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1 **Unconventional T cell recognition of an arthritogenic epitope of proteoglycan**  
2 **aggrecan released from degrading cartilage**

3

4 **Short title:** Unconventional T cell recognition of degrading cartilage [56 char/spaces]

5

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16

17 **Keywords:** Rodent, cartilage, T cells, Autoimmunity, Antigens/Peptides/Epitopes,  
18 Antigen Presentation and Processing.

19

20 **Abbreviations:**

21 **SUMMARY**

22 It has been proposed that peptide epitopes bind to MHC class II molecules to form  
23 distinct structural conformers of the same MHC-II-peptide complex termed type A and  
24 type B, and that the two conformers of the same peptide-MHC-II complex are  
25 recognized by distinct CD4 T cells, termed type A and type B T cells. Both types  
26 recognise short synthetic peptides but only type A recognise endosomally processed  
27 intact antigen. Type B T cells that recognise self peptides from exogenously degraded  
28 proteins have been shown to escape negative selection during thymic development  
29 and so have the potential to contribute to the pathogenesis of autoimmunity.

30 We generated and characterised mouse CD4 T cells specific for an arthritogenic  
31 epitope of the candidate joint autoantigen proteoglycan aggrecan. Cloned T cell  
32 hybridomas specific for a synthetic peptide containing the aggrecan epitope showed  
33 two distinct response patterns based on whether they could recognise processed  
34 intact aggrecan. Fine mapping demonstrated that both types of T cell recognised the  
35 same core epitope. The results are consistent with the generation of aggrecan-specific  
36 type A and type B T cells.

37 Type B T cells were activated by supernatants released from degrading cartilage  
38 indicating the presence of antigenic extracellular peptides or fragments of aggrecan.

39 Type B T cells could play a role in the pathogenesis of proteoglycan-induced arthritis  
40 in mice, a model for rheumatoid arthritis, by recognising extracellular peptides or  
41 protein fragments of joint autoantigens released by inflamed cartilage. [238w]

## 42 **Introduction**

43 Soon after the discovery that TCR recognize peptides bound to MHC molecules (1,2),  
44 it was shown that the local environment influences the conformation adopted when  
45 peptides bound to MHC-II (3,4). On this background, the results of a study of HEL  
46 presentation by the A<sup>k</sup> molecule suggested that MHC-II formed complexes with a  
47 single peptide in more than one conformation distinguishable by TCR (5-8). Similar  
48 observations were made for an E<sup>k</sup>-restricted epitope of HEL (9), and for autoantigens,  
49 binding to a number of H-2 A and E alleles and to HLA DR1 (9-16). More detailed  
50 investigation showed that a proportion of T cells from mice immunized with a synthetic  
51 HEL peptide recognized the peptide in vitro but not processed intact HEL, whereas  
52 epitope specific T cells from HEL-immunized mice recognized both synthetic peptide  
53 and processed HEL (9,17). The data were interpreted as showing the formation of two  
54 conformers, termed type A and type B peptide-MHC-II complexes, recognized by CD4  
55 T cells termed type A and type B T cells with specificity for one or other conformer  
56 (reviewed in (18-20)). Subsequent studies have shown that peptide-MHC complex  
57 formation is influenced by the mechanism of generation of the peptide epitope, so that  
58 the type A conformers are generated after processing of intact proteins in acidic  
59 endosomal compartments of APC and loading of newly synthesized MHC-II under the  
60 control of the chaperone DM (21). Alternatively, exogenous peptides were shown to  
61 bind to mature MHC-II molecules in early endosomes or at the APC surface in the  
62 absence of functional DM resulting in formation of a mixture of type A and type B T  
63 conformers (21). Infection of mice with *Salmonella typhimurium* has also been shown  
64 to bias HEL presentation by dendritic cells towards a type B T cell response by a  
65 Dectin-1 dependent mechanism (22,23). Furthermore, mice expressing endogenous  
66 HEL were used to show that type B T cells escape negative selection during thymic

67 development *in vivo*, whereas the majority of type A T cells are deleted (24), creating  
68 a scenario in which self-reactive type B T cells could persist in peripheral lymphoid  
69 organs. Type B T cells specific for the autoepitope of insulin 9-23/A<sup>97</sup> have been cloned  
70 from pancreatic islets, the site of autoimmune damage (16), and so localised where  
71 they could play a role in autoimmunity. However, the source of natural peptide ligands  
72 that preferentially activate type B T cells in autoimmune disease has not been  
73 reported.

74 The major cartilage component proteoglycan aggrecan is a candidate autoantigen in  
75 inflammatory arthritis (25) and immunization of BALB/c mice with aggrecan in adjuvant  
76 induces an inflammatory arthritis mediated by CD4 T cells specific for the  
77 immunodominant arthritogenic epitope 84-103 (26). In addition, extracellular  
78 proteolysis is a characteristic of inflammatory arthritis, resulting from upregulation of  
79 matrix degrading metallo-proteinases and increased degradation of cartilage matrix  
80 components including aggrecan (27). Thus we reasoned that the T cell repertoire  
81 includes type B T cells that may recognise extracellular aggrecan fragments released  
82 during inflammation. Here we demonstrate both type A and type B patterns of  
83 recognition amongst CD4 T cell hybridomas specific for the same core epitope of  
84 aggrecan. We further show that aggrecan-specific type B T cells are activated by  
85 exogenous antigenic ligands released from degrading cartilage. The results are a step  
86 towards determining whether type B T cells participate in proteoglycan-induced  
87 arthritis in mice, and more generally whether they contribute to the pathogenicity of  
88 rheumatoid arthritis and other autoimmune disease in man.

