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Primed B Cells Present Type-II Collagen to T Cells

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Development of type-II collagen (CII)-induced arthritis (CIA) is dependent on a T-cell mediated activation of autoreactive B cells. However, it is still unclear if B cells can present CII to T cells. To investigate the role of B cells as antigen-presenting cells (APCs) for CII, we purified B cells from lymph nodes of immunized and nonimmunized mice. These B cells were used as APC for antigen-specific T-cell hybridomas. B cells from naïve mice did present native, triple-helical, CII (nCII) but also ovalbumin (OVA) and denatured CII (dCII) to antigen-specific T-cell hybridomas. In addition, B cells primed with nCII or OVA, but not dCII, activated the antigen-specific T-cell hybridomas two to three times better than naïve B cells. We conclude that antigen-primed B cells have the capacity to process and present CII to primed T cells, and antigen-primed antigen-specific B cells are more efficient as APC than naïve B cells. We further conclude that B cells have the potential to play an important role as APC in the development of CIA.

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INTRODUCTION

Type-II collagen (CII) is the major protein component of cartilage, and immune recognition of CII plays a critical role in the development of collagen-induced arthritis (CIA) and may also play a role in rheumatoid arthritis (RA). CIA is a widely used model for RA in which B cells and antibodies are believed to play an important role [1–3]. However, we do not fully understand how the priming of CII autoimmunity occurs or the importance of various antigen-presenting cells (APCs) in the subsequent pathogenesis of CIA.

CIA is induced after an intradermal immunization with CII in adjuvant. Autoreactive T cells specific to CII are relatively resistant to activation as they have acquired partial tolerance [4]. Therefore, it is easier to use heterologous CII, which mainly activates T cells toward the administrated (nonself) CII and not to autologous CII. These T cells will lead to the activation of B cells producing arthritogenic antibodies to CII, which will subsequently contribute to the development of arthritis [2]. The CII-reactive B cells are apparently not tolerant, and are readily differentiated to produce pathogenic antibodies [5]. They recognize epitopes on the CII molecule that are strictly triple-helical and presented on autologous (mouse) CII but avoid epitopes shared with collagens that are systemically exposed [6]. Furthermore, it is also known that the activation of both CII-specific T cells and the susceptibility to CIA are major histocompatibility complex (MHC)-associated [7, 8]. This is explained by the observation that an immunodominant peptide, CII 260–270, has the capacity to bind to the MHC class-II molecule Aq. T-cell recognition of the Aq/CII-256–270 complex is crucial for immune activation and CIA development [9].

It is, however, unclear which type of APC has the capacity to process and present CII and subsequently activate the T cells. Collagen is handled differently by APC when compared with other antigens used, such as ovalbumin (OVA), because macrophages and not dendritic cell process and present collagen to T cells [10, 11]. It was also shown that the inability to process CII was due to the collagen's primary (sequential) structure (e.g. (Gly-Pro-X)n), as there were no differences in the presentation of native (nCII) or denatured CII (dCII) or if the epitope was inserted into type-I collagen [11]. The presence of dendritic cells is nevertheless believed to play an important role in the pathogenesis of RA [12, 13], a disease where CII is recognized as an autoantigen [12, 14, 15]. Possibly, monocytes/macrophages or B cells may be more

important as APC for CII in RA and in the animal models of RA.

In the priming of CII autoimmunity, the role of B cells is critical; therefore, it would be of importance to understand whether B cells can present CII. Clearly, the activation of CII-reactive B cells is T-cell dependent [16, 17]. Direct experiments on whether B cells can function as APCs have, however, not provided a clear answer. It has been reported that B cells are poor APCs for CII but not for other antigens [10, 18]. These studies have mainly used MHC class-II (Aq)-transfected B-cell lymphoma, and they cannot present collagen but can efficiently present OVA or collagen peptides, in similarity with dendritic cells. In addition, purified naïve or primed B cells have been reported to lack the capacity to stimulate CII-specific T cells [18]. This is, however, surprising in the light of the fact that B–T-cell interaction is thought to be crucial for the development of CIA. To clarify if B cells can present CII, and to investigate whether the presentation of CII operates through receptor-mediated uptake, we investigated the ability of purified B cells, both from nonimmunized and immunized mice, to process and present CII to CII-specific T-cell hybridomas.

