THE CD40 RECEPTOR

– TARGET, TOOL AND TECHNOLOGY

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ORIGINAL PAPERS

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II **Ellmark P, Ottosson C, Borrebaeck CAK, Malmborg-Hager AC, Furebring C.** Modulation of the CD40-CD40 ligand interaction using human anti-CD40 single-chain antibody fragments obtained from the n-CoDeR phage display library. *Immunology* 2002; 106:456-63.

III **Ellmark P, Esteban O, Furebring C, Malmborg Hager A-C , Ohlin M.** *In vitro* molecular evolution of antibody genes mimicking receptor revision. *Molecular Immunology* 2002; 39:349-56.

IV **Ellmark P, Furebring C , Borrebaeck CAK.** Pre-assembly of the extracellular domains of CD40 is not necessary for rescue of naive B-cells from anti-IgM induced apoptosis. *Submitted 2002*.

V **Ellmark P, Ohlin M, Borrebaeck CAK, Furebring C.** A novel selection system for identification of interacting protein pairs based on receptor activation. *Manuscript*

ABBREVIATIONS

Ab	antibody
ADCC	antibody dependent cellular cytotoxity
AID	activation-induced cytidine deaminase
APC	antigen presenting cell
BCR	B cell receptor
CD	cluster of differentiation
CDR	complementarity determining region
CTL	cytotoxic T lymphocytes
D	diversity
Da	Dalton
DC	dendritic cell
DNA	deoxiribonucleic acid
DR	death receptor
EAE	experimental autoimmune encephalomyelitis
Fab	fragment antigen binding
Fc	fragment crystalizable
FR	frame work region
g3p	gene 3 protein
GC	germinal centre
HAMA	human anti-mouse response
Hsp	heat shock protein
Ig	immunoglobulin
IGH	immunoglobulin heavy
IGL	immunoglobulin lambda
IL	interleukin
J	joining
Jak	janus kinase
mAb	monoclonal antibody
MS	multiple sclerosis
NF-κB	nuclear factor-kappaB

PCA	protein-fragment complementation assay
PCR	polymerase chain reaction
PLAD	pre-ligand assembly domain
RAG	recombination activating gene
RSS	recombination signal sequence
scFv	single chain fragment variable
SF	superfamily
SHM	somatic hypermutation
SPIRE	selection of protein interactions by receptor engagement
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TD	TRAF domain
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF	TNF receptor associated factor
V	variable
VH	variable heavy
VL	variable light
Y2H	yeast two-hybrid
Å	ångström

1 INTRODUCTION

The immune system is a sophisticated organisation of molecules, cells and tissues that acts in concert to protect us against different pathogens. It is composed of the evolutionary ancient innate compartment and the more complex adaptive compartment, where the latter is restricted to vertebrates. Together with the nerve system, the immune system is the major adaptive system in the body. These two systems (the "supersystems") act, and interact, with the main goal to maintain homeostasis.

Although the concept of immunity was known already in ancient Greece, it was Pasteur and colleagues who, at the end of the nineteenth century, gave born to the science of immunology by generalising Jenners use of vaccination. During the first half of the 20th century, the advancement of the discipline was stimulated by the controversy between adherents of the theory of cellular immunity and supporters of the humoral theory of immunity. The latter theory was most strongly advocated, resulting in enhanced focus on characterisation of antibodies, the key element of humoral immunity (Silverstein, 1999).

However, the extraordinary ability of the immune system to mount antibodies against almost every substance that it encounters, was not satisfactorily explained until MacFarlane Burnet presented the clonal selection theory of acquired immunity in the late 1950s (Burnet, 1959). He postulated that antibodies are cell surface receptors and that each individual cell (later known as B cells) display a single clonotype that upon binding to a specific antigen will proliferate and give rise to cells that produce antibodies. They also give rise to cells that are able to react upon repeated antigen encounter, i.e. memory cells. Furthermore, he incorporated Lederbergs suggestion that immunological tolerance is the result of clonal abortion of cells that react to self antigens in his theory. This theory was modified by Bretcher and Cohn (Bretscher and Cohn, 1970), who presented the two signal model 1969, in which they suggested that activation of a B cell require both recognition of an antigen via the B cell receptor (BCR) (signal one) and, subsequently, help (signal two) from another lymphocyte specific for that antigen. It was later found that the cells responsible

for providing this help was T-cells and that a B cell receptor, named CD40, plays a central role in transmitting the second activation signal to B cells. Laffererty and Cunningham expanded the hypothesis, and added a new cell type to the model, the antigen presenting cell (APC), which provides the T-cells with a second activation signal (Cunningham and Lafferty, 1977; Lafferty and Cunningham, 1975). It was later suggested that the APCs need to be activated via pattern recognition receptors (PRRs) to provide T-cells with a second signal (Janeway, 1989; Janeway, 1992). It is now evident that dendritic cells are the key APCs and play a central role in regulating and tuning the immune system (Banchereau et al., 2000).

Alongside with the increasing understanding of the immune system there have been ongoing efforts to explore this knowledge to develop novel techniques and more efficient therapies. Köhler and Milstein's development of the monoclonal antibody technology provided the research community with a source of well-defined and specific tools (Köhler and Milstein, 1975). However, the initial excitement over the possibilities to utilise this technology for antibody based treatment of human patients was hampered by the human anti-mouse antibody (HAMA) response that antibodies produced by this technique often elicit.

The development of the phage display technique (Smith, 1985), by which human antibodies can by displayed on the surface of phages (Barbas et al., 1991; McCafferty et al., 1990), enabled selection of specific, human, antibodies against target antigens. This started to restore the belief in therapeutic antibodies, and today the number of monoclonal antibodies in different stages of clinical trials are increasing rapidly (Borrebaeck and Carlsson, 2001).

Selection of antibodies from a phage display library mimics the adaptive immune response in that it relies on clonal selection and amplification of entities that recognise a certain antigen. Furthermore, there is a physical link between the gene that encodes the antigen binding protein (genotype) and the protein itself (phenotype). The combination of molecular libraries and selection systems where genotype and phenotype is linked, has proven to be very successful and a number of different techniques, beside the page display system, have been developed based on this principle, e.g. ribosomal display (Schaffitzel et al., 1999).

As implied by the title of this thesis, the CD40 receptor is the inter-connective theme that ties the different parts together. In PAPER I, some of the molecular mechanisms and requirements behind anti-CD40 antibody-mediated signalling have been elucidated. The results from PAPER I aided the development of human immuno-modulatory, anti-CD40 antibodies, described in PAPER II. We further utilised the antibodies obtained in PAPER II to analyse antibody evolution events (PAPER III). The molecular requirements for CD40 mediated stimulation was further investigated in PAPER IV. We showed that none of the extracellular domains of CD40 is critical for functional signalling. Finally, in PAPER V, we used the functional understanding of CD40 signalling to develop a eukaryote selection system, in which CD40 signalling is used to clonally select cells from a mammalian expression library.

2 THE CD40 RECEPTOR

2.1 Identification of CD40

CD40 was originally identified by an antibody raised against urinary bladder carcinoma that was found to bind B cells as well (Koho et al., 1984; Paulie et al., 1984). Today, CD40 is known to be expressed on a variety of cells in the immune system, e.g. on B cells, dendritic cells, basophils, eosinophils and monocytes. CD40 can, furthermore, be detected on endothelial cells, keratinocytes, smooth muscle cells, eptithelial cells and fibroblasts (Schonbeck and Libby, 2001; van Kooten and Banchereau, 1997; van Kooten and Banchereau, 2000). Although the CD40 ligand (CD40L), which also is known as CD154, is mainly expressed on activated T-cells it can also be found on basophils, eosinophils, monocytes, macrophages, dendritic cells, NK cells, B lymphocytes, platelets, mast cells, endothelial cells, smooth muscle cells and epithelial cells (Schonbeck and Libby, 2001). A common feature of all these cells is that the CD154 expression is non-constitutive, but can be rapidly induced upon activation.

2.2 CD40 plays a central role in the immune system

The pivotal importance of the CD40 molecule was recognised when it was discovered that X-linked hyper IgM syndrome is due to a detrimental mutation of the gene encoding CD154 (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthauer et al., 1993). This disease is characterised by defective isotype switching and lack of somatic mutation as well as deficient B cell memory (Notarangelo and Peitsch, 1996). It was found that CD40 signalling is critical for T cell dependent B cell activation (Foy et al., 1993). Furthermore, signalling via CD40 is necessary for subsequent steps in B lymphocyte activation, such as affinity maturation, isotype switching, generation of memory cells and germinal centre formation, although the latter may be a secondary effect (Siepmann et al., 2001). Stimulation of human B cells with anti-CD40 antibodies or recombinant CD154 has been shown to induce homeotypic aggregation and up-regulation of surface markers such as CD23, CD30, CD80,

CD86, Fas and major histocompatibility complex (MHC) II and soluble cytokines, e.g. IL-6, TNF-α and TNF-β (Schonbeck and Libby, 2001). Furthermore, CD40 is also critical for induction of cellular immunity and has a pro-inflammatory activity on e.g. monocytes and DC (Caux et al., 1994; Mackey et al., 1998).

It has recently been shown that CD40 also interacts with heat shock proteins (Hsp70) from both bacterial (Wang et al., 2001) and mammalian (Becker et al., 2002) sources. This type of heat shock proteins binds to a broad range of peptides during synthesis or under conditions of cellular stress. They can reach the extracellular space subsequent to necrosis or viral lysis and may serve as a carrier of antigenic peptides for cross-priming (Becker et al., 2002). The bacterial variant has been reported to upregulate chemokines, such as RANTES, MIP-1α and MIP-1β, upon binding to CD40 (Wang et al., 2001). Becker et al. (2002), furthermore, showed that mouse Hsp70, isolated from a tumour cell with bound tumour-associated peptide, can induce CD40 signalling in DC. Moreover, they suggested that peptide loading is required for this interaction. The Hsp70-peptide-CD40 interaction results in uptake of the Hsp70-peptide complex, which may result in a display of the loaded peptide on MHC class I or II.

2.3 Structure and molecular characteristics of CD40

CD40 is a 43-48 kDa glycoprotein composed of 277 amino acid residues (Braesch-Andersen et al., 1989). It belongs to the tumour necrosis factor receptor (TNFR) family, and as all members of this family the extracellular part of CD40 consist of several cysteine rich repeats. The four extracellular domains are composed of two types of modular units (Figure 1) (Naismith and Sprang, 1998; Singh et al., 1998) and each module is stabilised by one or two disulfide bonds. No crystal structure of CD40 has been reported, but several models of three of the extracellular domains have been proposed, using the known X-ray structure of TNFR as a template (Bajorath et al., 1995a; Bajorath et al., 1995b; Singh et al., 1998).

CD40 contains a single membrane spanning domain and expose the N-terminus of the protein to the exterior (type I membrane protein). Biochemical characterisation, suggests that CD40 is a hydrophobic protein with a quite acidic

pI of 3.2 (van Kooten and Banchereau, 2000). The cytoplasmic domain of CD40 contains several recognition sites for intermolecular proteins, e.g. tumour necrosis factor receptor associated factors (TRAFs) (Figure 1).

2.4 The CD40-CD154 interaction

The structure of the extracellular part of CD154 has been resolved by X-ray crystallography (Karpusas et al., 1995). It consists of two beta sheet with jellyroll topology that forms a symmetric homotrimer. The crystal structure of CD154 and the CD40 model has been used together with site-directed mutagenesis to identify five CD40 residues Y82, D84, N86, E74 and E117 and five CD154 residues K143, Y145, Y146, R203 and Q220 as important for the CD40-CD154 interaction (Bajorath and Aruffo, 1997; Bajorath et al., 1995a; Bajorath et al., 1995b). Thus the CD154 binding site has been shown to be located in the second and third domain of CD40. It has been suggested that polar interaction between the basic residues on the CD154 and the acidic residues on CD40 plays an important role for this interaction (Singh et al., 1998).

Notably, analysis of a structure model of clone F33, one of the single chain antibodies described in PAPER II, which almost totally abrogates the CD40-CD154 interaction, reveals that it does not contain any acidic residues in the central core of the binding site (Figure 2). Moreover, it has an unusually high estimated isoelectric point (theoretical pI 9.5) and it contain at least one centrally located basic residue, $R56_L$ (according to the IMGT nomenclature, (Lefranc, 2001). This residue has been reported to be the most frequent contact residue in CDRL2 (contact residue in 9/26 analysed antibodies) (MacCallum et al., 1996). From these observations, it may be hypothesised that the F33-CD40 interaction, at least partly, depends on similar charged residues as the CD40-CD154 interaction.

2.5 Intracellular signalling events

To transform the externally applied signal, resulting from oligomerisation of CD40, to an intracellular signal that ultimately can lead to a cellular response, CD40 depends on several proteins that can interact with different recognition motifs (Figure 1). The most important group of intracellular signalling proteins

that interacts with CD40 is the TRAF family, which is a group of adapter molecules with no intrinsic enzymatic activity. CD40 is known to bind directly to TRAF2, 3 and 6 and indirectly to TRAF1 and 5, although there is some controversies regarding the association of TRAF5 to CD40 (Ishida 1996, Nakano 1996, Bishop 2002). The TRAF2 and 3 binding motif on CD40 has been identified to a PVQET sequence, whereas TRAF6 binds to the QEPQEINF motif (Pullen et al., 1998).

The TRAFs are composed of a conserved C-terminal domain, TRAF domain (TD), and a less conserved N-terminal domain. The latter consists of one (TRAF1) or several (TRAF2-6) zinc finger structures and, in the case of TRAF2-6, a RING finger structure. This domain mediates interactions with different secondary signalling proteins (reviewed by Wajant et al., 2001 and (Inoue et al., 2000). Crystallographic analysis of the TD of the different TRAFs reveals a trimeric mushroom shaped structure where the N-terminal part, TRAF-N, forms a triple helical coiled coil and the C-terminal end, TRAF-C, forms a unique eight stranded anti-parallel beta sheet (Ahonen et al., 2002; McWhirter et al., 1999; Ni et al., 2000; Park et al., 1999). The N-terminal end of the TD also contains the CD40 binding site (Cheng and Baltimore, 1996; Cheng et al., 1995; Malinin et al., 1997; Rothe et al., 1996).

Affinity studies have shown that the TRAF-CD40 interaction depends on oligomerisation to form a stable signalling complex (Pullen et al., 1999). It has furthermore been shown that there are marked differences between the affinity to CD40 between the different TRAFs. Thereby they also seem to require different degree of oligomerisation to produce a stable interaction, and it has been suggested that the difference in affinity is the basis for the specificity of the TRAFs (Pullen et al., 1999). Furthermore, the different TRAFs may play distinct roles in different stages of B cell development. It has suggested that TRAF6 mediated signalling is crucial for affinity maturation and generation of long-lived plasma cells (Ahonen et al., 2002), whereas TRAF2 and 3 is essential for class switching (Jabara et al., 2002).

TRAF recruitment ultimately leads to activation of NF-κB, stress-activated protein kinase/c-jun amino terminal kinase (JNK/SAPK), p38 mitogen-activated protein kinase (MAPK) protein tyrosine kinases (PTK), phosphoinositide-3 (PI-

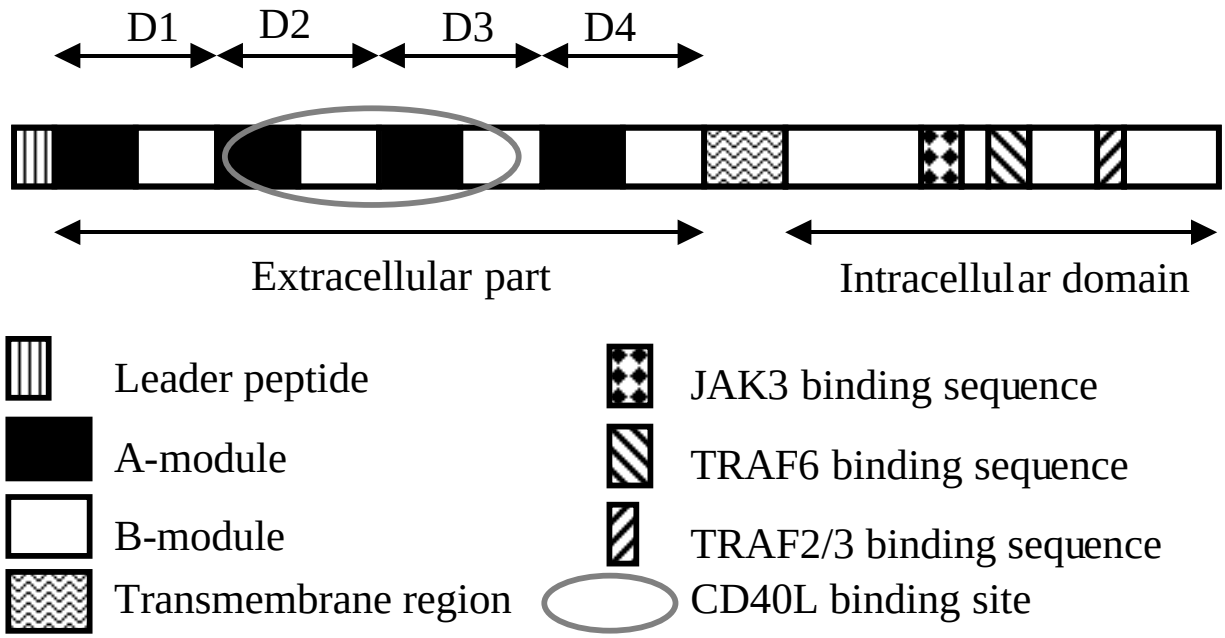

Figure 1. Schematic of the CD40 protein. The extracellular parts of CD40 is composed of four domains (D1-D4), which each consists of two modules. The approximate location of the CD154 binding site is depicted in the figure (a more detailed picture over the involved residues is presented in paper I). The putative interaction sites for the intracellular signalling intermediates that are known to interact directly with CD40 are also shown.

3) kinase and the extracellular signal-regulated mitogen activated protein kinase (ERK) (Berberich et al., 1996; Craxton et al., 1998; Grammer et al., 1998; Purkerson and Parker, 1998; van Kooten, 2000; van Kooten and Banchereau, 1997). Rescue from apoptosis requires a minimum of these signalling pathways, whereas stimulation of proliferation requires ERK, p38 and PI-3K pathways (Dadgostar et al., 2002).

