

# Targeting antigen to CD19 on B cells efficiently activates T cells

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## Abstract

**CD19 is a B cell-surface molecule that participates as an important regulatory signaling complex for antigen bound at the surface by Ig. Triggering of CD19 through its linkage with CD21 amplifies signals transduced through the Src family kinases and modulates B cell differentiation in response to antigen. This study examines the kinetics of antigen uptake and processing of antigen directly targeted to the CD19 protein on purified B cells. We have demonstrated that the antigen internalized within minutes through CD19 forms a cap at the B cell surface and can be found within lysosomes in the cytoplasm in 90 min. B cells acquiring antigen via CD19 express elevated levels of B7-1 and B7-2 co-stimulatory molecules. Moreover, antigen–anti-CD19 complexes administered intravenously bind B cells *in vivo* and activate antigen-specific T cells more efficiently than non-specific uptake and in a manner similar to antigen taken up through surface IgM on B cells. This work illustrates an important and previously unrecognized mechanism for targeting proteins to B lymphocytes for antigen presentation and activation of CD4 T cells.**

## Introduction

Antigen presentation to T lymphocytes can be accomplished through a number of mechanisms, most often from peptides presented by a number of cell types, notably dendritic cells, macrophages and B lymphocytes. B lymphocytes have the unique property of acquiring specific antigens from a milieu of proteins through their surface Ig (sIg). We and others have identified an important role for B cells in presenting auto-antigens in the development of autoimmune T cells and leading to tissue pathology (1–7). Indeed, antigen targeted to the BCR or other B cell-surface molecules, including FcR or transferrin receptor, can efficiently target antigen for processing and drive epitope spreading to both foreign and self-antigens (1, 2, 8, 9). In animal models of lupus autoimmunity, the absence of B cells prevents the spontaneous activation of autoreactive T cells (10). Conversely, B cell presentation of auto-antigens can activate specific subsets of autoreactive CD4 T cells in a manner not found with other professional antigen-presenting cells (APCs) such as dendritic cells (10).

B cells receive signals through a number of cell-surface molecules that are critical to their differentiation and de-

velopment. The CD19–CD21 complex provides co-receptor functions that are known to lower the threshold of antigenic stimulation through the BCR (11, 12). BCR and the CD19–CD21 complex work in concert to provide optimal intracellular signaling required for germinal center formation, Ig synthesis and secretion and the development and maintenance of B cell memory (13–15). The over-expression of CD19 can alter the development of B cell tolerance and lead to auto-antibody production (16, 17). CD19-deficient mice fail to develop germinal centers or normal B cell compartments, particularly B-1 B cells, and have <10% of the normal levels of serum IgG (18).

CD19 is expressed by all B cells from the pre-B cell stage until the development of plasma B cells. CD19 associates with CD21, CD81 and CD225 to form a multimeric signaling complex. CD19 mobilizes intracellular calcium and amplifies signals through intracellular MAP kinases (18–21). CD21 is a receptor for the complement cleavage product, C3d, and binds C3d–antigen complexes to the surface of B cells (12). While CD19 is found associated with CD21 at the B cell

surface, CD19 is also found independent of other proteins on the surface, indicating that it may have other unique ligand-binding and/or -signaling activities (16, 18, 22).

The present study has examined the ability of B cells to bind, process and present antigen through association with the CD19 surface protein. Antigen that is targeted to the CD19 protein rapidly forms a cap at the cell surface before it is taken up by the B cell into intracellular vesicles. The B cells up-regulate B7-1 and B7-2 co-stimulatory molecules and efficiently stimulate T cell activation both *in vitro* and *in vivo*. This work suggests that the CD19 protein may be an important surface molecule for the targeting of antigens and the subsequent amplification of antibody and T cell responses to foreign proteins. Alternatively, the ability to rapidly bind CD19 on circulating cells with toxin-linked antibody may be a mechanism by which to clear CD19-bearing lymphoma cells or to clear B cells in autoimmune responses (23, 24).

## Methods

### *Mice*

B10.BR AND transgenic mice expressing an  $\alpha/\beta$  TCR ( $V\alpha 11+$ ,  $V\beta 3+$ )-recognizing pigeon cytochrome c (PCC) p88–104 were a generous gift of Joe Craft (Yale University). AND transgenic mice were maintained as heterozygotes and confirmed by PCR screening of tail DNA. Wild-type B10.BR mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

### *PCC-anti-mouse CD19 complexes*

Purified PCC (Sigma Inc., St Louis, MO, USA) was biotinylated as previously described (10). Biotin-anti-mouse CD19 was purchased from BD-PharMingen (San Diego, CA, USA). The biotin-labeled PCC and anti-mouse CD19 were incubated with avidin (Pierce Chemical Co., Rockford, IL, USA) based on the molar ratio (40 : 1) for 2 h at room temperature. The reaction was terminated by adding PBS. The mixture of PCC-linked anti-CD19 was centrifuged through 100 kDa membranes (Millipore Corporation, Billerica, MA, USA) to remove free avidin and free PCC. Non-specific control complexes were synthesized as above using purified rat IgG2a complexed to PCC.

