Protein Engineering, Design & Selection vol. 25 no. 3 pp. 89–96, 2012
Published online January 10, 2012 doi:10.1093/proeng/gzr063

CD40/APC-specific antibodies with three T-cell epitopes loaded in the constant domains induce $CD4^+$ T-cell responses

Ingunn B.Rasmussen[1,2†], Inger Øynebråten[2†], Lene S.Høydahl[2], Morten Flobakk[1,5], Elin Lunde[1], Terje E.Michaelsen[3,4], Bjarne Bogen[2] and Inger Sandlie[1,2,6]

[1]Department of Molecular Biosciences, Centre for Immune Regulation, University of Oslo, PO Box 1041, Blindern, NO-0316 Oslo, Norway, [2]Department of Immunology, Centre for Immune Regulation, University of Oslo and Oslo University Hospital Rikshospitalet, PO Box 4950, Nydalen, 0424 Oslo, Norway, [3]Norwegian Institute of Public Health, Department of Bacteriology and Immunology, PO Box 4404, Nydalen, NO-0403 Oslo, Norway, [4]School of Pharmacy, Department of Chemical Pharmacy, University of Oslo, PO Box 1068, Blindern, NO-0316 Oslo, Norway and [5]Current address: BioInn 2317, Hamar, Norway

[6]To whom correspondence should be addressed.
E-mail: inger.sandlie@imbv.uio.no

Received May 31, 2011; revised November 1, 2011; accepted December 12, 2011

Edited by Sally Ward

$CD4^+$ T lymphocytes play a central role in the orchestration and maintenance of the adaptive immune response. Targeting of antigen to antigen presenting cells (APCs) increases peptide loading of major histocompatibility complex (MHC) class II molecules and $CD4^+$ T-cell activation. APCs have been targeted by APC-specific recombinant antibodies (rAbs) with single T-cell epitopes integrated in the constant region of the heavy chain (C_H). However, the strategy may be improved if several T-cell epitopes could be delivered simultaneously by one rAb. We here demonstrate that a single rAb can be loaded with multiple identical or different T-cell epitopes, integrated as loops between β-strands in C_H domains. One epitope was inserted in C_H1, while two were placed in C_H2 of IgG. T-cell proliferation assays showed that all three peptides were excised from loops and presented on MHC class II to T-cells. Induction of T-cell activation by each epitope in the multi-peptide rAb was as good, or even better, than that elicited by corresponding single-peptide rAbs. Furthermore, following DNA vaccination of mice with plasmids that encode CD40-specific rAbs loaded with either one or three peptides, T-cell responses were induced. Thus, integration of multiple epitopes in C_H region loops of APC-specific rAbs is feasible and may be utilized in design of multi-vaccines.

Keywords: antibodies/antigen presentation/epitope/T-cell activation/vaccine

Introduction

A central question in vaccine design is how to trigger a broad but specific immune response against a pathogen or a tumour antigen. In general, an efficient immune response is dependent on the contributions from B-cells, $CD8^+$ and $CD4^+$ T-cells. $CD4^+$ T-cells are important for interferon (IFN)-γ production and Ab formation, as well as long-term maintenance of $CD8^+$ memory cells. Furthermore, $CD4^+$ T-cells may exert cytolytic activity on MHC class II bearing target cells (Tite and Janeway, 1984; Bogen *et al.*, 1986). It is therefore important to increase our understanding of how $CD4^+$ T-cell function may be improved.

Targeting of whole antigen or antigenic peptides to antigen presenting cells (APCs) by way of APC-specific Abs improves MHC class II peptide loading and specific $CD4^+$ T-cell activation, and both chemical conjugation (Carayanniotis and Barber, 1987; Snider and Segal, 1987) and genetic linking (Baier *et al.*, 1995; Lunde *et al.*, 1999; Hawiger *et al.*, 2001) of antigen to Ab have been described. Abs are stable multi-domain proteins, and each Ab domain shares a characteristic structure, the Ig fold, consisting of anti-parallel β-strands that form two β-sheets stabilized by a conserved disulphide bond. The β-strands, A–F, are connected by loops, and six such loops exist in each of C_H1, C_H2 and C_H3 domains. These are numbered 1–6, starting from the N-terminal end of each domain (Fig. 1A).

We have previously described an approach where we substitute loop 6 (connecting strands F and G) in C_H1 with antigenic peptides that contain T-cell epitopes. Such molecules have further been equipped with variable (V) regions that confer specificity to surface molecules on APCs, such as IgD, MHC class II and CD14 (Lunde *et al.*, 1999; Rasmussen *et al.*, 2001; Lunde *et al.*, 2002; Schjetne *et al.*, 2005; Tunheim *et al.*, 2005). The final rAb targets the integrated T-cell epitope to the antigen-processing compartment of APCs, where the epitope is excised from the rAb, bound to MHC class II and transported to the cell surface for presentation to $CD4^+$ T-cells. This targeting strategy has generally increased the efficiency of T-cell stimulation four to five logs compared with synthetic peptides, on a molar basis. In each rAb we have previously introduced a single T-cell epitope derived from either the light (L) chain of the myeloma protein M315 (aa 91–101, $\lambda2^{315}$), ovalbumin (aa 323–339, OVA), HA (aa 110–120) or hen egg lysozyme (aa 46–61, HEL) (Lunde *et al.*, 1997; Eidem *et al.*, 2000; Rasmussen *et al.*, 2001).

The APC-specific Ab targeting strategy would, however, be more versatile if a single rAb molecule could carry more than one antigenic peptide. We have recently, in separate molecules, substituted every C_H loop in IgD-specific IgG

[†]I.B.R. and I.Ø. contributed equally to this work.