MATERIALS AND METHODS

Mice, antigens, culture medium and reagents. The (B10.Q × DBA/1) F1 and μMT-BQ mice were housed and bred in the animal facility of Medical Inflammation Research under defined specific pathogen conditions.

DBA/1 was originally obtained from Jackson Laboratories (Bar Harbor, ME,), and B10.Q was from Professor Jan Klein, Tübingen, Germany. A deleted immunoglobulin (IgM) gene (μMT) in a C57Bl/6 × 129 mouse was kindly provided by Dr Werner Müller (Cologne, Germany). The μMT was inserted in B10.Q (H-2q) mice through 10 back-crosses and subsequent intercrossing to provide homozygous B10.Q mice lacking B cells (μMT-BQ). Importantly, only offspring from homozygous mothers were used, as heterozygous mothers transfer antibodies and long-lived plasma cells to the offspring [19].

The presence of disrupted gene was investigated by polymerase chain reaction (PCR) analysis, and the absence of B cells by flow cytometry as described [20]. Mice of both strains were used when 8–12 weeks old. Rat CII was prepared from the Swarm chondrosarcoma [21] by limited pepsin digestion as described [22]. CII was used either as native or after heat denaturation at 50 °C for 30 min. OVA (grade-V) was purchased from Sigma chemical Co. (St Louis, MO, USA).

Anti-mouse B220 antibody RA3-6B2 conjugated to paramagnetic microbeads (Miltenyi Biotec, Bergisch, Gladbach, Germany) was used to enrich the B-cell population. Antibodies used for flow cytometry analysis of lymph node cells (LNCs) were anti-mouse CD8 (53–6.7) fluorescein isothiocyanate (FITC), CD4 (H129) phycoerythrin (PE), B220 (RA3–6B2) PE, B220 (RA3–6B2) Cy-chrome, 7.16.17 FITC and CD 11b (Mac 1) biotin-conjugated. All cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal calf serum (FCS), 10 μM β-mercaptoethanol, 10 mM HEPES, penicillin, L-glutamine and streptomycin in a humidified incubator at 37 °C in 7.5% CO_2.

Preparation of LNCs and purification of B cells. LNCs were prepared from (B10.Q × DBA/1) F1 immunized 11–12 days previously with 50 μg of either OVA or nCII or dCII emulsified in Freund's complete adjuvant (FCA) with H37Ra (Difco, Detroit, MI, USA) in each of the hind paws and at the base of the tail. B cells were separated from the LNCs by magnetic sorting.

The LNCs were separated using B220 RA3–6B2 (anti-B220) microbeads and MACS as described by the manufacturer (Miltenyi Biotec). The purity of the $B220^+$ cells was measured by flow cytometry, staining with B220 Cy-chrome-labelled antibodies, using FACS™ and FACStar™ and Cellquest™ software (BD PharMingen, San Diego, CA, USA).

Anti-CII-antibody enzyme-linked immunosorbent assay (ELISA). ELISA was used to determine anti-CII antibodies in the nCII-primed B-cell cultures. The flat-bottomed 96-well ELISA plates (Costar, Corning Inc., NY, USA) were coated with affinity-pure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), 5 μg/ml in phosphate-buffered saline (PBS) for 2 h in an incubator at 37 °C. One percent bovine serum albumin (BSA) (Sigma chemical Co.) in PBS was used for blocking of unspecific signals. After washing and adding the supernatants from the antigen-primed B-cell cultures, the plate was then incubated for another 2 h. Washing was repeated, and the plate was further incubated with a secondary antibody, peroxidase-conjugated affinity-pure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.), diluted 1 : 5000 in PBS and azino-diethylbenzthiazolinesulfate (ABTS) as substrate (Boehringer Mannheim, Mannheim, Germany), and the absorbance at 405 nm was recorded.

T-cell hybridomas. T-cell hybridomas used have been described in detail earlier [23, 24]. HCQ.10 responds to CII, positions 256–270, with a monosaccharide on lysine 264 which is the post-translational form, common in cartilage and dominant in the T-cell response [25]. The T-cell hybridoma HOB.6 responds to OVA. In the cell cultures, 50×10^3 T-cell hybridoma cells were co-cultured with APC and antigen in a total volume of 200 μl in flat-bottomed 96-well plates (Nunc, Roskilde, DK). In most experiments, the APCs were titrated while the amount of T-cell hybridomas and concentration of antigens were held constant. In order to show the sensitivity of the chosen antigen concentration, we also titrated the antigens while the amount of T-cell hybridomas and APCs was kept constant (Fig. 1).