89

## 90 **Materials and Methods**

### 91 *Reagents*

92 Proteoglycan aggrecan was purified from bovine nasal cartilage as previously  
93 described (28). The synthetic peptide <sup>84</sup>VLLVATE**GRVRVNSAY**QDK<sup>103</sup> of bovine  
94 aggrecan includes the core arthritogenic CD4 T cell epitope <sup>92</sup>GRVRVNSAY<sup>100</sup> ((29)  
95 where the sequence is numbered 73-81), see the Immune Epitope Data Base epitope  
96 ID 107090, 132189 and 137285 at <http://www.immuneepitope.org/>. The sequence of  
97 the aggrecan epitope is identical in human (Acc. No. P16112) and bovine (Acc. No.  
98 P13608) aggrecan but differs from mouse aggrecan by two amino acids  
99 (<sup>92</sup>GQVRVNSIY<sup>100</sup>, Acc. No. Q61282). Aggrecan peptide 84-103, and the same  
100 peptide modified by substitution of citrulline for arginine at position 93 (93cit95R), 95  
101 (93R95cit) or both (93cit95cit) compared to the native sequence (93R95R), were  
102 purchased from JPT Peptide Technologies GmbH, Berlin, Germany. Peptides for fine  
103 mapping were purchased from Proimmune (Oxford, UK), and represent staggered  
104 peptides of 14 amino acids offset by 1 amino acid inclusive of the aggrecan sequence  
105 84-108, and peptides with alanine substituted sequentially at positions 90 to 103,  
106 except glycine substituted for the alanine at position 89.

107 Bovine nasal cartilage and healthy human articular cartilage explants were prepared  
108 as described previously (30). Human cartilage from the femurs of rheumatoid arthritis  
109 patients undergoing surgery were obtained with local ethical approval. Briefly, cartilage  
110 was cut into 1 mm<sup>3</sup> fragments and 0.5 g of fragments transferred to 5ml serum-free  
111 medium (RPMI-1640 with 2mM glutamine, 30 µg/ml gentamycin and 0.05mM 2-  
112 mercaptoethanol) in wells of a 6 well plate. Plates were incubated in a humidified  
113 environment at 37°C with 5% CO<sub>2</sub>. The serum free medium was changed after 24  
114 hours and 1 ng/ml of recombinant human IL-1α and 10 ng/ml human Oncostatin-M  
115 (gifts from Dr. K. Ray, Glaxo-Smith-Kline, Stevenage) were added (30). Plates were  
116 further incubated at 37°C for up to 20 days and supernatants were removed and stored

117 at -30°C prior to use in T cell hybridoma assays.

118

#### 119 *Mice*

120 BALB/c mice were purchased from Charles River (Margate, UK) and the *in vivo*  
121 procedures described were performed under the terms of the Animals (Scientific  
122 Procedures) Act 1986 authorized by the Home Secretary, Home Office UK. Mice were  
123 immunized in one footpad with 25 µg of aggrecan or synthetic peptide in Titermax  
124 adjuvant (Sigma-Aldrich, Gillingham, UK) and popliteal lymph nodes were removed 7  
125 d later for ex-vivo assay.

126

#### 127 *Cells*

128 Culture medium reagents and other chemicals were purchased from Sigma-Aldrich  
129 (Gillingham, UK), unless otherwise stated. All cells were grown in culture medium  
130 consisting of RPMI 1640 containing 3 mM L-glutamine, 50 µM 2-ME, 10% v/v FBS  
131 (BioSera, Ringmer, UK) and 30 µg/ml gentamicin.

132 Macrophages were grown from femoral bone marrow cells of BALB/c mice by culture  
133 in bacteriological Petri dishes for 6 days in culture medium supplemented with 1 mM  
134 sodium pyruvate, 5% horse serum and 5% of a supernatant from the L929 fibroblast  
135 cell line as a source of M-CSF as described previously (31). Cells were activated by  
136 treatment with 1 ng/ml IFN-γ (R&D Systems, Abingdon, UK) overnight at 37°C in a  
137 humidified CO<sub>2</sub> incubator and shown by flow cytometry to be at least 90% positive for  
138 expression of the macrophage marker F4/80.

139 Dendritic cells (DC) were also grown from femoral bone marrow cells by culture for 10  
140 days in 6-well plates in culture medium supplemented with 20 ng/ml rGM-CSF,  
141 replenishing the supplemented medium every 3 days. Cells were then incubated with

142 0.2 µg/ml lipopolysaccharide to induce maturation, and shown by flow cytometry to be  
143 >80% DC for the dendritic cell marker CD11c.

144 The H-2<sup>d</sup> expressing B lymphoma cell line A20 (TIB-208) and macrophage cell line  
145 J774A.1 (TIB-67), were obtained from ATCC.

146 Stable T cell lines were derived from lymph node cells from peptide or aggrecan  
147 immunized BALB/c mice by 2 to 3 rounds of restimulation with synthetic 84-103  
148 peptide and irradiated spleen cells as APC, followed by expansion in rIL-2, before  
149 polyethylene glycol-mediated fusion with the TCR<sup>αβ</sup>- BW5147 leukaemic cell line, as  
150 described previously (32). The majority of T cell hybridomas were shown to respond  
151 to aggrecan peptide 84-103 and express CD4, CD3, and TCR<sub>αβ</sub> by flow cytometry.  
152 The following T cell hybridomas were used in this study: 2G9 (Vβ8.3), 1271b (Vβ8.2),  
153 1G2 (Vβ8.3), 2D7 (Vβ14).

