheral T cell tolerance and immunity.* J Mol Med, 2000. **78**(7): p. 363-71.
420. Molldrem, J.J., *et al.*, *Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia.* Nat Med, 2000. **6**(9): p. 1018-23.
421. Dudley, M.E., *et al.*, *Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes.* Science, 2002. **298**(5594): p. 850-4.
422. Hurwitz, A.A., E.D. Kwon, and A. van Elsas, *Costimulatory wars: the tumor menace.* Curr Opin Immunol, 2000. **12**(5): p. 589-96.
423. Dranoff, G., *et al.*, *Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3539-43.
424. Borges, L., *et al.*, *Synergistic action of fms-like tyrosine kinase 3 ligand and CD40 ligand in the induction of dendritic cells and generation of antitumor immunity in vivo.* J Immunol, 1999. **163**(3): p. 1289-97.
425. Chiodoni, C., *et al.*, *Dendritic cells infiltrating tumors cotransduced with granulocyte/macrophage colony-stimulating factor (GM-CSF) and CD40 ligand genes take up and present endogenous tumor-associated antigens, and prime naive mice for a cytotoxic T lymphocyte response.* J Exp Med, 1999. **190**(1): p. 125-33.
426. Simons, J.W., *et al.*, *Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer.* Cancer Res, 1997. **57**(8): p. 1537-46.
427. Soiffer, R., *et al.*, *Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13141-6.

428. Jaffee, E.M., *et al., Novel allogeneic granulocyte-macrophage colony-stimulating factor- secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation.* J Clin Oncol, 2001. **19**(1): p. 145-56.

429. Tao, M.H. and R. Levy, *Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma.* Nature, 1993. **362**(6422): p. 755-8.

430. Mahnke, K., *et al., The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments.* J Cell Biol, 2000. **151**(3): p. 673-84.

431. Overwijk, W.W., *et al., Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2982-7.

432. Bronte, V., *et al., Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo.* Cancer Res, 2000. **60**(2): p. 253-8.

433. Xiang, R., *et al., An autologous oral DNA vaccine protects against murine melanoma.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5492-7.

433a Zhu, D., *et al., DNA fusion vaccines against B-cell tumors.* Trends.Mol.Med, 2001. **7**(12): p.566-571

434. Reinhard, G., *et al., Generation of dendritic cell-based vaccines for cancer therapy.* Br J Cancer, 2002. **86**(10): p. 1529-33.

435. Lambert, L.A., *et al., Equipotent Generation of Protective Antitumor Immunity by Various Methods of Dendritic Cell Loading With Whole Cell Tumor Antigens.* J Immunother, 2001. **24**(3): p. 232-236.

436. Shimizu, K., *et al., Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein.* Cancer Res, 2001. **61**(6): p. 2618-24.

437. Song, W., *et al., Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity.* J Exp Med, 1997. **186**(8): p. 1247-56.

438. Specht, J.M., *et al., Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases.* J Exp Med, 1997. **186**(8): p. 1213-21.

439. Dyall, J., *et al., Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes.* Blood, 2001. **97**(1): p. 114-21.

440. Gong, J., *et al., Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2715-8.

441. Shu, S. and P. Cohen, *Tumor-Dendritic Cell Fusion Technology and Immunotherapy Strategies.* J Immunother, 2001. **24**(2): p. 99-100.