After 24 h in culture, 100 μl aliquots of the supernatants were removed and frozen at −20 °C. To measure the content of interleukin (IL)-2, the supernatants were thawed and cultured with 104 CTLL cells (IL-2-responsive murine T cells) in a total volume of 200 μl for 24 h, and then the cultures were pulsed with [^{3}H]-thymidine for an additional 15–18 h. The cells were harvested in a Micromate 196-cell harvester (Canberra Packard, Meriden, CT, USA) and the radioactivity determined in a Matrix™96 direct β counter (Canberra Packard). All results are mean ± standard deviation (SD) values of duplicate cultures. As a negative control, we used T-cell hybridomas incubated with antigen without APC. We also used another negative control with APC together with T-cell hybridomas without antigen. No detectable IL-2 was present in these cultures. All curves in the proliferation assays represent the biological means of several individual mice or of repeated experiments containing pooled cells from several mice in each experiment.

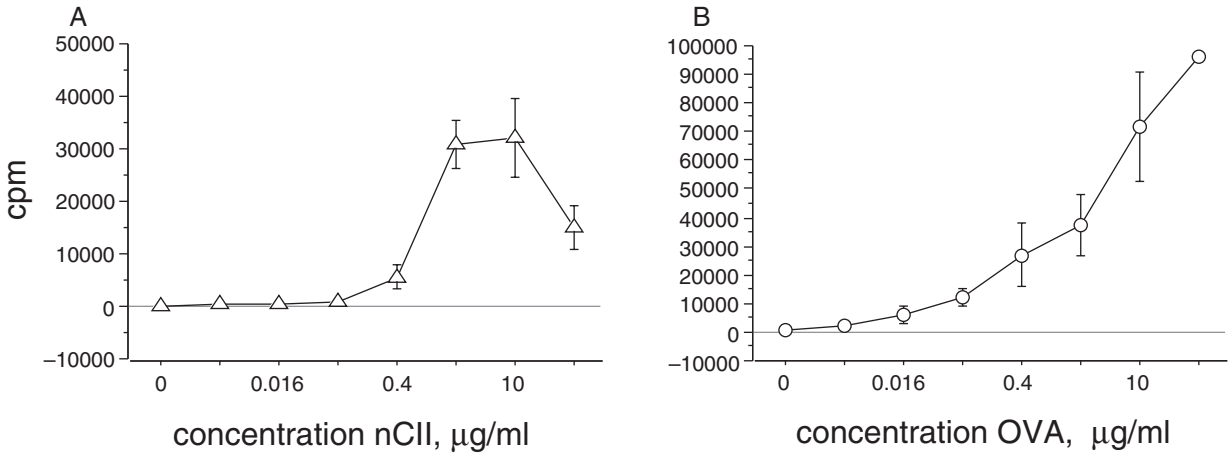

Fig. 1. Presentation of titrated antigens by antigen-primed B cells. Titrated concentrations of native type-II collagen (nCII) (△) and ovalbumin (OVA) (○) on a constant number of nCII and OVA-primed B cells (500×10^3 cells/well) and antigen-specific T-cell hybridomas. The antigen-primed B cells were incubated with the same antigen as they were immunized with. (A) The nCII-primed B cells were incubated with nCII. (B) The OVA-primed B cells were incubated with OVA. Level of interleukin (IL)-2 production of the T-cell hybridomas was determined as proliferation of CTLL cells. The curves represent biological means of two to four individually analysed mice.

RESULTS

Naïve B cells present CII, but less efficiently than CII-primed B cells

To analyse the ability of lymph node B cells to present CII, cells from popliteal and inguinal lymph nodes of naïve (B10.Q × DBA/1) F1 mice were collected. B cells were enriched by MACS separation using microbead-coupled anti-B220 antibody. The purity of the enriched cells (95–98%) was assessed by fluorescence-activated cell sorter (FACS). The remaining 2–5% were mostly macrophages, as they intensively expressed CD11b (data not shown) but could possibly also contain substantial numbers of B cells staining low for B220, as has been recently reported [26]. The B220-selected cells were loaded *in vitro* with either 10 μg/ml of nCII, i.e. triple-helical CII, or dCII, i.e. CII alpha-chain (dCII), and used as APCs for the HCQ.10 T-cell hybridomas. As a positive control, B cells were loaded with 10 μg/ml of OVA and used as APCs for the HOB.6 hybridoma. As shown in Fig. 2, the naïve B cells were able to present nCII, dCII and OVA for the antigen-specific T-cell hybridomas.