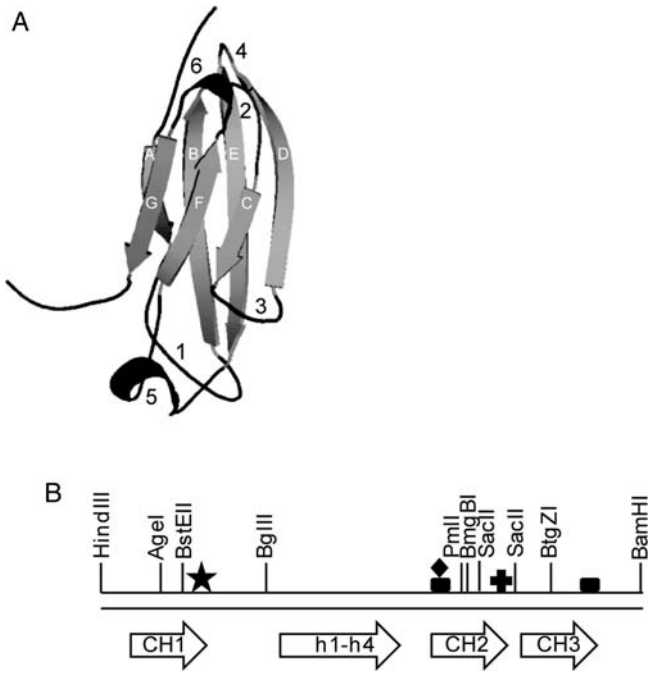

Fig. 1. Domain structure and H chain engineering. (**A**) Graphic representation of the C_H1 domain of human IgG. Loops are numbered 1–6, β-strands A–F. The figure was prepared using PyMOL v0.99 (DeLano Scientific LLC, CA, USA) from PDB ID: 1HZH (Saphire *et al.*, 2001). Secondary structure assignments are manually corrected based on the program Stride (Frishman and Argos, 1995). (**B**) Human Cγ gene (ID: X03604). Exons are marked as arrows. Restriction sites used for subcloning are indicated. Stars, diamonds, crosses and squares indicate the substitution with nt encoding OVA, HA, HEL and $\lambda2^{315}$, respectively.

with a model epitope derived from $\lambda2^{315}$ (aa 89–105). Eight loop positions were identified that could hold the inserted T-cell epitope while maintaining rAb secretion, binding to IgD on B cell APCs and ability to enhance stimulation of T-cells (Flobakk *et al.*, 2008). Here we have selected four of those eight loops which were identified as useful for peptide insertion, namely loop 6 in C_H1, loop1 and loop 6 in C_H2 and loop 6 in C_H3. These loops were substituted with the peptides described above, derived from OVA, HA, HEL as well as aa 89–105 $\lambda2^{315}$, and mutants were made that held one, two or more foreign peptide sequences. We found that mutants with one peptide in C_H1 in combination with two in C_H2 were secreted from producing cells and bound IgD. When mixed with irradiated spleen cells and specific T-cells *in vitro,* activation of T-cells with any of the three specificities was observed. We then constructed similar plasmids encoding recombinant antibodies (rAbs) with anti-CD40 specificity. The plasmids were given as DNA vaccines to sets of mice that raised specific T-cell responses.

Materials and methods

Production of mutant rAb

The gene encoding a G3m (b0) variant (Huck *et al.*, 1986), a gift from Dr M. P. LeFranc (IMGT, France), was template in all mutagenesis reactions, and the complete mutant H chains were assembled in the expression vector $pLNOH2_{IgD}$. Expression vectors $pLNOK_{IgD}$ and $pLNOH2_{IgD}$, which encode mouse IgD (mIgD) specific L and H chains,

Table I. Mutagenesis primers[a]

Primer	Sequence
1CH2HA(+)	GGA CCG TCA GTC TTC CTC TTC **TCA TTC GAA AGG TTC GAA ATA TTC CCA AAG GAA** GAG GTC ACG TGC GTG GTG GT
1CH2HA(−)	ACC ACC ACG CAC GTG ACC TC **TTC CTT TGG GAA TAT TTC GAA CCT TTC GAA TGA** G AAG AGG AAG ACT GAC GGT CC
CH2forw	TCA GCA CCT GAA CTC CTG GG
CH2rev	AGG AAG AAG GAG CCG TGC GA
nr6CH2HEL(−)	**GCG GCT GTT GAT CTG CAG GAT GCC GTA GTC GGT GCT GCC GTC GGT GTT** GGA GAC CTT GCA CTT GTA CT
nr6CH2HEL(+)	**AAC ACC GAC GGC AGC ACC GAC TAC GGC ATC CTG CAG ATC AAC AGC CGC** ATC GAG AAA ACC ATC TCC AAA AC
HELdir	TCC TGC AGA TCA ACA GCC GC

[a]Nt encoding epitopes are in bold. Underline indicates a silent SspI site.

respectively, have been described (Lunde *et al.*, 1999). Vectors encoding human IgG3 (hIgG3) H chain genes with aa 323–339 from OVA substituted with loop 6 C_H1 or $\lambda2^{315}$ in loop 1 C_H2 and loop 6 C_H3 have been described (Rasmussen *et al.*, 2001; Flobakk *et al.*, 2008). Loop 1 in C_H2 was substituted with nucleotide (nt) encoding aa110–120 from HA by QuikChange mutagenesis (Stratagene). A silent SspI site was introduced for the identification of plasmids harbouring the mutation. Loop 6 in C_H2 was substituted with aa 46–61 from HEL by polymerase chain reaction SOEing. Primers are presented in Table I. Screening for mutants was performed with the HEL specific primer HELdir and the primer CH2rev. Genes encoding multiple epitopes were constructed by subcloning using restriction sites AgeI, BglII, BmgBI, BstEII, BtgZI, HindIII, PmlI and SacII (Fig. 1B). All mutant H chain genes assembled in $pLNOH2_{IgD}$ were co-transfected with $pLNOK_{IgD}$ in 293E cells as described (Berntzen *et al.*, 2005). The cloning of V region genes that confer specificity for mouse CD40 (mCD40) has been described (Schjetne *et al.*, 2007). The mutant H chain genes were assembled in $pLNOH2_{CD40}$ and co-transfected with $pLNOK_{CD40}$ in 293E cells. Supernatants were harvested as described (Berntzen *et al.*, 2005).

Ammonium sulphate precipitation

Protein in supernatants was precipitated by a 1 : 1 addition of a saturated ammonium sulphate solution. After 20 min room temperature (RT) incubation, the precipitates were centrifuged at 20 000 *g* for 10 min. Pellets were dissolved in 2 ml dH_2O and dialysed three times with phosphate-buffered saline (PBS) and twice with fresh cell culture media at 4°C.

Antibodies

The mAb HP-6050 (Sigma-Aldrich) is specific for the hIgG3 hinge. The two mAbs 132c8-bio and HP-6050 were compared for binding to all four human IgG isotypes side by side in enzyme-linked immunosorbent assay (ELISA), and found to bind hIgG3 hinge in a similar fashion. Sheep polyclonal Ab s303 is specific for human IgG Fab and has been described (Aaberge *et al.*, 1992). Soluble mIgD was produced as described (Loset *et al.*, 2004). Recombinant mCD40/Fc Ig chimeric protein was obtained from R&D Systems.

Analysis of secreted rAb by sandwich ELISA

Microtiter plates were coated with human IgG Fab specific Ab s303 (2 μg/ml), soluble mIgD (4 μg/ml), 5-iodo-4-hydroxy-3-nitrophenactetyl conjugated bovine serum albumin (BSA-NIP) (1 μg/ml) or mCD40/Ig (4 μg/ml). Supernatants were applied to each well and detected with hIgG3 hinge-specific mAb 132c8-bio (1 : 8000) or anti-hIgG3-bio (clone HP6050, Sigma).