442. Thurner, B., *et al.*, *Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma.* J Exp Med, 1999. **190**(11): p. 1669-78.
443. Holtl, L., *et al.*, *Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells.* J Urol, 1999. **161**(3): p. 777-82.
444. Zitvogel, L., E. Angevin, and T. Tursz, *Dendritic cell-based immunotherapy of cancer.* Ann Oncol, 2000. **11**(Suppl 3): p. 199-205.
445. Parkhurst, M.R., *et al.*, *Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues.* J Immunol, 1996. **157**(6): p. 2539-48.
446. Dyall, R., *et al.*, *Heteroclitic immunization induces tumor immunity.* J Exp Med, 1998. **188**(9): p. 1553-61.
447. Slansky, J.E., *et al.*, *Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex.* Immunity, 2000. **13**(4): p. 529-38.
448. Townsend, S.E. and J.P. Allison, *Tumor rejection after direct costimulation of CD8+ T cells by B7- transfected melanoma cells.* Science, 1993. **259**(5093): p. 368-70.
449. Chen, L., *et al.*, *Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4.* Cell, 1992. **71**(7): p. 1093-102.
450. Kim, J.J., *et al.*, *Engineering of in vivo immune responses to DNA immunization via codelivery of costimulatory molecule genes.* Nat Biotechnol, 1997. **15**(7): p. 641-6.
451. Agadjanyan, M.G., *et al.*, *CD86 (B7-2) can function to drive MHC-restricted antigen-specific CTL responses in vivo.* J Immunol, 1999. **162**(6): p. 3417-27.
452. Weinberg, A.D., *et al.*, *Engagement of the OX-40 receptor in vivo enhances antitumor immunity.* J Immunol, 2000. **164**(4): p. 2160-9.
453. Vonderheide, R.H., *et al.*, *Phase I study of recombinant human CD40 ligand in cancer patients.* J Clin Oncol, 2001. **19**(13): p. 3280-7.
454. Molenaar, W.M., H. Ellens, and H.M. van den Berg, *Characterization of a high-grade malignant murine B-cell lymphoma and a study of its dissemination pattern after intraperitoneal or intravenous inoculation.* Invasion Metastasis, 1985. **5**(4): p. 206-17.
455. Cobb, L.M., *et al.*, *Characterisation of a new murine B cell lymphoma.* Br J Cancer, 1986. **54**(5): p. 807-18.
456. Illidge, T., *et al.*, *A new in vivo and in vitro B cell lymphoma model, pi-BCL1.* Cancer Biother Radiopharm, 2000. **15**(6): p. 571-80.
457. Sanderson, S. and N. Shastri, *LacZ inducible, antigen/MHC-specific T cell hybrids.* Int Immunol, 1994. **6**(3): p. 369-76.

458. Rock, K.L., L. Rothstein, and S. Gamble, *Generation of class I MHC-restricted T-T hybridomas.* J Immunol, 1990. **145**(3): p. 804-11.

459. Karttunen, J., S. Sanderson, and N. Shastri, *Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens.* Proc Natl Acad Sci U S A, 1992. **89**(13): p. 6020-4.

459a Peto R., *Editorial: Guidelines on the analysis of tumour rates and death rates in experimental animals.* Br.J.Cancer, 1974. **29**(2): p.101-105

460. Tutt, A.L., *et al.*, *T cell immunity to lymphoma following treatment with anti-CD40 monoclonal antibody.* J Immunol, 2002. **168**(6): p. 2720-8.

461. Tutt, A.L., *et al.*, *Monoclonal antibody therapy of B cell lymphoma: signaling activity on tumor cells appears more important than recruitment of effectors.* J Immunol, 1998. **161**(6): p. 3176-85.

462. Todryk, S.M., *et al.*, *CD40 ligation for immunotherapy of solid tumours.* J Immunol Methods, 2001. **248**(1-2): p. 139-47.

463. van Mierlo, G.J., *et al.*, *CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5561-6.

464. Townsend, S.E., and J.P. Allison, *Tumor Rejection After Direct Costimulation of CD8+ T-cells by B7-Transfected Melanoma Cells.* Science, 1993. **259**: p.368-370.

465. Leach, D.R., Krummel, M.F., and Allison, J.P., *Enhancement of Antitumor Immunity by CTLA-4 Blockade.* Science, 1996. **271**: p.1734-1736.

466. Couderc, B., *et al.*, *Enhancement of antitumor immunity by expression of CD70 (CD27 ligand) or CD154 (CD40 ligand) costimulatory molecules in tumor cells.* Cancer Gene Ther, 1998 **5**(3): p.163-75

467. Tamada, K., *et al.*, *Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway.* Nat. Med., 2000. **6**: p.283-289

468. Szabo, S.J., *et al.*, *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.

469. Afkarian, M., *et al.*, *T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells.* Nat Immunol, 2002. **3**(6): p. 549-57.

470. Turner, J.G., *et al.*, *Anti-CD40 antibody induces antitumor and antimetastatic effects: the role of NK cells.* J Immunol, 2001. **166**(1): p. 89-94.

471. Shuford, W.W., *et al.*, *4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses.* J Exp Med, 1997. **186**(1): p. 47-55.

472. Iyoda, T., *et al.*, *The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo.* J Exp Med, 2002. **195**(10): p. 1289-302.