Janus kinase 3 (JAK3) has been shown to interact with a proline motif in the intracellular region of CD40 (Hanissian and Geha, 1997) and it is phosphorylated upon CD40 stimulation, resulting in the subsequent phosphorylation of STAT3, STAT5a (Hanissian and Geha, 1997; Revy et al., 1999) and STAT6 (Karras et al., 1997). The JAK3 pathway seems to be important for activation of monocytes and dendritic cells (Revy et al., 1999; Saemann et al., 2002) although it seems to be of little importance in CD40 mediated B cell activation (Jabara et al., 1998).

3 SIGNAL INITIATION VIA CD40

3.1 Oligomerisation of CD40

By comparing the ability of soluble monomeric, dimeric and trimeric CD40 variants to activate NF-κB in the cytosol, it has been shown that the minimal requirement to induce a signal through CD40 is the formation of a receptor dimer (Werneburg et al., 2001), although trimerisation generates a stronger signal. Haswell et al. has shown that the strength of the receptor mediated signal correlates with increasing valency of an extracellularly applied ligand (Haswell et al., 2001). Furthermore, the functional consequences of CD40 signalling also depends on the cell-type and the differentiation stage of the cell (van Kooten and Banchereau, 2000). In addition, the level of cross-linking that is required to initiate a certain signalling pathway varies between different cell types (Fanslow et al., 1994). It has been suggested that ligation via trimeric CD154 expressed on a cell membrane is required to activate some particular signalling pathways e.g. IL-6 production in naive B cells (Baccam and Bishop, 1999).

It has been demonstrated that the stimulatory capacity of different anti-CD40 antibodies depends on the location of the epitope on CD40 (Björck et al., 1994; Challa et al., 1999; Pound et al., 1999; Sakata et al., 2000). Pound et al. has suggested that rescue from apoptosis is essentially epitope independent, whereas the induction of proliferation is more epitope dependent (Pound et al., 1999). Furthermore, it has been shown that the functional activity of anti-mouse CD40 antibodies correlates with the ability to inhibit CD154 binding to CD40 expressing cells (Barr and Heath, 2001). However, in PAPER I and II we show that this correlation is only valid when one particular blocking method is used. Moreover, it can be concluded from these studies that other factors than direct epitope masking influence the results from these assays. Steric hindrance, conformational changes as well as CD40-complex formation induced by the first applied molecule may influence the ability of the second molecules to bind. The proposed CD40 signalling model, which will be discussed more in detail in the next section, does however predict that the orientation of the epitope in relation to the CD154 epitope is important for signal transmission. However, it is

probably not important which of the extracellular domains of CD40 the binding epitope is located on.

It has been suggested that binding of CD154 induces a conformational change in CD40, which is necessary for signal transmission (Barr and Heath, 2001; Fanslow et al., 1994; Kaykas et al., 2001; Ledbetter et al., 1997; Pound et al., 1999). This would indeed predict that the location of the epitope in relation to the CD154 binding site is critical, since conformational changes in the protein structure often depends on interaction between the paratope and residues in the epitope. This theory is mostly based on the observation that monomeric molecules such as scFv (single chain fragment variable) or soluble monomeric CD154 can be used to stimulate CD40 signalling. However, the molecules used in these studies have a tendency to spontaneous form oligomers (Karpusas et al., 1995; Gilliland et al., 1996). Moreover, in PAPER IV we show that none of the extracellular domains are essential for signalling via human CD40 in a murine B cell line (WEHI 231). We, furthermore, show that CD40 mediated rescue of WEHI 231 cells is comparable between cells that display wild-type CD40 and those that display CD40 variants, where two of the extracellular domains have been removed. Taken together, our results indicate that conformational changes are unlikely to be necessary for CD40 signalling.

3.2 The role of pre-ligand association of CD40

Several members of the TNFR superfamily spontaneously form oligomers on the cell surface even in the absence of a ligand. Papoff et al. first showed that oligomerisation of CD95 (Fas) depends on the distal extracellular domain (Papoff et al., 1999). This was later confirmed (Siegel et al., 2000a) and it was also showed that TNFR1 and TNFR2 have a similar oligomerisation domain. The domain that mediates the interaction has been named pre-ligand assembly domain (PLAD) (Chan et al., 2000a). They, furthermore, showed that CD40 and Death receptor 4 (DR4) also is specifically pre-ligand associated in the membrane (Chan et al., 2000a), as detected by FRET analysis. Moreover, it has been known for long that CD40 purified from normal B cells, under non-reducing conditions, forms dimers and multimers (Braesch-Andersen et al., 1989).

Although it has not been shown which CD40-domain that is responsible for pre-ligand assembly, it has been proposed that PLAD is located in the distal domain (D1) based on homology studies (Locksley et al., 2001). The group of Leonardo, furthermore, showed that PLAD is necessary for ligand binding to TNFR I and II and Fas, even though PLAD seems to be physically distinct from the ligand contact domains (Siegel et al., 2000a; Chan et al., 2000a). We have demonstrated that the CD40-CD154 interaction also require the distal domain (D1) of CD40 (PAPER I), although this domain does not contain any of the residues that are known to be important for CD154 binding. In conclusion, there are some evidence for a model in which members of the TNFR-SF form oligomers in the membrane prior to ligand binding without initiating any signals and that this oligomerisation is necessary for interaction with its cognate ligand. Hence, ligand induced signalling cannot only be a result of oligomerisation of the receptor. It rather implies that ligand binding induce a change in the arrangement of the receptor that allow adapter molecules e.g. TRAFs to bind. The advantages that this pre-assembly system provides to the TNFR-SF is, however, not fully understood. It may be suggested that this mechanism is used to separate the intracellular domains of CD40 prior to ligation, in order to minimise spontaneous signal initiation (Chan et al., 2000b). Furthermore, it may be a route to down-regulate the susceptibility for ligand induced signalling by assembly of non-functional receptors that lack the intracellular part together with functional receptors, as suggested by Papoff et al. (1999). An alternative explanation is that ligand binding induce the formation of a super cluster (Chan et al., 2000a; Chan et al., 2000b; Locksley et al., 2001; Siegel et al., 2000a; Siegel and Lenardo, 2001), which in turn initiate intracellular signalling events. However, it has not been investigated to what extent the PLAD effects signalling via TNFR-SF members.

In PAPER IV we provide evidence suggesting that the distal domains (where the PLAD is assumed to be located) is not necessary for CD40 signal initiation in a naive B cell line, since both domain 1 and 2 of CD40 can be removed without effecting the ability to promote proliferation. We further confirm this in PAPER V, where we show that the extracellular domains also can be replaced by a scFv. These results also imply that PLAD-mediated formation of supercluster

is not involved in CD40 signalling. It can, however, not be excluded that some of the CD40 signalling pathways require the PLAD. However, it has been demonstrated that induction of proliferation require most of the important pathways e.g. the ERK, p38 and the PI-3K pathway (Dadgostar et al., 2002), indicating that our assay is highly relevant.

3.3 CD40 in lipid rafts

It was recently found that CD40 depends on the microenvironment of lipid rafts to function optimally (Grassme et al., 2002c; Hostager et al., 2000; Kaykas et al., 2001; Pham et al., 2002; Vidalain et al., 2000), although one group has reported conflicting results (Malapati and Pierce, 2001). Lipid rafts (also denoted membrane rafts) are cholesterol and glycosphingolipid enriched micro domains that serve as a site for assembly of several receptor complexes of central importance for the immune system. When for instance a T cell interacts with an APC the interacting receptors are enriched in membrane rafts and subsequently form an ordered polarised cluster in the interacting surface between the cells, termed the immunological synapse (Bromley et al., 2001). Certain molecules, such as proteins that contain glycosylphosphatidylinositol (GPI)-anchored proteins and double acetylated proteins have a permanent affinity for lipid rafts. Other receptors, e. g. the T cell receptor (TCR) and the BCR (Brown, 2002; Dykstra et al., 2001; Pierce, 2002; Simons and Toomre, 2000) can be recruited to rafts after stimulation, while some molecules are permanently excluded from the rafts. It has been shown that engagement of CD40 results in the translocation of CD40, as well as of TRAF2 and 3 to lipid rafts (Hostager et al., 2000; Vidalain et al., 2000), although they partially exists in small lipid rafts (elementary rafts) even prior to ligation. It has, furthermore, been shown that active formation of CD154 clusters is required for subsequent formation of CD40 cluster (Grassme et al., 2002a), which in turn is central for initiation of CD40 signalling.

The results from the proliferation assay and the selections from the model libraries, described in PAPER V, shows that a protein fused to the transmembrane and cytosolic part of CD40 can act as a mock CD40 receptor, and transmit a rescue signal in a mouse B cell line (WEHI 231). In this study (PAPER V), the

rescue signal is provided by another fusion protein, which also contain the transmembrane and intracellular part of CD40 (mock ligand). The mock ligand is displayed on the surface of a fibroblast cell line (3T3). Furthermore, a CD40 variant that can function as a ligand when displayed on fibroblasts, can function as a receptor when displayed on B cells. This shows that although neither of the CD40 variants spontaneously forms signalling complexes when transfected into B cells, they are still able to induce the formation of such complexes when transfected into fibroblasts.

Several possible explanations for this dual nature of the CD40 variants may be envisaged. It has recently been shown that active formation of CD154 cluster is required for subsequent formation of CD40 clusters, which is central for initiation of intracellular signalling events. It may thus be suggested, that the CD40 variants become self-activated in the fibroblasts and thereby form lipid raft-associated complexes, which in turn can induce oligomerisation of a mock receptor displayed on a B cell. (Grassme et al., 2002b). In fact, it has been shown that high expression levels of CD40 can lead to ligand independent activation and spontaneous formation of CD40 enriched rafts (Kaykas et al., 2001). It may thus be suggested that the CD40 variants become self-activated in fibroblasts and not in the B cells, since difference in mean flourescence signal between fibroblasts and B cells, transfected with the same CD40 variant, shows that the display level is higher in the fibroblasts (PAPER V).

However, there are other possible explanations to the fact that the CD40 variants can act both as a receptor and as a ligand when displayed on B cells or fibroblasts, respectively. It has been suggested that receptors that are involved in lipid raft formation, prior to activation exists in elementary rafts that only contain a few receptors (Dykstra et al., 2001; Simons and Toomre, 2000). Ligation of these receptors is suggested to lead to aggregation of the small rafts, leading to formation of larger membrane raft clusters. It is thus possible that the interaction between CD40 variants on fibroblasts respectively WEHI 231 cells can stabilise the formation of successively larger cluster. This process may be enhanced by the increased concentration of raft-associated proteins in the cell-cell interaction surface (Dykstra et al., 2001). It may thus be suggested that, when a CD40 variant displayed on a B cell interacts with a complementary

CD40 variant on a fibroblast, signals are transmitted into both of the cell types. If this is the case, the CD40 variants simultaneously act as mock-receptors and mock-ligands. This may be possible to utilise for development of novel library versus library screening methods and is further discussed in section 7.4.

4 CD40 AS A TARGET FOR IMMUNOTHERAPY

The central role of the CD40-CD154 dyad in the adaptive immune response makes it an ideal target for immunotherapy. Modulation of CD40 signalling has been used to block CD40 signalling in e.g. chronic inflammatory conditions or subsequent to transplantation. In other approaches, agonistic anti-CD40 antibodies have been used to stimulate the immune system in e.g. cancer therapy and vaccination formulations.

4.1 CD40 based therapy in clinical inflammatory conditions

The main focus of therapeutic intervention of CD40 signalling in clinical inflammation has been on experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple sclerosis (MS). MS is an inflammatory disease characterised by demyelinating plaques dominated by autoreactive activated T cells, monocytes and macrophages (Karp et al., 2000; Karp et al., 2001; Laman et al., 1998; Pouly and Antel, 1999; 't Hart et al., 2001). Antibody producing B cells as well as some autoreactive antibodies has also been detected in the plaques (Genain et al., 1999; Pouly and Antel, 1999; von Budingen et al., 2002). Although the involvement of the CD40-CD154 interaction in the pathology of MS has not been fully elucidated, it has been suggested to be important for several of the critical events during the progression of the disease, e.g. priming of autoreactive B cell, activation of T cells, activation of monocytes and macrophages in the lesions (Laman et al., 1998; Pouly and Antel, 1999; 't Hart et al., 2000).

Two different approaches to disrupt the CD40-CD154 interaction in EAE have been evaluated, either using antibodies against CD154 (Gerritse et al., 1996) or CD40 (Boon et al., 2001; Boon et al., 2002; Laman et al., 2002). They have both shown some promising pre-clinical results and one anti-CD40 antibody is currently in phase I/II studies for treatment of Crohn's Disease, which also is a clinical inflammatory condition. The mechanisms behind these effects are however not entirely elucidated and it is not clear to what extent the antibodies used in these studies also induce signalling in cells that display

CD154 or CD40, respectively. Antibodies towards CD40 is known to induce CD40 signalling in human B cells to different extent (Barr and Heath, 2001; Björck et al., 1994; Challa et al., 1999; Kwekkeboom et al., 1993; Pound et al., 1999). In addition, antibodies against CD154 has been shown to stimulate T cells in some experimental settings (Blotta et al., 1996; Grassme et al., 2002a; Koppenhoefer et al., 1997). The murine anti-CD40 mAb 5D12 that has been used in several pre-clinical models of MS (de Boer et al., 1992; Kwekkeboom et al., 1993) has been referred to as an antagonistic antibody though it clearly have an effect on CD40 signalling (Kwekkeboom et al., 1993 and PAPER I). Moreover, it has been shown by Mauri et al. (2000) that agonistic antibodies have a beneficial clinical effect on certain chronic autoimmune inflammatory processes.

Thus, these investigations indicate that anti-CD40 antibodies, which by them self have a proinflammatory effect on CD40 expressing cells, might have a therapeutic effect on autoimmune conditions. This is so, in spite of the fact that such inflammatory diseases often benefit from suppression of the adaptive immune system, which, in turn, depends on the CD40-CD154 interaction. The mechanism behind this, seemingly conflicting observation, has not been clarified, although it has been suggested that modulations of the function of DCs or antigen specific B cells may play a role (Mauri et al., 2000; Zanelli and Toes, 2000). There is, however, another explanation. It has been shown that also agonistic anti-CD40 antibodies block proliferation induced by membrane bound CD154 significantly, despite their ability to induce proliferation on their own (Kwekkeboom et al., 1993). As discussed above, the magnitude of CD40 signalling subsequent to cross-linking, depends on the mode by which it is ligated. Several signalling pathways require stimulation via cell surface expressed CD154 and thus the stimulating effect of an agonistic CD40 antibody may be neglectable compared to a functional, T cell-mediated, CD154-CD40 interaction. Furthermore, the formation of a functional immunologic synapse involves signalling via several other co-stimulatory molecules. Also agonistic anti-CD40 antibodies may interrupt this process by blocking the CD40-CD154 interaction, and thereby suppress autoantigen-specific activation of the adaptive compartment. Hence, it may be suggested that agonistic antibodies can block

scFv clone	CDRH3	k_d ($x10^{-3}$/s)	KD (nM)	Domain	Activation of B cells	Ability to block the CD40-CD154 interaction
A24	ARAPVDYSNPSGMDV	0.4	10	D1*	(+)	+++
A43	ARAPVDYSNPSGMDV	0.6	35	D1*	(+)	+++
A49	ARAPVDYSNPSGMDV	1.1	44	D1*	(+)	+++
A54	ARAPVDYSNPSGMDV	0.9	40	D1*	(+)	+++
B44	ARILRGGS---GMDL	0.9	60	D1/B2	++++	+++
C27	ARADWEYYYY-GMDV	6.6	16	D1/B2	++	+++
D13	ARHIYPW----GMDV	0.6	600	D1*	+++	++
E30	ARMTPWYY---GMDV	5.8	3.6	D1/B2	++++	+++
F33	ARGWLL-------DY	0.4	3.1	D2/B1	+++	++++

Table 1. Characteristics of the anti-CD40 antibody fragments described in PAPER II. + 5-20%, ++20-40%, +++ 60-80 %, ++++80-100%.
* These clones only binds CD40 displayed without the detection tag (AD2)

specific APC driven T-cell activation in autoimmune inflammatory conditions, despite their systemic immuno-stimulatory effect.

Still, for treatment of inflammatory conditions it would probably be advantageous to use an anti-CD40 antibody that can block the CD40-CD154 interaction and at the same time minimally affect CD40-displaying cells. To this end, we have selected fully human anti-CD40 antibodies that have a very low stimulatory effect on B cell proliferation (PAPER II). Although neither of them are totally antagonistic, the type A anti-CD40 (Table 1) scFv require unusually high concentrations to activate B cell proliferation. These antibodies have been produced in full IgG format, preliminary data indicates that these characteristics are maintained (data not shown). It remains to analyse to what extent they are capable to block T cell induced B cell proliferation.

4.2 CD40 in cancer treatment

During the evolution of a tumour, cancer cells has to evolve mechanisms to escape immune surveillance, which normally would eradicate cells with malignant transformations (Diehl et al., 2000). As CD40 plays a central role in the immune system it may be possible to use antibodies against CD40 to redirect the immune response towards the evolving tumour (Costello et al., 1999).

Furthermore, several haematological malignancies express CD40, which makes direct targeting with anti-CD40 antibodies possible. One of the mechanisms by which tumour cells evade the immune system is to downregulate expression of MHC I and II as well as costimulatory proteins and adhesion molecules (Restifo et al., 1993), thereby preventing triggering of tumour specific cytotoxic T lymphocytes (CTLs). It has been shown that CD40 treatment of e.g. follicular lymphoma can induce expression of these molecules and restore the reactivity of CTLs (Costello et al., 1999). Furthermore, agonistic CD40 antibodies has been used to successfully eradicate tumours in mice (Francisco et al., 2000; French et al., 1999; Fujita et al., 2001).

Anti-CD40 therapy can also lead to effective treatment of CD40 negative tumours (Tutt et al., 2002; van Mierlo et al., 2002), possibly by inducing a strong CTL response. It has been suggested that systemic CD40 stimulation result in activation of dendritic cells, which then more efficiently can cross-prime cancer specific $CD8^+$ cytotoxic T cells and thereby bypass the need for $CD4^+$ T helper cells (Diehl et al., 2000; Ribas et al., 2001). It has, furthermore, been suggested that differentiation of $CD8^+$ T cells directly depend on CD40 mediated signalling, where $CD4^+$ T helper cells provide the CD40 ligand, *in vivo* (Bourgeois et al., 2002).

