Full title: Antigen targeting to MHC class II with SMEZ-2 M1, a superantigen-based vaccine carrier

Running Title: Antigen targeting to MHC class II

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Abstract

SMEZ-2 is a streptococcal superantigen that primarily stimulates human T cells bearing $\mathrm{V}_\beta 8$ and mouse T cells bearing $\mathrm{V}_\beta 11$. Mutagenesis of T cell receptor (TCR) binding residues (W75L.K182Q.D42C) produced a mutant called M1 that was $>10^5$-fold less active towards human peripheral blood lymphocytes and splenocytes from transgenic mice that express human CD4 and either human HLA-DR3-DQ2 or HLA-DR4-DQ8. Similarly, cytokine production in response to M1 in lymphocyte culture was rendered undetectable and no change was observed in the frequency of $\mathrm{V}_\beta 11$ bearing T cells in mice receiving M1. M1 toxoid was tested as a potential vaccine conjugate. Vaccination with 1-10 $\mu$g M1:ovalbumin resulted in more rapid and quantitatively higher levels of anti-ovalbumin IgG, with endpoint titers 1,000-10,000 fold greater than animals immunised with unconjugated ovalbumin. Substantially higher levels of anti-ovalbumin IgG were observed in mice transgenic for human MHC class II. Substitution of M1 with an MHC class II binding mutant (DM) eliminated enhanced immunity, suggesting that M1 enhanced the delivery of antigen via MHC class II+ antigen presenting cells that predominate within lymphoid tissue. Immunisation of animals with M1: ovalbumin $\text{323-339}$ peptide conjugate generated 100-fold higher levels of anti-peptide IgG compared to animals immunized with peptide alone. Coupling of a TCR defective superantigen toxoid presents a new strategy for conjugate vaccines with the additional benefit of targeted delivery to MHC class II bearing cells.
Introduction

Superantigens from *S. pyogenes* are a structurally conserved family of proteins (16) with the common ability to cross-link MHC class II outside the peptide binding domain and the TCR causing massive T cell proliferation and systemic cytokine mediated shock (11, 25).

Superantigens are highly mitogenic to T cells from many species but sensitivity and toxicity is typically reduced by several orders of magnitude in mice compared to man (34). To better mimic the extreme sensitivity of human to superantigens, mice transgenic for human HLA genes have been used that recapitulate many of the symptoms of superantigen-mediated human toxinoisis (10, 36, 40, 47).

Bacterial superantigens have evolved over time to become exquisitely specific for components of the human immune system, raising the possibility that with appropriate modification they could be used as the basis for immune-targeting therapeutics in the same way that tetanus toxoid and other toxoids have been used with great success in conjugate vaccines (1, 15). The specificity of superantigens for MHC class II is of particular interest, as MHC class II is a molecule expressed on professional antigen presenting cells such as Dendritic cells, B cells and macrophages. MHC class II is central to the initiation of antigen-specific immune responses by CD4+ T helper cells and the subsequent development of humoral and cellular immunity. Immature antigen presenting cells, particularly Dendritic cells, continually cycle MHC class II between the cell membrane and endosomes, where antigen sampling determines whether the endosome contents go on to late endosomes/multivesicular bodies, lysosomes or are recycled (44). Targeting antigens directly to the MHC class II pathway enables delivery of material directly into the antigen
processing pathway of antigen presenting cells. This approach has been tested using antibody specific for MHC class II(6) or with ‘Troybodies’, recombinant MHC class II antibody which has been modified to incorporate MHC class II-restricted T cell epitopes (30).

To establish whether a TCR defective superantigen toxoid might enhance immunogenicity of coupled antigens by MHC class II targeting, we modified the most potent superantigen from *S. pyogenes*, Streptococcal Mitogenic Exotoxin Z (SMEZ-2) (34) to produce a mutant (M1). The M1 protein targets all subsets of Dendritic Cells in the mouse and is highly effective at eliciting an enhanced cellular response to coupled antigen both *in vitro* and *in vivo* when administered in conjunction with the adjuvant α-galactosylceramide(13). We have thoroughly characterised the M1 protein to confirm that it is defective in TCR binding and devoid of *in vitro* and *in vivo* superantigen activity. *In vivo* studies designed to further assess the safety and utility of M1 *in vivo* demonstrated that conjugating antigen to M1 stimulated significantly enhanced antibody responses in both wildtype and sensitive ‘humanised’ transgenic mice expressing human HLA-DR3-DQ2 or HLA-DR4-DQ8.
Materials and Methods

Superantigens

Cloning and sequencing of the smez-2 gene encoding SMEZ-2 from *S. pyogenes* strain 2035 has been described elsewhere (34). Mutations in the TCR or MHC class II binding sites were introduced by site-directed mutagenesis using pGEX utility primers (5'-TCAGAGGGTTTTCACCGTC-3' and 5'ACCATCCTCCAAAATCGG-3') and sets of mis-matched overlapping primers in a two-step overlap PCR (17) (Table 1). Overlap PCR products were cloned into the pGEX-3C expression vector using the restriction enzyme sites included in the pGEX utility primers. Genes were sequenced and expressed in *Escherichia coli* DH5α cells as GST fusion proteins. Cultures were grown at 28°C in terrific broth, and induced for 3–4 h after adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Two-step purification involved affinity chromatography using glutathione agarose, cleavage with 3C protease, and finally cation exchange chromatography using carboxy methyl sepharose (Amersham Pharmacia, UK) as described previously (34).

Site-specific mutagenesis of amino acid residues critical to TCR binding in SMEZ-2 were inferred by structural analogy to similar positions in the structure of SEC3 co-crystallized with TCR (26). A combination of two amino acid mutants (W75L, K182Q) showed greater than five orders of magnitude less potency in human T cell stimulation assays compared to the parent molecule. These were combined with a third mutation that substituted position D42 with a cysteine. This residue was chosen because it is highly solvent exposed and central to the TCR binding site, making it ideal for direct coupling of peptides and proteins.
well away from the MHC class II binding located on the opposite face of the SMEZ-2 molecule (Figure 1).