Western blot

rAb samples were separated on Criterion XT Precast Gels (Bio-Rad) and blotted onto an Immobilon^{TM}-p polyvinylidene difluoride membrane (Sigma-Aldrich) at 20 V for 30 min. Membranes were probed with biotinylated mouse αhIgG3 (HP-6050) (Sigma-Aldrich), horseradish peroxidase-conjugated streptavidin (GE Healthcare) and ECL Plus Western Blotting Detection Reagents kit (GE Healthcare), and were analysed using the software ImageQuant TL v2003.02 (GE Healthcare). Individual Ab concentrations were normalized to a known concentration of purified hIgG3.

Cell lines and mice

DO11.10 OVA$^{323-339}$-specific T-cell receptor (TCR) transgenic mice on a BALB/c background (Murphy *et al.*, 1990), and the λ2^{315}-specific TCR transgenic mice on a BALB/c background (Bogen *et al.*, 1992) have been described. BALB/c and C3H mice were from Taconic (Ry, Denmark). The study was approved by the Norwegian Animal Research Committee (Oslo, Norway). The cell line 293E (CRL-10852) was obtained from American type culture collection (ATCC). The T-cell hybridoma 3A9 that recognizes aa 46–61 of HEL in context of MHC class II I-A^k (Allen and Unanue, 1984) was a gift from Dr R N Germain (NIAID, USA). Spleen cells derived from BALB/c (H-2^d, IgH^a) or C3H (H-2^k, IgH^a) mice were used as APC. The interleukin (IL)-2 dependent T-cell line CTLL-2 was purchased from ATCC.

Peptides

OVA (323–339) and HEL (46–61) peptides were from GenScript (Piscataway, NJ, USA). A synthetic peptide corresponding to aa 89–107 of λ2^{315} of the myeloma protein M315 was obtained from NeoMPS (Groupe SNPE, France).

In vitro *T-cell proliferation assays*

Samples of 5×10^5 irradiated (2000 cGy) BALB/c spleen APC were cultured with 10^5 LN cells from DO11.10 mice or mice transgenic for T-cell receptors of the CD4$^+$ T-cell clone 4B2A1 (Bogen *et al.*, 1992) and various amounts rAb and synthetic peptides in triplicates for 72 h. The cultures were then pulsed with 1 μCi [^{3}H]dThd for 16–24 h, harvested, and counted using the TopCount NXT scintillation counter (GMI, MN, USA). Detection of IL-2 in supernatants was performed in plates coated with JES6-1A12 (2 μg/ml). JES6-5H4 (1 μg/ml) was used as detection Ab for IL-2. Standard curves were prepared from a 2-fold dilution starting at 2 ng/ml of rIL-2 (BD Pharmingen).

Cytokine secretion assay

Portions of spleen cells from C3H mice (irradiated at 2000 cGy) 5×10^5 per well and 3A9 hybridoma cells, 5×10^4 cells per well, were added in triplicates. RAb and synthetic aa 46–61 HEL peptides were added in 5-fold series and the plates were incubated at 37°C for 48 h. Supernatants were diluted 1 : 3, 1 : 30 and 1 : 300 and portions of 5×10^3 CTLL-2 cells added to each well. A standard of IL-2 were included. After incubation at 37°C for 20–24 h, 1 μCi [^{3}H]dThd was added followed by incubation, cell harvest and scoring as above.

DNA vaccination and electroporation

Plasmids were purified by EndoFree Qiagen kit (Germantown, MD, USA) and diluted in 0.9% NaCl. The DNA-solution (70 μl) was a mixture of four plasmids: 100 μg αmCD40IgG-pLNOH$_2$, 100 μg αmCD40-pLNOκ, 50 μg pUMVC1-mGM-CSF (Aldevron) and 50 μg mMIP-1α-pLNOH$_2$. Immediately after injection, electroporation was performed by the use of the Elgen electroporator device equipped with a caliper electrode (Elgen, Inovio Biomedical Co., PA, USA). The settings were: bipolar pulses of 100 mV × 0.2 ms with pulse sequence and pulse sequence train being 1000 and 10, respectively.

In an alternative protocol, DNA was delivered intradermally according to the procedure described by Roos *et al.* (2006). The DNA-solution (28 μl) contained 28 μg encoding αCD40IgG-pLNOH$_2$, 28 μg αCD40-pLNOH$_2$κ, 14 μg pUMVC1-mGM-CSF and 14 μg mMIP-1α-pLNOH$_2$. Immediately after injection, electrodes were placed over the injection site and voltage was applied (two pulses, 1125 V/cm, 50 μs+8 pulses, 275 V/cm, 10 ms). Electroporation was performed by the PA-4000S-Advanced PulseAgile Rectangular Wave Electroporation System (Cyto Pulse Sciences, Inc.).

Enzyme-linked immunospot assay

OVA- and HEL-specific cellular immune responses in vaccinated mice were assessed by IFNγ- and IL-4-enzyme-linked immunospot (ELISPOT) assays. Multi-ScreenHTS-IP Filter Plates (Millipore) were pre-treated as suggested by the manufacturer. The plates were coated with 75 μl 11B11 (10 μg/ml) or AN-18 (10 μg/ml) for detection of IL-4 and IFNγ, respectively. After incubation at 4°C overnight, the plates were washed three times in 150 μl sterile PBS before adding splenocytes that were incubated together with OVA or HEL peptide at 37°C for ∼26 h. After washing in PBS (3 × 150 μl), adherent cells were lyzed by water before one wash with 150 μl PBS containing 0.01% Tween 20 and one wash in PBS. Biotinylated anti-IL-4 (75 μl, 1.5 μg/ml, BVD6-24G2, BD Pharmingen, San Jose, CA, USA) or anti-IFNγ (1.5 μg/ml, XMG1.2) was added overnight (4°C). Streptavidin conjugated with alkaline phosphatase (1 : 3000, Amersham Pharmacia Biotech, Amersham, UK) was added for 1–2 h at RT before development of the spots by 5-bromo-4-chloroindol-3-yl phosphate/nitro blue tetrazolium buffer solution (Zymed, Carlsbad, CA, USA). Some experiments were performed using the The ELISpot^{PRO} kit and The ELISpot^{PLUS} kit from Mabtech (Mabtech AB, Sweden). The reaction was stopped by tap water and the plates were air dried before the number of spots was determined electronically by Zeiss KS-EliSpot-401 instrument.

Statistical analyses

Immune responses among groups of mice are presented as means with SEM. *P* values were calculated by use of the

two-tailed *t* test in GraphPad Prism version 4.03. *P* values <0.05 were considered significant.

