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Short communication

A humanized HLA-DR4 mouse model for autoimmune myocarditis

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ABSTRACT

Myocarditis, the principal cause of dilated cardiomyopathy and heart failure in young adults, is associated with autoimmunity to human cardiac α-myosin (hCAM) and the DR4 allele of human major histocompatibility II (MHCII). We developed an hCAM-induced myocarditis model in human HLA-DR4 transgenic mice that lack all mouse MHCII genes, demonstrating that immunization for 3 weeks significantly increased splenic T-cell proliferative responses and titres of IgG1 and IgG2c antibodies, abolished weight gain, provoked cardiac inflammation and significantly impaired cardiac output and fractional shortening, by echocardiography, compared to adjuvant-injected mice. Neither cardiac dilatation nor fibrosis occurred at this time point but prolonging the experiment was associated with mortality. Treatment with mixtures of hCAM derived peptides predicted to have high affinity for DR4 significantly preserved ejection fraction and fractional shortening. Our new humanized mouse model of autoimmune cardiomyopathy should be useful to refine hCAM-derived peptide treatment.

1. Introduction

Myocarditis is the leading cause of heart failure in people under 40 years of age. Viral (frequently Coxsackievirus B3, Parvovirus B19, adenoviruses or herpes viruses), bacterial (e.g. *Corynebacterium diphtheriae*, *Staphylococcus aureus*, *Borrelia burgdorferi* or *Ehrlichia* species) and some parasitic (e.g. *Trypanosoma cruzi*) infections are implicated in acute myocarditis, which becomes chronic and progresses towards dilated cardiomyopathy (DCM) in one fifth of cases [1]. Many chronic patients have autoimmunity to cardiac muscle derived antigens [2], including (in 41%) cardiac specific human α-myosin (hCAM) [3]. As with other autoimmune diseases, there is familial aggregation, autoantibody presence in unaffected relatives [4] and association with specific MHCII genotypes [5]. A recent meta-analysis of 19 independent studies totalling 1378 cases and 10,383 controls demonstrated a statistically elevated frequency of the human leukocyte antigen HLA-DR4 allele [5]. Myocarditis and DCM can be induced in genetically susceptible rodent strains by immunization with hCAM in complete Freund's adjuvant (CFA) but humanized mouse models are needed to evaluate immunotherapies because they bear human rather than mouse MHCII genes. Mice transgenic for the HLA-DQ8 allele on a non-obese, diabetic (NOD) background (so-called NOD.DQ8 mice) develop spontaneous autoimmune myocarditis [6]. However, there is no evidence of increased antibody levels or propensity for DCM in diabetic humans or those carrying the DQ8 allele [5]. Consequently, we studied humanized HLA-DR4 transgenic mice that lack all mouse MHCII genes on a metabolically normal background (henceforth referred to as DR4 mice) [7]. DR4 mice immunized with hCAM developed myocarditis that could be ameliorated by treatment with hCAM-derived peptides.

2. Material and methods

2.1. Human cardiac α-myosin (hCAM)

Heart tissue was obtained from organ donors giving informed consent under research ethical committee approval (NHS South West – Frenchay Ref No 08/H0107/48) and was stored according to the regulations of the UK Human Tissue Act 2004. hCAM was extracted from homogenised hearts by high-salt precipitation, as described previously [8] and its purity was confirmed by SDS polyacrylamide gel electrophoresis.