Expression and Purification of Vβ8.1/Vα1.2 TCR and HLA-DR1

The Drosophila melanogaster S2 cell line was transfected with calcium phosphate/DNA crystals using 10 µg pRmHa-3 Vβ8 and pRmHa-3 Vα1.2 DNA each (5) and 1 µg of pBS-hsPuro DNA (from Dr. Klaus Karjaleinen, Basel Institute for Immunology, Switzerland). Resistant cells were selected with 100 µg/ml Puromycin (Sigma Aldrich, Sydney, Australia). Expression of the soluble, native Vβ8 TCR was confirmed by intracellular FACS analysis and by ELISA. After induction of expression with 1 mM copper sulfate, S2 cell supernatants were concentrated with 40% v/v saturated (NH₄)₂SO₄. After dialysis into PBS pH 8.0, TCR was purified using W4F.5B Ab-affinity chromatography (2), and concentrated to 1 mg/ml. S2 cells expressing soluble HLA-DR1 were a gift from Dr. Dennis Zaller (Merck Research Laboratories, Rahway, NJ). The HLA-DR1 heterodimer was produced as previously described (46), and loaded with the FluHA 308-319 peptide (Chiron Mimotopes, Sydney, Australia).

Biacore Analysis

The binding of superantigens to human Vβ8.1 TCR and HLA-DR1 was measured by surface plasmon resonance using a Biacore 2000 system (Biacore AB, Uppsala, Sweden). SMEZ-2 wildtype and mutants were immobilized on the dextran matrix of CM5 sensor chips with an Amine Coupling kit (Biacore). All superantigens were coupled in 10 mM sodium acetate buffer, optimal pre-concentrations were 10 µg/ml of SMEZ-2 wildtype at 6
pH 4.5, 15 µg/ml of M1 and 10 µg/ml of DM at pH 4.0, and 20 µg/ml of M2 at pH 5.0. 10 mM HEPES, 0.15 M NaCl, and 0.005 % v/v P20 surfactant (HBS-P, degassed and filtered) was used as a continuous running buffer for all binding studies. The Vβ8/α1 TCR and HLA-DR1 (loaded with FluHA 308-319 peptide) were diluted in running buffer. Binding was monitored at 25°C for 3 min at a constant flow rate of 20 µl/min. The activity of the sensor surface was analyzed using a triplicate injection of purified Vβ8/α1 TCR at 1 mg/ml. Subsequent binding experiments used a two-fold dilution series of TCR from 500 µg/ml to 7.8 µg/ml. Binding of HLA-DR1 to SMEZ-2 wildtype and mutants was performed at 10 and 1 µM in running buffer. Sensor chip surfaces were regenerated using a triplicate injection pulse of 10 mM HCl for TCR and 50 mM H₃PO₄ for DRI. Sensorgram data were analyzed with the BIAevaluation software version 3.1.1 (Biacore): Inline reference subtraction was used during binding studies that removed background/bulk shift responses producing binding curves of response difference between reference and ligand flow cell. Overlay plots were produced by alignment of time and response axes for the curves.

Proteins and peptides

Ovalbumin (OVA) and OVA_{323-339} was purchased from Sigma Aldrich. The OVA_{323-339} peptide (37, 38) was custom synthesised to >95% purity and included a cysteine at the N-terminus to permit coupling of the peptide to the M1 carrier. Proteins were labelled using Fluorescein isothiocyanate N-hydroxysuccinimide ester (Sigma Aldrich) or a Cy5 bifunctional reactive dye according to the manufacturer’s instructions (Amersham Pharmacia, UK).
Mice
Female C57BL/6 and BALB/c mice 6 - 8 weeks old were purchased from the VJU, University of Auckland, New Zealand. IAE-/DR4-DQ8/hCD4(7) and IAE-/DR3-DQ2/hCD4(8) (shortened to DR4-DQ8 and DR3-DQ2 hereafter) transgenic mice were kindly supplied by Professor James McCluskey, University of Melbourne, Australia.

Immunisation experiments with transgenic mice and controls used age-matched male and female mice aged ~ 4 months. C57BL/6 control mice for the transgenic studies were from the VJU or generously provided by The Malaghan Institute, Wellington, New Zealand.

Animals were housed and cared for under SPF conditions in accordance with The Animal Welfare Act (1999) and institutional guidelines provided by the University of Auckland Animal Ethics Committee, which reviewed and approved these experiments.

For vaccination studies, coupled protein or peptide antigens were diluted in sterile PBS, emulsified 1:1 in Incomplete Freund’s Adjuvant (IFA; Sigma) and delivered subcutaneously into the nape of the neck. Blood was sampled from the tail vein at regular intervals, collected in Microvette 500 serum-gel tubes (Sarstedt, Germany), processed and stored at -20º C.

Cell culture
Human PBMC and murine splenocytes were prepared as outlined elsewhere(45). For proliferation assays, 1 x 10^5 PBMC and 2 x 10^5 splenocytes/well were cultured in serial dilutions of recombinant superantigens and proliferation quantified by adding 0.25 μCi [³H] thymidine (Amersham, USA) per well on day 3. Approximately 16 hours later cells were
harvested onto glass filter mats and [3H] thymidine uptake quantified using a Beta scintillation counter (all from Wallac OY, Finland).

The acute monocytic leukemia cell line THP-1 (ATCC, USA) was maintained as per ATCC recommendations and activated by combining 0.5 x 10⁶ cells/ml and 200 U/ml IFN-γ for 2 days to up-regulate MHC class II expression. Cells were incubated with 1 μM of FITC conjugated protein for 20 min at 4°C to measure MHC class II binding by flow cytometry. Dendritic cells were derived from the bone marrow of C56BL/6 mice as described by Inaba et al., (22) and semi-adherent cells used on day 6. All cell culture materials were purchased from Invitrogen, New Zealand.

Microscopy

IFN-γ activated THP-1 cells were stained with 1 μM M1-FITC for 20 minutes at 4°C, washed in PBS and incubated at 37°C for 0, 30, or 120 min in culture medium. Cells were then washed in PBS, fixed in 4% paraformaldehyde (5 min, 37°C) and mounted onto poly-L-lysine coated glass slides. Images were captured on a Nikon E600 fluorescence microscope. Dendritic cells were triple-stained in PBS/ 0.5% BSA/ 5% normal rabbit serum/ 5% normal mouse serum with anti-mouse I-A/I-E, N418-FITC, and M1-Cy5. Cells were washed twice in PBS and incubated for 10 min at 4°C with streptavidin-Cy3 (Amersham Pharmacia, UK). Dendritic cells were then either left on ice or incubated at 37°C for 0.5 h prior to fixation with 4% paraformaldehyde. Fixed cells were mounted on poly-L-lysine coated glass slides with fluorescent mounting media (DAKO, Germany). Images were captured on a Leica TC SP2 confocal microscope.
In vivo localization of iodinated Superantigens.