154

#### 155 *Lymph node proliferation assay*

156 Popliteal lymph nodes were removed 7 d after footpad immunisation of BALB/c mice.  
157 Popliteal lymph node cells were cultured in the presence or absence of a range of  
158 doses of aggrecan or synthetic peptides for 72 h at 3 x 10<sup>5</sup> per well in 96-well round  
159 bottom microtiter plates. 5 KBq per well of tritiated thymidine (NET027A005MC, sp.  
160 act. 0.074 TBq/mM, Perkin-Elmer, Cambridge, UK) was added for the final 16 h of  
161 culture before harvesting on glass fibre membranes. Radioactivity was quantitated  
162 using a liquid scintillation counter (Perkin Elmer Microbeta Trilux) and results are  
163 presented as mean cpm ± SEM. Responses two-fold above medium only controls  
164 were considered significant.

165

#### 166 *T cell hybridoma assay*



167 Antigen presenting cells ( $4 \times 10^4$ /well macrophages, A20 B cells or DC) were plated in  
168 medium with or without a range of doses of aggrecan or synthetic peptide, or diluted  
169 cartilage explant supernatants in triplicate wells and incubated for 3 h. T cell hybridoma  
170 cells ( $4 \times 10^4$ /well) were added and plates were incubated for a further 24 h and then  
171 frozen. Responses of T cell hybridomas were determined as the amount of IL-2  
172 released in a bioassay measuring proliferation of IL-2 dependent CTLL-2 cells. CTLL-  
173 2 cells ( $3 \times 10^4$ /well) were incubated in triplicate wells of flat-bottomed 96-well  
174 microtiter plates in the presence of T cell hybridoma culture supernatants diluted 1:2.  
175 Plates were incubated for 24 h and labelled with tritiated thymidine and results  
176 expressed as described for the lymph node proliferation assay above. Experiments  
177 were repeated no less than three times.

178

## 179 **Results**

### 180 *Lymph node proliferation responses of aggrecan and peptide immunised mice*

181 BALB/c mice were immunised with bovine aggrecan or with the synthetic peptide  
182 representing amino acids 84-103 of aggrecan, containing the major arthritogenic CD4  
183 T cell epitope, and proliferation to aggrecan and peptide was recalled in vitro (Fig. 1).  
184 Mice immunized with aggrecan responded to both peptide and aggrecan to a similar  
185 extent, confirming the immunodominance of the response to the arthritogenic epitope  
186 contained within peptide 84-103 (Fig. 1a). Mice immunized with peptide responded to  
187 both peptide and aggrecan, but the response to peptide was of a greater magnitude  
188 (Fig 1b), suggesting more T cells responded to peptide alone than to both peptide and  
189 aggrecan. The specificity of response for the immunizing antigens was confirmed by  
190 the lack of response in HBSS immunized mice (Fig. 1a). One interpretation of the  
191 results is that peptide-immunisation induces two populations of peptide-specific T cells

192 differing in their ability to recognize processed aggrecan, reminiscent of the type A and  
193 type B T cell responses reported for epitopes of HEL (18).

194

#### 195 *Patterns of recognition of T cell hybridomas to aggrecan and peptide*

196 To investigate further we cloned and immortalised T cells from peptide 84-103  
197 immunised mice and measured their responses to peptide and aggrecan. Of 23 84-  
198 103-specific T cell hybridomas generated from peptide-immunized mice, 9 responded  
199 to peptide and aggrecan and 14 responded to peptide only, consistent with the  
200 generation of cloned T cells with type A and type B recognition patterns. Dose  
201 response titrations for presentation of aggrecan and peptide by bone marrow-derived  
202 macrophages, dendritic cells (DC) and immortalised A20 B cells to representative T  
203 cell hybridomas are shown in Fig. 2. T cell hybridomas 2G9 (Fig. 2a,e) and 1271b (Fig.  
204 2b,f) responded equally well to aggrecan and peptide when presented by either  
205 macrophages or DC, typical of type A T cells. T cell hybridomas 1G2 (Fig. 2c,g) and  
206 2D7 (Fig. 2d,h) responded to peptide but not to aggrecan presented by macrophages  
207 or DC over the same antigen dose range. Although A20 B cells process and present  
208 aggrecan less efficiently, the same pattern was still observed, in that T cell hybridomas  
209 2G9 and 1271b responded to peptide and aggrecan (figs. 2i,j), but hybridomas 1G2  
210 and 2D7 responded to peptide only (Fig. 2k,l). All four peptide specific hybridomas  
211 clearly showed one or other of the two recognition patterns, independent of the type  
212 of APC, with no intermediate patterns of response.

213

#### 214 *Fine specificity of aggrecan epitope recognition*

215 To establish whether the patterns of recognition could be accounted for by the  
216 presence of more than one epitope within peptide 84-103, the fine specificity of type

217 A and type B T cell hybridomas was determined using overlapping peptides staggered  
218 by one amino acid (Fig. 3A), as well as peptides in which each position was serially  
219 substituted with alanine (Fig. 3B). T cell hybridomas 2G9, 1271b, 1G2 and 2D7  
220 differed in the requirement for the presence of particular amino acids of the core  
221 epitope and flanking amino acids (Fig. 3A), ranging from maximum response to 86-99  
222 to 92-105 staggered peptides (2G9) to maximum response to only 89-102 (2D7), but  
223 responses of all four T cell hybridomas tested centred on peptide 89-102.  
224 Fine specificity differences were also observed in the responses to serially alanine  
225 substituted peptides (Fig. 3B). A clear dependence on arginine at position 95 and  
226 asparagine at position 97 was observed, suggesting that the P4 and P6 MHC contact  
227 residues of the core epitope, defined previously as P1-P9 <sup>92</sup>GRVRSNSAY<sup>100</sup> (29), is a  
228 common feature of all T cell hybridomas studied. Thus the response to staggered and  
229 substituted peptide sets showed that T cells with both the type A and type B recognition  
230 patterns focussed on a single core epitope binding to A<sup>d</sup> in the same register.