CD40 antibodies may also have a direct apoptotic effect on CD40 expressing tumours (Tong et al., 2000). However, on certain types of CD40 positive lymphomas, CD40 stimulation have a proliferative effect (Andersen et al., 2000; Challa et al., 2002), indicating that treatment is only feasible on certain $CD40^+$ lymphomas.

In conclusion, tumour therapy with anti-CD40 antibodies may be a promising approach, although there sometimes is a finely tuned balance between growth inhibitory and growth promoting capabilities that have to be considered before therapy can be feasible. We have selected and characterised several agonistic anti-CD40 fragments of interest, e.g. clone B44, E30 and F33 (PAPER II). Clone B44 has been produced in an IgG format and preliminary analysis of its stimulatory capacity shows that its capacity to activate human B cells is comparable to the well characterised mouse anti-CD40 antibodies, G28-5 and

EA5 (PAPER I). Further analysis of these clones may reveal a potential candidate for clinical trials.

4.3 Vaccination and transplantation

There are also other possible applications for modulation of CD40 signalling, e.g. to induce transplant tolerance. Interruption of the CD40-CD154 interaction with anti-CD154 antibodies have successfully been used to prevent graft rejection in both mice (Larsen et al., 1996a; Larsen et al., 1996b; Parker et al., 1995) and primates (Kirk et al., 1999). In addition, it has recently been shown that anti-CD40 antibodies successfully extend renal allograft survival in a primate model (Pearson et al., 2002).

Moreover, it has been shown that a non-immunogenic, non-viable *Listeria monocytogenes* could be converted to an immunogenic vaccine by simultaneous delivery of an activating anti-CD40 mAb (Rolph and Kaufmann, 2001). This indicates that co-administration of anti-CD40 antibodies might be useful for future development of novel vaccine formulations. Furthermore, systemic anti-CD40 treatment have been shown to increase the immune response to certain pathogens (Haas et al., 2001).

Tumour vaccination with viral vectors that contains DNA sequences encoding tumour associated antigens (Pardoll, 1998), provide another route to induce activation of the cellular immune system. Dendritic cells (DC) are the most promising target for adenovirus based vaccination protocols (Song et al., 1997). In fact, Gruijl et al. (2002) has recently shown that it is possible to target adenovirus to DC by armouring the virus particle with anti-CD40 antibodies. They demonstrated that, in addition to a more specific delivery of the adenovirus, the engineered virus also induced CD40 mediated activation and maturation of the DC.

5 ANTIBODY DEVELOPMENT

Antibodies (immunoglobulins) are key soluble effector molecules of the adaptive immune response and the immune system can generate specific antibodies to almost any target structure. Upon binding to an antigen, antibodies can exert several different effects, e.g. phagocytosis of the antigen (Gessner et al., 1998; Indik et al., 1995; Raghavan and Bjorkman, 1996), activation of cytotoxic cells (ADCC) (Sulica et al., 2001), binding to complement (Kishore and Reid, 2000), and neutralisation of virus and toxins (Burton et al., 2001; Burton et al., 2000), but also induce allergic responses (Stephen and Lantz, 1999). In addition, antibody molecules have structural features that allow them to penetrate, e.g. the placenta, providing passive protection of the neonate (Raghavan and Bjorkman, 1996).

5.1 Basic antibody structure

The basic quaternary structure of the immunoglobulin molecule is composed of four polypeptide chains; two identical, larger, chains (heavy chains) and two identical, smaller chains (light chains). The heavy and light chains form three functional units, two antigen binding structures (Fabs) and one effector structure (Fc). These units are linked together via flexible linkers in an Y- or T-shaped configuration (Alzari et al., 1988; Amsel and Poljak, 1979; Edmundson et al., 1995; Padlan, 1994).

The Fab parts of the antibody molecule are composed of two domains from the heavy chain and two from the light chain, whereas the Fc part consists of two or three domains from each of the heavy chains. The idiotypic sequence variability that determines the antibody specificity is confined to the N-terminal domain of the heavy and the light chains, and these domains are denoted variable heavy (VH) domain and variable light (VL) domain respectively (Frazer and Capra, 1999). Remaining domains, in which the sequence only differs between the different isotypes and allotypes, are named constant domains (C). There are five different classes of heavy chains (α, μ, γ, δ and ε) and two classes of light chains (λ and κ).

Beside the non-covalent intra- and inter-domain interactions that stabilise the antibody structure, inter-chain disulphide bonds covalently link the heavy chains to each other. The Fab structure is furthermore stabilised by a cysteine bridge between the constant domain of the light chain and the first constant domain of the heavy chain. Antibody domains adopt a structure known as the immunoglobulin fold, which consists of a compressed antiparallel beta-barrel built up by two beta-sheets (Poljak et al., 1973; Schiffer et al., 1973) and is usually stabilised by a intrachain disulphide bridge. Variable domains fold slightly different then the constant domains. One of the two beta-sheets of the variable domains contains five beta-strands, compared to three in the constant domains, which results in the formation of three connecting loops in each end of the beta-barrel. Most of the sequence variability in the V-domains is restricted to the loops that face the exterior (Wu and Kabat, 1970). These complementarity determining regions (CDRs), determine the shape and charge of the antigen-binding site (Davies and Cohen, 1996; Davies et al., 1990; Webster et al., 1994; Wilson and Stanfield, 1993; Wilson and Stanfield, 1994).

5.2 Generation of diversity

The ability to generate antibodies against almost any molecule depends on the huge diversity in the variable domains. In mice and man, this variability is initially created by recombination of different germline gene segments, named variable (V), diversity (D) and joining (J) segment (Tonegawa, 1983). Successful recombination of both the heavy and the light chain is required for the development of a mature B cell (Meffre et al., 2000). V(D)J recombination is a specific process that occurs between sequences that contain complementary recombination signal sequences (RSS) (Max et al., 1979; Sakano et al., 1979). Initiation of the recombination process requires upregulation of recombination activating genes-1 and 2 (RAG-1 and RAG-2) (Oettinger et al., 1990; Schatz and Baltimore, 1988), which introduce double stranded DNA breaks between the V, D and J segments. Several non-homologous DNA end-joining proteins (Bassing et al., 2002) subsequently join the DNA ends of the gene segments. Part of the diversity of the novel variable genes is created by the combination of different V(D)J germline gene segments. Additional diversity is introduced by

imprecise DNA nicking resulting in different reading frame, nucleotide deletions and introduction of non-germline encoded (N) (Alt and Baltimore, 1982; Gilfillan et al., 1993) and palindromic (P) nucleotides (Lewis, 1994; Wuilmart et al., 1977).

5.3 Secondary recombination – Receptor editing

The development of mature B cells requires productive V(D)J rearrangement of the light chain and the heavy chain (Meffre et al., 2000; Rajewsky, 1996). However, if the initial rearrangement is non-productive or the resulting antibody is autoreactive, the immature B cell can be rescued by secondary rearrangements, known as receptor editing (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). Especially the Ig-κ locus is ideally organised for receptor editing. Secondary rearrangements can occur in a single step between remaining upstream Vκ genes and downstream Jκ genes since the RSS are compatible even after the primary recombination (Nemazee, 2000; Nemazee and Weigert, 2000).

5.4 Somatic hypermutation – stepwise mutations

Activation of a mature B cell by a T cell dependent antigen initiates molecular and cellular processes that further diversify the antibody sequence. These processes results in affinity maturation of the antibody (Dörner et al., 1998b; Tarlinton and Smith, 2000) and occur in germinal centres, which develops in secondary lymphoid organs subsequent to antigen stimulation (Berek et al., 1991). The somatic hypermutation (SHM) process specifically targets sequences in, or nearby, the variable region (Winter and Gearhart, 1998). Although the mechanism controlling this is not fully elucidated, it appears as though both cis and trans acting sequences are involved in this process (Papavasiliou and Schatz, 2002b). SHM is initiated by a DNA single and/or double strand break followed by error prone reparation of the gene (Martin and Scharff, 2002a; Papavasiliou and Schatz, 2002b). This process depends, albeit only loosely, on the sequence, as specific hot spot motifs seem to exist (Rogozin and Kolchanov, 1992).

The SHM process may result in amino acid substitutions as well as insertions and deletions (de Wildt et al., 1999b; Goossens et al., 1998; Lantto and Ohlin, 2002a; Ohlin and Borrebaeck, 1998; Wilson et al., 1998). It has recently been

shown that the somatic hypermutation process depends on an activation-induced cytidine deaminase (AID), a crucial step towards understanding somatic hypermutation (Arakawa et al., 2002; Bross et al., 2002; Faili et al., 2002; Martin and Scharff, 2002b; Muramatsu et al., 1999; Nagaoka et al., 2002; Papavasiliou and Schatz, 2002a; Petersen-Mahrt et al., 2002; Rada et al., 2002; Yoshikawa et al., 2002). AID is, furthermore, critical for other diversification processes, such as class-switching (Muramatsu et al., 2000; Okazaki et al., 2002) and gene conversion (Arakawa et al., 2002; Harris et al., 2002).

5.4 Somatic hypermutation – Receptor revision

Although still controversial, an additional diversification process, named receptor revision, has been suggested to occur in germinal centres (in man and mouse) leading to further diversification of the evolving antibody. In contrast to receptor editing, which is tolerance driven and takes place in the bone marrow (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993), receptor revision is suggested to be a part of the somatic mutation process and contribute to the affinity maturation process (Fink and McMahan, 2000; Nemazee and Weigert, 2000). Initial evidence for secondary recombinations in the GCs came from reports showing that RAG1 and RAG2 are upregulated in a subset of germinal centre B cells (Han et al., 1997; Han et al., 1996; Hikida et al., 1997; Hikida et al., 1998). It has, furthermore, been shown that both the light chain (Brard et al., 1999; de Wildt et al., 1999a) and the heavy chain (Itoh et al., 2000; Wilson et al., 2000) can be rearranged during this process.

During secondary rearrangements of the light chain, a V segment can directly recombine with a new J segment due to the asymmetry of the RSS of the V and the J segment (Bassing et al., 2002). In contrast, the primary VH recombination results in the deletion of all remaining D segments and subsequent secondary rearrangements cannot utilise the classical recombination signal sequences (Nemazee 2000). Instead those secondary recombinations rely on cryptic RSS, mainly embedded in the framework region 3 (FR3) (Chen et al., 1995). Secondary rearrangements of the heavy chain also differs from light chain rearrangements, in that parts of the original gene is retained, in particular CDR3,

which often is the key sequence element that determine antigen specificity (Xu and Davis, 2000).

It has not been clarified to what extent receptor revision shapes the antibody repertoire during the affinity maturation process. Some reports have questioned the relevance of the V(D)J recombination in GCs, since cells that up-regulate RAG1 and 2 display a immature phenotypical profile (Hikida et al., 1997; Yu et al., 1999). However, even if receptor revision is a rare event (Goossens et al., 2001) there is today convincing evidence that it indeed occurs in the periphery (Bellan et al., 2002; de Wildt et al., 1999a; Hikida et al., 1998; Itoh et al., 2000; Wilson et al., 2000). It is, however, difficult to determine the frequency of receptor revision events, since they are troublesome to detect in many instances (Wilson et al., 2000). Furthermore, the high probability of a non-productive rearrangement, resulting in the deletion of the B cell clone, may result in that most rearranged clones are not found.

5.5 Anti-CD40 antibodies as a tool to study affinity maturation events

In PAPER III, we used CD40 antibodies as a tool to investigate affinity maturation events *in vitro*. A scFv phage display library was created based on four of the previous selected anti-CD40 antibodies (type A antibodies, PAPER II), using a CDR shuffling approach (Jirholt et al., 1998; Söderlind et al., 1999). CDRH1 and 2 and CDRL1-3 was allowed to vary, mainly by introducing sequences from the n-CoDeR library (Söderlind et al., 2000), which allows for introduction of point mutations, insertions and deletions, as well as larger sequence modifications that resemble receptor revision events. Thereby, all components of the variability that is created *in vivo,* during diversity driven evolution of antibodies, were used to create the library. As in most cases of heavy chain revision *in vivo*, the CDRH3 sequence was retained in the library, which may result in maintained epitope specificity, whereas other parts of the VH and VL genes were allowed to vary. We found that after competitive selection for CD40, using a method that mimic the affinity driven selection process in GC (Hawkins et al., 1992), there had been a selective advantage for a distinct shift in CDRH2. This shift in sequence, which has a distinct similarity to

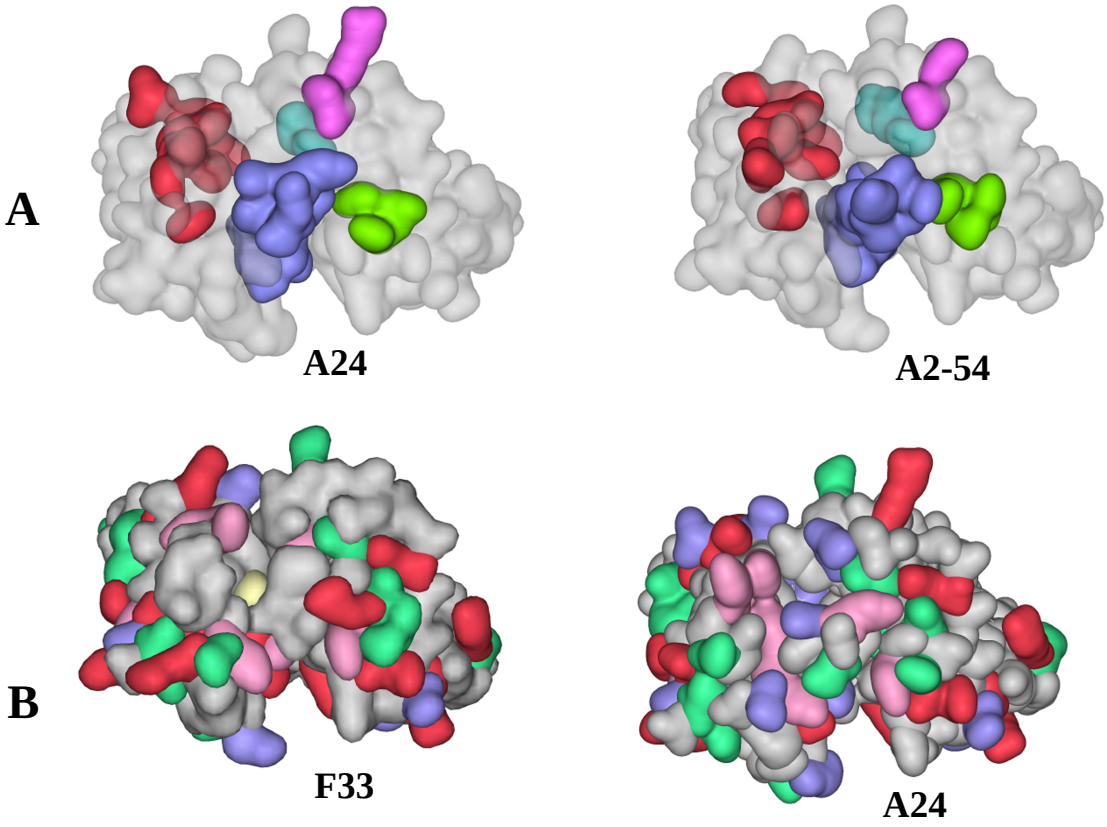

Figure 2. Structure models of anti-CD40 scFv. Figure A) shows a structure model of one of the original anti-CD40 scFv, A24, and one of the evolved clones A2-54 (WAM model using dead end elimination side chain building method and VFF screening method, http://antibody.bath.ac.uk/index.html). The CDRH3 sequence, which is identical in both of the scFv, is depicted in blue. Beside CDRH3, all other residues that are identical are illustrated with a transparent surface, coloured grey. The residues that are illustrated in red show the differences in CDRH2, whereas differences in CDRL1 are shown in purple, CDL2 in green and CDRL3 in green blue. This picture clearly shows that the majority of the differences in the evolved clones are due to the distinct shift in CDRH2 sequence.

In figure B), the antigen binding site of the scFv that almost completely blocks the CD40-CD154 interaction, F33 (WAM model using dead end elimination side chain building method and accessibility profile screening method), is compared with A24. Basic residues (Lysine and Arginine) are depicted in red, whereas acidic residues (glutamate and aspartate) are depicted in blue. Histidines are illustrated in yellow, tyrosine residues in pink and glutaimine and aspargine in green. Remaining residues are depicted in grey. As shown in the model, F33 contains no acidic residues in the central antigen binding site (compare with A24). It may be suggested that this further imply that F33 utilise similar residues as CD154 for binding to CD40.

receptor revision of VH *in vivo,* was mainly to a sequence type that originates from a germline allele that is more infrequent in the unselected library.

Receptor revision give rise to a relatively large modification of the antibody gene, which may result in development of self-reactive specificities. Indeed, autoreactivity has been reported to be associated with this process (Brard et al., 1999; Dörner et al., 1998a; Itoh et al., 2000; Klonowski et al., 1999). However, receptor revision may also provide evolving B cells with properties that are sufficiently valuable to compensate for the increased risk of developing autoreactive receptors. In fact George and Gray have suggested that receptor revision may provide a mean to escape from local affinity optima, which would be impossible to accomplish by stepwise mutations (George and Gray, 1999). In PAPER III we have shown that receptor revision like events, where a large segment of the V gene is exchanged in the selected repertoire, indeed may significantly alter the association and dissociation rate constant profiles of evolving antibodies. The antibody fragment sequences obtained, after the selections from the shuffled library that was used for this study, is very unlikely to be obtained using other *in vitro* protocols, such as repeated rounds of error-prone PCR. The latter type of process generates, and accumulates, point mutations, which most likely would have given rise to low affinity intermediates, which would be lost during the selection process.

In conclusion, our data shows that receptor revision-like events may confer selective advantage to an evolving antibody, thereby allowing it to bridge gaps in sequence space that would be difficult to cross by stepwise mutations. Our data reinforce the suggestion that receptor revision provides the immune system with a valuable complement to point mutations and insertions and deletions for generation of diversity.