To obtain antigen-primed B cells, mice were immunized with nCII, dCII or OVA in FCA, and 11–12 days later, LNCs from draining lymph nodes were prepared and purified as described above; again 95% of the cells were B cells. The antigen-primed B cells were subsequently incubated with antigen-specific T-cell hybridomas and with 10 μg/ml of either nCII, dCII or OVA. The results showed that B cells needed to be primed in order to efficiently present antigens, at least in the context of native proteins, as dCII was not able to induce a strong clonal expansion of the relevant B cells (Fig. 2). This supports earlier observations that B cells preferentially recognize conformational epitopes on the triple-helical structures of collagen and not the denatured collagen alpha-chains [6, 27].

Antigen-primed B cells can also present irrelevant proteins

In Fig. 3, we used purified B cells from mice immunized with nCII, dCII or OVA. The antigen-primed B cells were stimulated *in vitro* with either the same antigen (10 μg/ml) used for immunization or with an irrelevant antigen, and tested using T-cell hybridoma specific for the antigen. When stimulated with the immunized antigen *in vitro*, there was a strong proliferation whereas stimulation with irrelevant antigens was two to three times weaker but still significantly over the background. The unspecific proliferation was similar to the proliferation caused by naïve B cells. The irrelevant antigens may have been picked up unspecifically by endocytosis. It is probable that B cells present antigens captured both by membrane-bound Ig and by the receptor-nonspecific uptake on MHC class-II. However, the Ig receptor-mediated uptake was more efficient as we observed an enhanced efficiency in presentation if the B cells were stimulated with the immunized antigen, but a significantly weaker response with the irrelevant antigen, arguing for a role of antigen-specific B cells.

CII-reactive antibodies do not enhance the macrophage presentation of CII

The difference observed between the primed and naïve B cells could possibly be owing to the increased amounts of CII antibodies, which may aid the antigen-presentation capacity of the possibly contaminating macrophage population,