20 μg recombinant superantigens were radioiodinated by the chloramine T method as previously described (27). Excess $^{125}$I was removed from labeled superantigen by size exclusion chromatography using Sephadex G25 columns. $^{125}$I-superantigen was mixed with cold superantigen to a final concentration of 10 μCi/ml. 1 μCi/mouse corresponding to 10 μg or 0.4 nmol total superantigen was injected subcutaneously into C57BL/6 mice. Lymph nodes, spleen, lung, liver, and kidney were collected after 24 h and measured in an auto gamma counter, counts for organs were normalized with respect to weight.

Cytokine assays

Human PBMCs ($1 \times 10^5$) from five healthy volunteers were stimulated with 100 ng/ml recombinant superantigen and supernatants harvested after 72 h. Cytokine levels were determined by Human Cytokine LINCOplex Kit (LINCO Research, USA). Evaluation of the data was performed by a 5-parameter logistic curve-fitting method for calculating the cytokine concentration using the Luminex software 100 IS.

Mice were given a 10 μg dose of SMEZ-2 wildtype, M1 or PBS via the intraperitoneal route to determine whether the SMEZ-2 M1 carrier elicited a proinflammatory cytokine response in vivo. After 3 hours blood was sampled from the tail vein and processed for serum. Serum was diluted 1/3 for assay with a Mouse Inflammation Kit (BD Biosciences) and cytokine concentrations determined by a 4-parameter logistic curve-fitting method using FCAP Array Software (BD Biosciences).
Cell associated IL-4 and IFN-γ production was measured with an ELISPOT set (BD Biosciences) as per the manufacturer’s instructions. Splenocytes were cultured in triplicate at 1 and 2 x 10^5 cells/well for 16 hours in the presence of 100 μg/ml OVA. Control wells were set up in parallel and stimulated with 0.5 μg/ml Concanavalin A (Sigma) or medium alone to confirm that the number of functional cells per well was equivalent across all individual mice and to establish background numbers of cytokine secreting cells. The number of Spot Forming Cells (SFC) per well was determined manually by light microscopy (20x magnification). The medium control values were subtracted from OVA stimulated values and used to calculate the number of Spot Forming Cells (SFCs) per 10^6 splenocytes.

Flow cytometry

Cells were counted, suspended at 10^6 cells/tube and labelled for 20 min on ice with directly conjugated antibodies to cell surface markers (BD Biosciences). Labelled cells were washed and resuspended in PBS-2% foetal bovine serum/0.02% NaN₃ supplemented with 0.5 μg/ml propidium iodide (Invitrogen) to enable selection of viable cells. Cells were analysed with a FACScan or LSR II flow cytometer equipped with CellQuest or FACSDiva software (BD Biosciences).

Conjugation of peptide and proteins to carrier

Recombinant SMEZ-2 M1 and DM (0.2 mM) were reduced for 1 hour at RT with 0.1 M Dithiothreitol (DTT) and buffer exchanged into 0.1 M Tris, pH 8.0 immediately prior to conjugation to remove DTT. Synthetic peptide was solubilised in 0.1% acetic acid and conjugated to purified SMEZ-2 M1 or DM at a 10:1 molar ratio by overnight incubation.
with 200 mM Tris.HCl, pH 8; 2 μM CuSO₄ to promote disulphide bond formation between the exposed cysteine residue on the M1 carrier and cysteine present on the N-terminus of the OVA323-339 peptide. Free peptide was removed by buffer exchange through a 5 K cut-off Vivaspin concentrator (Vivascience, Germany). Conjugated peptide was concentrated by buffer exchange into 0.1 M Tris, pH 8.0, filter sterilized (0.22 μM) and stored at 4ºC, peptides remained stably coupled for some months after conjugation.

Peptide conjugation was confirmed by 17% SDS-PAGE(24) and coomassie blue staining. M1 and M1:OVA323-339 conjugates were transferred onto Nitrocellulose membrane (PALL Life Sciences) (43) and probed with serum from OVA immunised mice diluted 1:500. The membrane was stripped and re-probed with a mouse anti-M1 monoclonal antibody at 0.2 μg/ml. Both primary antibodies were detected using anti-mouse IgG:HRP (Jackson ImmunoResearch) at 0.08 μg/ml and ECL western blotting substrate (Pierce). Images were acquired using a FujiFilm LAS-3000. M1:OVA323-339 conjugates were run through a Superdex 200 5/150 GL (GE Healthcare, USA) analytical column under reducing (1 mM DTT) and non-reducing conditions. A₂₈₀ data was collected using FPLC.

OVA was stably cross-linked to SMEZ-2 M1 or DM with Sulfo-SMCC (Pierce Chemicals No. 22322). M1 and DM protein (0.2 mM) was reduced with DTT as above and DTT removed on a G10 desalting column (Bio-Rad, Australia). Sulfo-SMCC was combined with OVA at a molar ratio of 10:1 at RT for 1 hour. Free sulfo-SMCC was removed using a G10 desalting column and the derivatised OVA protein immediately incubated overnight with the reduced SMEZ-2 M1 and DM at a molar ratio of 1:1. The cross-linked proteins were
separated from uncoupled protein by FPLC size exclusion chromatography (Superdex 200 10/30 GL column, GE Pharmaceuticals, USA). High molecular weight fractions were combined and concentrated to ~2 mg/ml (20 nM) by ultrafiltration. Endotoxin was removed from the M1:OVA and DM:OVA conjugates by Triton X-114 extraction (28) and confirmed by Limulus amoebocyte lysate assay. The conjugates were sterilized through a 0.22 μM filter and stored in aliquots at 4°C.

Assessment of specific serum Ab responses

Maxisorp microtitre plates (Nunc, Denmark) were coated with 0.5 μg or 0.25 μg per well protein or peptide in PBS. The plates were blocked with 2% BSA in PBS, washed with 0.05% Tween-20 in PBS and bound antibody detected using HRP conjugated anti-mouse IgG, IgG1 (Serotec, UK) or IgG2c (Jackson ImmunoResearch, PA) and OPD substrate. Serial 2-fold dilutions of 0.1 μg per well mouse anti-OVA IgG1 Ab (OVA-14; Sigma Aldrich) or mouse anti-M1 IgG1 Ab were used as a standard in some assays. Plates were read at 490 nm and a 4-parameter fit of the standard curve produced with KC4 software (Bio-Tek Instruments, Inc). Endpoint titres were used to determine anti-OVA IgG Ab levels in transgenic and control mice, starting at a 1/100 dilution. The endpoint = (2 x A490) + (2 SD) of a baseline serum sample.
$P$ values were calculated using Prism® Version 5.02 (GraphPad Software, Inc, California). The tests applied are detailed in the figure legends. A $P$ value of 0.05 was considered to be statistically significant.
Results