231

#### 232 *T cell hybridoma recognition of citrullinated aggrecan peptides*

233 Different patterns of recognition of the aggrecan epitope could result from post-  
234 translational modifications such as citrullination of one or both of the arginines within  
235 the aggrecan <sup>92</sup>GRVRSNSAY<sup>100</sup> epitope as a result of the activity of peptidyl arginyl  
236 deaminases known to be present both within Rheumatoid synovium (33) and within  
237 the endosomal system of APC (34). We tested synthetic 84-103 peptides in which  
238 arginine at position 93 (93cit, P2 of the core epitope) or 95 (95cit, P4 of the core  
239 epitope), or both (93cit95cit), were substituted with citrulline, and compared  
240 recognition by type A and type B T cell hybridomas using the macrophage cell line  
241 J774A.1 as APC (Fig. 4). Both type A and type B hybridomas recognised the

242 unmodified peptide (93R95R) (Fig. 4). All hybridomas also recognized the peptide  
243 citrullinated at position 93, and both type A hybridomas, but neither type B, responded  
244 to the peptide citrullinated at position 95, all in the sub-micromolar dose range. None  
245 of the hybridomas recognised the doubly modified peptide 93cit95cit. The similar  
246 responses of all hybridomas to the peptide citrullinated at position 93, excludes the  
247 possibility that the different recognition patterns resulted from this post translational  
248 modification. The response of type A but not type B T cell hybridomas to the peptide  
249 citrullinated at position 95 could represent an alternative to conformational isomerism  
250 in explanation of type B recognition.

251

#### 252 *T cell hybridoma recognition of supernatants from degrading cartilage*

253 Cartilage turnover is catalysed *in vivo* by metallo-proteinase enzymes that degrade  
254 aggrecan and other matrix components, the activity of which can be induced by the  
255 presence of inflammatory cytokines IL-1 $\alpha$  and oncostatin-M (30). We recapitulated this  
256 milieu of degradation products *in vitro* by incubating bovine and human cartilage  
257 explants with these inflammatory cytokines and removing supernatants for use as  
258 antigen in T cell assays. Supernatants were added to antigen presentation assays with  
259 type A and type B T cell hybridomas and bone marrow macrophages as APC. Type A  
260 T cell hybridoma 1271b responded to doses of less than 1% of cartilage supernatants  
261 and responses increased proportional to the incubation time. Responses to  
262 supernatants reached the magnitude of the peptide control by 10 days of cartilage  
263 incubation (Fig. 5a), consistent with the kinetics of aggrecan release in this system  
264 (30). T cell hybridoma 1271b also responded optimally to doses of less than 1% of the  
265 three supernatants from degrading human cartilage, reaching the magnitude of the  
266 peptide control (Fig. 5b).

267 The two type B T cell hybridomas 1G2 and 2D7 also recognised supernatants from  
268 degrading bovine and human cartilage explants, but much less efficiently than the type  
269 A T cell hybridoma 1271b (Fig. 5a,b). Responses of both type B T cell hybridomas  
270 required doses greater than 1% of supernatant and cartilage incubation for 20 days,  
271 and were of a low magnitude compared to the peptide control (Fig. 5a). Both type B T  
272 cell hybridomas also responded to all three supernatants from degrading human  
273 cartilage (Fig. 5b). Despite the low magnitude of responses, the data suggest that  
274 aggrecan-derived type B ligands are released and available for presentation during  
275 cartilage degradation. Relatively small amounts of type B T cell ligands compared to  
276 intact aggrecan could stimulate type B T cells that have escaped thymic deletion, when  
277 type A T cells would be scarce due to their more complete deletion.

278

## 279 **Discussion**

280 We show here that T cell hybridomas specific for a major arthritogenic epitope of the  
281 cartilage component proteoglycan aggrecan express TCR that display recognition  
282 patterns characteristic of type A and type B T cells. This pattern has previously been  
283 defined for epitopes of HEL, cytochrome-c, E $\alpha$ , A $\beta^k$ , myelin basic protein and insulin,  
284 and includes epitopes binding to a range of mouse MHC class II A and E alleles as  
285 well as to HLA-DR1 (8-13,15,16,18,35). Our data is the first report of type B T cells  
286 restricted by A $^d$ , and the first for type B T cell recognition of a candidate joint  
287 autoantigen. The number of different antigens and MHC alleles that have been studied  
288 suggest that type B T cell recognition is a universal property of TCR-peptide-MHC  
289 class II interactions. The most likely explanation for the different patterns of recognition  
290 is that type A and type B T cells recognise distinct conformational isomers of a single  
291 peptide-MHC complex (18), a conclusion supported by biochemical data (11,36-38),

292 but no x-ray crystallographic evidence to date. However, a similar recognition pattern  
293 could result from a number of alternative mechanisms (discussed in (18)): (i)  
294 Contamination of peptides during synthesis or storage leading to the presence of  
295 peptides of different specificities, although we have used freshly dissolved peptides  
296 obtained from more than one commercial supplier to minimise this possibility. (ii) Free  
297 N- or C-terminal amino acids flanking the epitope in shorter synthetic peptides dictating  
298 distinct patterns of TCR fine specificity, excluded here by the observation that type B  
299 T cells recognised a number of adjacent staggered peptides with different terminal  
300 amino acids. (iii) Post-translational modification of amino acids leading to distinct TCR  
301 specificities. We investigated the response of T cell hybridomas to the aggrecan  
302 peptide 84-103 modified by citrullination at position 93, 95 or both. The responses of  
303 type A and B T cells were similar to peptides citrullinated at position 93 or at positions  
304 93 and 95, but type A cross reacted with the peptide citrullinated at position 95  
305 whereas type B T cells did not. This could be a result of the reduced sensitivity of type  
306 B T cell recognition but is unlikely to explain the difference in type A and type B  
307 recognition. All T cell hybridomas cross reacted between unmodified 84-103 and the  
308 peptide citrullinated at position 93, and none of the hybridomas recognised the doubly  
309 citrullinated peptide, so that citrullination could not explain the type A and type B T cell  
310 recognition patterns observed. (iv) Different populations of T cells specific for  
311 overlapping dominant and cryptic epitopes present on the same peptide, for which  
312 there are precedents (39-42), that would result in the differential recognition of intact  
313 protein by T cells against the two epitopes. The T cell hybridomas used here were  
314 shown to be specific for the same core aggrecan epitope 84-103 by fine mapping with  
315 staggered and substituted peptide sets, and only a single epitope was localised to this  
316 region in extensive epitope mapping of aggrecan in BALB/c mice (43).