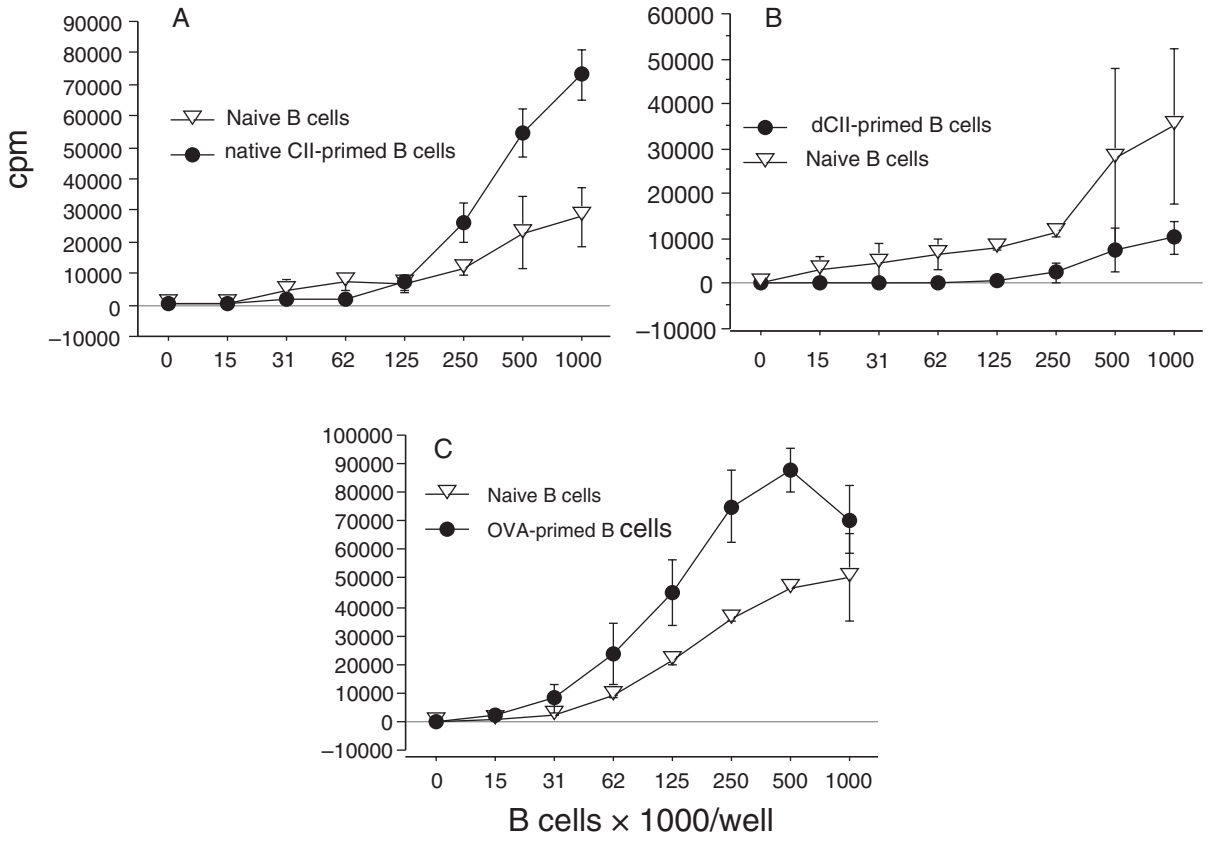

Fig. 2. Presentation of antigen by naïve and antigen-primed B cells. Titrated number of naïve B cells (▽) and antigen-primed B cells (●) were incubated with a constant dose of antigen (10 μg/ml) and antigen-specific T-cell hybridomas. The antigen-primed B cells were incubated with the same antigen as they were immunized with. (A) The B cells were incubated with native type-II collagen (nCII). (B) The B cells were incubated with denatured CII (dCII). (C) The B cells were incubated with ovalbumin (OVA). Level of interleukin (IL)-2 produced by the T-cell hybridomas was determined as proliferation of CTLL cells. The results are biological means of two to five repeated experiments, each experiment containing pooled cells from several mice.

thereby giving a greater efficiency in presentation. An enhanced antigen-specific antigen-presentation function by macrophages may be explained by Fc receptor-mediated uptake. This was clearly a possibility, as we regularly find levels of anti-CII IgG antibodies (approximately 2 μg/ml) in cultures of B cells incubated with nCII and T-cell hybridomas. Enhanced antigen presentation by dendritic cells and macrophages via Fc receptors has been observed by several groups [28, 29]. To address this possibility, we immunized (B10.Q × DBA/1) F1 mice, and 11 days later isolated B cells from the draining lymph nodes. The B cells were stimulated with 10 μg/ml of nCII and the T-cell hybridoma, HCQ.10. After 24 h, the supernatants, containing anti-CII autoantibodies, were collected and stored at −20 °C. B cell-deficient mice (μMT-BQ) were then immunized with nCII, and LNCs were collected on days 11 and 12, and stimulated with nCII *in vitro*. We dissolved the LNC (10^6 cells/well), containing mostly macrophages, in the thawed supernatant containing anti-CII antibodies, 2 μg/ml. The presentation efficiency of this macrophage culture was then tested using 10 μg/ml of nCII and the T-cell hybridoma HCQ.10, which was kept constant. The efficiency of macrophage antigen presentation was measured after 24 h using the CTLL assay on the supernatants. In spite of anti-CII antibodies, presentation of CII by macrophages was not augmented (Fig. 4).

DISCUSSION

B cells play an important role in the development of CIA, but their activation is dependent on cooperation with CII-specific T cells. Here we have shown that B cells present CII to T cells through receptor-mediated uptake of triple-helical CII. This receptor-mediated uptake has been observed for many other antigens [30, 31] but has not been obvious for a triple-helical collagen structure such as CII and will be of critical importance for understanding the development of CIA.

It is well established that B cells are potent APCs for T cells [32–34]. The most efficient antigen-presentation pathway is through antigen uptake by the Ig receptor on the