6 GENERATION OF HUMAN ANTIBODIES FOR THERAPEUTIC APPLICATIONS

Human antibodies carry several molecular characteristics that make them highly suitable for therapeutic applications (Borrebaeck and Carlsson, 2001; de Kruif et al., 1996; Glennie and Johnson, 2000). Highly specific antibodies with high affinity can easily be made towards almost any molecular structure, which makes targeting of widely different pathogens such as tumour cells, snake toxins and viruses possible. Furthermore, naked antibodies, i.e. unconjugated antibodies, contain an intrinsic effector domain that can recruit natural effector mechanisms, like ADCC via the Fc part (Clynes et al., 2000). Moreover, the bivalent nature of the antibody molecule (IgG) may result in direct killing of tumour cells by receptor cross-linking (Shan et al., 1998). The Fc part also confers a prolonged serum half-life to the antibody molecule (Raghavan and Bjorkman, 1996; Shan et al., 1998).

6.1 Human monoclonal antibodies

The advent of monoclonal antibody technology (Köhler and Milstein, 1975) resulted in the first clinical trial of a monoclonal antibody (mAb) for cancer therapy in 1979 (Nadler et al., 1980). However, it was not until 1986 that the first mAb (OKT3) was approved by the Food and Drug Administration (FDA) in USA. Most of the problems associated with the earlier attempts to use mouse mAbs in therapy was due to the human anti-mouse antibody (HAMA) response that most patients developed, which made repetitive treatment difficult (Khazaeli et al., 1994). Furthermore, these antibodies displayed a short biological half-life and low ability to recruit effector functions (Borrebaeck et al., 1993). Attempts have been made to circumvent this by making chimeric antibodies, i.e. exchanging part of the mouse antibody with human sequences (Morrison et al., 1984; Wu et al., 1999). However, it was the introduction of phage display of large human antibody libraries that made production of fully human antibodies feasible (de Haard et al., 1999; Huls et al., 1999; Knappik et

al., 2000; Sheets et al., 1998; Söderlind et al., 2000; Vaughan et al., 1996). This technology development has resulted in several human antibodies in different stages of clinical trials, and is likely to have a huge impact on future development of both analytical tools and therapeutic candidates.

In the studies described in PAPER II and III we have used a human antibody library (n-CoDeR) (Söderlind et al., 2000) to select and evolve antibodies against CD40. This large, non-immunised, fully human scFv library provides several advantages when developing novel therapeutics (Carlsson and Söderlind, 2001). n-CoDeR has been constructed by insertion of CDRs, obtained by PCR amplification from human peripheral B cells, into a single human VH-VL scaffold (IGHV3-23*01 and IGLV1-47*01). Thereby, all sequences in the library have been proof-read, implicating that all the individual sequences have been evolved and developed in the context of the human immune system prior to assembly. However, the assembly of the fragments into a whole scFv may create new T cells epitopes, although probably not to any greater extent. In fact, it has been shown that clones from the n-CoDeR library display even fewer potential T cell epitopes than normal human variable genes of IgG type, when analysed by peptide threading (Söderlind et al., 2000).

The recombination of CDRs from different germline alleles, which the n-CoDeR concept depends on, allowed us to select a varied panel of scFv with fully human sequences against human CD40 (PAPER II). This further indicates that the CDR shuffling approach generates totally novel specificities, since it is very unlikely that any of the sequences in the FR or CDRs of these scFv have been part of an anti-CD40 binding antibody *in vivo*.

When considering utilisation of binders obtained from scFv libraries in a therapeutic situation, the scFv format might actually have an advantage over the whole antibody format. The smaller size of the scFv may lead to more efficient tissue and tumour penetration, and it may be easier and cheaper to produce (Adams and Schier, 1999), although a major problem is the very short half-lifes of the small fragments. In most instances, however, the effector functions of the Fc part as well as the bivalency of a whole IgG molecule are of clinical advantage (Borrebaeck and Carlsson, 2001; Winter et al., 1994). Therefore, we have converted and produced some of the anti-CD40 antibodies described in

PAPER II and III (A24, B44, F33 and A2-54) into an IgG format. Although this molecular format is commonly used for therapeutic antibodies, it may be advantageous to engineer the Fc part, e.g. by altering the affinity profile for the different Fc receptors (Clynes et al., 2000). Alternatively, in the case of stimulatory CD40 antibodies, it may be advantageous to increase the valency further, using other isotypes (Boel, 2000) or different recombinant techniques (Chapman et al., 1999; Plückthun and Pack, 1997) to increase the stimulatory capacity (Haswell et al., 2001).

6.2 Antibodies binding to CD40

The mouse anti-CD40 antibody, mAb 5D12, that is profiled in PAPER I have shown promising results in pre-clinical models of multiple sclerosis (Boon et al., 2001; Laman et al., 2002). However, this antibody is of mouse origin, which hamper its therapeutic efficiency in humans as outlined above. The investigation in PAPER I and (Barr and Heath, 2001; Challa et al., 1999; Pound et al., 1999), suggested that the specific epitope, to which an antibody is directed, may influence the function of anti-CD40 antibodies. Thus, we attempted to enrich for human anti-CD40 clones by performing an, additional, competitively elution (Meulemans et al., 1994), using mAb 5D12 to enrich for anti-CD40 clones with similar binding pattern as 5D12, and thereby similar characteristics. To elute such binders, 5D12 mAb was added to phages bound to CD40 antigen in a large molar excess, during the fourth round of selection.

The sequence of the scFv clones obtained from the competitive elution that bound strongly to cell surface displayed CD40, was either of type A or identical with clone B44 (Table 1). Although, these scFv clones also were frequently found, when using the standard, trypsin-based, elution, both clone B44 and the type A antibodies were approximately two times more frequent, using competitive elution. When analysing these scFv in a blocking experiment it was found that 5D12 almost completely block B44 binding to CD40 expressing cells, as expected. Interestingly, the 5D12 mAb did not block the type A clones at all, which is highly unexpected. Furthermore, the domain-mapping analysis (PAPER I and II) shows that clone B44 and 5D12 binds to the same, deleted, CD40 variant, whereas the type A antibodies only bind to wild type CD40

(unpublished data). This further reinforces the conclusions drawn in PAPER I that the outcome of blocking experiments, not only depends on the exact location of the epitopes. There are alternative approaches to determine epitope location, e.g. alanine scanning mutagenesis. However, this method may also mapp the epitope to residues that are not involved in binding, since alanine residue substitutions may affect the structure of a protein both locally and globally (Greenspan and Di Cera, 1999).

The anti-CD40 antibodies described in PAPER II recognise at least three different epitopes on CD40, as determined by domain mapping. Comparison with the domain mapping results presented in PAPER I, revealed at least one new specificity, not shared by any of the mouse antibodies characterised in PAPER I. This epitope must be located near the distal end, since even the addition of a short peptide tag (13 amino acids) abrogates the binding. From the domain mapping analysis, this epitope seems to be shared between clone D13 and the type A clones.

Interestingly, the blocking analysis, using 5D12 mAb, indicates that the type A antibodies detect a unique epitope, since 5D12 blocks binding of D13 to CD40 rather well, in contrast to the type A clones (see above). Thus their binding patterns differ despite that they bind to the same CD40 variant as D13, in the domain mapping analysis. This may indicate that the low activating character of the type A antibodies actually are a result of a specific binding epitope.

The surface of the accessible part of the extracellular domains of CD40 can be estimated to 8000-9300 $Å^2$ (based on SwissPdbViewer calculation for the three distal domains). This can be compared to the buried surface area of a protein-antibody interaction that is about 600-900 $Å^2$ (Braden and Poljak, 1995; Davies and Cohen, 1996). Hence the CD40 molecule theoretically could harbour approximately 10 non-overlapping antibody binding sites. However, sterical hindrance, due to the large size of the antigen-binding domain of the antibody, probably limits the possible number of binding sites. Still it is remarkable that almost all of the mouse antibodies investigated in PAPER I affect each other's binding. This may depend on a low number of accessible epitopes on the CD40-antigen used for the generation of antibodies. It may also depend on restrictions

in the murine germline genes that favours a response against a limited numbers of epitopes, similar to what Lantto et al has suggested (2002), for antibodies that recognise antigenic domain 2 on glycoprotein B of cytomegalovirus.

6.3 Antibody evolution and affinity maturation *in vitro*

From a clinical point of view, it is generally advantageous that a therapeutic antibody exhibits a high affinity to the target antigen (Adams et al., 1998a; Adams et al., 1998b; McCall et al., 2001; Roovers et al., 1998), although extremely high affinity can impair tumour penetration (Adams et al., 2001). It has been shown that affinity maturation *in vivo* reaches an affinity plateau. This is mainly due to restrictions imposed by the selection mechanisms in the germinal centre reaction, mainly by the requirement to internalise the BCR/antigen complex, since antibody-antigen interaction with a half-life much longer than the time necessary for uptake do not have any competitive advantage. (Batista and Neuberger, 1998; Foote and Eisen, 1995). *In vitro* affinity maturation techniques does, however, not suffer from these restrictions (Foote and Eisen, 2000). Today, *in vitro* affinity maturation techniques can break the *in vivo* affinity ceiling and a number of different technologies have been developed to accomplish this, e.g. phage display (Hawkins et al., 1992; Parmley and Smith, 1988), ribosomal display (Hanes et al., 2000; Schaffitzel et al., 1999), bacterial display (Daugherty et al., 1998) and yeast display (Boder et al., 2000).

There are several routes to diversify an antibody gene in order to create improved antibodies prior to selection, using any of the methods described above. Directed point mutations, error prone PCR (Leung et al., 1989) and chain shuffling (Stemmer, 1994) acts randomly on the entire sequence, whereas CDR shuffling (Jirholt et al., 1998) allows for targeting of mutations, insertions and deletions, as well as larger modifications, to the CDRs. The latter method may provide some advantages over the random approaches, since it has been shown that the CDRs tolerate larger modifications than the framework (Dörner et al., 1998a). However, affinity maturation studies where error prone PCR has been used to create variability have revealed that several of the clones with approved affinity contained mutations outside the CDRs (Daugherty et al., 2000; Hawkins

et al., 1993). Using CDR shuffling, these mutation would not have been found. It is, however, indeed possible to combine the different methods, though the addition of randomly introduced mutations to the diversification process will increase the probability of introducing new T cell epitopes. The novel combinations of germline gene CDRs in the n-CoDeR library may, furthermore, also have influenced the high affinity of the obtained binders (Borrebaeck and Ohlin, 2002).

It has been suggested that the average affinity of clones selected from a molecular library correlates with the number of independent clones in the library (Griffiths et al., 1994). Analysis of the anti-CD40 scFv clones that were selected from the n-CoDeR library (PAPER II) confirms this notion, since several of them display high affinities (nanomolar range).

In addition to the insights into the processes that governs antibody evolution, the study described in PAPER III resulted in affinity maturation of the type A, anti-CD40 antibodies. In fact, the CDR shuffling approach that was used (PAPER III) is an efficient route to generate antibodies with higher affinity (Jirholt et al., 2002). The selection method we applied resembles the affinity selection method described by Hawkins et al. (1992), which is designed to select for improved overall affinity. However, the washing step in this scheme, which is a necessary step of the method, confers a competitive advantage for clones with slow dissociation rate constant. Despite this, the clones selected in PAPER III displayed a markedly enhanced association rate compared to the original scFv clones (8-36 times). The overall affinity improvement was however only approximately two-fold between scFv clone A24 and A2-54, due to an increased dissociation rate of the evolved clones. It will be interesting to investigate if these evolved scFv clones display similar ability to induce CD40 signalling, or if the shift in dissociation rate and association rate also effect this process.

Manivel et al. (2000) has suggested that the qualitative differences between antibodies from primary and the secondary response are mainly restricted to the association step. The increased association rate in the secondary response is due to stabilisation of the paratope and is accomplished by favourable entropy changes. It is possible that a similar conformational stabilisation of the paratope account for the increased association rate in our secondary repertoire (PAPER

III), since many of the mutations in the secondary repertoire are located outside of the central core of the putative binding site (Figure 2) (Tomlinson et al., 1996). The structure model presented in Figure 2 also indicates that these changes have an effect on the conformation of CDRH3.

7 IDENTIFICATION OF PROTEIN INTERACTIONS

The completion of the HUGO project and other large-scale DNA sequencing project has made many complete genome sequences available. Although this accomplishment by it self is extremely remarkable, it's just a first step towards understanding of the cellular and intracellular processes that all living organisms depend on. The next step toward this goal is to determine the biological function of the gene products, i.e. the proteins. This has resulted in the new emerging field of Proteomics, the large-scale analysis of proteins (Auerbach et al., 2002; Pandey and Mann, 2000). As the function of a protein in many cases depends on its interactions with other molecules, analysis of protein interactions is highly relevant. However, the large size of different proteoms (10^5-10^6) has created a need for novel high through put methods for screening of proteins. These novel methods for screening of protein interactions may also have further applications for e.g. cancer target identification.

There are several different approaches for protein-protein identification. One alternative is to use methods where interacting molecules are purified and the protein sequence is analysed using mass spectroscopy. Another possibility is to use methods based on enrichments of interacting pairs from library systems, where the target proteins is physically linked to the gene that encodes it, e.g. phage display and yeast two hybrid system.

7.1 Mass spectroscopy based identification of proteins

The first step in mass spectroscopy based analysis of protein-protein interactions is purification of the target proteins. This can be achieved by separation and detection on two-dimensional gels, or by immunoprecipitation, or variants of the latter (Figeys et al., 2001). Immunoprecipitation can be accomplished in several different ways using both scFv and IgG antibody format as bait (Kaelin et al., 1991; Schneider et al., 1982). It may, however, be difficult to obtain pure samples of proteins that are expressed in low amounts, are poorly soluble or

binds with low affinity. An alternative high-thoughput and promising approach to obtain proteins for subsequent analyses by mass spectroscopy is to use protein chips (Borrebaeck et al., 2001; Steinhauer et al., 2002).

7.2 Library selection approaches

Library screening systems based on surface display on a filamentous bacteriophage that infect *Escherichia coli (E. coli)*, phage display (reviewed by Hill and Stockley, 1996) have had a huge impact on the field of life science during the last decades. The success of the phage display system is mainly due to the nature of the expression system, which ensures a physical link between the displayed protein and the corresponding gene. This allows for efficient selection, amplification and, in the end, retrieval of a selected entity. However, the phage display system is restricted by a number of intrinsic features (Rhyner et al., 2002). All displayed proteins must cross the bacterial inner membrane without disrupting it, which limits the potential size of the molecule, and confers problems with proteins that contains hydrophobic patches. Furthermore, proteins expressed in E. coli lack post-translational modifications and several protein types do not fold properly. Moreover, the most efficient phage display systems are based on a fusion with the g3p coat protein, which require aminoterminal cloning that make translation of full length genes impossible. This problem, which may limit the functional size of the library, can be circumvented, by using the c-jun/c-fos zipper interaction to provide a covalent linkage between C-terminal cloned cDNA and g3p (Crameri and Suter, 1993). Alternatively, the cDNA library may be displayed on gp6, which allows c-terminal cloning (Hufton et al., 1999). There have also been other attempts to directly link protein interactions with infectivety, e.g. SAP (Dueñas and Borrebaeck, 1994) and SIP (Krebber et al., 1995), however these methods do not seem to be generally applicable, and are best suited for high affinity interactions (Nilsson et al., 2002). Furthermore, de Wildt et al. (2002) has recently described a novel selection system for library versus library sceening, named SAC (selection by avidity capture). In this system, a library of receptors are fused to the g3p and co-expressed with a library of ligands in *E. coli*. Phages that are produced in clones, where the ligand can interact with the receptor, are selectively

immunoprecipitated and arrayed. The gene that encodes the receptor and the ligand are selectively retrieved by growth on medium containing double antibiotic resistance (one for the gene that encodes the receptor and one for the gene that encodes the ligand).

Two-hybrid systems provide another route to library selections. Fields and Song (1989) developed the original yeast two hybrid system (Y2H), using the fact that several transcription factors can be subdivided into two distinct domains. In the Y2H-system, a "bait" molecule is fused to the DNA binding part, whereas a "prey" molecule is fused to the activation domain of the transcription factor. In cells in which the bait binds to the prey, the function of the transcription factor is restored, and by coupling the reconstitution of the transcription factor to a reporter gene, interacting pairs can be selected. A disadvantage with the Y2H is that it generates several false positives, mainly due to intrinsic transcriptional activity. Also, the posttransciptional machinery in yeast differs from mammalian cells (Wallach et al., 1998), which may hamper functional expression of some proteins. Furthermore, the yeast two-hybrid system is best suited for proteins that are expressed intracellularly and proteins that e.g. contain hydrophobic transmembrane domains cannot be translocated into the nucleus.

There are several alternative two hybrid system, which utilise reconstitution of a protein that confers some kind of selectable phenotype, e.g. RRS (Ras recruitment system) (Broder et al., 1998). RRS depends on relocalisation of Ras to the yeast surface by protein interaction between a membrane anchored bait and a prey fused to mammalian Ras, which induce cell growth at 36°C. This system has also been reversed to allow for membrane proteins to be used as baits (Hubsman et al., 2001). The G protein based screening system use a similar strategy aimed at membrane proteins (Ehrhard et al., 2000). However, the most widely used alternative is the split-ubiquitin system (Johnsson and Varshavsky, 1994). Here the bait and prey-molecule are fused to one domain each of ubiquitin, where an introduced mutation has abolished the natural binding between the halves. An interaction between the bait and the prey restore the ubiquitin structure, which then is recognised by a protease that release a reporter protein from one of the halves. These protein-fragment complementation assays

(PCAs) has been further developed by the group of Michnick (Pelletier et al., 1998). Their system has been designed for library versus library selections (Pelletier et al., 1999) and has also been adapted for identification of protein interactions in mammalian cells (Remy and Michnick, 1999; Galarneau et al., 2002). However, PCA are best suited for analysis of proteins that express well in the cytosol.

7.3 A novel selection technology based on the CD40 receptor - Selection of Protein Interactions by Receptor Engagement (SPIRE).

In PAPER V, we have described a novel selection system in which molecular libraries displayed on the surface of a mammalian cell line are enriched, using Selection of Protein Interaction by Receptor Activation (SPIRE). SPIRE aims at solving several of the problems discussed in the previous section, i. e. folding, post-translational modifications et c. In this system a library of "mock-receptors" (preys) are fused to the transmembrane and cytoplasmic domains of CD40 (CD40TC). These constructs are transfected into WEHI-231 cells, a naïve murine B cell line that undergoes apoptosis when treated with anti-IgM antibodies. If the prey molecule, displayed on cells from this library, can interact with a bait molecule they are rescued and allowed to proliferate. In PAPER V, we have applied SPIRE on two model libraries and one cDNA library. Therein, we clearly show that SPIRE can be used to enrich clones that express a target molecule.