TCR defective SMEZ-2 retains MHC class II binding

Surface plasmon resonance was used to compare the direct binding of SMEZ-2 and mutants M1, M2 and DM to soluble forms of human Vβ8/Vα1.2 TCR and HLA-DR1 FluHA₃₀₈-₃₁₉. SMEZ-2, M1, M2 and DM were coupled to separate dextran sensor chips at 2138, 2579, 3535 and 2232 RU respectively. Soluble hVβ8/Vα1.2 TCR binding to immobilised SMEZ-2 was characterised by fast association and slow dissociation, consistent with other studies of direct binding of soluble TCR to superantigens (26). For simplicity, only the response to the highest TCR concentration (1mg/ml) is shown (Figure 2a). At this concentration soluble Vβ8/Vα1.2 TCR bound to SMEZ-2 with 2000 RU while M1 and M2 bound with approximately 400 RU. At 250 µg/ml of TCR, binding to M1 or M2 was undetectable (not shown) while SMEZ-2 bound at 400 RU. No difference was observed for wild-type SMEZ-2, M1 or M2 binding to DR1 + FluHA₃₀₈-₃₁₉ at either 1 or 10 µM (Figure 2b).

Binding of MHC class II to the DM mutant was undetectable. Addition of 1 µM EDTA to the analyte destroyed binding of soluble DR1 + FluHA₃₀₈-₃₁₉ to SMEZ-2 and mutants confirming that binding was mediated by zinc (not shown). These studies verified the intended mutations of M1 and the independence of TCR and MHC class II binding sites on the molecule. The M1 and M2 TCR- mutants of SMEZ-2 showed comparable specificities in this assay and the M1 protein, which had 3 mutated TCR-binding residues, was selected for further investigation.
SMEZ-2 M1 binds to MHC class II and is internalised.

M1 was examined for its ability to bind to IFN-γ activated MHC class II⁺ THP-1 cells. M1-FITC bound to IFN-γ treated THP-1 cells and was effectively blocked by an excess of the parental SMEZ-2, but not by DM (Figure 2c). A low level of binding to THP-1 cells was also observed with the control protein OVA (Figure 2c). Fluorescence microscopy allowed direct visualization of surface stained THP-1 cells with M1-FITC at time zero followed by internalization after incubation at 37°C for 30 minutes (Figure 2d). By 2 hours, all M1-FITC was located within intra cellular vesicles (Figure 2d). A similar result was observed with murine C57BL/6 bone marrow derived dendritic cells (BMDC) (Figure 2e). Triple colour staining of M1-FITC, CD11c, and MHC Class II showed that M1 and MHC Class II were internalized into the same intracellular vesicle in BMDC and were superimposed within 30 minutes. This confirmed that M1 initiated and remained bound to MHC class II during internalisation into what is presumed to be the MII-C compartment (Figure 2f).

SMEZ-2 M1 does not stimulate T cell proliferation

SMEZ-2 is the most potent superantigenic mitogen towards human T cells with a calculated P₅₀ of 0.2 pg/ml (Figure 3a) (34). M1 was 10⁵-fold less active with only minimal proliferation detected at the highest concentration of 10 ng/ml (Figure 3a), while the DM mutant lacking MHC class II binding displayed no proliferative response to human PBMC at any concentration (Figure 3a). SMEZ-2 was 10⁴-fold less potent towards C57BL/6 murine splenocytes; requiring 1 ng/ml to generate a half-maximal proliferative response (Figure 3b). This reflected a reduced affinity for murine MHC class II that was...
substantially corrected in splenocytes expressing either DR3-DQ2 (Figure 3c) or DR4-DQ8 (Figure 3d). These cells required 10 pg/ml of SMEZ-2 to generate half-maximal proliferation, although the absolute cell proliferation was substantially less than in human splenocytes, perhaps reflecting a lower frequency of SMEZ-2 responsive T cells (Figure 3b). M1 and DM displayed no measureable proliferative activity toward splenocytes from C57BL/6 or C57BL/6 human class II transgenic mice (Figure 3b-d).

SMEZ-2 M1 does not stimulate cytokine responses or expansion of T cells
SMEZ-2 elicits a substantial in vitro cytokine response from human PBMC, stimulating very high levels (>2000 pg/ml) of IL-2, IL-6 and IFN-γ and moderate levels of IL-10 and TNF-α (Figure 4a). In contrast, PBMC stimulated with M1 produced only background cytokine levels confirming that TCR engagement was essential for cytokine production.

In vivo cytokine responses to SMEZ-2 were elevated >2-fold (TNF-α), >4-fold (CCL2) and >10-fold (IL-6) in DR3-DQ2 and DR4-DQ8 transgenic mice relative to the parental C57BL/6 mice, confirming the importance of human MHC class II and human CD4 in maximal cytokine production (Figure 4b-d). The highest level of all three cytokines was detected in the serum from DR4-DQ8 mice treated with SMEZ-2 (Figure 4d). Exposure of these mice to equivalent amounts of M1 did not elicit any significant cytokine response (Figures 4b-d).

SMEZ-2 activates murine T cells expressing the mVβ11 TCR, but not the mVβ8 TCR (36, 45). The proportion of Vβ11 TCR⁺ CD4⁺ and CD8⁺ T cells was thus increased 2-4 fold in
the DR3-DQ2 and DR4-DQ8 mice 3 days after exposure to SMEZ-2, while the frequency of Vβ8 TCR+ T cells remained unchanged (Figure 4e-f). In comparison, transgenic mice treated with M1 showed no detectable increase in Vβ11 TCR+ CD4 or CD8 T cells. Together these data confirmed that M1 was inactive as a T cell mitogen in human MHC class II/CD4 transgenic mice.