Results

Secretion of single-loop replacement rAb

Loops between β-strands in all three Ab C_H domains can be replaced with the $\lambda2^{315}$ T-cell epitope (Flobakk *et al*., 2008). To study if other T-cell epitopes may be inserted, we replaced four different loops, namely loop 6 in C_H1, loops 1 and 6 in C_H2 as well as loop 6 in C_H3 with model epitopes from OVA, HA, HEL and $\lambda2^{315}$. Loop 6 C_H1 was replaced with OVA derived peptide, loops 1 and 6 C_H2 with HA and HEL peptides, respectively and loop 6 C_H3 with the $\lambda2^{315}$ epitope (Figs 1B and 2A). Resulting rAbs with mIgD specificity were produced in 293E cells from expression vectors pLNOH2 (encoding H chain) and pLNOK (encoding L chain) as previously described (Berntzen *et al*., 2005). The resulting rAb had the expected specificity as shown in ELISA (data not shown). Moreover, the indicated loops could be substituted with maintenance of secretion, albeit at levels reduced to 20–55% of wild type (wt) level (Fig. 2B). Thus, all these T-cell epitopes may be introduced into the loops chosen in a single replacement format.

Secretion of multi-loop replacement rAb

We then constructed rAbs which carried combinations of two or three peptides from OVA, HA, HEL and $\lambda2^{315}$ simultaneously substituted in loops 6 C_H1, 1 C_H2, 6 C_H2 and 6 C_H3.

Upon producing rAb transfectants, we found that rAbs with one epitope in C_H1 and a second in C_H2 or C_H3 were secreted, however at reduced levels at ∼15% compared with wt. This also applied to a rAb with two epitopes in C_H2, and one with three epitopes; one in C_H1 and two in C_H2. Neither of five rAb with epitopes in both C_H2 and C_H3 was secreted (Fig. 2B).

To rule out that the different aa sequences of the four T-cell epitopes chosen were responsible for the effects seen, the experiment was repeated for double- and triple-peptide rAb that carried more than one copy of the $\lambda2^{315}$ epitope in loop 6 C_H1, 6 C_H2 and 6 C_H3. Again, rAbs with one epitope in C_H1 and a second in either C_H2 or C_H3 were secreted (10–20% of wt), while rAbs with epitope in both C_H2 and C_H3 were not. Furthermore, a mutant with the $\lambda2^{315}$ peptide in both loops 1 and 6 of C_H2 was tested, and found to be secreted at the same level as the rAb with HA and HEL epitopes in these loops (Fig. 2C). Based on these results, we excluded replacement of loop 6 C_H3 from our multi-vaccine design, and focused our work on a triply substituted rAb with OVA in loop 6 C_H1, $\lambda2^{315}$ in loop 1 C_H2 and HEL in loop 6 C_H2. This, as well as a second, similarly substituted triple mutant rAb with OVA, HA and HEL was secreted at a level of 15% of wt (Fig. 2B).

Triple-epitope rAb stimulate specific T-cells with efficiencies similar to that of single-peptide rAb

Supernatants from cells producing rAbs were ammonium sulphate precipitated and dialysed in fresh medium, as described in Materials and methods (Berntzen *et al*., 2005). Samples were then run on sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotted to demonstrate that all rAbs had the correct mass of ∼165 kDa and thus consisted of disulfide linked H and L chains, H_2L_2 (Fig. 2D). The final

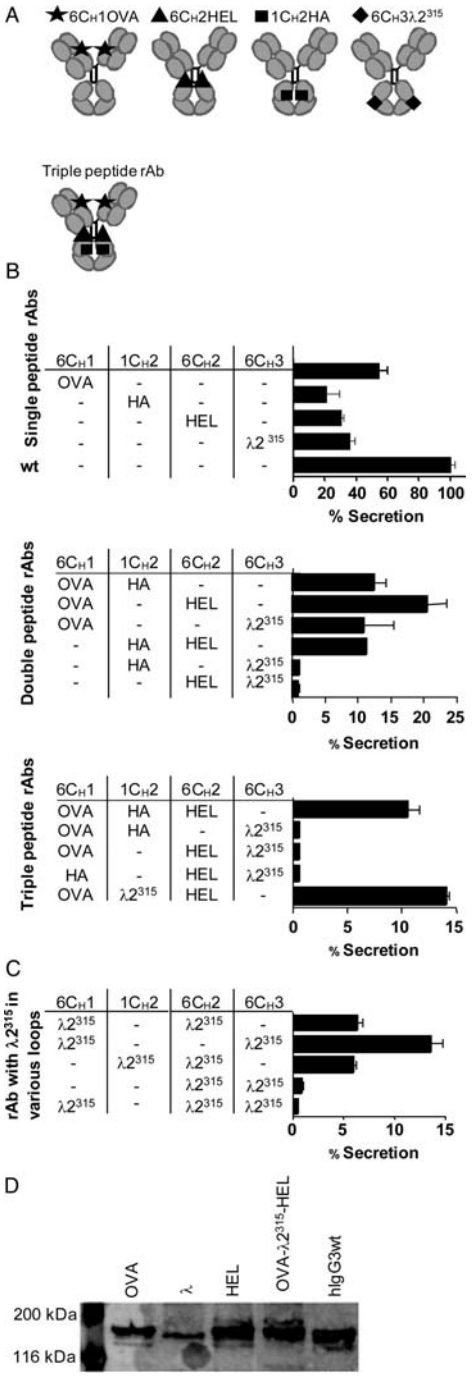

Fig. 2. Multiple epitope insertions in mIgD-specific rAbs, and maintenance of secretion. (**A**) Four loops, loop 6 in C_H1, loop 1 and 6 in C_H2 and loop 6 in C_H3 of IgG, were substituted with OVA, HA, HEL and $\lambda2^{315}$ peptides as indicated. (**B**) Level of IgG secretion compared with transfectants producing wt IgG, is indicated below each singly substituted rAb (mean ± standard deviation). Four loops, 6 C_H1, 1 C_H2, 6 C_H2 and 6 C_H3 in IgG, were used for simultaneous substitution with up to three peptides as indicated. Secretion by transfectants of double- and triple-peptide rAb as % of wt, determined by an IgG-specific ELISA, is shown. Error bars indicate SD of triplicates. (**C**) Multiple insertions of the $\lambda2^{315}$-peptide per IgG molecule, as indicated. Secretion by transfectants of double- and triple $\lambda2^{315}$-peptide IgG3 as % of wt. (**D**) Western blot of ammonium sulphate precipitated rAb with a hIgG3 hinge-specific Ab (HP-6050).

concentrations of rAbs were adjusted according to the results from the ELISAs and the western blot, to ensure that close to identical amounts of rAbs were tested in T-cell proliferation assays.