473. Huleatt, J.W. and L. Lefrancois, *Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and CD8+ T cells in vivo.* J Immunol, 1995. **154**(11): p. 5684-93.
474. McKnight, A.J., *et al.*, *Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family.* J Biol Chem, 1996. **271**(1): p. 486-9.
475. Havenith, C.E., *et al.*, *Separation of alveolar macrophages and dendritic cells via autofluorescence: phenotypical and functional characterization.* J Leukoc Biol, 1993. **53**(5): p. 504-10.
476. Karni, A., *et al.*, *IL-18 is linked to raised IFN-gamma in multiple sclerosis and is induced by activated CD4(+) T cells via CD40-CD40 ligand interactions.* J Neuroimmunol, 2002. **125**(1-2): p. 134-40.
477. Berard, F., *et al.*, *Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells.* J Exp Med, 2000. **192**(11): p. 1535-44.
478. Feuerer, M., *et al.*, *Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow.* Nat Med, 2001. **7**(4): p. 452-8.
479. Lechler, R., *et al.*, *The contributions of T-cell anergy to peripheral T-cell tolerance.* Immunology, 2001. **103**(3): p. 262-9.
480. Stevanovic, S., *Identification of tumour-associated T-cell epitopes for vaccine development.* Nat Rev Cancer, 2002. **2**(7): p. 514-20.
481. Wong, F.S., *et al.*, *Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library.* Nat Med, 1999. **5**(9): p. 1026-31.
482. Kataoka, T., *et al.*, *Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity.* J Immunol, 1996. **156**(10): p. 3678-86.
483. Blazar, B.R., *et al.*, *Ligation of 4-1BB (CDw137) regulates graft-versus-host disease, graft- versus-leukemia, and graft rejection in allogeneic bone marrow transplant recipients.* J Immunol, 2001. **166**(5): p. 3174-83.
484. Claudia, M., Lennart, T., and Londei, M., *Therapeutic activity of agonisitic monoclonal antibodies against CD40 in a chronic autoimmune inflammatory process.* Nat Med, 2000. **6**(6):p. 673-9.
485. Zanelli, E., and Toes, R.E.M., *A dual function for CD40 agonists.* Nat Med, 2000. **6**(6): p.629-30.
486. Fillatreau, S. *et al.*, *B cells regulate autoimmunity by provision of IL-10.* Nat Imm, 2002. **3**(10): p.944-950.
487. Ochsenbein, A.F., *et al.*, *Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction.* Nature, 2001. **411**(6841): p. 1058-64.

488. Nguyen, L.T., *et al.*, *Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance.* J Exp Med, 2002. **195**(4): p. 423-35.
489. Shurin, M.R., *et al.*, *Inhibition of CD40 expression and CD40-mediated dendritic cell function by tumor-derived IL-10.* Int J Cancer, 2002. **101**(1): p. 61-8.
490. Chen, W., *et al.*, *Identification of a gag-encoded cytotoxic T-lymphocyte epitope from FBL- 3 leukemia shared by Friend, Moloney, and Rauscher murine leukemia virus-induced tumors.* J Virol, 1996. **70**(11): p. 7773-82.
491. Green, W.R., *Cytotoxic T lymphocytes to endogenous mouse retroviruses and mechanisms of retroviral escape.* Immunol Rev, 1999. **168**: p. 271-86.
492. Feenstra, A., *et al.*, *In vitro methylation inhibits the promotor activity of a cloned intracisternal A-particle LTR.* Nucleic Acids Res, 1986. **14**(10): p. 4343-52.
493. Florl, A.R., *et al.*, *DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas.* Br J Cancer, 1999. **80**(9): p. 1312-21.
494. McGrath, M.S., G. Tamura, and I.L. Weissman, *Receptor mediated leukemogenesis: murine leukemia virus interacts with BCL1 lymphoma cell surface IgM.* J Mol Cell Immunol, 1987. **3**(4): p. 227-42.
495. Golgher, D., *et al.*, *Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens.* Eur J Immunol, 2002. **32**(11): p. 3267-75.
496. den Boer, A.T., *et al.*, *Longevity of antigen presentation and activation status of APC are decisive factors in the balance between CTL immunity versus tolerance.* J Immunol, 2001. **167**(5): p. 2522-8.
497. Pardoll, D.M., *Inducing autoimmune disease to treat cancer.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5340-2.
498. Ghetie, V. and E.S. Ward, *Multiple roles for the major histocompatibility complex class I- related receptor FcRn.* Annu Rev Immunol, 2000. **18**: p. 739-66.