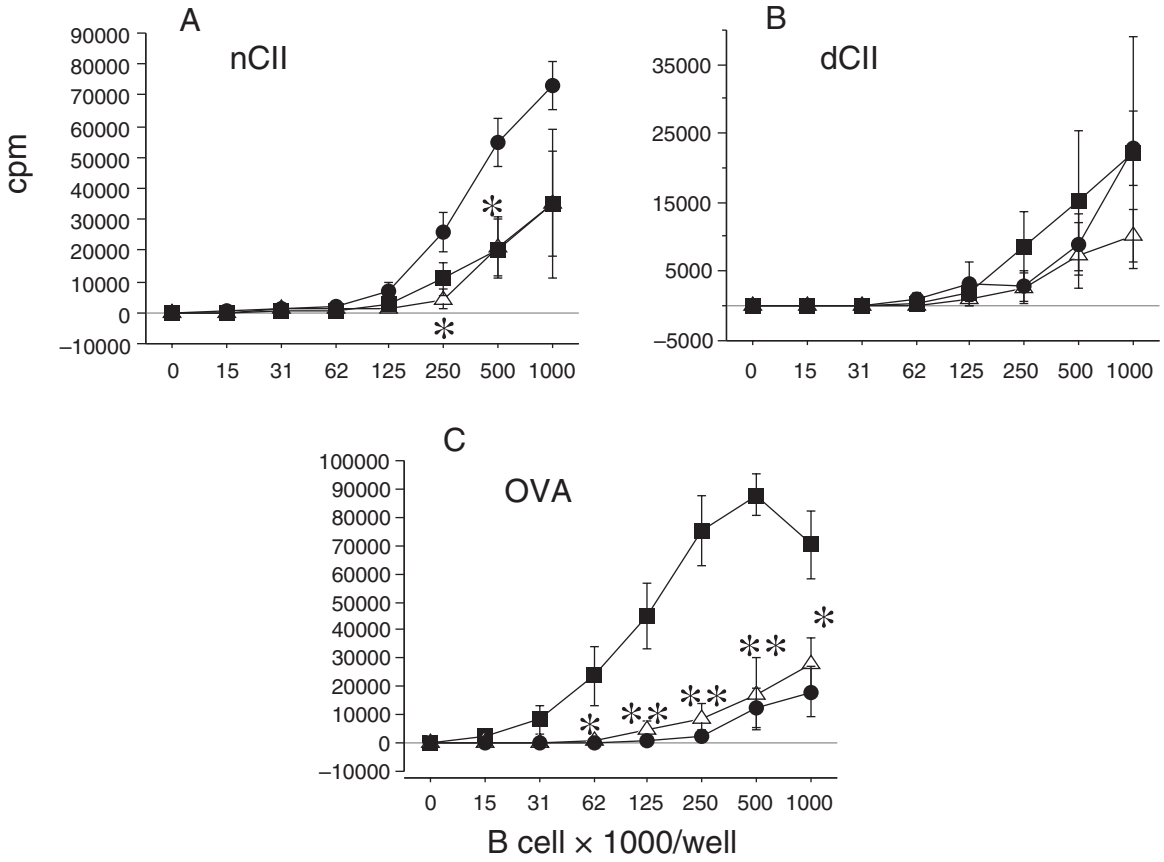

Fig. 3. B cells preferentially present the antigen they are primed with except for denaturated type-II collagen (dCII). The mice were immunized with either native CII (nCII) (A) or dCII (B) or OVA (C) in FCA. After 11–12 days, draining lymph node cells (LNCs) were positively selected on B220 expression and used as antigen-presenting cells (APCs). The selected B cells were incubated with 10 μg/ml of the same antigen as immunized but also with the irrelevant antigen, with nCII (●) or dCII (△) or OVA (■). HCQ.10 T-cell hybridomas were used with nCII- and dCII-loaded B cells and HOB.6 with OVA-loaded B cells. (A–C) The interleukin (IL)-2 produced by the T-cell hybridomas was determined as proliferation of CTLL cells, and the cpm (counts per minute) was counted. $* = P < 0.05$; $** = P < 0.01$. *Analysis of variance:* The curves are biological means of three to five repeated experiments, each experiment containing pooled cells from several mice.

cell surface, where B cells specifically capture the native antigen. The amount of antigen required for effective presentation is less when targeted via surface Ig than if the antigen is taken up by endocytosis [33, 35]. In addition, the processing of antigens captured by surface Ig is more rapid [36] and utilizes a different processing pathway, as compared with more nonspecific endocytic uptake [37].

The *in vivo* role of B cells as APC in priming naïve, activated and memory T cells is still unclear. Furthermore, the role of B cells will most likely differ depending on factors such as antigen, status of the T cells and the context of costimulation. Importantly, it is not yet clear whether B cells could prime and/or tolerize against self-antigens, during a chronic and possibly perpetuating autoimmune disease course.