To detect rAb-induced T-cell proliferation, LN T-cells from DO11.10 TCR transgenic mice were used. These cells recognize the OVA peptide in complex with MHC class II I-A^d. Likewise, the $\lambda2^{315}$ peptide in complex with MHC class II I-E^d were recognized by LN T-cells from mice transgenic for the TCR of the $CD4^+$ T-cell clone 4B2A1. Furthermore, the T-cell hybridoma 3A9 was employed which recognizes the HEL peptide in complex with MHC class II I-A^k. In each case, titrated amounts of single- or multi-epitope rAb were mixed with BALB/c or C3H spleen cells and specific T-cells. Proliferation of LN cells was measured as incorporation of [^{3}H] dThd, while IL-2 secretion by activated 3A9 hybridoma cells was measured by incorporation of [^{3}H] dThd into proliferating IL-2 dependent CTLL-2 cells (Fig. 3).

The dose–response curves (Fig. 3A–C) illustrate that triple-epitope Abs are equal or better than single-epitope rAbs in their ability to stimulate specific T-cells. These results indicate that the loading of an Ab with multiple immunogenic peptides is feasible, and that peptide excision, presentation and the subsequent T-cell activation induced by the individual peptides is not reduced by the presence of the additional epitope substitutions.

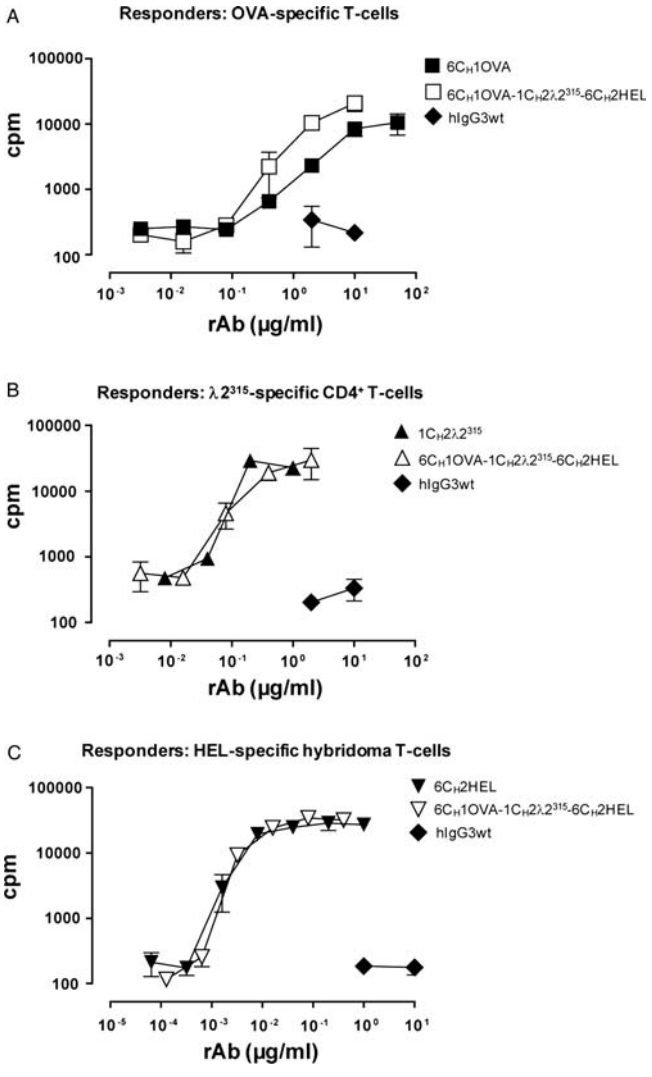

Fig. 3. Ability of anti-mIgD rAb with one or three peptide-substitutions to induce proliferation of specific $CD4^+$ T-cells. Irradiated BALB/c (**A** and **B**) or C3H (**C**) spleen cells were mixed with the indicated rAb, or wt IgG and specific T-cells (OVA: D011.10 cells; $\lambda2^{315}$: 4B2A1 cells; HEL: 3A9 cells). Activation was measured in *A* and *B* as incorporation of ^{3}[H]dThd in T-cells, in *C* as ability of supernatants to support proliferation of IL-2 dependent CTLL-2 cells (^{3}[H]dThd incorporation). Error bars indicate SD of triplicates. The graphs are derived from one representative experiment.

Mouse CD40-specific rAbs enhance in vitro *proliferation of antigen-specific $CD4^+$ T-cells*

The above experiments were designed for testing of multiple loop replacements *in vitro*. To prepare for *in vivo* studies, the mIgD-specific V regions were replaced with V regions conferring anti-mCD40 specificity and rAbs were successfully produced as above (Rolink *et al.*, 1996; Schjetne *et al.*, 2007).

Specificity was demonstrated using recombinant mCD40/Fc chimeric Ab or BSA-NIP as antigens in ELISA. As shown in Fig. 4A, all the anti-mCD40 rAbs bound to the capture Ab, but not to control BSA-NIP. The converse was true for anti-NIP rAb used as non-targeted control in the cellular assays (see below).

In a first set of experiments, we compared the ability of mCD40 specific rAbs and non-targeted rAbs with OVA peptide in loop 6 C_H1 to induce *in vitro* T-cell proliferation.

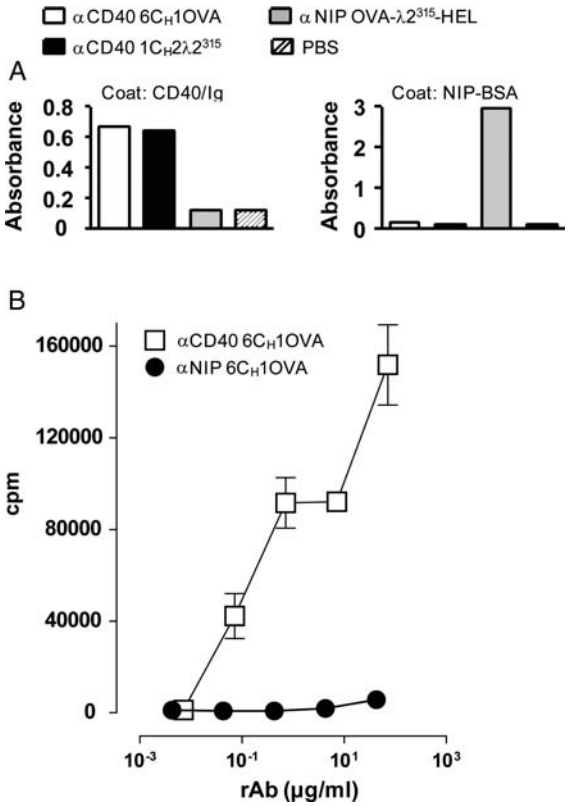

Fig. 4. Specificity and *in vitro* antigenicity of αmCD40 rAbs. (**A**) Binding of mCD40-specific or NIP-specific rAbs to mCD40-Ig fusion protein (left) or BSA-NIP (right), in ELISA. (**B**) Proliferation of OVA-specific D011.10 $CD4^+$ T-cells in presence of irradiated spleen APC and mCD40-specific or NIP-specific rAbs with the OVA-peptide loaded in loop 6 C_H1.