317 As type B T cells recognise peptides but not intact protein antigens after intracellular  
318 processing, we propose that the peptide ligands required for type B T cell activation  
319 are generated by extracellular degradation during the inflammatory response. It is  
320 known that peptides in the extracellular milieu directly bind MHC class II on the APC  
321 surface (44) or in early endosomes (45-49). Treatment of DC with TLR ligands or IFN-  
322  $\alpha$  *in vitro* (50) or their administration *in vivo* (51) leads to presentation of epitopes from  
323 intact proteins to type B T cells probably as a result of extracellular processing,  
324 although the mechanisms were not investigated. However, a source of natural ligands  
325 that activate type B T cells has not been previously reported.

326 Hyaline cartilage turnover in healthy synovial joints is mediated by a family of metallo-  
327 proteases, including aggrecanases, and these enzymes contribute to the cartilage loss  
328 observed in inflammatory arthritis (27). Extracellular matrix degradation products have  
329 been identified in synovial fluid from patients with arthritis (52-54), and fragments of  
330 collagen and hyaluronan have been shown to contribute to the inflammatory response  
331 (55). These extracellular antigenic fragments are presumed to be the basis for the  
332 proposal that 'remnant epitopes' are generated from cartilage components in the  
333 extracellular space that are then presented to T cells as a mechanism that perpetuates  
334 autoimmunity (56-58). Whether it is type A or type B T cells which participate in this  
335 response has not been investigated to date. We used bovine and human cartilage  
336 explants treated with pro-inflammatory cytokines to mimic cartilage loss during  
337 inflammatory arthritis, and showed that the supernatants generated could activate  
338 aggrecan-specific type B T cells, suggesting degrading cartilage releases aggrecan  
339 peptides that are available for presentation to type B T cells. The responses were  
340 detectable only after extended periods of incubation of cartilage explants and the  
341 magnitude of response was also low compared to the responses of type A T cells, the

342 latter probably reflecting recognition of processed intact aggrecan known to be  
343 released by degrading cartilage (52-54). The data suggest that type B T cell ligands  
344 are less abundant in degrading cartilage than intact aggrecan, and are more likely to  
345 be rapidly degraded. It has also been shown that capture and presentation of  
346 extracellular peptide epitopes by surface MHC class II is inefficient (45,59) but DM  
347 independent (47,48), and so would be permissive for type B T cell recognition.  
348 Type B T cells recognising exogenous self antigens have been suggested to persist  
349 in the periphery due to escape from negative selection during thymic development,  
350 circumstances in which type A T cells would be largely deleted. Consequently we  
351 propose that type B T cells recognizing cartilage antigens may represent a significant  
352 and even dominant contribution to the T cell response in autoimmune arthritis (15, 20).  
353 Increased turnover of cartilage due to chronic trauma or reactive inflammation caused  
354 by infection could release peptides or fragments from cartilage autoantigens above a  
355 threshold required for type B T cell activation. However, it remains to be determined  
356 whether type B T cells perform regulatory functions, provide help for pathogenic  
357 autoantibody responses, or differentiate into IFN- $\gamma$  and IL-17 producing effector T cells  
358 implicated in joint damage in the animal models of arthritis, in RA in humans, and in  
359 other automimmune diseases.



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364

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577 **Figure legends**

578

579 **Figure 1. Lymph node responses to aggrecan and peptide.** BALB/c mice were  
580 immunized with 25 µg aggrecan (a), or 100 µg peptide (b) in adjuvant, and 7 days later  
581 proliferation of PLN cells was assayed by tritiated thymidine incorporation in response  
582 to a dose range of peptide (circles) or aggrecan (triangles). Proliferation of PLN from  
583 HBSS-immunized mice was used as a negative control (open symbols in a), and  
584 responses of PLN to 5 µg/ml concanavalin A (con A) as a positive control (closed  
585 squares). Results are expressed as mean cpm ± SEM of 5 mice.

586

587 **Figure 2. T cell hybridoma responses to peptide and aggrecan.**  $4 \times 10^4$  per well of  
588 T cell hybridomas 2G9 (a,e,i), 1271b (b,f,j), 1G2 (c,g,k) or 2D7 (d,h,l) were incubated  
589 for 24 h with  $4 \times 10^4$  per well of either bone marrow derived macrophages, bone  
590 marrow-derived DC, or the B lymphoma line A20 as APC, and a range of doses of  
591 aggrecan or aggrecan peptide 84-103. Responses were measured as cytokine release  
592 into the supernatant in a bioassay with IL-2 dependent CTLL-2 cells as described in  
593 Materials and Methods, and results are shown as cpm ± SEM for triplicate wells for a  
594 representative of at least two different experiments for each hybridoma.