Subtractive selection of tumour specific antibodies, using phage display libraries is an attractive method to develop novel candidates for tumour therapy (de Kruif et al., 1995; Huls et al., 1999; Marks et al., 1993). By making subtractive pre-selections against normal cells, followed by selection against the target tumour, a panel of specific antibodies can be relatively easily achieved. It is furthermore possible to select for tumour epitopes that mediates internalisation, which may make them suitable for targeted gene delivery (Larocca et al., 1998; Poul et al., 2000; Poul and Marks, 1999). To further validate the potential tumour candidates it is necessary to identify the tumour epitope, even though this may be difficult and time consuming (Roovers et al., 2001). SPIRE has the potential to provide a high-thoughput solution to this task.

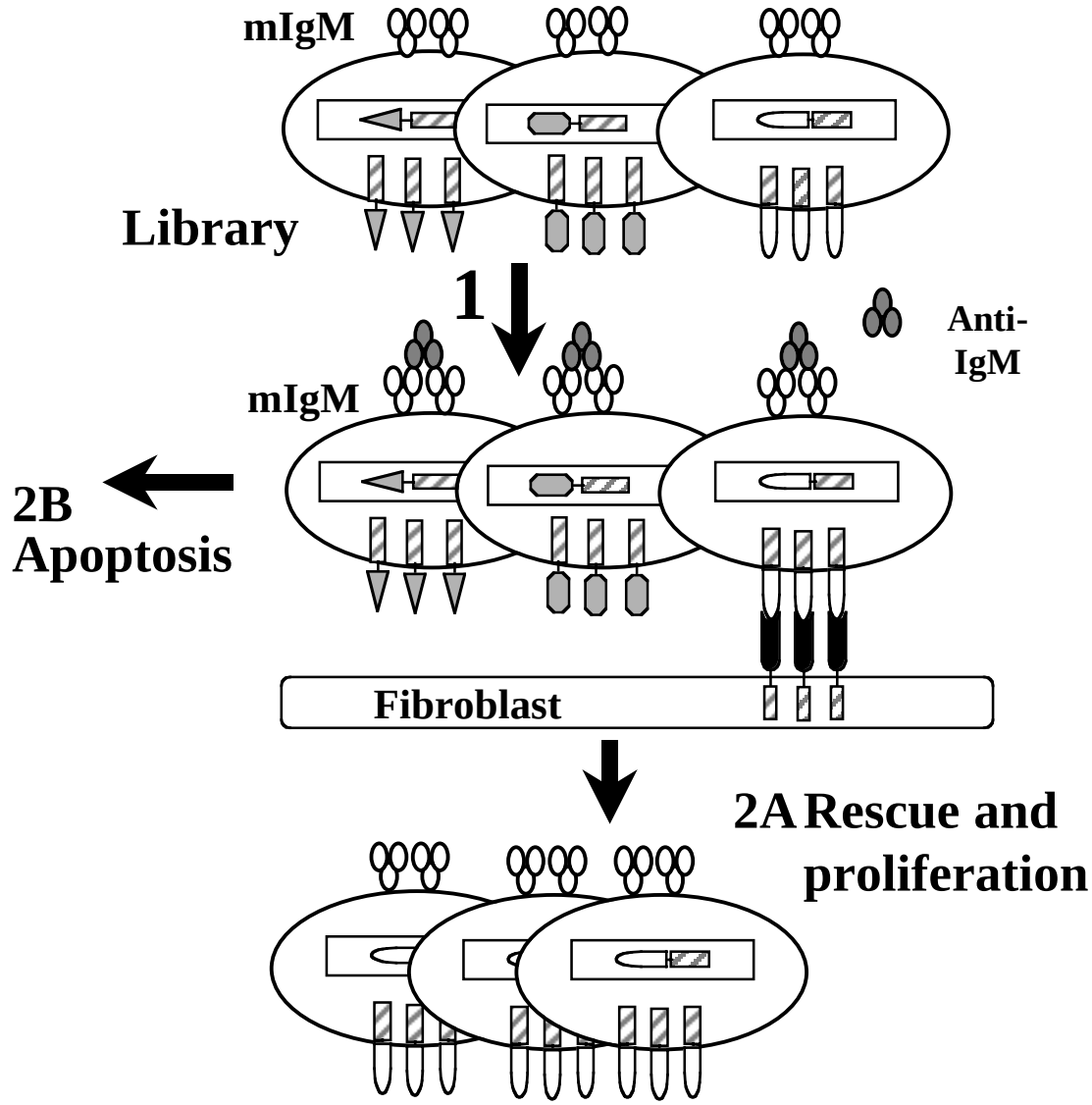

Figure 3 Selection of Protein Interactions by Receptor Engagement (SPIRE). A molecular library ( ) is fused to the transmembrane and cytoplasmic domains of CD40 (CD40TC), depicted as a hatched box. Stable transfected of the library (boxed figure) is made into WEHI 231 , which express membrane bound IgM on the surface (mIgM), depicted as a schematic, unfilled, antibody.

Step 1. Anti-IgM antibody (grey), which induce programmed cell death, apoptosis, in the cells, is added to the library. The cells are cultured on irradiated fibroblasts (3T3), which express a bait molecule (depicted as black figures on the fibroblast).

Step 2A. Cells expressing a mock-receptor (prey) that can interact with the bait molecule (mock-ligand) are rescued from apoptosis and start to proliferate. Rescued cells are expanded and analysed and subjected to further rounds of selections. Remaining cells (**Step 2B**), which do not receive a rescue signal through the CD40 fusion protein will eventually die.

SPIRE is designed to allow for utilisation of scFv as bait molecules and allows for rapid conversion of antibody fragments obtained from phage display libraries to a format suitable for mammalian selection. Beside the membrane displayed bait approach described in PAPER V, it may also be possible to use whole antibodies to stimulate specific clones. We have previously successfully used antibodies to rescue WEHI-231 cells that express truncated CD40 variants from apoptosis (PAPER IV), which indicates that this format also can be used for selection. In addition, preliminary data indicates that it also is possible to use soluble scFv, in combination with a secondary cross-linking antibody to induce proliferation in transfected WEHI-231 cells (unpublished data).

The applicability of the approach to use 3T3 fibroblasts transfected with CD40 fusion proteins to stimulate proliferation of transfected WEHI-231 cells was also confirmed with an, additional, antibody fragment fused to CD40TC and displayed on 3T3 cells. This construct contains a scFv that recognise human CD40 (B44) (Table I) fused to the transmembrane and cytosolic part of CD40. These 3T3 cells were successfully used to stimulate transgenic WEHI-231 cells that express human CD40 and also to induce proliferation in human peripheral B-lymphocytes (unpublished data).

7.4 Further development of SPIRE

SPIRE has in its current status, an additional application beside to screen human cDNA libraries for clones that express a target antigen as outlined in PAPER V. An alternative application is to use the method for molecular evolution of complex proteins of eukaryot origin. It may provide a valuable alternative to other selection technologies, when working with proteins that only fold properly in mammalian cells, or require specific mammalian post translational modifications to function correctly.

In PAPER V we show that the interacting pair, AE11-CD40TC and D2/B1-AD2, fused to the transmembrane and intracellular part of CD40 can function either as a ligand when transfected in 3T3 cells or as a mock receptor when transfected into WEHI-231. This indicates that these CD40 variants can transmit signals simultaneously into the 3T3 cells and into the WEHI-231 cells. This may be utilised in a library versus library selection approach.

The problem with low functionality of cDNA libraries, described in PAPER V, could be solved by pre-selection of the library, using a flow-cytometry based approach, prior to SPIRE selections as outlined in PAPER V. Alternatively, it may be possible to enrich for open reading frames, using bacterial systems (Davis and Benzer, 1997) and thereby utilise the high transformation capacity of *E. coli*, prior to transfection into WEHI-231 cells. This group fused an antibiotic resistance gene to the C-terminal of the library construct. Thereby, only clones with a full open reading frame produce a functional resistance marker, and these can be selected by culturing them on the appropriate antibiotics. That method could be further developed by inserting a gene that encodes a protease cleavage site between the cDNA and the reporter gene. Thereby, the protein that confers antibiotic resistance will be cleaved of from the cDNA-encoded protein, which may reduce problems associated with biased enrichment of proteins that are prone to form inclusion bodies. A similar enrichment system has also been described in yeast (Holz et al., 2001).

Another approach to obtain highly functional libraries has been invented by, Jacobs et al., 1997 and Klein et al., 1996, who has devised a strategy to enrich for secreted proteins. By using a library vector without a built in leader sequence, only cDNA clones from exported proteins will be secreted. Selection of secreted clones was made by fusing the cDNA product to a protein that needs to be secreted to allow cell survival in a certain environment. It may be possible to use SPIRE in a similar approach for identification of exported proteins.

CONCLUDING REMARKS

CD40 is a cell surface receptor of pivotal importance that is expressed on several of the cells in the immune system. It is critical for many important events, such as T cell dependent B cell activation, isotype switching, somatic mutation and generation of B cell memory. The central role of CD40 in the immune system makes it an ideal target for antibody based immunotherapy. This led us to characterise a panel of monoclonal anti-CD40 antibodies. In PAPER I, we investigated their cellular activation potential and analysed to what extent this correlates with their affinity, epitope specificity and domain recognition profile. The antibody profiles we obtained in this first study may be valuable for understanding of the mechanisms that influence the therapeutic capacity of these antibodies. In fact, one of the antibodies that we investigated is currently in phase I/II trials.

However, all of the antibodies that we characterised in PAPER I are of mouse origin, which probably limits their clinical efficiency, due to the human anti-mouse response that most patients develop against such antibodies. Therefore we selected a set of human anti-CD40 antibodies, which are described in PAPER II, from a recombinant antibody gene library. These antibodies display a wide variety of distinct properties, which may make them a valuable source when evaluating therapeutic candidates for *in vivo* trials.

In PAPER III, we have used some of the anti-CD40 antibodies described in PAPER II to create an antibody library that was utilised to investigate antibody evolution *in vitro*. The results from this study showed that events, which resembles receptor revision, i.e. secondary rearrangements of antibody genes in the periphery, may provide an evolving antibody with competitive advantages during a selection process that is similar to the affinity maturation process *in vivo*. Our data reinforce the suggestion that receptor revision is an important complement to point mutations and insertions and deletions in the somatic hypermutation process that occur in germinal centres.

It has been suggested that members of the TNFR family pre-associate in the membrane via one of the extracellular domains, the pre-ligand assembly domain (PLAD). Therefore, in PAPER IV, we investigated the functional role of the

different domains of CD40, in a B cell model system. The results from this study showed that neither of the extracellular domains is essential for signal transduction and, furthermore, implies that conformational changes play no critical role for the CD40 signalling pathway.

Based on the findings that all of the extracellular part of CD40 can be replaced with retained signalling capacity, we developed a novel selection method, named Selection of Protein Interactions by Receptor Engagement (SPIRE). In PAPER V, we demonstrated that this selection system can be used for clonal enrichment of cells that display a mock-CD40 receptor, used as prey, on the surface by interaction with a certain bait protein. Thus, SPIRE allows for clonal selection of interacting protein pairs in a mammalian environment. SPIRE may have several different applications such as identification of tumour target molecules or for molecular evolution of complex proteins.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar arbetar konstant med att skydda kroppen från olika föremål, t. ex. bakterier, virus, gifter och cancertumörer, som kan orsaka skada eller sjukdom. För att kunna försvara kroppen mot sådana föremål så har immunförsvaret tillgång till flera, oerhört effektiva, vapen. Det viktigt att immunförsvaret känner igen, och endast angriper, skadliga föremål, så att inte dessa vapen attackerar de normala cellerna och vävnaderna i kroppen. För att immunförsvarets olika beståndsdelar skall kunna bestämma om, och hur, dom skall reagera på exempelvis ett virus, krävs det att de olika cellerna i immunsystemet kommunicerar med varandra. Denna kommunikation sker bland annat via receptorer som finns på cellernas yta. En av immunförsvarets viktigaste receptorer kallas CD40, och den återfinns på flera av de celler som ingår i vårt immunsystem. I den här avhandlingen sammanfattas ett antal studier, där CD40 använts och undersökts, dels som målmolekyl för att ta fram och studera nya antikroppar, dels som verktyg för att studera en immunologisk process, och slutligen för att utveckla en ny biomedicinsk teknologi.

I den första artikeln har vi undersökt ett antal antikroppar som binder till CD40. Anledning till att sådana antikroppar är intressanta, är att de skulle kunna användas som ett framtida läkemedel för behandling av exempelvis olika cancerformer och av multipel skleros (MS). De antikropparna vi undersökte i den första studien var ursprungligen framtagna från en mus. Detta innebär att det mänskliga immunförsvaret ”ser” dessa antikroppar som något främmande, och tror därför att de kan vara skadliga. Därmed kommer immunförsvaret att snabbt göra sig av med antikropparna, vilket gör dem olämpliga för utveckling av nya behandlingsmetoder för människor. Emellertid kunde de kunskaper om antikroppar mot CD40 som vi fick från den första studien användas i den andra studien, där vi, med hjälp av moderna biotekniska metoder, tog fram ”mänskliga” antikroppar. I och med att dessa antikroppar är ”mänskliga”, så kommer vårt immunförsvar att, med stor sannolikhet, inte se dem som något främmande, utan accepterar dem som kroppsegna antikroppar. Vidare så visade

vi i den artikeln att dessa nya antikroppar har intressanta egenskaper som kan visa sig vara värdefulla för utveckling av framtida läkemedel.

De antikroppar som vi tagit fram i den andra artikeln, användes i den tredje studien för att undersöka de processer som är involverade i antikroppsutveckling i kroppen. Vi använde oss då av en teknik som liknar den normala antikroppsutvecklingen i kroppen. De data som framkom från den studien, visar att det i vissa fall är fördelaktigt för utvecklingen av en antikropp att stora delar av den gen som beskriver hur antikroppen skall se ut byts ut mot ett annat, liknande gensegment. Våra resultat stödjer därmed den teori som föreslår att sådana förändringar av antikroppsgener är en viktig del av utvecklingen av antikroppar i vår kropp.

I den fjärde studien undersökte vi hur de delar av CD40 som finns på utsidan av cellerna påverkar dess funktion som receptor. Anledningen till at vi intresserade oss för det var att en del grupper har föreslagit att en dessa delar är viktig för kommunikationen genom CD40 in i cellen. Våra data visade att man kan ta bort i princip alla de yttre delarna av CD40 receptorn och ändå skicka signaler via den, om man bara ersätter dem med ett litet igenkänningsfragment.

I den sista artikeln beskriver vi utvecklingen av en ny biomedicinsk teknik som vi kallat för SPIRE. Förkortningen står för Selection of Protein Interactions by Receptor Engagement som kan översättas med ”Selektion av proteininteraktioner genom receptor engagemang”. I den studien utnyttjade vi resultaten från den fjärde studien som visade att de yttre delarna av CD40 kan ersättas. I SPIRE byts den yttre delen av CD40 mot ett stort ”bibliotek” av olika, okända, molekyler, som därmed kan fungera som ”låtsasreceptorer”. Från detta bibliotek kan man sedan selektera fram de celler som har en viss låtsasreceptor på ytan. SPIRE-tekniken kan komma att få ett brett användningsområde, exempelvis för att utvecklingen av nya läkemedel.

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REFERENCES

Adams, G. P. and Schier, R., 1999. Generating improved single-chain Fv molecules for tumor targeting. J Immunol Methods. 231, 249-260.

Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D. and Weiner, L. M., 1998a. Increased affinity leads to improved selective tumor delivery of single- chain Fv antibodies. Cancer Res. 58, 485-490.

Adams, G. P., Schier, R., McCall, A. M., Crawford, R. S., Wolf, E. J., Weiner, L. M. and Marks, J. D., 1998b. Prolonged in vivo tumour retention of a human diabody targeting the extracellular domain of human HER2/neu. Br J Cancer. 77, 1405-1412.

Adams, G. P., Schier, R., McCall, A. M., Simmons, H. H., Horak, E. M., Alpaugh, R. K., Marks, J. D. and Weiner, L. M., 2001. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. Cancer Res. 61, 4750-4755.

Ahonen, C., Manning, E., Erickson, L. D., O'Connor, B., Lind, E. F., Pullen, S. S., Kehry, M. R. and Noelle, R. J., 2002. The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. Nat Immunol. 3, 451-456.

Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Disteche, C. M., Simoneaux, D. K. and et al., 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science. 259, 990-993.

Alt, F. W. and Baltimore, D., 1982. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci U S A. 79, 4118-4122.

Alzari, P. M., Lascombe, M.-B. and Poljak, R. J., 1988. Three-dimesional structure ofantibodies. Annual review of Immunology. 6, 555-580.

Amsel, L. M. and Poljak, R. J., 1979. Three-dimesional structure of immunoglobulins. Annual review of Biochemistry. 48, 961-997.

Andersen, N. S., Larsen, J. K., Christiansen, J., Pedersen, L. B., Christophersen, N. S., Geisler, C. H. and Jurlander, J., 2000. Soluble CD40 ligand induces selective proliferation of lymphoma cells in primary mantle cell lymphoma cell cultures. Blood. 96, 2219-2225.

Arakawa, H., Hauschild, J. and Buerstedde, J. M., 2002. Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion. Science. 295, 1301-1306.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M. and et al., 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. Cell. 72, 291-300.

Auerbach, D., Thaminy, S., Hottiger, M. O. and Stagljar, I., 2002. The post-genomic era of interactive proteomics: Facts and perspectives. Proteomics. 2, 611-623.

Baccam, M. and Bishop, G. A., 1999. Membrane-bound CD154, but not CD40-specific antibody, mediates NF- kappaB-independent IL-6 production in B cells. Eur J Immunol. 29, 3855-3866.

Bajorath, J. and Aruffo, A., 1997. Construction and analysis of a detailed three-dimensional model of the ligand binding domain of the human B cell receptor CD40. Proteins. 27, 59-70.