BALB/c spleen cells were used as APCs and LN cells from DO11.10 mice as responder cells. As illustrated by the dose–response curves in Fig. 4B, the mCD40 targeted rAbs were $>10^3$ more efficient than the non-targeted control.

Targeting of single- and triple-peptide containing rAb to mCD40 induces an antigen-specific Th1 response in mice

Given the finding that the rAbs stimulated specific T-cells *in vitro* (Fig. 4), we wanted to investigate whether they could induce antigen-specific T-cell responses *in vivo*. We have previously shown that CD40-specific proteins with the $\lambda 2^{315}$ epitope induce T-cell responses (Schjetne *et al.*, 2007). As $\lambda 2^{315}$ may be considered a relatively weak T-cell antigen (Bogen *et al.*, 1986), the response elicited in normal mice was minimal. With mice adoptively transferred with LN-cells from $\lambda 2^{315}$-specific transgenic mice, the proliferative responses of specific T-cells were high (Schjetne *et al.*, 2007). In the present study, we examined responses of non-transgenic OVA- and HEL-specific $CD4^+$ T-cells in normal mice.

We took advantage of the finding that injection of DNA L and H chain encoding genes, combined with electroporation, results in secretion of functional Abs (Tjelle *et al.*, 2004). Both single- and triple-epitope mCD40-specific rAbs were delivered as DNA into the muscle (i.m.) or into the dermis (i.d.). Plasmids encoding mGM-CSF and mMIP-1α were delivered together with the rAbs in each case in order to recruit APC and promote maturation (McKay *et al.*, 2004).

C3H mice were immunized i.m. with plasmids encoding anti-mCD40 rAb with single (HEL) or triple (OVA-λ-HEL) peptides. Splenocytes were harvested after 19 days, and responded, however, weakly, to HEL peptide. A mixed Th1/Th2 response was observed as the single- and triple-peptide rAb enhanced the number of HEL-specific IFNγ- and IL-4-producing T-cells (Fig. 5A and data not shown). Plasmids encoding mGM-CSF and mMIP-1α or NaCl did not elicit specific T-cell responses following i.m. delivery in BALB/c (data not shown). In another set of experiments, BALB/c mice were immunized i.d. with plasmids encoding anti-mCD40 rAb containing single (OVA) or triple (HEL-λ-OVA) epitopes and splenocytes and lymph node cells were harvested after 21 days. Mice given NaCl and mice given mGM-CSF and mMIP-1α did not show peptide-specific responses. By contrast, plasmids encoding anti-mCD40 rAb containing single (OVA) or triple (HEL-λ-OVA) elicited significant peptide-specific Th1 responses (Fig. 5B). The results taken together suggest that the Ab format can be utilized for delivery of single- and triple epitopes and induce antigen-specific responses *in vivo*.

Discussion

We have previously constructed APC-specific rAbs, so-called 'Troybodies', with single T-cell epitopes replacing loops between β-strands in their C_H domains. For vaccination purposes, however, it is often desirable to generate a broad immune response induced by several different sequences. This could be achieved by the production and use of mixes of rAb that carry different foreign sequences, or by the design of rAb that carry more than one foreign sequence. The latter approach would avoid competition for the target on APC and would be a more simple vaccine formulation. We have therefore explored this possibility.

We have recently shown that many but not all loops in all three C_H domains can be substituted with the aa 89–105 $\lambda 2^{315}$ T-cell epitope, with maintenance of Ab secretion from

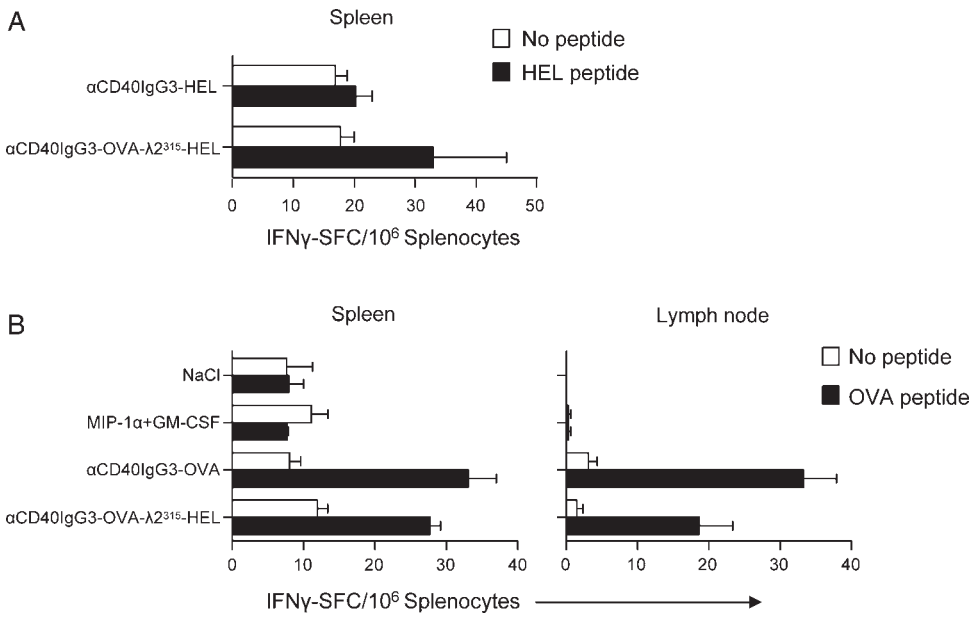

Fig. 5. CD40-targeted rAbs with peptide substitutions, delivered as naked DNA, induce antigen specific T-cell responses *in vivo*. (**A**) C3H mice were immunized i.m. with plasmids encoding mCD40-specific rAbs loaded with one (HEL) or three (HEL-λ-OVA) antigenic peptides, together with mGM-CSF and mMIP-1α. After 19 days, spleens were harvested and the splenocytes cultivated with HEL peptide in an IFN-γ-specific ELISPOT assay. Mean values are presented with error bars indicating SEM. Four mice were included per group. (**B**) BALB/c mice were immunized i.d. with plasmids encoding mCD40-specific rAbs with one (OVA) or three (HEL-λ-OVA) antigenic peptides, mGM-CSF, and mMIP-1α. After 21 days, harvested splenocytes and lymph node cells were cultivated with OVA peptide in an IFN-γ-specific ELISPOT assay. Mean values are presented with error bars indicating SEM. Four mice were included per group.

producing cells and presentation of the grafted T-cell epitope after processing in targeted APC (Flobakk *et al.*, 2008). More specifically, from a total of 18 different rAb, 8 were both secreted well and found to induce T-cell activation. These had the peptide in either of loops 6 C_H1, all in C_H2 or 6 C_H3.