595

596 **Figure 3. Fine mapping T cell hybridoma responses to peptide.** T cell hybridomas  
597 2G9, 1271b, 1G2 and 2D7 were assayed for responses to 10 µM doses of the  
598 staggered (row A) or alanine substituted (row B) peptides shown, using bone marrow  
599 derived macrophages as APC as in the legend to Fig. 2, and results are shown as cpm  
600 ± SEM for triplicate wells. Controls were T cells and APC without peptide (0 in A and  
601 B), and 200 nM aggrecan (aggrecan in A), or 10 µM unsubstituted peptide (89-103 in

602 B).

603

604 **Figure 4. Hybridoma responses to citrullinated peptide 84-103.** T cell hybridomas  
605 2G9 (a), 1271b (b), 1G2 (c) or 2D7 (d) were assayed as described in the legend to  
606 Fig. 2, except that  $4 \times 10^4$  per well of the macrophage cell line J774A.1 were added as  
607 APC, and the antigen for the dose titrations were the native aggrecan peptide 84-103  
608 (93R95R), or the same peptide with citrulline substituted for the arginine at position 93  
609 (93cit95R), position 95 (93R95cit), or both (93cit95cit).

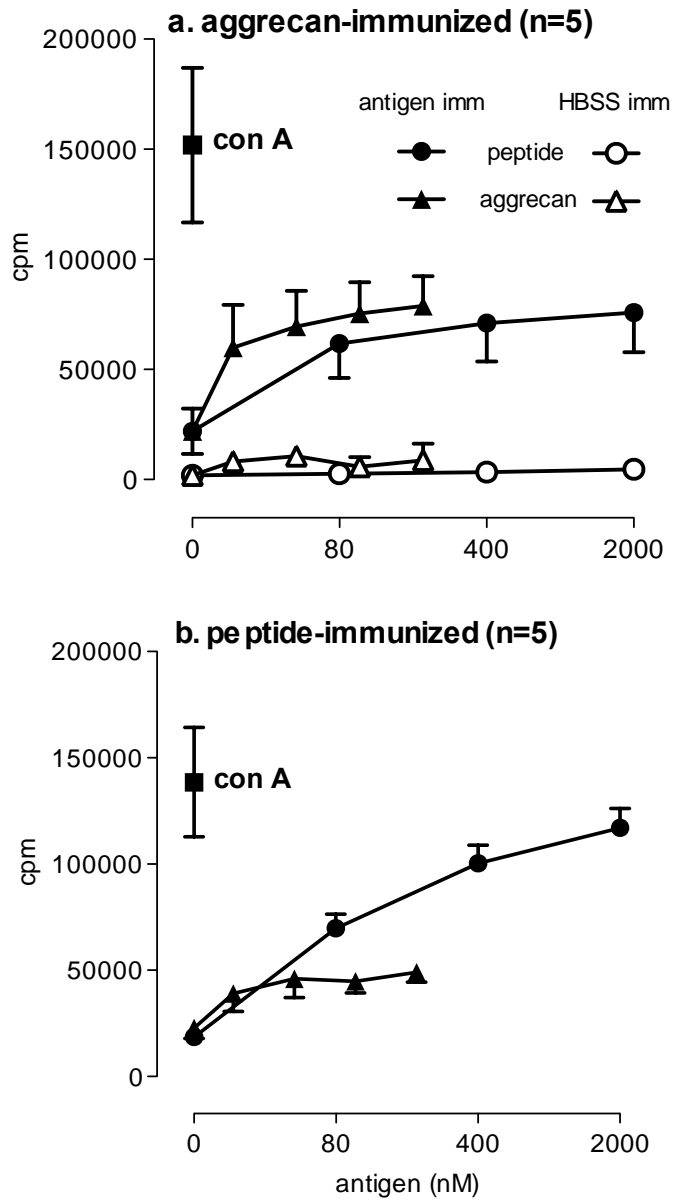
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611 **Figure 5. T cell hybridoma responses to supernatants from degrading cartilage**  
612 **explants.** T cell hybridomas 1271b (a,d), 1G2 (b,e) and 2D7 (c,f) were assayed for  
613 responses to (column A) supernatants collected from bovine cartilage explants after  
614 1, 5, 10 15 and 20 days of culture (showing one experiment of three), or (column B)  
615 supernatants collected from human articular cartilage explants after days 15 days of  
616 culture (showing supernatants from three different experiments HAC1-3). Preparation  
617 of supernatants is described in Materials and Methods, and supernatants were titrated  
618 and shown as percent of the total culture volume (the average protein concentration  
619 3.07 mg/ml for human and 1.5 mg/ml for bovine cartilage explant supernatants, of  
620 which the majority is known to be aggrecan (30)). Bone marrow derived macrophages  
621 were used as APC and results are shown as cpm  $\pm$  SEM for triplicate wells. Controls  
622 included 1  $\mu$ M peptide 84-103 (84-103) and absence of peptide (0).