SMEZ-2 wild-type and M1 but not the DM are transported to the lymph node

SEA and SEB are detectable from as early as 6h in the lymph nodes and spleen after intraperitoneal injection of mice, with an increase of total DC numbers and an increase of co-stimulatory molecules on DC in the T cell areas (32, 48). To follow the in vivo trafficking of SMEZ-2 and its mutants, SMEZ-2, M1, M2 and DM were radioiodinated and injected subcutaneously into C57BL/6 mice. Tissue distribution of SMEZ-2 was determined by removing organs 24 hours after injection and counting in a gamma counter then adjusting for organ weight. Figure 5a shows that SMEZ-2 concentrated in the draining lymph node with a 4- to 8-fold higher specific level compared to spleen, kidney, lung or liver. M1 and M2 also concentrated into the draining lymph nodes, although the ratios compared to other organs were reduced to between 2- and 5-fold (Figure 5b & c). DM was evenly distributed throughout all tissues indicating that MHC class II binding was essential for the concentration of M1 in lymphoid tissue (Figure 5d). The substantially higher ratio of wild-type SMEZ-2 in draining lymph nodes compared to M1 and M2 suggested that T cell activation also contributed to lymphoid entrapment. These data indicate that M1 conjugates injected into mice are likely to concentrate antigen to secondary lymph nodes.
Conjugation of proteins/peptides to SMEZ-2 M1

Synthetic peptides were conjugated to M1 or DM via the cysteine residue at position D42C using a simple reversible disulphide oxidation that provided a fixed superantigen:peptide molar ratio of 1:1. Peptide conjugation was confirmed by 17% SDS-PAGE, with conjugate preparations running at a slightly higher molecular weight than monomeric M1 (Figure 6a, lanes A1, 2). Minimal quantities of dimeric M1/DM protein (~50 kDa) were evident, suggesting a high conjugation efficiency. When peptide was added at a 10-fold molar excess, the reaction was routinely >90% efficient.

Due to the small difference in molecular weight (~2 kDa) between the M1 and M1:OVA\textsubscript{323-339} proteins, conjugation was verified by western blotting. Samples were transferred to a nitrocellulose membrane and probed with anti-OVA antiserum (Figure 6a, lanes B1, 2), then stripped and re-probed with M1 monoclonal antibody (Figure 6a, lanes C1, 2). Bound antibody was detected with anti-mouse IgG:HRP to confirm that OVA\textsubscript{323-339} peptide was incorporated into the M1:OVA\textsubscript{323-339} conjugate. Additionally, conjugates were excised from an SDS-PAGE, processed for LC-MS mass spectrometry and the most abundant sequences found to match published sequences for SMEZ-2 and an in-house database containing the modified OVA\textsubscript{323-339} peptide sequence (p<0.05, data not shown). M1:OVA\textsubscript{323-339} preparations were also run through an analytical sizing column and absorbance measured at A\textsubscript{280} to demonstrate that the non-reduced sample comprised of a single dominant peak (M1:OVA\textsubscript{323-339}), whilst the reduced sample had two peaks representing free M1 protein and an OVA\textsubscript{323-339} peptide peak (Figure 6b).
The chemical cross-linker sulfo-SMCC was used to couple OVA protein and this process was approximately 30-50% efficient and produced conjugates containing M1:OVA at a ratio of 1:1 and 2:1 with an average molecular weight of approximately 70 kDa (Figure 6c).

SMEZ-2 M1 enhances Ab responses to conjugated peptide

Initial vaccination studies were performed in BALB/c mice using OVA protein coupled to the M1 carrier and demonstrated that a single injection with 10 μg M1:OVA in IFA produced significantly enhanced anti-OVA IgG responses and required chemical coupling of M1 to OVA (data not shown). Later studies indicated that the carrier effect of M1 was more significant in C57BL/6 mice, a strain that produces a very weak antibody response to OVA in IFA.

Further vaccination studies were performed in C57BL/6 mice, testing the ability of M1 to enhance IgG production to a B-cell peptide epitope OVA323-339. A single vaccination with M1: OVA323-339 in IFA produced significantly higher specific antibody responses after 25 days (median =100 µg/ml) and 42 days (median = 90 µg/ml) (Figure 7a) compared to mice immunised with peptide alone in IFA (median = 0.5 µg/ml) at 42 days. Importantly the absolute amount of coupled peptide was 3-fold less (~0.35 µg) in the conjugate relative to the control animals (1 µg), showing that M1 induced higher responses with less antigen.

C57BL/6 mice vaccinated with the non-targeting control preparation DM:OVA323-339 in IFA developed no detectable response, confirming the requirement for MHC class II binding.
Anti-M1 Ab responses do not reduce the activity of the M1 carrier

The M1 protein by itself is immunogenic and stimulates a strong antibody response in mice (Figure 7b) and humans (35). To determine whether pre-existing anti-M1 antibodies altered the M1 carrier effect by blocking binding to MHC class II, C57BL/6 mice were first immunized with M1 or PBS then later vaccinated with M1:OVA conjugate. Comparable levels of anti-OVA IgG developed in both groups of mice which all had similar levels of pre-existing anti-M1 antibody (Figure 7b). However the range of anti-OVA IgG in the M1 sero-converted group was substantially larger (n=7, range =100 - 40,000 µg/ml) than in the sham pre-immunised group (n=7, range = 800 - 2000 µg/ml). This indicated that anti-M1 antibody did not prevent the M1 carrier effect and in some animals, actually dramatically enhanced the response.

SMEZ-2 M1 enhances Ab responses in DR3-DQ2 and DR4-DQ8 transgenic mice

Superantigens have higher affinity towards human MHC class II than mouse. Vaccination studies were therefore performed in C57BL/6 mice transgenic for human CD4 and either DR3-DQ2 or DR4-DQ8 and compared to parental C57BL/6. Mice were immunised subcutaneously once with 10 µg of antigen in IFA and circulating specific serum anti-OVA IgG measured at regular intervals. All mice vaccinated with M1:OVA rapidly developed high levels of anti-OVA IgG as early as 2 weeks post vaccination. At week 2 C57BL/6 mice displayed a median anti-OVA IgG end-point titre of 1,000 (Figure 8a), DR3-DQ2 mice an endpoint titre of 10,000 (Figure 8b) and DR4-DQ8 mice an endpoint titre of 80,000 (Figure 8c). By week 5 these levels had increased 5-fold (DR4-DQ8 and DR3-DQ2 Figure 8b,c) and 40-fold (C57BL/6 Figure 8a). The DR3-DQ2 mice ultimately displayed the
highest median endpoint titre of 200,000 (Figure 8c). In contrast, vaccination with OVA alone was less effective with 3/5 C57BL/6, 1/5 DR3-DQ2 and 3/5 DR4-DQ8 mice failing to develop any detectable anti-OVA IgG response by week 5 (Figure 8a-c). A comparative assessment of specific IgG1 and IgG2c subtype (31) responses to M1:OVA in the vaccinated animals showed that the subtype was strongly biased towards IgG1 (Figure 8d), suggesting that M1 conjugation promotes a strong Th2 type response (39).

The M1 carrier stimulates enhanced in vitro, but not in vivo, cellular responses

BALB/c mice were immunized subcutaneously with 10 μg OVA in IFA (weeks 0, 3) to generate OVA responder cells which were then tested for proliferation responses to OVA protein or the equivalent quantity of OVA coupled to M1. In vitro delivery of OVA protein coupled to M1 enhanced T cell sensitivity to OVA by >10,000-fold compared to OVA alone, M1 + OVA or OVA coupled to SMEZ-2 DM (Figure 9a). Proliferating cells comprised of both CD4+ and CD8+ T cells and the proportion of these cells was similar irrespective of whether the stimulus was M1:OVA or OVA alone (data not shown).