2.1.1. Induction of experimental autoimmune myocarditis

DR4 mice (aged 6–8 weeks) were bred by Apitope International NV under licence from the originator, Dr. L Fugger (MRC Human Immunology Unit, University of Oxford, UK). Mouse transgenic for multiple copies of the HLA-DRA*0101 and HLA-DRB1*0401 alleles were generated as described [7] and bred onto the MHCII$^{\Delta/\Delta}$ strain (which lacks all the mouse MHCII genes [9]) on a C57black/6 background for at least 10

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generations. Breeding and experimental procedures were conducted according to the Guide for the care and use of laboratory animals, eighth edition (2011) (http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf) and under the approval of UK Home Office licence 30/3195. Purified hCAM (2 mg/ml) was emulsified 1:1 in complete Freund's adjuvant (CFA; Difco #263810) boosted with 8 mg/ml of heat-killed *Mycobacterium tuberculosis H37 Ra* (hkMTB; Difco #231141) and 100 μg myosin/mouse was injected subcutaneously. On day 0 and 2, 200 ng of pertussis toxin (Sigma Aldrich) in 0.5 ml of phosphate buffered saline (PBS) was injected intraperitoneally to each mouse as a second adjuvant [10]. A control group received PBS in the same boosted CFA as well as pertussis toxin.

2.2. Echocardiographic analysis of left ventricle function

Anaesthesia was essential to locate the probe accurately and avoid motion during measurements. 3 weeks after immunization, mice were anaesthetised by intraperitoneal injection of 250 mg/kg of tribromoethanol (Avertin; Sigma Aldrich). A left ventricle short-axis view at the papillary muscle level was obtained in M-Mode using a Vevo 770 echocardiography system (Visual Sonics; Toronto, Canada) just before terminating the mice. Because anaesthesia reduces heart rate, all the functional measurements were obtained at between 400 and 500 beats per minute to avoid any artefactual differences.

2.3. Histopathological analysis of cardiac inflammation and fibrosis

Hearts were fixed for 24 h in 10% (v/v) formalin in PBS. After embedding in paraffin wax, sections (5 μm) were stained with haemotoxylin and eosin. Images were captured by Aperio slide scanner (Leica Biosystems; Nussloch, Germany) and areas of inflammatory cell infiltration were defined blindly and quantified using Image J (Open Source) software. Masson's trichrome staining was used to detect fibrosis (blue colour).

2.4. Anti-hCAM antibody and T-cell proliferation measurements

Serum concentrations of serum CSF2, IFNγ, IL1β, IL2, IL6, IL10 and TNFα were measured by multiplex cytokine analysis (Merck Millipore, Watford, UK) according to the manufacturer's instructions. Serum anti-hCAM antibody levels were measured by ELISA on Nunc Immuno MaxiSorp 96-well flat bottom plates coated with 100 μl of 10 μg/ml purified hCAM in 0.05 M carbonate-bicarbonate buffer (pH 9.0). Bound antibodies were detected with 1:1000 of goat anti-mouse IgG1 or IgG2c secondary antibodies conjugated to alkaline phosphatase (Abcam; Cambridge, UK). For the hCAM-reactive T-cell proliferation assay [11], Spleens were mechanically disrupted in X-VIVO 15 medium (Lonza; Manchester, UK) supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, filtered through a 40 μm cell strainer and the cells collected by centrifugation at 300 × g for 5 min at 4 °C. After treatment with red blood cell lysis buffer (Sigma Aldrich; Poole, UK) cells were washed in PBS and resuspended in supplemented X-VIVO 15 medium. Splenocytes were cultured in 96-well plates (5×10^5 cells/well) for 72 h at 37 °C at 5% CO_2 in the presence of purified hCAM or concanavalin A. Cells were then pulsed with 0.5 μCi/well of ^{3}H-thymidine for 18 h before harvesting onto glass fibre filters (Cox Scientific; Northants, UK) and placing into a 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer; Massachusetts, USA).

2.5. In silico prediction and application of hCAM-specific peptides

Linear peptide epitopes binding to DR4 locus with the highest affinity were predicted by the online ProPred, NetMHCII and IEDB algorithms and synthetic peptides (minimum 95% purity) were obtained from GenScript (New Jersey, USA). Their antigenicity was screened on hCAM sensitized splenic T-cells using the forementioned ^{3}H-thymidine incorporation assay. The best inducers (pool(1): YHIFYQILSNKKPEL, PHIFSISDNAYQYML, VNPYKWLPVYNAEVV and pool(2): RVQLLHSQNTSLINQ, EATLQHEATAAALRK, KSSLKLMATLFSSYA) were subcutaneously injected in PBS at equal mass ratios (with PBS alone as control) into 6–8 weeks old mice every 2 days starting from 0.1 μg/mouse (total). The dose was escalated 10-fold until 100 μg/mouse was injected 3 times and then every 4 days until the end of the experiment [12].