623

624 Figure 1. Lymph node responses to peptide 84-103 and aggrecan

625

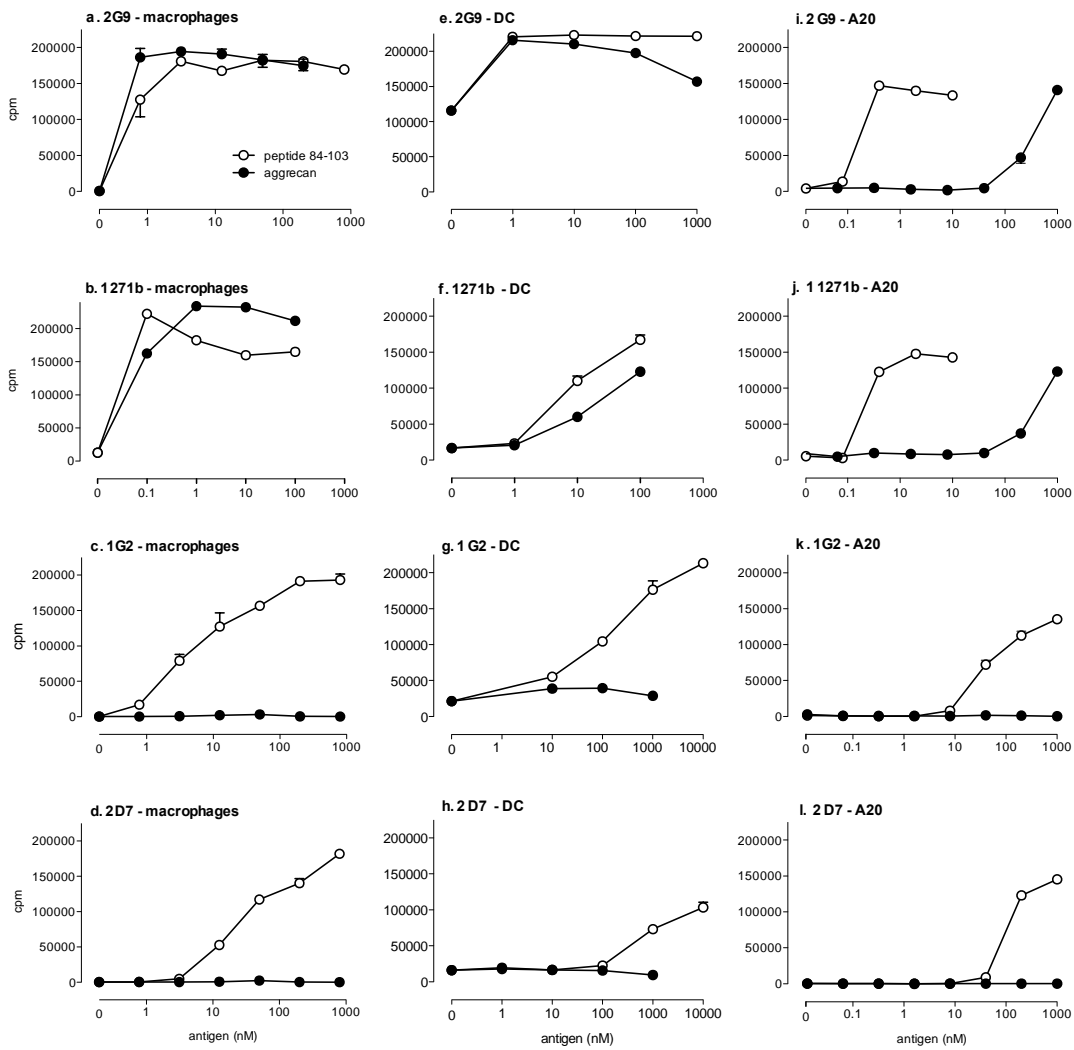


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628 Figure 2. T cell hybridoma responses to peptide 84-103 and aggrecan

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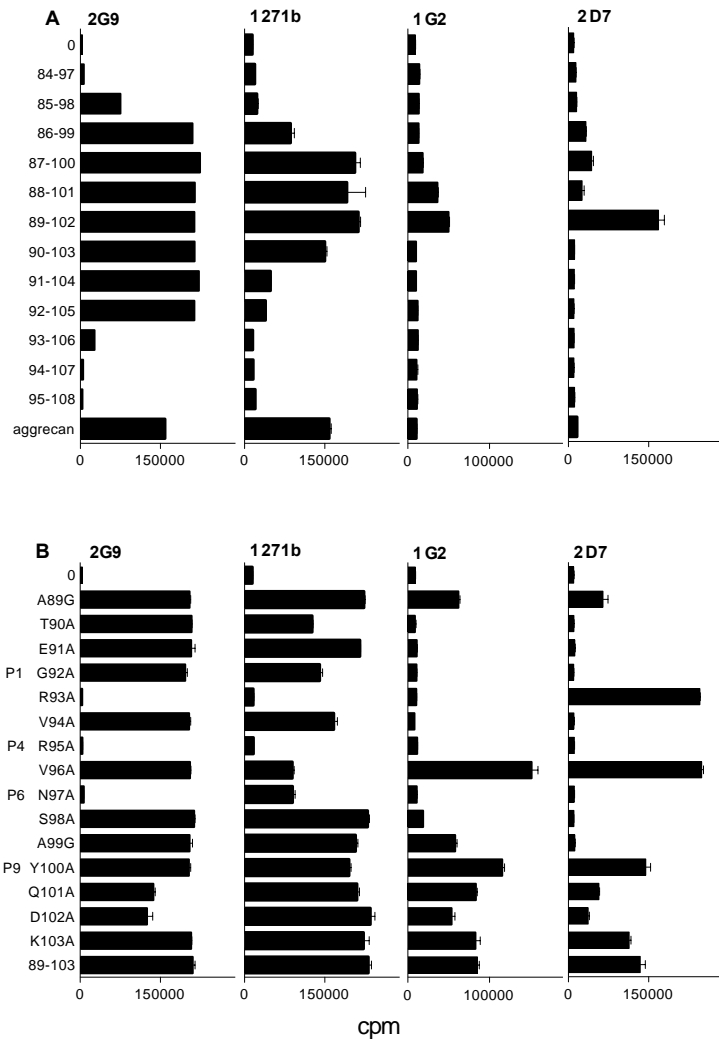
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632 Figure 3. Fine mapping T cell hybridoma responses to peptide 84-103.