Murine splenocytes (including those from MHC class II transgenic animals) were harvested from multiple studies to measure specific T cell recall responses in M1:OVA immunized mice, relative to OVA control animals. The T cell proliferation responses in OVA immunized mice were equivalent to those of the M1:OVA immunized animals in all studies, as represented in Figure 9b. OVA-specific IL-4 and IFN-γ splenocyte responses were measured by ELISPOT, with no striking differences observed between M1:OVA and OVA vaccinated mice (Figure 9c). Thus although the M1 carrier dramatically enhanced
cellular responses to OVA \textit{in vitro}, this was not matched by a corresponding increase in OVA-specific T cell expansion \textit{in vivo} despite a significantly enhanced and sustained T dependent anti-OVA IgG1 response.
Vaccination is a highly successful strategy for stimulating protective immunity to microbial pathogens, however there are numerous diseases which lack effective vaccines including parasitic and bacterial diseases prevalent in the tropics (20) and influenza (14). Vaccine development has progressed from being based on heat killed whole organisms or attenuated strains, which are intrinsically immunogenic, to refined preparations based on protein, peptide or carbohydrate-based immunogens. A limitation of using defined vaccine preparations is that they are often poorly immunogenic, necessitating the need for an adjuvant, carrier or specialized delivery system (33). An approach with considerable potential is direct delivery of materials to antigen presenting cells by targeting cell surface molecules, which has been demonstrated with live vectors or specific targeting of receptor ligands and surface receptors (for example CD40, Gb3 and C-type lectins) with antibody (42).

We describe an alternative approach using a detoxified superantigen to target coupled antigen to MHC class II on antigen presenting cells in vitro and in vivo. The key residues required for binding of the TCR and MHC class II were identified based on the published structure of SEC3 (26), substituted and confirmed to eliminate superantigen associated biological activities. Removal of the capacity to bind the TCR alone is sufficient to ablate mitogenic activities and release of proinflammatory cytokines responsible for superantigen toxicity.
Wild-type SMEZ-2 mainly stimulates hVβ8 bearing T cells and is the most potent superantigen identified in humans (34). Elimination of only two residues in the TCR binding site of SMEZ-2 produced M1 that was >10⁴-fold less active in human T cell proliferation studies and failed to elicit any observable human T cell responses except at the highest concentration tested. This highlights the exquisitely small variations in TCR affinity that lead to either profound superantigen T cell activation or nothing at all. Some residual affinity of M1 towards soluble hVβ8 was detected by Surface Plasmon Resonance biosensor analysis but only at high ligand concentrations. This weak binding was clearly not sufficient to translate into T cell activation under normal physiological concentrations. Moreover, the antigen coupling point introduced on M1 is a cysteine deliberately engineered in the centre of the TCR binding site to ensure it does not interfere with MHC class II binding on the opposite face of M1, making it impossible for M1 to engage with TCR Vβ while antigen is bound. Surface Plasmon resonance studies also demonstrated that M1 retained specificity for MHC class II and this was comparable to wildtype SMEZ-2. Specific binding to MHC class II was also evident on both murine and human antigen presenting cells, leading to rapid internalisation and co-localisation with MHC class II protein in endocytic vesicles of dendritic cells. Antigen coupled to M1 is taken into a different intracellular compartment than antigen alone and it has been speculated that it is taken up as part of the MHC class II recycling pathway (13). Lymph node tracking studies in mice showed that M1 was enriched in lymph nodes, which contain high numbers of MHC class II⁺ antigen presenting cells, corroborating data showing that M1 binds efficiently to all murine MHC class II⁺
antigen presenting cells, including all dendritic cell subsets in mice (13). M1 binding to
dendritic cells in vivo does not elicit maturation of these cells (13).

In common with other superantigens, SMEZ-2 is much less active on mouse T cells. While
the molecular basis for this potency is not entirely understood, it is most likely to be a
consequence of the combined high affinities of SMEZ-2 for a peptide dependent subset of
HLA-DQ molecules and the human Vβ8 TCR polypeptide. The potency of SMEZ-2
increased approximately 100-fold in proliferation assays using cells from DR3-DQ2 or
DR4-DQ8 transgenic mice, confirming the preference of SMEZ-2 for human DR/DQ
proteins over mouse I-A/I-E. Production of the inflammatory cytokines CCL2, IL-6 and
TNF-α in response to in vivo exposure to wild-type SMEZ-2 was also enhanced by up to
10-fold in DR3-DQ2 or DR4-DQ8 transgenic animals relative to non-transgenic animals.
Selective expansion of Vβ11+ T cells three days after exposure to wild-type SMEZ-2(36, 45) was only evident in transgenic mice, reiterating the role of human MHC class II in
maximal superantigen response. We speculate that the potency of SMEZ-2 could further
approach that seen in humans if the mice were also transgenic for the human Vβ8 T cell
receptor gene.

A strong, long lasting IgG1 antibody response resulted from a single subcutaneous injection
of M1:OVA or M1:OVA323-339 conjugate in IFA. This was due entirely to the MHC class II
binding of M1 because the mutant DM, which is defective in both TCR and MHC class II
binding, displayed no enhanced immunity when coupled to OVA. Moreover, the effect was
dependent on the physical attachment of OVA to M1. Immunization with unconjugated
OVA and M1 was also ineffective. Mice transgenic for human MHC class II clearly responded more rapidly to the OVA:M1 conjugate and developed higher titers of anti-OVA antibody.

A potential limitation of a microbial based conjugate is either pre-existing or developing antibodies in the recipient that might neutralize binding. M1 by itself was highly immunogenic, particularly in BALB/c mice. Nevertheless, BALB/c mice pre-immunized and displaying strong anti-M1 sero-conversion, still developed an equivalent anti-OVA response when immunized with OVA:M1 conjugate compared to naïve mice. Thus the effect of M1 conjugation was not compromised by pre-existing immunity. Notably, normal human sera capable of neutralizing T cell proliferation in response to SMEZ-2 wildtype protein was unable to inhibit the binding of SMEZ-2 to MHC class II, suggesting that the immunodominant epitopes recognized by these antibodies are separated from the MHC class II binding site (Jacelyn Loh, personal communication). Wildtype SMEZ-2 is a highly potent superantigen on human cells but also efficiently targets MHC class II from other species such as the mouse, suggesting that the M1 carrier will be highly efficacious in humans despite variability in HLA type.