Bajorath, J., Chalupny, N. J., Marken, J. S., Siadak, A. W., Skonier, J., Gordon, M., Hollenbaugh, D., Noelle, R. J., Ochs, H. D. and Aruffo, A., 1995a. Identification of residues on CD40 and its ligand which are critical for the receptor-ligand interaction. Biochemistry. 34, 1833-1844.

Bajorath, J., Marken, J. S., Chalupny, N. J., Spoon, T. L., Siadak, A. W., Gordon, M., Noelle, R. J., Hollenbaugh, D. and Aruffo, A., 1995b. Analysis of gp39/CD40 interactions using molecular models and site- directed mutagenesis. Biochemistry. 34, 9884-9892.
Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K., 2000. Immunobiology of dendritic cells. Annu Rev Immunol. 18, 767-811.
Barbas, C. F., 3rd, Kang, A. S., Lerner, R. A. and Benkovic, S. J., 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A. 88, 7978-7982.
Barr, T. A. and Heath, A. W., 2001. Functional activity of CD40 antibodies correlates to the position of binding relative to CD154. Immunology. 102, 39-43.
Bassing, C. H., Swat, W. and Alt, F. W., 2002. The mechanism and regulation of chromosomal V(D)J recombination. Cell. 109 Suppl, S45-55.
Batista, F. D. and Neuberger, M. S., 1998. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. Immunity. 8, 751-759.
Becker, T., Hartl, F. U. and Wieland, F., 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J Cell Biol. 158, 1277-1285.
Bellan, C., Lazzi, S., Zazzi, M., Lalinga, A. V., Palummo, N., Galieni, P., Marafioti, T., Tonini, T., Cinti, C., Leoncini, L., Pileri, S. A. and Tosi, P., 2002. Immunoglobulin gene rearrangement analysis in composite hodgkin disease and large B-cell lymphoma: evidence for receptor revision of immunoglobulin heavy chain variable region genes in Hodgkin-Reed- Sternberg cells? Diagn Mol Pathol. 11, 2-8.
Berberich, I., Shu, G., Siebelt, F., Woodgett, J. R., Kyriakis, J. M. and Clark, E. A., 1996. Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. Embo J. 15, 92-101.
Berek, C., Berger, A. and Apel, M., 1991. Maturation of the immune response in germinal centers. Cell. 67, 1121-1129.
Björck, P., Braesch-Andersen, S. and Paulie, S., 1994. Antibodies to distinct epitopes on the CD40 molecule co-operate in stimulation and can be used for the detection of soluble CD40. Immunology. 83, 430-437.
Blotta, M. H., Marshall, J. D., DeKruyff, R. H. and Umetsu, D. T., 1996. Cross-linking of the CD40 ligand on human CD4+ T lymphocytes generates a costimulatory signal that up-regulates IL-4 synthesis. J Immunol. 156, 3133-3140.
Boder, E. T., Midelfort, K. S. and Wittrup, K. D., 2000. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. Proc Natl Acad Sci U S A. 97, 10701-10705.
Boon, L., Brok, H. P., Bauer, J., Ortiz-Buijsse, A., Schellekens, M. M., Ramdien-Murli, S., Blezer, E., van Meurs, M., Ceuppens, J., de Boer, M., t Hart, B. A. and Laman, J. D., 2001. Prevention of experimental autoimmune encephalomyelitis in the common marmoset (Callithrix jacchus) using a chimeric antagonist monoclonal antibody against human CD40 is associated with altered B cell responses. J Immunol. 167, 2942-2949.
Boon, L., Laman, J. D., Ortiz-Buijsse, A., den Hartog, M. T., Hoffenberg, S., Liu, P., Shiau, F. and de Boer, M., 2002. Preclinical assessment of anti-CD40 Mab 5D12 in cynomolgus monkeys. Toxicology. 174, 53-65.
Borrebaeck, C. A. K. and Ohlin, M., 2002. Evolving Novel Therapeutic Antibodies in Vitro. Nature Biotechnology. in press.
Borrebaeck, C. A. K. and Carlsson, R., 2001. Human therapeutic antibodies. Curr Opin Pharmacol. 1, 404-408.
Borrebaeck, C. A.K., Ekstrom, S., Hager, A. C., Nilsson, J., Laurell, T. and Marko-Varga, G., 2001. Protein chips based on recombinant antibody fragments: a highly sensitive approach as detected by mass spectrometry. Biotechniques. 30, 1126-1130, 1132.

Borrebaeck, C. A. K., Malmborg, A. C. and Ohlin, M., 1993. Does endogenous glycosylation prevent the use of mouse monoclonal antibodies as cancer therapeutics? Immunol Today. 14, 477-479.
Bourgeois, C., Rocha, B. and Tanchot, C., 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science. 297, 2060-2063.
Braden, B. C. and Poljak, R. J., 1995. Structural features of the reactions between antibodies and protein antigens. Faseb J. 9, 9-16.
Braesch-Andersen, S., Paulie, S., Koho, H., Nika, H., Aspenstrom, P. and Perlmann, P., 1989. Biochemical characteristics and partial amino acid sequence of the receptor-like human B cell and carcinoma antigen CDw40. J Immunol. 142, 562-567.
Brard, F., Shannon, M., Prak, E. L., Litwin, S. and Weigert, M., 1999. Somatic mutation and light chain rearrangement generate autoimmunity in anti-single-stranded DNA transgenic MRL/lpr mice. J Exp Med. 190, 691-704.
Bretscher, P. and Cohn, M., 1970. A theory of self-nonself discimination. Science. 169, 1042-1049.
Broder, Y. C., Katz, S. and Aronheim, A., 1998. The ras recruitment system, a novel approach to the study of protein- protein interactions. Curr Biol. 8, 1121-1124.
Bromley, S. K., Burack, W. R., Johnson, K. G., Somersalo, K., Sims, T. N., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. and Dustin, M. L., 2001. The immunological synapse. Annu Rev Immunol. 19, 375-396.
Bross, L., Muramatsu, M., Kinoshita, K., Honjo, T. and Jacobs, H., 2002. DNA double-strand breaks: prior to but not sufficient in targeting hypermutation. J Exp Med. 195, 1187-1192.
Brown, D., 2002. Structure and function of membrane rafts. Int J Med Microbiol. 291, 433-437.
Burnet, F. M. (1959) The clonal selection theory of acquired immunuity. Cambridge: The University Press, London.
Burton, D. R., Saphire, E. O. and Parren, P. W., 2001. A model for neutralization of viruses based on antibody coating of the virion surface. Curr Top Microbiol Immunol. 260, 109-143.
Burton, D. R., Williamson, R. A. and Parren, P. W., 2000. Antibody and virus: binding and neutralization. Virology. 270, 1-3.
Carlsson, R. and Söderlind, E., 2001. n-CoDeR concept: unique types of antibodies for diagnostic use and therapy. Expert Rev Mol Diagn. 1, 102-108.
Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I. and Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. J Exp Med. 180, 1263-1272.
Challa, A., Eliopoulos, A. G., Holder, M. J., Burguete, A. S., Pound, J. D., Chamba, A., Grafton, G., Armitage, R. J., Gregory, C. D., Martinez-Valdez, H., Young, L. and Gordon, J., 2002. Population depletion activates autonomous CD154-dependent survival in biopsylike Burkitt lymphoma cells. Blood. 99, 3411-3418.
Challa, A., Pound, J. D., Armitage, R. J. and Gordon, J., 1999. Epitope-dependent synergism and antagonism between CD40 antibodies and soluble CD40 ligand for the regulation of CD23 expression and IgE synthesis in human B cells. Allergy. 54, 576-583.
Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L. and Lenardo, M. J., 2000a. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 288, 2351-2354.
Chan, K. F., Siegel, M. R. and Lenardo, J. M., 2000b. Signaling by the TNF receptor superfamily and T cell homeostasis. Immunity. 13, 419-422.

Chapman, A. P., Antoniw, P., Spitali, M., West, S., Stephens, S. and King, D. J., 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat Biotechnol. 17, 780-783.
Chen, C., Nagy, Z., Prak, E. L. and Weigert, M., 1995. Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing. Immunity. 3, 747-755.
Cheng, G. and Baltimore, D., 1996. TANK, a co-inducer with TRAF2 of TNF- and CD 40L-mediated NF-kappaB activation. Genes Dev. 10, 963-973.
Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S. and Baltimore, D., 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science. 267, 1494-1498.
Clynes, R. A., Towers, T. L., Presta, L. G. and Ravetch, J. V., 2000. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med. 6, 443-446.
Costello, R. T., Gastaut, J. A. and Olive, D., 1999. What is the real role of CD40 in cancer immunotherapy? Immunol Today. 20, 488-493.
Crameri, R. and Suter, M., 1993. Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. Gene. 137, 69-75.
Craxton, A., Shu, G., Graves, J. D., Saklatvala, J., Krebs, E. G. and Clark, E. A., 1998. p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. J Immunol. 161, 3225-3236.
Cunningham, A. J. and Lafferty, K. J., 1977. A simple, conservative explanation of the H-2 restriction of interactions between lymphocytes. Scand J Immunol. 6, 1-6.
Dadgostar, H., Zarnegar, B., Hoffmann, A., Qin, X. F., Truong, U., Rao, G., Baltimore, D. and Cheng, G., 2002. Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes. Proc Natl Acad Sci U S A. 99, 1497-1502.
Daugherty, P. S., Chen, G., Iverson, B. L. and Georgiou, G., 2000. Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. Proc Natl Acad Sci U S A. 97, 2029-2034.
Daugherty, P. S., Chen, G., Olsen, M. J., Iverson, B. L. and Georgiou, G., 1998. Antibody affinity maturation using bacterial surface display. Protein Eng. 11, 825-832.
Davies, D. R. and Cohen, G. H., 1996. Interactions of protein antigens with antibodies. Proc Natl Acad Sci U S A. 93, 7-12.
Davies, D. R., Padlan, E. A. and Sheriff, S., 1990. Antibody-antigen complexes. Annu Rev Biochem. 59, 439-473.
Davis, C. A. and Benzer, S., 1997. Generation of cDNA expression libraries enriched for in-frame sequences. Proc Natl Acad Sci U S A. 94, 2128-2132.
de Boer, M., Conroy, L., Min, H. Y. and Kwekkeboom, J., 1992. Generation of monoclonal antibodies to human lymphocyte cell surface antigens using insect cells expressing recombinant proteins. J Immunol Methods. 152, 15-23.
de Gruijl, T. D., Luykx-de Bakker, S. A., Tillman, B. W., van den Eertwegh, A. J., Buter, J., Lougheed, S. M., van der Bij, G. J., Safer AM, H. H. J., Curiel, D. T., Scheper, R. J., Pinedo, H. M. and Gerritsen, W. R., 2002. Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after in situ delivery of CD40-targeted adenoviral vectors. J Immunol. 169:5322-31, 5322-5331.
de Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P., de Bruine, A. P., Arends, J. W. and Hoogenboom, H. R., 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem. 274, 18218-18230.
de Kruif, J., Terstappen, L., Boel, E. and Logtenberg, T., 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci U S A. 92, 3938-3942.

de Kruif, J., van der Vuurst de Vries, A. R., Cilenti, L., Boel, E., van Ewijk, W. and Logtenberg, T., 1996. New perspectives on recombinant human antibodies. Immunol Today. 17, 453-455.
de Wildt, R. M., Hoet, R. M., van Venrooij, W. J., Tomlinson, I. M. and Winter, G., 1999a. Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. J Mol Biol. 285, 895-901.
de Wildt, R. M., Tomlinson, I. M., Ong, J. L. and Holliger, P., 2002. Isolation of receptor-ligand pairs by capture of long-lived multivalent interaction complexes. Proc Natl Acad Sci U S A. 99, 8530-8535.
de Wildt, R. M., van Venrooij, W. J., Winter, G., Hoet, R. M. and Tomlinson, I. M., 1999b. Somatic insertions and deletions shape the human antibody repertoire. J Mol Biol. 294, 701-710.
Diehl, L., Den Boer, A. T., van der Voort, E. I., Melief, C. J., Offringa, R. and Toes, R. E., 2000. The role of CD40 in peripheral T cell tolerance and immunity. J Mol Med. 78, 363-371.
DiSanto, J. P., Bonnefoy, J. Y., Gauchat, J. F., Fischer, A. and de Saint Basile, G., 1993. CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. Nature. 361, 541-543.
Dörner, T., Brezinschek, H. P., Foster, S. J., Brezinschek, R. I., Farner, N. L. and Lipsky, P. E., 1998a. Delineation of selective influences shaping the mutated expressed human Ig heavy chain repertoire. J Immunol. 160, 2831-2841.
Dörner, T., Foster, S. J., Farner, N. L. and Lipsky, P. E., 1998b. Somatic hypermutation of human immunoglobulin heavy chain genes: targeting of RGYW motifs on both DNA strands. Eur J Immunol. 28, 3384-3396.
Duenas, M. and Borrebaeck, C. A., 1994. Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. Biotechnology (N Y). 12, 999-1002.
Dykstra, M., Cherukuri, A. and Pierce, S. K., 2001. Rafts and synapses in the spatial organization of immune cell signaling receptors. J Leukoc Biol. 70, 699-707.
Edmundson, A. B., Guddat, L. W., Rosaur, R. A., Andersen, K. N., Shan, L. and Fan, Z.-C. (1995) Three-dimensional Aspects of IgG Structure and Function. In *The Antibodies* (Edited by Zanetti M. and Capra J. D.), Vol. 1, p. 41-100. Harwood Academic publishers GmbH, Luxemburg.
Ehrhard, K. N., Jacoby, J. J., Fu, X. Y., Jahn, R. and Dohlman, H. G., 2000. Use of G-protein fusions to monitor integral membrane protein-protein interactions in yeast. Nat Biotechnol. 18, 1075-1079.
Faili, A., Aoufouchi, S., Gueranger, Q., Zober, C., Leon, A., Bertocci, B., Weill, J. C. and Reynaud, C. A., 2002. AID-dependent somatic hypermutation occurs as a DNA single-strand event in the BL2 cell line. Nat Immunol. 3, 815-821.
Fanslow, W. C., Srinivasan, S., Paxton, R., Gibson, M. G., Spriggs, M. K. and Armitage, R. J., 1994. Structural characteristics of CD40 ligand that determine biological function. Semin Immunol. 6, 267-278.
Fields, S. and Song, O., 1989. A novel genetic system to detect protein-protein interactions. Nature. 340, 245-246.
Figeys, D., McBroom, L. D. and Moran, M. F., 2001. Mass spectrometry for the study of protein-protein interactions. Methods. 24, 230-239.
Fink, P. J. and McMahan, C. J., 2000. Lymphocytes rearrange, edit and revise their antigen receptors to be useful yet safe. Immunol Today. 21, 561-566.
Foote, J. and Eisen, H. N., 1995. Kinetic and affinity limits on antibodies produced during immune responses. Proc Natl Acad Sci U S A. 92, 1254-1256.

Foote, J. and Eisen, H. N., 2000. Breaking the affinity ceiling for antibodies and T cell receptors. Proc Natl Acad Sci U S A. 97, 10679-10681.
Foy, T. M., Shepherd, D. M., Durie, F. H., Aruffo, A., Ledbetter, J. A. and Noelle, R. J., 1993. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. J Exp Med. 178, 1567-1575.
Francisco, J. A., Donaldson, K. L., Chace, D., Siegall, C. B. and Wahl, A. F., 2000. Agonistic properties and in vivo antitumor activity of the anti-CD40 antibody SGN-14. Cancer Res. 60, 3225-3231.
Frazer, J. K. and Capra, J. D. (1999) Immunoglobulins: Structure and function. In *Fundamental Immunology* (Edited by Paul W. E.). Lippincott-Raven Publishers, Philadelphia.
French, R. R., Chan, H. T., Tutt, A. L. and Glennie, M. J., 1999. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nat Med. 5, 548-553.
Fujita, N., Kagamu, H., Yoshizawa, H., Itoh, K., Kuriyama, H., Matsumoto, N., Ishiguro, T., Tanaka, J., Suzuki, E., Hamada, H. and Gejyo, F., 2001. CD40 ligand promotes priming of fully potent antitumor CD4(+) T cells in draining lymph nodes in the presence of apoptotic tumor cells. J Immunol. 167, 5678-5688.
Galarneau, A., Primeau, M., Trudeau, L. E. and Michnick, S. W., 2002. beta-Lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions. Nat Biotechnol. 20, 619-622.
Gay, D., Saunders, T., Camper, S. and Weigert, M., 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. J Exp Med. 177, 999-1008.
Genain, C. P., Cannella, B., Hauser, S. L. and Raine, C. S., 1999. Identification of autoantibodies associated with myelin damage in multiple sclerosis. Nat Med. 5, 170-175.
George, A. J. and Gray, D., 1999. Receptor editing during affinity maturation. Immunol Today. 20, 196.
Gerritse, K., Laman, J. D., Noelle, R. J., Aruffo, A., Ledbetter, J. A., Boersma, W. J. and Claassen, E., 1996. CD40-CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. Proc Natl Acad Sci U S A. 93, 2499-2504.
Gessner, J. E., Heiken, H., Tamm, A. and Schmidt, R. E., 1998. The IgG Fc receptor family. Ann Hematol. 76, 231-248.
Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C. and Mathis, D., 1993. Mice lacking TdT: mature animals with an immature lymphocyte repertoire. Science. 261, 1175-1178.
Gilliland, L. K., Norris, N. A., Marquardt, H., Tsu, T. T., Hayden, M. S., Neubauer, M. G., Yelton, D. E., Mittler, R. S. and Ledbetter, J. A., 1996. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. Tissue Antigens. 47, 1-20.
Glennie, M. J. and Johnson, P. W., 2000. Clinical trials of antibody therapy. Immunol Today. 21, 403-410.
Goossens, T., Brauninger, A., Klein, U., Kuppers, R. and Rajewsky, K., 2001. Receptor revision plays no major role in shaping the receptor repertoire of human memory B cells after the onset of somatic hypermutation. Eur J Immunol. 31, 3638-3648.
Goossens, T., Klein, U. and Kuppers, R., 1998. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci U S A. 95, 2463-2468.
Grammer, A. C., Swantek, J. L., McFarland, R. D., Miura, Y., Geppert, T. and Lipsky, P. E., 1998. TNF receptor-associated factor-3 signaling mediates activation of p38 and Jun N-