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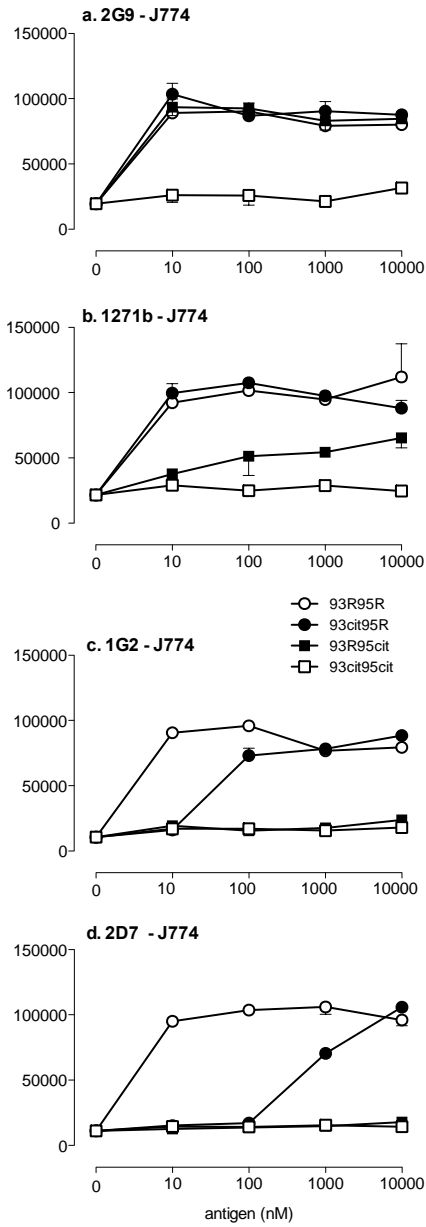
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639 Figure 4. Hybridoma responses to citrullinated peptides.

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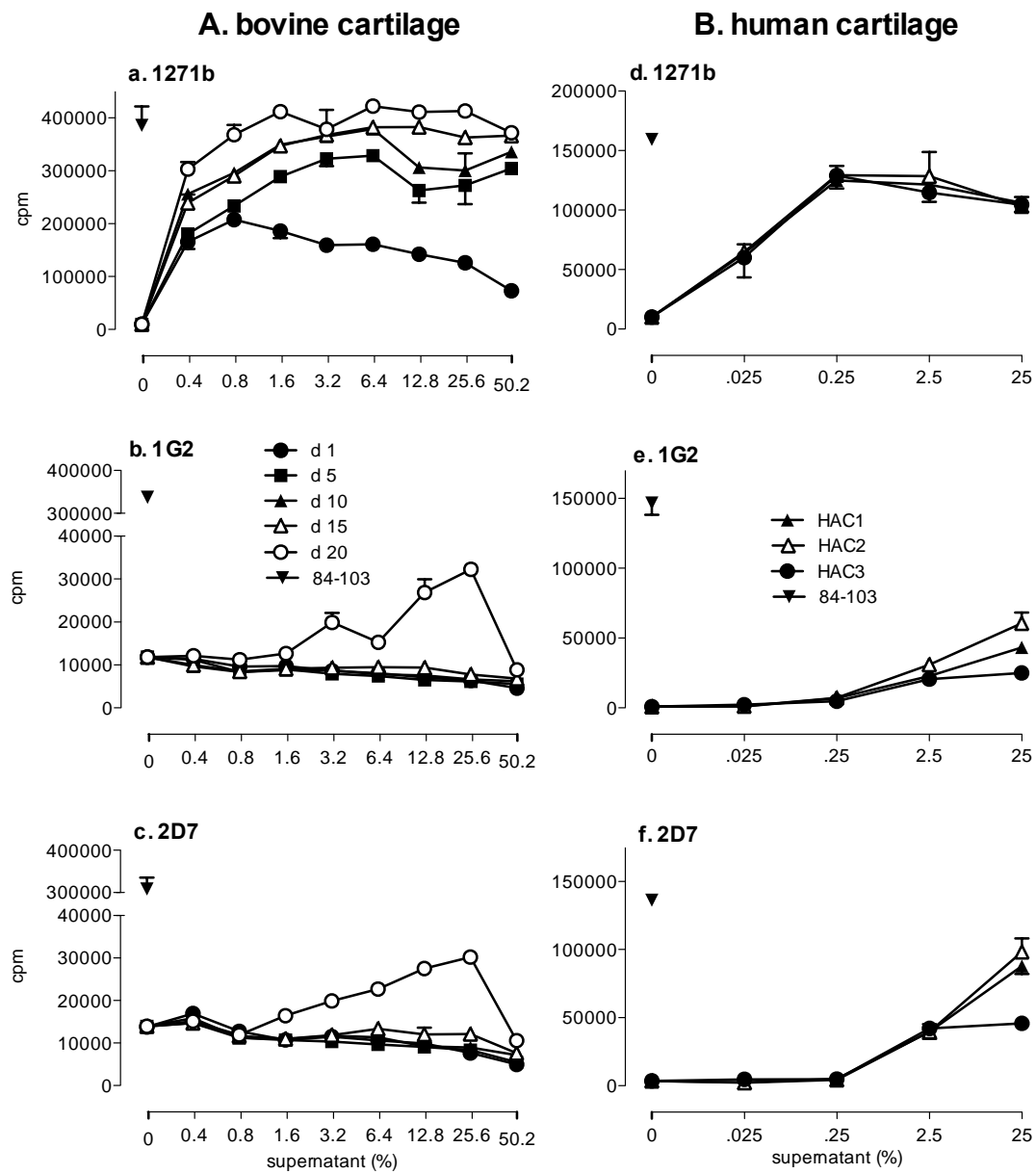
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643 Figure 5. T cell hybridoma responses to supernatants from cartilage explants

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