Despite in vitro studies showing stimulation of greatly enhanced cellular proliferation of antigen-specific responder cells with M1:OVA, vaccination with M1:OVA or M1: OVA323-339, failed to drive an observable in vivo T cell response. In these studies IFA was used to deliver antigen. IFA is a water:oil emulsion that forms a slow release depot, rather than directly targeting particular cell types or skewing towards Th1 or Th2 immunity(9).
dominant subclass in the antibody response to OVA:M1 was IgG1, suggesting a predominantly Th2 type response. Stimulation of strong cellular immunity probably requires additional innate Toll like Receptor (TLR) mediated signals not provided in IFA. It is worth noting that most antigen-targeting studies have used TCR transgenic animals such as the OT-I and OT-II system for OVA257-264 and OVA323-339 respectively (4, 18) in order to increase precursor frequency sufficiently to observe T cell expansion. In a previous study, M1:OVA induced strong anti-tumour immunity in OT-I and OT-II mice when combined with the potent iNKT cell ligand α-Galactosylceramide (13). These studies clearly demonstrate that targeted mutation of the TCR binding site in the SMEZ-2 superantigen detoxified the protein, whilst retaining its capacity to bind MHC class II. Targeting of antigen to MHC class II bearing cells has been shown previously using chemical coupling to anti-MHC class II antibodies (6) or by engineering T epitopes into the sequence of anti-MHC class II antibodies (29). Here we demonstrate a different strategy using conjugation to SMEZ-2 toxoid. This approach offers the advantage of cross-reactivity with both murine and human MHC class II, which permits experimentation in both systems. It is highly likely, given the DR3-DQ2 and DR4-DQ8 mice appear to have an intermediate response to SMEZ-2 wt, that the M1 carrier would be more effective in humans. The recombinant SMEZ-2 M1 protein can be readily produced in large quantities and is known to be temperature and protease resistant (3, 12, 19), hence is suited to in vivo applications. The mutations selected completely ablated superantigen associated biological activities in vivo both in wildtype and ‘humanised’ mice, resulting in the need for an adjuvant to demonstrate utility as a vaccine carrier. An alternative approach might be to
partially retain superantigen-like activity, which is likely to produce an adjuvant effect, however this strategy risks the prospect of residual toxic superantigen activities. The M1 carrier merits further assessment to determine whether it is compatible with a variety of adjuvants. It may prove particularly useful in settings where elevated antibody responses are associated with protection, such as influenza (23) and *Streptococcus pyogenes* (21). Vaccination with M1:peptide produced significantly elevated antibody responses with 3-fold less peptide, thus it may have potential in the context of epidemic influenza, in which ‘antigen sparing’ may be a necessity to ensure sufficient vaccine coverage (41). Targeting antigen via MHC class II to antigen presenting cells is an effective strategy to concentrate antigen to lymphoid organs where it can be more efficiently presented to B cells to stimulate rapid and enhanced humoral immunity to conjugated peptide or protein antigen.

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FPLC data on the M1:OVA<sub>323-339</sub> conjugate. Martin Middleditch from the University of Auckland Centre for Genomics and Proteomics arranged the mass spectrometry analysis and guidance on interpreting the data. The mouse anti-M1 monoclonal Ab was produced by Anuruddika Fernando. Surface Plasmon Resonance studies were performed by the University of Auckland Biacore Facility.

Professor John D Fraser holds a patent on the use of the SMEZ-2 M1 protein as a vaccine carrier.


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exotoxin Z is the major immunoactive agent of Streptococcus pyogenes. J Immunol 169:2561-2569.


Table 1. Sets of mis-matched primers used to create the M1, M2 and DM mutants of SMEZ-2.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
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<tbody>
<tr>
<td>W75L&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5'-CCATTTGATTGAACTATTTATC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATAAAATAGCTAAATCAAATGG-3'</td>
</tr>
<tr>
<td>K182Q&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5'-GATATAGAGATCAAGAAAGTATC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATACTTTCTTGATCTCTATTC</td>
</tr>
<tr>
<td>D42C1&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>5'-GATGTTAGATGTGCTAGAGATTTC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTCTAGCACACATCTACATCAAGTTTC-3'</td>
</tr>
<tr>
<td>Y18A&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>5'-CGATTGTAGCTGAATATTCAGATATAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAATATTCAGCTACAATCGTACTATAG-3'</td>
</tr>
<tr>
<td>H202A.D204A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-GGGGCTTTAGCTATAGAAATTGACTCC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGCTAAAGCCCCAAATTTATCTATATTG-3'</td>
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<sup>a</sup>SMEZ-2 M1 mutant was created by mutating 3 TCR-binding residues (W57.L.K182.Q.D42.C). This mutant bound to MHC class II, but not the TCR.

<sup>b</sup>SMEZ-2 M2 mutant was created by mutating 4 TCR-binding residues (Y18.A.W75.L.K182.Q.D42.C). This mutant bound to MHC class II, but not the TCR.