terminal kinase, cytokine secretion, and Ig production following ligation of CD40 on human B cells. J Immunol. 161, 1183-1193.
Grassme, H., Bock, J., Kun, J. and Gulbins, E., 2002a. Clustering of CD40 ligand is required to form a functional contact with CD40. J Biol Chem. 277, 30289-30299.
Grassme, H., Bock, J., Kun, J. and Gulbins, E., 2002b. Clustering of CD40 ligand is required to form a functional contact with CD40. J Biol Chem. 13, 13.
Grassme, H., Jendrossek, V., Bock, J., Riehle, A. and Gulbins, E., 2002c. Ceramide-rich membrane rafts mediate CD40 clustering. J Immunol. 168, 298-307.
Greenspan, N. S. and Di Cera, E., 1999. Defining epitopes: It's not as easy as it seems. Nat Biotechnol. 17, 936-937.
Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J. and et al., 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. Embo J. 13, 3245-3260.
Haas, K. M., Taylor, K. A., MacHugh, N. D., Kreeger, J. M. and Estes, D. M., 2001. Enhancing effects of anti-CD40 treatment on the immune response of SCID- bovine mice to Trypanosoma congolense infection. J Leukoc Biol. 70, 931-940.
Han, S., Dillon, S. R., Zheng, B., Shimoda, M., Schlissel, M. S. and Kelsoe, G., 1997. V(D)J recombinase activity in a subset of germinal center B lymphocytes. Science. 278, 301-305.
Han, S., Zheng, B., Schatz, D. G., Spanopoulou, E. and Kelsoe, G., 1996. Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. Science. 274, 2094-2097.
Hanes, J., Schaffitzel, C., Knappik, A. and Pluckthun, A., 2000. Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. Nat Biotechnol. 18, 1287-1292.
Hanissian, S. H. and Geha, R. S., 1997. Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. Immunity. 6, 379-387.
Harris, R. S., Sale, J. E., Petersen-Mahrt, S. K. and Neuberger, M. S., 2002. AID is essential for immunoglobulin V gene conversion in a cultured B cell line. Curr Biol. 12, 435-438.
Haswell, L. E., Glennie, M. J. and Al-Shamkhani, A., 2001. Analysis of the oligomeric requirement for signaling by CD40 using soluble multimeric forms of its ligand, CD154. Eur J Immunol. 31, 3094-3100.
Hawkins, R. E., Russell, S. J., Baier, M. and Winter, G., 1993. The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen. The interaction of mutant D1.3 antibodies with lysozyme. J Mol Biol. 234, 958-964.
Hawkins, R. E., Russell, S. J. and Winter, G., 1992. Selection of phage antibodies by binding affinity. Mimicking affinity maturation. J Mol Biol. 226, 889-896.
Hikida, M., Mori, M., Kawabata, T., Takai, T. and Ohmori, H., 1997. Characterization of B cells expressing recombination activating genes in germinal centers of immunized mouse lymph nodes. J Immunol. 158, 2509-2512.
Hikida, M., Nakayama, Y., Yamashita, Y., Kumazawa, Y., Nishikawa, S. I. and Ohmori, H., 1998. Expression of recombination activating genes in germinal center B cells: involvement of interleukin 7 (IL-7) and the IL-7 receptor. J Exp Med. 188, 365-372.
Hill, H. R. and Stockley, P. G., 1996. Phage presentation. Mol Microbiol. 20, 685-692.
Holz, C., Lueking, A., Bovekamp, L., Gutjahr, C., Bolotina, N., Lehrach, H. and Cahill, D. J., 2001. A human cDNA expression library in yeast enriched for open reading frames. Genome Res. 11, 1730-1735.
Hornung, M., Lindemann, D., Kraus, C., Peters, A. and Berberich, I., 1998. The CD40 TRAF family member interacting motif carries the information to rescue WEHI 231 cells from anti-IGM-induced growth arrest. Eur J Immunol. 28, 3812-3823.

Hostager, B. S., Catlett, I. M. and Bishop, G. A., 2000. Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. J Biol Chem. 275, 15392-15398.
Hubsman, M., Yudkovsky, G. and Aronheim, A., 2001. A novel approach for the identification of protein-protein interaction with integral membrane proteins. Nucleic Acids Res. 29, E18.
Hufton, S. E., Moerkerk, P. T., Meulemans, E. V., de Bruine, A., Arends, J. W. and Hoogenboom, H. R., 1999. Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. J Immunol Methods. 231, 39-51.
Huls, G. A., Heijnen, I. A., Cuomo, M. E., Koningsberger, J. C., Wiegman, L., Boel, E., van der Vuurst de Vries, A. R., Loyson, S. A., Helfrich, W., van Berge Henegouwen, G. P., van Meijer, M., de Kruif, J. and Logtenberg, T., 1999. A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat Biotechnol. 17, 276-281.
Indik, Z. K., Park, J. G., Hunter, S., Mantaring, M. and Schreiber, A. D., 1995. Molecular dissection of Fc gamma receptor-mediated phagocytosis. Immunol Lett. 44, 133-138.
Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S. and Yamamoto, T., 2000. Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp Cell Res. 254, 14-24.
Itoh, K., Meffre, E., Albesiano, E., Farber, A., Dines, D., Stein, P., Asnis, S. E., Furie, R. A., Jain, R. I. and Chiorazzi, N., 2000. Immunoglobulin heavy chain variable region gene replacement As a mechanism for receptor revision in rheumatoid arthritis synovial tissue B lymphocytes. J Exp Med. 192, 1151-1164.
Jabara, H., Laouini, D., Tsitsikov, E., Mizoguchi, E., Bhan, A., Castigli, E., Dedeoglu, F., Pivniouk, V., Brodeur, S. and Geha, R., 2002. The Binding Site for TRAF2 and TRAF3 but Not for TRAF6 Is Essential for CD40-Mediated Immunoglobulin Class Switching. Immunity. 17, 265.
Jabara, H. H., Buckley, R. H., Roberts, J. L., Lefranc, G., Loiselet, J., Khalil, G. and Geha, R. S., 1998. Role of JAK3 in CD40-mediated signaling. Blood. 92, 2435-2440.
Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., LaVallie, E. R., Merberg, D., Spaulding, V., Stover, J., Williamson, M. J. and McCoy, J. M., 1997. A genetic selection for isolating cDNAs encoding secreted proteins. Gene. 198, 289-296.
Janeway, C. A., Jr., 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 54, 1-13.
Janeway, C. A., Jr., 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today. 13, 11-16.
Jirholt, P., Borrebaeck, C. A. K. and Ohlin, M., 2002. Antibody evolution from the centre to the periphery: applied to a human antibody fragment recognising the tumour-associated antigen mucin-1. J Mol Biol. 318, 407-416.
Jirholt, P., Ohlin, M., Borrebaeck, C. A. K. and Söderlind, E., 1998. Exploiting sequence space: shuffling in vivo formed complementarity determining regions into a master framework. Gene. 215, 471-476.
Johnsson, N. and Varshavsky, A., 1994. Split ubiquitin as a sensor of protein interactions in vivo. Proc Natl Acad Sci U S A. 91, 10340-10344.
Kaelin, W. G., Jr., Pallas, D. C., DeCaprio, J. A., Kaye, F. J. and Livingston, D. M., 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell. 64, 521-532.
Karp, C. L., Biron, C. A. and Irani, D. N., 2000. Interferon beta in multiple sclerosis: is IL-12 suppression the key? Immunol Today. 21, 24-28.

Karp, C. L., van Boxel-Dezaire, A. H., Byrnes, A. A. and Nagelkerken, L., 2001. Interferon-beta in multiple sclerosis: altering the balance of interleukin-12 and interleukin-10? Curr Opin Neurol. 14, 361-368.
Karpusas, M., Hsu, Y. M., Wang, J. H., Thompson, J., Lederman, S., Chess, L. and Thomas, D., 1995. 2 A crystal structure of an extracellular fragment of human CD40 ligand. Structure. 3, 1031-1039.
Karras, J. G., Wang, Z., Huo, L., Frank, D. A. and Rothstein, T. L., 1997. Induction of STAT protein signaling through the CD40 receptor in B lymphocytes: distinct STAT activation following surface Ig and CD40 receptor engagement. J Immunol. 159, 4350-4355.
Kaykas, A., Worringer, K. and Sugden, B., 2001. CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. Embo J. 20, 2641-2654.
Khazaeli, M. B., Conry, R. M. and LoBuglio, A. F., 1994. Human immune response to monoclonal antibodies. J Immunother. 15, 42-52.
Kirk, A. D., Burkly, L. C., Batty, D. S., Baumgartner, R. E., Berning, J. D., Buchanan, K., Fechner, J. H., Jr., Germond, R. L., Kampen, R. L., Patterson, N. B., Swanson, S. J., Tadaki, D. K., TenHoor, C. N., White, L., Knechtle, S. J. and Harlan, D. M., 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nat Med. 5, 686-693.
Kishore, U. and Reid, K. B., 2000. C1q: structure, function, and receptors. Immunopharmacology. 49, 159-170.
Klein, R. D., Gu, Q., Goddard, A. and Rosenthal, A., 1996. Selection for genes encoding secreted proteins and receptors. Proc Natl Acad Sci U S A. 93, 7108-7113.
Klonowski, K. D., Primiano, L. L. and Monestier, M., 1999. Atypical VH-D-JH rearrangements in newborn autoimmune MRL mice. J Immunol. 162, 1566-1572.
Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A. and Virnekas, B., 2000. Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. J Mol Biol. 296, 57-86.
Koho, H., Paulie, S., Ben-Aissa, H., Jonsdottir, I., Hansson, Y., Lundblad, M. L. and Perlmann, P., 1984. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. I. Determination of the selectivity of six antibodies by cell ELISA and immunofluorescence. Cancer Immunol Immunother. 17, 165-172.
Koppenhoefer, U., Brenner, B., Lang, F. and Gulbins, E., 1997. The CD40-ligand stimulates T-lymphocytes via the neutral sphingomyelinase: a novel function of the CD40-ligand as signalling molecule. FEBS Lett. 414, 444-448.
Korthauer, U., Graf, D., Mages, H. W., Briere, F., Padayachee, M., Malcolm, S., Ugazio, A. G., Notarangelo, L. D., Levinsky, R. J. and Kroczek, R. A., 1993. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. Nature. 361, 539-541.
Krebber, C., Spada, S., Desplancq, D. and Pluckthun, A., 1995. Co-selection of cognate antibody-antigen pairs by selectively-infective phages. FEBS Lett. 377, 227-231.
Kwekkeboom, J., De Boer, M., Tager, J. M. and De Groot, C., 1993. CD40 plays an essential role in the activation of human B cells by murine EL4B5 cells. Immunology. 79, 439-444.
Köhler, G. and Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256, 495-497.
Lafferty, K. J. and Cunningham, A. J., 1975. A new analysis of allogeneic interactions. Aust J Exp Biol Med Sci. 53, 27-42.

Laman, J. D., De Boer, M. and Hart, B. A., 1998. CD40 in clinical inflammation: from multiple sclerosis to atherosclerosis. Dev Immunol. 6, 215-222.
Laman, J. D., Hart, B. A., Brok, H., Meurs Mv, M., Schellekens, M. M., Kasran, A., Boon, L., Bauer, J., Boer Md, M. and Ceuppens, J., 2002. Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). Eur J Immunol. 32, 2218-2228.
Lantto, J., Lindroth, Y. and Ohlin, M., 2002. Non-germ-line encoded residues are critical for effective antibody recognition of a poorly immunogenic neutralization epitope on glycoprotein B of human cytomegalovirus. Eur J Immunol. 32, 1659-1669.
Lantto, J. and Ohlin, M., 2002a. Functional consequences of insertions and deletions in the complementarity determining regions of human antibodies. J Biol Chem. 16, 16.
Lantto, J. and Ohlin, M., 2002b. Uneven distribution of repetitive trinucleotide motifs in human immunoglobulin heavy variable genes. J Mol Evol. 54, 346-353.
Larocca, D., Witte, A., Johnson, W., Pierce, G. F. and Baird, A., 1998. Targeting bacteriophage to mammalian cell surface receptors for gene delivery. Hum Gene Ther. 9, 2393-2399.
Larsen, C. P., Alexander, D. Z., Hollenbaugh, D., Elwood, E. T., Ritchie, S. C., Aruffo, A., Hendrix, R. and Pearson, T. C., 1996a. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. Transplantation. 61, 4-9.
Larsen, C. P., Elwood, E. T., Alexander, D. Z., Ritchie, S. C., Hendrix, R., Tucker-Burden, C., Cho, H. R., Aruffo, A., Hollenbaugh, D., Linsley, P. S., Winn, K. J. and Pearson, T. C., 1996b. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature. 381, 434-438.
Ledbetter, J. A., Francisco, J. A., Siegall, C. B., Gilliland, L. K., Hollenbaugh, D., Aruffo, A., Siadak, A. W., Mischel-Petty, N., Grosmaire, L. S., Gordon, M. L., Brown, T. J., Moran-Davis, P., Mittler, R. S., Kiener, P. A. and Nadler, S. G., 1997. Agonistic activity of a CD40-specific single-chain Fv constructed from the variable regions of mAb G28-5. Crit Rev Immunol. 17, 427-435.
Lefranc, M. P., 2001. IMGT, the international ImMunoGeneTics database. Nucleic Acids Res. 29, 207-209.
Leung, D. W., Chen, E. and Goeddel, D. V., 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique. 1, 11–15.
Lewis, S. M., 1994. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol. 56, 27-150.
Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C. and Kain, S. R., 1998. Generation of destabilized green fluorescent protein as a transcription reporter. J Biol Chem. 273, 34970-34975.
Locksley, R. M., Killeen, N. and Lenardo, M. J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 104, 487-501.
MacCallum, R. M., Martin, A. C. and Thornton, J. M., 1996. Antibody-antigen interactions: contact analysis and binding site topography. J Mol Biol. 262, 732-745.
Mackey, M. F., Gunn, J. R., Maliszewsky, C., Kikutani, H., Noelle, R. J. and Barth, R. J., Jr., 1998. Dendritic cells require maturation via CD40 to generate protective antitumor immunity. J Immunol. 161, 2094-2098.
Malapati, S. and Pierce, S. K., 2001. The influence of CD40 on the association of the B cell antigen receptor with lipid rafts in mature and immature cells. Eur J Immunol. 31, 3789-3797.
Malinin, N. L., Boldin, M. P., Kovalenko, A. V. and Wallach, D., 1997. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature. 385, 540-544.

Manivel, V., Sahoo, N. C., Salunke, D. M. and Rao, K. V., 2000. Maturation of an antibody response is governed by modulations in flexibility of the antigen-combining site. Immunity. 13, 611-620.
Marks, J. D., Ouwehand, W. H., Bye, J. M., Finnern, R., Gorick, B. D., Voak, D., Thorpe, S. J., Hughes-Jones, N. C. and Winter, G., 1993. Human antibody fragments specific for human blood group antigens from a phage display library. Biotechnology (N Y). 11, 1145-1149.
Martin, A. and Scharff, M. D., 2002a. AID and mismatch repair in antibody diversification. Nat Rev Immunol. 2, 605-614.
Martin, A. and Scharff, M. D., 2002b. Somatic hypermutation of the AID transgene in B and non-B cells. Proc Natl Acad Sci U S A. 99, 12304-12308.
Mauri, C., Mars, L. T. and Londei, M., 2000. Therapeutic activity of agonistic monoclonal antibodies against CD40 in a chronic autoimmune inflammatory process. Nat Med. 6, 673-679.
Max, E. E., Seidman, J. G. and Leder, P., 1979. Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene. Proc Natl Acad Sci U S A. 76, 3450-3454.
McCafferty, J., Griffiths, A. D., Winter, G. and Chiswell, D. J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature. 348, 552-554.
McCall, A. M., Shahied, L., Amoroso, A. R., Horak, E. M., Simmons, H. H., Nielson, U., Adams, G. P., Schier, R., Marks, J. D. and Weiner, L. M., 2001. Increasing the affinity for tumor antigen enhances bispecific antibody cytotoxicity. J Immunol. 166, 6112-6117.
McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R. and Alber, T., 1999. Crystallographic analysis of CD40 recognition and signaling by human TRAF2. Proc Natl Acad Sci U S A. 96, 8408-8413.
Meffre, E., Casellas, R. and Nussenzweig, M. C., 2000. Antibody regulation of B cell development. Nat Immunol. 1, 379-385.
Meulemans, E. V., Slobbe, R., Wasterval, P., Ramaekers, F. C. and van Eys, G. J., 1994. Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. J Mol Biol. 244, 353-360.
Morrison, S. L., Johnson, M. J., Herzenberg, L. A. and Oi, V. T., 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A. 81, 6851-6855.
Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. and Honjo, T., 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 102, 553-563.
Muramatsu, M., Sankaranand, V. S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N. O. and Honjo, T., 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem. 274, 18470-18476.
Nadler, L. M., Stashenko, P., Hardy, R., Kaplan, W. D., Button, L. N., Kufe, D. W., Antman, K. H. and Schlossman, S. F., 1980. Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. Cancer Res. 40, 3147-3154.
Nagaoka, H., Muramatsu, M., Yamamura, N., Kinoshita, K. and Honjo, T., 2002. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. J Exp Med. 195, 529-534.
Naismith, J. H. and Sprang, S. R., 1998. Modularity in the TNF-receptor family. Trends Biochem Sci. 23, 74-79.
Nemazee, D., 2000. Receptor editing in B cells. Adv Immunol. 74, 89-126.