Introduction of multiple epitopes, either in different units (Fab or Fc), different domains or within the same domain, would greatly benefit vaccine design, but could adversely affect Ab stability and rAb secretion from producing cells as well as intracellular processing. To test the feasibility of multi-epitope insertion into a single IgG, we selected four loops, namely loop 6 in all three C_H domains as well as loop 1 in C_H2 for substitution with four different T-cell epitopes, $\lambda2^{315}$, HA, HEL and OVA. The four chosen loops are solvent exposed, located towards the outer edge of their respective domains, and do not participate in interdomain interactions. Moreover, these four loops tolerated single insertion of the $\lambda2^{315}$ peptide as judged by secretion of rAb.

We substituted loop 6 C_H1 with the OVA peptide, loop 1 C_H2 with the HA peptide and loop 6 C_H2 with the HEL peptide. Secretion of all these singly substituted rAb by transfectants was compared with that of wt IgG, and all were secreted, but less efficiently than the wt. This is consistent with previous findings, where the $\lambda2^{315}$ peptide was substituted in the same locations (Flobakk *et al.*, 2008). The $\lambda2^{315}$ peptide in its original position is a complementary-determining region loop flanked by β-strands, the OVA peptide is mostly a β-ladder (Stein *et al.*, 1991), the HEL peptide in its native position has three turns and two β-strands (Maenaka *et al.*, 1995), while the HA peptide has an unknown structure. Compared with the $\lambda2^{315}$ peptide, either of the latter non-Ab peptides most likely introduces stronger tension on the domain structure, which might reduce stability and affect secretion of whole rAb. However, level of secretion seems to be more influenced by localization of loop replacement than by the sequence of the epitope.

Table II. Summary of results

rAb variant	Secretion from transfected cells[a]	T-cell proliferation *in vitro*[b]	T-cell response *in vivo*[c]
Single peptide rAb			
$6C_H1$	+++	Yes	Yes
$1C_H2$	++	Yes	
$6C_H2$	++	Yes	Yes
$6C_H3$	++		
Double peptide rAb			
$6C_H1$ and $1C_H2$	++		
$6C_H1$ and $6C_H2$	++		
$6C_H1$ and $6C_H3$	++		
$1C_H2$ and $6C_H2$	+		
$1C_H2$ and $6C_H3$	+		
$6C_H2$ and $6C_H3$	−		
Triple peptide rAb			
$6C_H1$, $1C_H2$ and $6C_H2$	++	Yes	Yes
$6C_H1$, $1C_H2$ and $6C_H3$	−		
$6C_H1$, $6C_H2$ and $6C_H3$	−		

[a]Secretion levels: +++ indicates secretion levels at 50–100%; ++ and + indicates 10–49 and 1–9%, respectively, and − indicates <1% of wt level.
[b]Determined by incorporation of ^{3}[H]dThd.
[c]Based on number of peptide-responsive T-cells determined by ELISPOT assays on splenocytes harvested from immunized mice.

As for the feasibility of multi-epitope insertions into a single rAb, several conclusions can be made. First, 6 C_H3 loop replacement was poorly compatible with simultaneous loop replacements in C_H2 (Table II). A reason for this might be that the two chains of the Fc fragment are connected through a conserved C_H3–C_H3 interface (Dall'Acqua *et al.*, 1998), and the accumulated tension caused by multiple loop substitutions most likely disrupts the C_H3–C_H3 interface. Thus, we avoided C_H3 insertions in our multi-epitope rAb. Second, the destabilizing effect of the substitutions was additive, even if the peptides were located in different domains and different parts, such as Fab (C_H1) and Fc (C_H2 and C_H3). This rule of thumb seems general and not dependent on specific peptide sequences used for loop replacements. Thus, we produced secretion competent rAbs that carried three epitopes, one in the Fab fragment, and two in C_H2 in the Fc fragment.

For APC targeting and peptide loading purposes, target cell killing should be avoided, and lack of Ab effector functions might well be an advantage. Interesting in this respect, is the fact that rAbs with single epitopes in either loop of C_H2 do not bind FcγRIIA (15). We here find that the C_H2 domain has the potential to carry at least two peptides, which could to a large extent abrogate effector functions of rAb such as complement activation and antibody-dependent cell cytoxicity.

For a rAb loaded with multiple antigenic peptides to be beneficial as a vaccine reagent, every loaded peptide should be presented by APC from its position within the APC-specific rAb. Therefore, we performed T-cell proliferation assays to evaluate whether and to what extent the triple-peptide rAb induced activation of specific T-cells. The results were compared with those obtained for three singly substituted peptide rAb. We found that all three peptides within the same multi-epitope rAb were presented to T-cells and induced T-cell activation at least as efficiently as did single-epitope rAb. It should be noted that IgD-targeting of singly substituted rAbs enhanced presentation $\times10^3$ compared with non-targeted NIP-specific equivalents (Lunde *et al.*, 1999; Rasmussen *et al.*, 2001). As IgD-specific multi- and singly substituted rAb had similar dose–response curves, an enhancement of T-cell responses by 10^3, obtained by targeting to IgD, is likely, also for the multi-epitope rAbs. These results showed that multi-insertion does not diminish the presentation of each epitope, which is important for vaccine design.

The target molecule on the APC must be internalized for efficient processing, peptide loading and presentation to specific T-cells. Furthermore, binding should preferably induce maturation of the target APC. The rAb used in the present *in vitro* assays are specific for IgD on mouse B-cells. Although targeting to this receptor *in vitro* induces strong T-cell responses, IgD is not an optimal target *in vivo*. However, the discovery of Ag uptake receptors on DCs, such as DEC-205, DC-SIGN, CD40 and others (reviewed in Tacken *et al.*, 2007), has enabled the engineering of anti-receptor rAbs which specifically target DCs. Here we chose CD40 as target in *in vivo* studies. CD40 is a co-stimulatory molecule which belongs to the tumour necrosis factor receptor family and is expressed on many cells including monocytes, DCs, epithelial cells and endothelial cells (van Kooten and Banchereau, 2000). On DCs, the ligation of CD40 induces cellular

maturation and activation as manifested by increased surface expression of co-stimulatory and MHC molecules, production of pro-inflammatory cytokines such as IL-12 and enhanced T-cell activation (Cella *et al.*, 1996). We produced a series of plasmids with genes encoding CD40-specific single- and triple-peptide loaded rAbs. The rAbs were tested in *in vitro* assays, and we found that targeting of rAb to CD40 on APCs increased T-cell responses by $>10^3$ compared with non-targeted (NIP-specific) controls.