Experiments using mice lacking B cells have shown that different antigens are handled differently. Immunization with certain antigens including OVA has been reported to lead to a weaker T-cell response in B cell-deficient mice [38]. However, immunization with most other antigens, including CII, was equally effective in priming T-cell responses in the absence of B cells [20, 39]. This does not exclude B cells from priming naïve T cells but argues for the T-cell response to these antigens to be primed by other APCs, such as macrophages and dendritic cells. It has been reported that B cells containing certain self-antigens (cytochrome c) can prime naïve T cells [40]. However, Hagerty [41] and Stockinger [42] found that other self antigens, such as complement C5 or haemoglobin, cannot be presented at all by B cells *in vivo*, in contrast to foreign antigen such as OVA.

In fact, it has been shown that the outcome of B-cell priming of naïve T cells normally leads to tolerance rather than immune activation, and that only already-primed T cells can be fully activated by B cells [43–45]. Interactions of

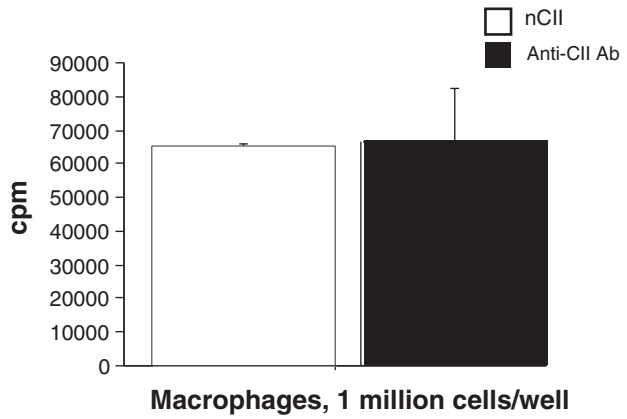

Fig. 4. Anti-collagen type-II (CII) antibodies produced by CII-primed B cells did not augment macrophage presentation of native CII (nCII). nCII-primed macrophages from B cell-deficient mice (μMTBQ) were diluted in medium containing anti-CII antibodies (2 μg/ml) and incubated with nCII (10 μg/ml) and a constant amount of antigen-specific T-cell hybridomas HCQ.10. The interleukin (IL)-2 produced by the stimulated T-cell hybridomas was determined as proliferation of CTLL cells. The results are means of two repeated experiments, each experiment containing pooled cells from several mice.

already-primed T cells with antigen-specific B cells may have an essential role both for the rapid expansion of antigen-specific T cells [46] as well as for the triggering of B cells to enter the germinal centre and to differentiate into plasma cells.

One unusual property of collagen is that dendritic cells (believed to be the main APC to prime T cells) are inefficient in presenting CII. Moreover, the observation that B cell-deficient mice give a strong T-cell response to CII [20] argues that APCs additional to B cells can prime CII-reactive T cells, presumably macrophages. Observations that B-cell lymphomas displayed the same features as dendritic cells in regard to collagen presentation, i.e. inefficient in presenting collagen molecules but efficient in presenting collagen peptides, OVA [47] or its own Ig-receptors [48], have led to the belief that CII is not presented by B cells. Confirming this view, it was reported that both naïve and primed-B cells, purified from cathepsin-S-deleted and wildtype DBA/1 mice, were inefficient in presenting CII to CII-specific T-cell hybridomas [18].

However, it is difficult to accept that B cells are unable to present CII, given the crucial role of B cells in the induction of the very strong and T-cell dependent B-cell response after immunization with CII. It is therefore important to understand the specific role of B cells *in vivo* for the further analysis of the pathogenesis of CIA which is an important model for RA. To re-analyse the role of B cells as APC for CII, we selected a (B10.Q × DBA/1) F1 mouse, known to have a strong B-cell response to CII. We also selected two equally sensitive T-cell hybridomas for the read out, one specific for OVA as a positive control and the other CII-specific, detecting the major CII peptide CII256-270 with a monosaccharide on lysine 264 bound to the Aq molecule expressed by (B10.Q × DBA/1) F1 mice. In addition, care was taken in the isolation of a pure population of B cells using a one-step positive selection. The results showed that antigen-primed B cells have the capacity to present CII. As expected, the antigen-primed B cells were more efficient than the naïve B cells, suggesting that both sIg-mediated uptake and endocytotic uptake were operating. However, we did not address whether B cells could prime naïve T cells *in vitro* or *in vivo* or already-activated T cells *in vivo*. This has been shown to occur using other self- and nonself antigens, and it appears that the efficiency and outcome of such interactions vary greatly between the various antigens used.

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