Nemazee, D. and Weigert, M., 2000. Revising B cell receptors. J Exp Med. 191, 1813-1817.
Ni, C. Z., Welsh, K., Leo, E., Chiou, C. K., Wu, H., Reed, J. C. and Ely, K. R., 2000. Molecular basis for CD40 signaling mediated by TRAF3. Proc Natl Acad Sci U S A. 97, 10395-10399.
Nilsson, N., Karlsson, F., Rakonjac, J. and Borrebaeck, C. A., 2002. Selective infection of E. coli as a function of a specific molecular interaction. J Mol Recognit. 15, 27-32.
Notarangelo, L. D. and Peitsch, M. C., 1996. CD40lbase: a database of CD40L gene mutations causing X-linked hyper- IgM syndrome. Immunol Today. 17, 511-516.
Oettinger, M. A., Schatz, D. G., Gorka, C. and Baltimore, D., 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. 248, 1517-1523.
Ohlin, M. and Borrebaeck, C. A.K., 1998. Insertions and deletions in hypervariable loops of antibody heavy chains contribute to molecular diversity. Mol Immunol. 35, 233-238.
Okazaki, I. M., Kinoshita, K., Muramatsu, M., Yoshikawa, K. and Honjo, T., 2002. The AID enzyme induces class switch recombination in fibroblasts. Nature. 416, 340-345.
Padlan, E. A., 1994. Anatomy of the antibody molecule. Mol Immunol. 31, 169-217.
Pandey, A. and Mann, M., 2000. Proteomics to study genes and genomes. Nature. 405, 837-846.
Papavasiliou, F. N. and Schatz, D. G., 2002a. The activation-induced deaminase functions in a postcleavage step of the somatic hypermutation process. J Exp Med. 195, 1193-1198.
Papavasiliou, F. N. and Schatz, D. G., 2002b. Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. Cell. 109 Suppl, S35-44.
Papoff, G., Hausler, P., Eramo, A., Pagano, M. G., Di Leve, G., Signore, A. and Ruberti, G., 1999. Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. J Biol Chem. 274, 38241-38250.
Pardoll, D. M., 1998. Cancer vaccines. Nat Med. 4, 525-531.
Park, Y. C., Burkitt, V., Villa, A. R., Tong, L. and Wu, H., 1999. Structural basis for self-association and receptor recognition of human TRAF2. Nature. 398, 533-538.
Parker, D. C., Greiner, D. L., Phillips, N. E., Appel, M. C., Steele, A. W., Durie, F. H., Noelle, R. J., Mordes, J. P. and Rossini, A. A., 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. Proc Natl Acad Sci U S A. 92, 9560-9564.
Parmley, S. F. and Smith, G. P., 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene. 73, 305-318.
Paulie, S., Koho, H., Ben-Aissa, H., Hansson, Y., Lundblad, M. L. and Perlmann, P., 1984. Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. II. Identification of the cellular target structures by immunoprecipitation and SDS-PAGE analysis. Cancer Immunol Immunother. 17, 173-179.
Pearson, T. C., Trambley, J., Odom, K., Anderson, D. C., Cowan, S., Bray, R., Lin, A., Hollenbaugh, D., Aruffo, A., Siadak, A. W., Strobert, E., Hennigar, R. and Larsen, C. P., 2002. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. Transplantation. 74, 933-940.
Pelletier, J. N., Arndt, K. M., Pluckthun, A. and Michnick, S. W., 1999. An in vivo library-versus-library selection of optimized protein- protein interactions. Nat Biotechnol. 17, 683-690.
Pelletier, J. N., Campbell-Valois, F. X. and Michnick, S. W., 1998. Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. Proc Natl Acad Sci U S A. 95, 12141-12146.
Petersen-Mahrt, S. K., Harris, R. S. and Neuberger, M. S., 2002. AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. Nature. 418, 99-103.

Pham, L. V., Tamayo, A. T., Yoshimura, L. C., Lo, P., Terry, N., Reid, P. S. and Ford, R. J., 2002. A CD40 Signalosome anchored in lipid rafts leads to constitutive activation of NF-kappaB and autonomous cell growth in B cell lymphomas. Immunity. 16, 37-50.
Pierce, S. K., 2002. Lipid rafts and B-cell activation. Nat Rev Immunol. 2, 96-105.
Plückthun, A. and Pack, P., 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology. 3, 83-105.
Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizacklerley, R. P. and Saul, F., 1973. Three-dimensional structure of the Fab' Fragment of a human immunoglobulin at 2.8-Å resolution. Proc Natl Acad Sci. 70, 3305-3310.
Poul, M. A., Becerril, B., Nielsen, U. B., Morisson, P. and Marks, J. D., 2000. Selection of tumor-specific internalizing human antibodies from phage libraries. J Mol Biol. 301, 1149-1161.
Poul, M. A. and Marks, J. D., 1999. Targeted gene delivery to mammalian cells by filamentous bacteriophage. J Mol Biol. 288, 203-211.
Pouly, S. and Antel, J. P., 1999. Multiple sclerosis and central nervous system demyelination. J Autoimmun. 13, 297-306.
Pound, J. D., Challa, A., Holder, M. J., Armitage, R. J., Dower, S. K., Fanslow, W. C., Kikutani, H., Paulie, S., Gregory, C. D. and Gordon, J., 1999. Minimal cross-linking and epitope requirements for CD40-dependent suppression of apoptosis contrast with those for promotion of the cell cycle and homotypic adhesions in human B cells. Int Immunol. 11, 11-20.
Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J. and Kehry, M. R., 1999. High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. Biochemistry. 38, 10168-10177.
Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J. and Kehry, M. R., 1998. CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. Biochemistry. 37, 11836-11845.
Purkerson, J. M. and Parker, D. C., 1998. Differential coupling of membrane Ig and CD40 to the extracellularly regulated kinase signaling pathway. J Immunol. 160, 2121-2129.
Rada, C., Jarvis, J. M. and Milstein, C., 2002. AID-GFP chimeric protein increases hypermutation of Ig genes with no evidence of nuclear localization. Proc Natl Acad Sci U S A. 99, 7003-7008.
Radic, M. Z., Erikson, J., Litwin, S. and Weigert, M., 1993. B lymphocytes may escape tolerance by revising their antigen receptors. J Exp Med. 177, 1165-1173.
Raghavan, M. and Bjorkman, P. J., 1996. Fc receptors and their interactions with immunoglobulins. Annu Rev Cell Dev Biol. 12, 181-220.
Rajewsky, K., 1996. Clonal selection and learning in the antibody system. Nature. 381, 751-758.
Remy, I. and Michnick, S. W., 1999. Clonal selection and in vivo quantitation of protein interactions with protein-fragment complementation assays. Proc Natl Acad Sci U S A. 96, 5394-5399.
Restifo, N. P., Kawakami, Y., Marincola, F., Shamamian, P., Taggarse, A., Esquivel, F. and Rosenberg, S. A., 1993. Molecular mechanisms used by tumors to escape immune recognition: immunogenetherapy and the cell biology of major histocompatibility complex class I. J Immunother. 14, 182-190.
Revy, P., Hivroz, C., Andreu, G., Graber, P., Martinache, C., Fischer, A. and Durandy, A., 1999. Activation of the Janus kinase 3-STAT5a pathway after CD40 triggering of human monocytes but not of resting B cells. J Immunol. 163, 787-793.

Rhyner, C., Kodzius, R. and Crameri, R., 2002. Direct selection of cDNAs from filamentous phage surface display libraries: potential and limitations. Curr Pharm Biotechnol. 3, 13-21.
Ribas, A., Butterfield, L. H., Amarnani, S. N., Dissette, V. B., Kim, D., Meng, W. S., Miranda, G. A., Wang, H. J., McBride, W. H., Glaspy, J. A. and Economou, J. S., 2001. CD40 cross-linking bypasses the absolute requirement for CD4 T cells during immunization with melanoma antigen gene-modified dendritic cells. Cancer Res. 61, 8787-8793.
Rogozin, I. B. and Kolchanov, N. A., 1992. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. Biochim Biophys Acta. 1171, 11-18.
Rolph, M. S. and Kaufmann, S. H., 2001. CD40 signaling converts a minimally immunogenic antigen into a potent vaccine against the intracellular pathogen Listeria monocytogenes. J Immunol. 166, 5115-5121.
Roovers, R. C., Henderikx, P., Helfrich, W., van der Linden, E., Reurs, A., de Bruine, A. P., Arends, J. W., de Leij, L. and Hoogenboom, H. R., 1998. High-affinity recombinant phage antibodies to the pan-carcinoma marker epithelial glycoprotein-2 for tumour targeting. Br J Cancer. 78, 1407-1416.
Roovers, R. C., van der Linden, E., de Bruine, A. P., Arends, J. W. and Hoogenboom, H. R., 2001. Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma. Eur J Cancer. 37, 542-549.
Rothe, M., Xiong, J., Shu, H. B., Williamson, K., Goddard, A. and Goeddel, D. V., 1996. I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. Proc Natl Acad Sci U S A. 93, 8241-8246.
Saemann, M., Kelemen, P., Zeyda, M., Bohmig, G., Staffler, G. and Zlabinger, G., 2002. CD40 triggered human monocyte-derived dendritic cells convert to tolerogenic dendritic cells when JAK3 activity is inhibited. Transplant Proc. 34, 1407.
Sakano, H., Huppi, K., Heinrich, G. and Tonegawa, S., 1979. Sequences at the somatic recombination sites of immunoglobulin light- chain genes. Nature. 280, 288-294.
Sakata, N., Hamelmann, E., Siadak, A. W., Terada, N., Gerwins, P., Aruffo, A., Johnson, G. L. and Gelfand, E. W., 2000. Differential regulation of CD40-mediated human B cell responses by antibodies directed against different CD40 epitopes. Cell Immunol. 201, 109-123.
Schaffitzel, C., Hanes, J., Jermutus, L. and Pluckthun, A., 1999. Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. J Immunol Methods. 231, 119-135.
Schatz, D. G. and Baltimore, D., 1988. Stable expression of immunoglobulin gene V(D)J recombinase activity by gene transfer into 3T3 fibroblasts. Cell. 53, 107-115.
Schiffer, M., Girling, R. L., Ely, K. R. and Edmundson, A. B., 1973. Structure of a lambda-type Bence-Jones protein at 3,5 Å resolution. Biochemistry. 12, 4620-4631.
Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U. and Greaves, M. F., 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. J Biol Chem. 257, 10766-10769.
Schonbeck, U. and Libby, P., 2001. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci. 58, 4-43.
Shan, D., Ledbetter, J. A. and Press, O. W., 1998. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood. 91, 1644-1652.
Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindquist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J. C., Marks, J. D. and Lindqvist, E., 1998. Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc Natl Acad Sci U S A. 95, 6157-6162.

Siegel, R. M., Chan, F. K., Chun, H. J. and Lenardo, M. J., 2000a. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol. 1, 469-474.
Siegel, R. M., Frederiksen, J. K., Zacharias, D. A., Chan, F. K., Johnson, M., Lynch, D., Tsien, R. Y. and Lenardo, M. J., 2000b. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science. 288, 2354-2357.
Siegel, R. M. and Lenardo, M. J., 2001. To B or not to B: TNF family signaling in lymphocytes. Nat Immunol. 2, 577-578.
Siepmann, K., Skok, J., van Essen, D., Harnett, M. and Gray, D., 2001. Rewiring of CD40 is necessary for delivery of rescue signals to B cells in germinal centres and subsequent entry into the memory pool. Immunology. 102, 263-272.
Silverstein, A. M. (1999) The History of Immunology. In *Fundamental Immunology* (Edited by Paul W. E.). Lippincott-Raven Publishers, Philadelphia.
Simons, K. and Toomre, D., 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 1, 31-39.
Singh, J., Garber, E., Van Vlijmen, H., Karpusas, M., Hsu, Y. M., Zheng, Z., Naismith, J. H. and Thomas, D., 1998. The role of polar interactions in the molecular recognition of CD40L with its receptor CD40. Protein Sci. 7, 1124-1135.
Smith, G. P., 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science. 228, 1315-1317.
Söderlind, E., Ohlin, M. and Carlsson, R., 1999. Complementarity-determining region (CDR) implantation: a theme of recombination. Immunotechnology. 4, 279-285.
Söderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Åberg, A. M., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R. and Borrebaeck, C. A. K., 2000. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat Biotechnol. 18, 852-856.
Song, W., Kong, H. L., Carpenter, H., Torii, H., Granstein, R., Rafii, S., Moore, M. A. and Crystal, R. G., 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. J Exp Med. 186, 1247-1256.
Steinhauer, C., Wingren, C., Malmborg Hager, A.-C. and Borrebaeck, C. A. K., 2002. Single Framework recombinant antibody fragments designed for protein chip applications. Biotechniques supplement "High-throuhgput proteomics: Protein Arrays". in press.
Stemmer, W. P., 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci U S A. 91, 10747-10751.
Stephen, J. G. and Lantz, C. S. (1999) *Allergy*. Lippincott-Raven Publishers, Philadelphia.
Sulica, A., Morel, P., Metes, D. and Herberman, R. B., 2001. Ig-binding receptors on human NK cells as effector and regulatory surface molecules. Int Rev Immunol. 20, 371-414.
t Hart, B. A., Brok, H. P., Amor, S. and Bontrop, R. E., 2001. The major histocompatibility complex influences the ethiopathogenesis of MS-like disease in primates at multiple levels. Hum Immunol. 62, 1371-1381.
t Hart, B. A., van Meurs, M., Brok, H. P., Massacesi, L., Bauer, J., Boon, L., Bontrop, R. E. and Laman, J. D., 2000. A new primate model for multiple sclerosis in the common marmoset. Immunol Today. 21, 290-297.
Tarlinton, D. M. and Smith, K. G., 2000. Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. Immunol Today. 21, 436-441.
Tiegs, S. L., Russell, D. M. and Nemazee, D., 1993. Receptor editing in self-reactive bone marrow B cells. J Exp Med. 177, 1009-1020.

Tomlinson, I. M., Walter, G., Jones, P. T., Dear, P. H., Sonnhammer, E. L. and Winter, G., 1996. The imprint of somatic hypermutation on the repertoire of human germline V genes. J Mol Biol. 256, 813-817.
Tonegawa, S., 1983. Somatic generation of antibody diversity. Nature. 302, 575-581.
Tong, A. W., Seamour, B., Chen, J., Su, D., Ordonez, G., Frase, L., Netto, G. and Stone, M. J., 2000. CD40 ligand-induced apoptosis is Fas-independent in human multiple myeloma cells. Leuk Lymphoma. 36, 543-558.
Tutt, A. L., O'Brien, L., Hussain, A., Crowther, G. R., French, R. R. and Glennie, M. J., 2002. T cell immunity to lymphoma following treatment with anti-CD40 monoclonal antibody. J Immunol. 168, 2720-2728.
Uetz, P., 2002. Two-hybrid arrays. Curr Opin Chem Biol. 6, 57-62.
van Kooten, C., 2000. Immune regulation by CD40-CD40-l interactions - 2; Y2K update. Front Biosci. 5, D880-693.
van Kooten, C. and Banchereau, J., 1997. Functions of CD40 on B cells, dendritic cells and other cells. Curr Opin Immunol. 9, 330-337.
van Kooten, C. and Banchereau, J., 2000. CD40-CD40 ligand. J Leukoc Biol. 67, 2-17.
van Mierlo, G. J., den Boer, A. T., Medema, J. P., van der Voort, E. I., Fransen, M. F., Offringa, R., Melief, C. J. and Toes, R. E., 2002. 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. 99, 5561-5566.
Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. and Johnson, K. S., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat Biotechnol. 14, 309-314.
Vidalain, P. O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D. and Manie, S., 2000. CD40 signaling in human dendritic cells is initiated within membrane rafts. Embo J. 19, 3304-3313.
von Budingen, H. C., Hauser, S. L., Fuhrmann, A., Nabavi, C. B., Lee, J. I. and Genain, C. P., 2002. Molecular characterization of antibody specificities against myelin/oligodendrocyte glycoprotein in autoimmune demyelination. Proc Natl Acad Sci U S A. 99, 8207-8212.
Wajant, H., Henkler, F. and Scheurich, P., 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. Cell Signal. 13, 389-400.
Wallach, D., Boldin, M. P., Kovalenko, A. V., Malinin, N. L., Mett, I. L. and Camonis, J. H., 1998. The yeast two-hybrid screening technique and its use in the study of protein-protein interactions in apoptosis. Curr Opin Immunol. 10, 131-136.
Wang, Y., Kelly, C. G., Karttunen, J. T., Whittall, T., Lehner, P. J., Duncan, L., MacAry, P., Younson, J. S., Singh, M., Oehlmann, W., Cheng, G., Bergmeier, L. and Lehner, T., 2001. CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity. 15, 971-983.
Webster, D. M., Henry, A. H. and Rees, A. R., 1994. Antibody-antigen interactions. Current opinion in structural biology. 4, 123-129.
Werneburg, B. G., Zoog, S. J., Dang, T. T., Kehry, M. R. and Crute, J. J., 2001. Molecular characterization of CD40 signaling intermediates. J Biol Chem. 18, 18.
Wilson, I. A. and Stanfield, R. L., 1993. Antibody-antigen interactions. Current opinion in structural biology. 3, 113-118.
Wilson, I. A. and Stanfield, R. L., 1994. Antibody-antigen interactions: new structures and new conformational changes. Current opinion in structural biology. 4, 857-867.

Wilson, P. C., de Bouteiller, O., Liu, Y. J., Potter, K., Banchereau, J., Capra, J. D. and Pascual, V., 1998. Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. J Exp Med. 187, 59-70.
Wilson, P. C., Wilson, K., Liu, Y. J., Banchereau, J., Pascual, V. and Capra, J. D., 2000. Receptor revision of immunoglobulin heavy chain variable region genes in normal human B lymphocytes. J Exp Med. 191, 1881-1894.
Winter, D. B. and Gearhart, P. J., 1998. Dual enigma of somatic hypermutation of immunoglobulin variable genes: targeting and mechanism. Immunol Rev. 162, 89-96.
Winter, G., Griffiths, A. D., Hawkins, R. E. and Hoogenboom, H. R., 1994. Making antibodies by phage display technology. Annu Rev Immunol. 12, 433-455.
Wu, H., Nie, Y., Huse, W. D. and Watkins, J. D., 1999. Humanization of a murine monoclonal antibody by simultaneous optimization of framework and CDR residues. J Mol Biol. 294, 151-162.
Wu, T. T. and Kabat, E. A., 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. J Exp Med. 132, 211-250.
Wuilmart, C., Urbain, J. and Givol, D., 1977. On the location of palindromes in immunoglobulin genes. Proc Natl Acad Sci U S A. 74, 2526-2530.
Xu, J. L. and Davis, M. M., 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. Immunity. 13, 37-45.
Yoshikawa, K., Okazaki, I. M., Eto, T., Kinoshita, K., Muramatsu, M., Nagaoka, H. and Honjo, T., 2002. AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. Science. 296, 2033-2036.
Yu, W., Nagaoka, H., Jankovic, M., Misulovin, Z., Suh, H., Rolink, A., Melchers, F., Meffre, E. and Nussenzweig, M. C., 1999. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature. 400, 682-687.
Zanelli, E. and Toes, R. E., 2000. A dual function for CD40 agonists. Nat Med. 6, 629-630.