The plasmids were then delivered to two different mouse strains, BALB/c and C3H as naked DNA. Delivery of the DNA vaccines into mice caused weak $CD4^+$ T-cell responses, which nevertheless could be detected by ELISPOT assays. Future experiments will unravel how the responses may be enhanced.

Acknowledgements

We are grateful to Dr Keith Thompson for providing and dissecting C3H mice, and Peter O. Hofgaard, Hilde Omholt and Elisabeth Vikse for technical assistance.

Funding

The work was supported by the Norwegian Cancer Society (I.B.R. and M.F.) and the Research Council of Norway (I.Ø., L.S.H. and E.L.).

References

Aaberge,I.S., Michaelsen,T.E., Rolstad,A.K., Groeng,E.C., Solberg,P. and Lovik,M. (1992) *Infect. Immun.*, **60**, 4146–4153.
Allen,P.M. and Unanue,E.R. (1984) *J. Immunol.*, **132**, 1077–1079.
Baier,G., Baier-Bitterlich,G., Looney,D.J. and Altman,A. (1995) *J. Virol.*, **69**, 2357–2365.
Berntzen,G., Lunde,E., Flobakk,M., Andersen,J.T., Lauvrak,V. and Sandlie,I. (2005) *J. Immunol. Methods*, **298**, 93–104.
Bogen,B., Gleditsch,L., Weiss,S. and Dembic,Z. (1992) *Eur. J. Immunol.*, **22**, 703–709.
Bogen,B., Malissen,B. and Haas,W. (1986) *Eur. J. Immunol.*, **16**, 1373–1378.
Carayanniotis,G. and Barber,B.H. (1987) *Nature*, **327**, 59–61.
Cella,M., Scheidegger,D., Palmer-Lehmann,K., Lane,P., Lanzavecchia,A. and Alber,G. (1996) *J. Exp. Med.*, **184**, 747–752.
Dall'Acqua,W., Simon,A.L., Mulkerrin,M.G. and Carter,P. (1998) *Biochemistry*, **37**, 9266–9273.
Eidem,J.K., Rasmussen,I.B., Lunde,E., Gregers,T.F., Rees,A.R., Bogen,B. and Sandlie,I. (2000) *J. Immunol. Methods*, **245**, 119–131.
Flobakk,M., Rasmussen,I.B., Lunde,E., Frigstad,T., Berntzen,G., Michaelsen,T.E., Bogen,B. and Sandlie,I. (2008) *J. Immunol.*, **181**, 7062–7072.
Frishman,D. and Argos,P. (1995) *Proteins*, **23**, 566–579.
Hawiger,D., Inaba,K., Dorsett,Y., Guo,M., Mahnke,K., Rivera,M., Ravetch,J.V., Steinman,R.M. and Nussenzweig,M.C. (2001) *J. Exp. Med.*, **194**, 769–779.
Huck,S., Fort,P., Crawford,D.H., Lefranc,M.P. and Lefranc,G. (1986) *Nucleic Acids Res.*, **14**, 1779–1789.
Loset,G.A., Roux,K.H., Zhu,P., Michaelsen,T.E. and Sandlie,I. (2004) *J. Immunol.*, **172**, 2925–2934.
Lunde,E., Bogen,B. and Sandlie,I. (1997) *Mol. Immunol.*, **34**, 1167–1176.
Lunde,E., Munthe,L.A., Vabo,A., Sandlie,I. and Bogen,B. (1999) *Nat. Biotechnol.*, **17**, 670–675.
Lunde,E., Western,K.H., Rasmussen,I.B., Sandlie,I. and Bogen,B. (2002) *J. Immunol.*, **168**, 2154–2162.
Maenaka,K., Matsushima,M., Song,H., Sunada,F., Watanabe,K. and Kumagai,I. (1995) *J. Mol. Biol.*, **247**, 281–293.
McKay,P.F., Barouch,D.H., Santra,S., Sumida,S.M., Jackson,S.S., Gorgone,D.A., Lifton,M.A. and Letvin,N.L. (2004) *Eur. J. Immunol.*, **34**, 1011–1020.
Murphy,K.M., Heimberger,A.B. and Loh,D.Y. (1990) *Science*, **250**, 1720–1723.
Rasmussen,I.B., Lunde,E., Michaelsen,T.E., Bogen,B. and Sandlie,I. (2001) *Proc. Natl Acad. Sci. USA*, **98**, 10296–10301.
Rolink,A., Melchers,F. and Andersson,J. (1996) *Immunity*, **5**, 319–330.
Roos,A.K., Moreno,S., Leder,C., Pavlenko,M., King,A. and Pisa,P. (2006) *Mol. Ther.*, **13**, 320–327.
Saphire,E.O., Parren,P.W., Pantophlet,R., *et al.* (2001) *Science*, **293**, 1155–1159.
Schjetne,K.W., Fredriksen,A.B. and Bogen,B. (2007) *J. Immunol.*, **178**, 4169–4176.
Schjetne,K.W., Thommesen,J.E., Fredriksen,A.B., Lunde,E., Sandlie,I. and Bogen,B. (2005) *Eur. J. Immunol.*, **35**, 3142–3152.
Snider,D.P. and Segal,D.M. (1987) *J. Immunol.*, **139**, 1609–1616.
Stein,P.E., Leslie,A.G., Finch,J.T. and Carrell,R.W. (1991) *J. Mol. Biol.*, **221**, 941–959.
Tacken,P.J., de Vries,I.J., Torensma,R. and Figdor,C.G. (2007) *Nat. Rev. Immunol.*, **7**, 790–802.
Tite,J.P. and Janeway,C.A, Jr. (1984) *Eur. J. Immunol.*, **14**, 878–886.
Tjelle,T.E., Corthay,A., Lunde,E., Sandlie,I., Michaelsen,T.E., Mathiesen,I. and Bogen,B. (2004) *Mol. Ther.*, **9**, 328–336.
Tunheim,G., Schjetne,K.W., Fredriksen,A.B., Sandlie,I. and Bogen,B. (2005) *J. Leukoc. Biol.*, **77**, 303–310.
van Kooten,C. and Banchereau,J. (2000) *J. Leukoc. Biol.*, **67**, 2–17.