2.6. Statistical analysis

Discrete variables were examined using Fisher's exact test. Kolmogorov-Smirnov tests ($n = 5–7$) or D'Agostino tests ($n > 8$) were applied to test normality and data expressed as the mean ± SD (Standard Deviation). A two-tailed unpaired Student's *t*-test or a Wilcoxon non-parametric test was used, as appropriate. For more than two groups, a one-way or two-way ANOVA was performed, followed by a Dunnett or Bonferroni post-test. In all cases, the values were considered significant if the two-tailed probability $p < 0.05$.

3. Results

3.1. Effect of hCAM immunization on DR4 mice

Addition of hCAM significantly increased proliferation relative to medium controls of splenic T-cells from DR4 mice injected with hCAM/CFA (but not PBS/CFA) and pertussis toxin similar to positive control ConA (Fig. 1A). Evidently, subcutaneous hCAM evoked a strong T-cell mediated immune response. Immunization with hCAM greatly increased serum anti-hCAM IgG1 and IgG2c antibody levels, which were undetectable in the PBS/CFA treated mice (Fig. 1B, C), indicating a strong B-cell response also. When serum concentrations of CSF2, IFNγ, IL1β, IL2, IL6, IL10 and TNFα were measured by multiplex cytokine analysis, only IL6 concentration was significantly increased from 58 ± 8 to 133 ± 28 pg/ml ($p = 0.025$, $n = 5$). DR4 mice gained 2.4 g in weight 3 weeks after PBS/CFA immunization but hCAM-immunized mice stopped gaining weight and became significantly lighter than PBS/CFA controls (Fig. 1D), suggestive of generalised malaise. No PBS/CFA treated mice (0/5) had histological evidence of leukocyte infiltration or myocyte damage (Fig. 1E, a), indicating absence of spontaneous autoimmune myocarditis at 9–11 weeks. By contrast, all hCAM-immunized mice (5/5) showed histological evidence of inflammation and myocardial necrosis ($p = 0.0079$, Fisher's exact test). This affected both atria and ventricles (e.g. Fig. 1E, b) and averaged approximately 30% of the area of sections. None of the hearts from PBS/CFA injected control mice or myosin injected animals showed any fibrosis using Masson's trichrome staining (0/5). In a separate study, 3/5 immunized and 0/5 control mice died when the experiment lasted longer than 3 weeks. More mortality data were not collected for ethical reasons. In another pilot study, myocarditis did not occur in the absence of pertussis toxin. Most importantly, hCAM immunization plus pertussis impaired left ventricular function, significantly decreasing ejection fraction (44 ± 5 vs 77 ± 3%; $p = 0.003$; $n = 5$) and fractional shortening (22 ± 8 vs 48 ± 2%; $p = 0.006$; $n = 5$) (Fig. 1F) and increasing end systolic dimensions (2.8 ± 0.4 vs 1.8 ± 0.2 mm; $p = 0.039$; $n = 5$, Fig. 1G) compared to PBS/CFA treated mice. However, end-diastolic dimensions were not significantly increased, despite a weak trend ($p = 0.25$, $n = 5$), implying no cardiac dilatation (Fig. 1G). Hence hCAM immunization caused autoimmune myocarditis with impairment of left ventricular function, which did not progress to significant DCM or fibrosis after 3 weeks.

3.2. Feasibility study of hCAM-specific peptide treatment

Autoantigen-derived peptides with high affinities for MHCII can provoke immunomodulation and hence desensitize T-cell responses in models of other autoimmune diseases [13]. To establish the feasibility