<sup>c</sup>SMEZ-2 DM mutant was created by mutating two TCR-binding residues (Y18.A.D42.C) and two MHC class II-binding residues (H202.A.D204.A). This mutant no longer bound to MHC class II or the TCR.
Figure 1. Location of residues involved in TCR binding (Y18,D42,W75,K182) and MHC class II binding (D202,H204). Placement of the antigen coupling site (D42C) interferes with TCR binding while leaving the MHC class II binding site free.
Figure 2. Superantigen mutants no longer bind to the TCR but retain specificity for MHC class II and are internalised into APC. (a, b) SMEZ-2 wt, M1, M2 and DM were immobilized on a dextran chip of a Biacore biosensor. As analytes (a) 1 mg/ml soluble Vβ8/α1 TCR and (b) 1 µM and 10 µM HLA-DR1 were injected at a flow rate of 20 µl/min. Curves represent triplicate injections with binding in relative response units (RU), with the background binding to a plain dextran surface subtracted. (c) FACS analysis of SMEZ-2 M1 binding to THP-1 cells. 1 µM of FITC conjugated protein was incubated with 1x10^5 THP-1 cells for 20 min at 4°C. Competition with unlabeled superantigen occurred at a 10:1 molar ratio where indicated. Cells were washed and analyzed by flow cytometry. (d) SMEZ-2 M1 binding of THP-1 cells imaged on a Nikon E600 fluorescence microscope. THP-1 cells were incubated with 1 µM FITC conjugated SMEZ-2 M1 for 20 min at 4°C before washing and incubating at 37°C in media for 0, 30, or 120 min. (e, f) Murine BMDC were incubated for 10 min at 4°C with fluorescent-labeled anti-CD11c, SMEZ-2 M1, and anti-MHC class II. Dendritic cells were washed and analyzed by confocal microscopy before (e) or after (f) a further incubation for 30 min at 37°C.
Figure 3. SMEZ-2 M1 does not stimulate T cell proliferation from human or murine immune cells. (a) Human PBMC, or (b) murine C57BL/6, (c) humanised DR3-DQ2 or (d) DR4-DQ8 transgenic splenocytes were cultured with graded doses of SMEZ-2 wt, M1 and DM for 3 days and proliferation quantified by uptake of tritiated thymidine over the final 16 hours of the culture period. Murine data are the mean ± SD from 10 individual mice per figure, combined from 5 independent experiments. Human PBMC (mean ± SD from triplicate wells) from a single healthy donor are shown as a point of comparison.
Figure 4(a-d). SMEZ-2 M1 fails to stimulate cytokine production or *in vivo* expansion of V\(\beta^{11+}\) T cells. (a) Human PBMCs were incubated with 100 ng/ml SMEZ-2 wt, 100 ng/ml M1 or no Ag. The cytokine response was quantified in culture supernatants after 72 h at 37°C. Results are shown as the mean concentration ± SD of five donors. (b – d) The serum cytokine response was measured 3 hours after intraperitoneal injection with 10 μg SMEZ-2, M1 or PBS in age-matched wildtype C57BL/6 (b), DR3-DQ2 (c) and DR4-DQ8 (d) mice. Data are presented as the mean ± SD for n=2 mice per treatment per strain, a total of n=18 mice were used per study. These data are representative of two independent experiments.
Figure 4 (e-f). Three days after injection of 10 μg SMEZ-2, SMEZ-2 M1 or PBS T cell populations were enumerated by combining data from whole spleen cell counts with flow cytometric analysis: (e) CD4^+Vβ8^+, (f) CD8^+Vβ8^+, (g) CD4^+Vβ11^+, (h) CD8^+Vβ11^+. The fold change in T cell numbers was determined relative to the PBS control group. Data were combined from two independent experiments (n=4 per treatment group; n=36 mice in total). Each point represents a single mouse and the horizontal bar is the median value. P values were calculated using a one-tailed Mann-Whitney test.
Figure 5. In vivo trafficking of superantigens. C57BL/6 mice (n=2) were injected s.c. with 0.4 nmol $^{125}$I labelled superantigen: (a) SMEZ-2 wildtype; (b) SMEZ-2 M1; (c) SMEZ-2 M2; and (d) SMEZ-2 DM. 24 hours later, mice were sacrificed and lymph nodes, spleen, kidney, lung, and liver were collected and the $^{125}$I incorporation measured in a gamma counter. Data are normalized with respect to the weight of organs; each point represents a single mouse and the horizontal line is the median value. Data are representative of two experiments (n=8 mice per experiment).
Figure 6. OVA_{323-339} peptide and OVA protein can be chemically conjugated to the M1 carrier. (a) Conjugation efficiency was checked by 17% SDS-PAGE in non-reducing sample buffer and protein detected using Coomassie Blue (A). The identity and co-localisation of the OVA_{323-339} (B) and M1 (C) was confirmed by western blotting. Lane 1: M1; 2: M1:OVA_{323-339}. (b) To further verify chemical coupling of peptide to conjugate, samples were run through an analytical sizing column in the presence or absence of 1 mM...
DTT and the number of peaks assessed at $A_{280}$. (c) Representative fractions of conjugated protein before, during and at the end of purification by size exclusion were run using 12% SDS-PAGE in non-reducing sample buffer and stained with Coomassie Blue. OVA:M1 protein conjugates are indicated by a bracket. Lane 1: Prior; 2: OVA:M1 conjugates; 3: OVA:M1 and free OVA; 4: unconjugated OVA and M1.
Figure 7. The M1 carrier enhances antibody responses to coupled peptide and is effective in the presence of specific anti-M1 antibodies. (a) C57BL/6 mice were vaccinated once with M1:OVA323-339, DM:OVA323-339 or peptide alone, all emulsified in IFA. IgG responses are shown as whisker plots, showing the min to max range of data points and the median value. Data are representative of two independent experiments each containing n=5 mice per group and n=15 mice in total. (b) BALB/c mice were immunized with 10 μg M1 or PBS in IFA, followed by vaccination with 10 μg M1:OVA in IFA on weeks 3 and 6. Serum samples (n=7/group) were collected for assay by ELISA for M1 antibody (squares) on week 3 and OVA antibody (triangles) on week 8. Data are combined from two independent experiments (n=28 mice in total). Each individual point represents a single mouse and the horizontal line is the median value. P values were calculated using a Kruskall-Wallace test with Dunn’s procedure.
Figure 8. The M1 carrier enhances anti-OVA Ab responses in wt and transgenic mice. (a) C57BL/6, (b) humanised DR3-DQ2 or (c) DR4-DQ8 transgenic mice were given a single subcutaneous injection with 10 μg OVA alone or 10 μg OVA coupled to M1, emulsified in IFA. The development of a circulating Ab response to OVA was determined by ELISA at 2, 3, 5 and 8 weeks post-immunisation. (d) The ratio of IgG1/IgG2c Ab was measured by ELISA on week 8. All ELISA data are displayed as whisker plots, showing the min to max range of data points and the horizontal line represents the median value. Data shown contain n=5 mice/treatment group and n=30 mice in total and are representative of two independent experiments. *P* values were calculated using a one-tailed Mann-Whitney test.
Figure 9. Conjugation of Ag to the M1 carrier enhances in vitro, but not in vivo, Ag-specific cellular responses. (a) Splenocytes from OVA immunized BALB/c mice were cultured with graded doses of OVA, DM:OVA, M1, M1 + OVA and M1:OVA. Proliferation was quantified by the addition of $[^3]H$thymidine for the final 16 hours of the 72 hour culture period. Data are the mean +/- SD of triplicate wells and are representative of two experiments. (b,c) Splenocytes were harvested from C57BL/6 mice 8 weeks after a single immunisation with 10 $\mu$g OVA or M1:OVA. (b) Thymidine uptake was used to assay for proliferation in response to graded doses of OVA. Each mouse was assayed individually (n=5/group; n=10 mice in total per experiment) and data are presented as the mean +/- SD (c) ELISPOT was used to measure cytokine responses in response to 100 $\mu$g/ml OVA, each point represents a single mouse (n=5/group; n=10 mice in total per experiment) with the median value represented as a horizontal line. All data are representative of two or more independent experiments.