### *PCC-anti-mouse IgM complexes*

Biotinylated PCC was incubated with 0.1 mg of succinimidyl 4*N*-maleimidomethyl cyclohexane-1-carboxylate (Pierce Chemical Co.) per milligram of PCC for 1 h at room temperature with rotation. Rat anti-mouse IgM (IgG2a isotype; Pierce Chemical Co.) was reduced in 20 mM dithiothreitol per milliliter of antibody at room temperature for 30 min. The PCC together with reduced antibody were incubated at room temperature for 1 h and the reaction was terminated by adding 34  $\mu$ g of *N*-ethylmaleimide. PCC-linked anti-IgM was separated from free PCC by centrifugation through filters as above (50 kDa cutoff). In some experiments as indicated, anti-CD19 antibody was also chemically cross-linked with biotinylated PCC. Control, non-specific complexes were synthesized with purified rat IgG2a as described above.

### *Flow cytometry of antigen-antibody binding to B cells*

Splenocytes were prepared from B10.BR mice and incubated with FcR-blocking antibody (anti-CD16; BD-PharMingen) on ice for 30 min followed by incubation with PCC-anti-CD19, PCC-anti-IgM or biotin-PCC on ice for 30 min. Cell suspensions were washed and stained with streptavidin-PE and B220-CyChrome. In some experiments as indicated, the expression of B7-1 and B7-2 was examined by flow cytometry (anti-B7-1 and anti-B7-2 from Promega, Inc., Madison, WI, USA) after targeting of antigen to CD19 or IgM. All samples were analyzed on a FACSCalibur flow cytometry (BD Biosciences, Mountain View, CA, USA). The data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

### *Antibodies and immunofluorescence*

Splenic B cells were purified using anti-mouse CD43 microbeads (Miltenyi Biotec, Auburn, CA, USA) as previously described according to the manufacturer's instructions. Purified B cells (>95% purity as verified by flow cytometry) were incubated with PCC-anti-CD19 followed by streptavidin-PE or anti-IgM-FITC at 37°C for 10 min, 30 min or 90 min, as indicated in the figures. Images were photographed by digital microscopy (Carl Zeiss, Inc., Thornwood, NY, USA). For colocalization studies, purified B cells were incubated with 1  $\mu$ g of biotinylated PCC-anti-CD19 for 30 min at 37°C followed by Streptavidin-PE (SA-PE) (BD-PharMingen). Cells ( $0.5-2 \times 10^5$ ) in serum-free RPMI 1640 were allowed to adhere to 1% alcian blue (Sigma Inc.)-coated cover slips at 37°C, 5% CO<sub>2</sub>, for 30 min. Cells were fixed in 4% PFA in PBS at room temperature for 15 min and permeabilized in RPMI 1640 containing 10% goat serum (Invitrogen, Carlsbad, CA, USA), 10 mM HEPES (Invitrogen, Carlsbad, CA, USA), 10 mM glycine and 0.05% saponin (Sigma). mAbs were utilized for staining. Lysosomes were detected using rat anti-mouse LAMP-2/Igp-B mAb GL2A7 and anti-I-A<sup>k</sup> (25). Secondary antibodies used were goat anti-rat Ig Alexa-488 (Invitrogen, Carlsbad, CA, USA) and goat anti-mouse Ig Cy5 (Jackson ImmunoResearch, West Grove, PA, USA). Cover slips were mounted in ProLong media (Molecular Probes Inc.) and fluorescence was analyzed on a Zeiss confocal microscope (0.3- to 0.6- $\mu$ m optical sections) using LSM 510 software. Projections of 4–8 z-sections or single slices are shown in figures.

### *In vitro activation of PCC-specific T cells by PCC-anti-CD19 or PCC-anti-IgM complexes*

AND transgenic CD4 T cells were purified by negative selection using anti-mouse CD8, CD11b, Class II and CD8 microbeads (Miltenyi Biotec). The purity was assessed by FACS (>95%). AND transgenic T cells ( $2 \times 10^5$  per well) were co-cultured with  $5 \times 10^5$  irradiated purified B cells (2500 rads) in the presence of PCC-anti-CD19 or PCC-anti-IgM complexes or free PCC. All assays were performed with triplicate samples and incubated for 3 days. Lymphocyte proliferation was assessed by [<sup>3</sup>H]thymidine incorporation (1.0  $\mu$ Ci per well; ICN Chemicals, Irvine, CA, USA) during the last 18 h of culture. Sample wells were harvested onto filters, and incorporated radioactivity was counted in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD, USA).

*In vivo targeting of antigen to B cells with PCC–anti-CD19 or PCC–anti-IgM*

B10.BR mice were intravenously (i.v.) administered 10 µg of PCC–anti-mouse CD19 or PCC–anti-mouse IgM complexes or biotin–PCC as control. The peripheral blood was harvested with heparin at the indicated time points in the figures. PBMCs were enriched by Ficoll-Hypaque density separation. Cells were mixed with FcR-blocking anti-CD16 antibody followed by incubation with streptavidin–PE and B220–CyChrome (BD-PharMingen). Analytical flow cytometry was conducted on a FACSCalibur and analyzed using FlowJo (Tree Star Inc.) software.

*In vivo activation of T cells by B cells pulsed with PCC–anti-CD19 or PCC–anti-IgM*

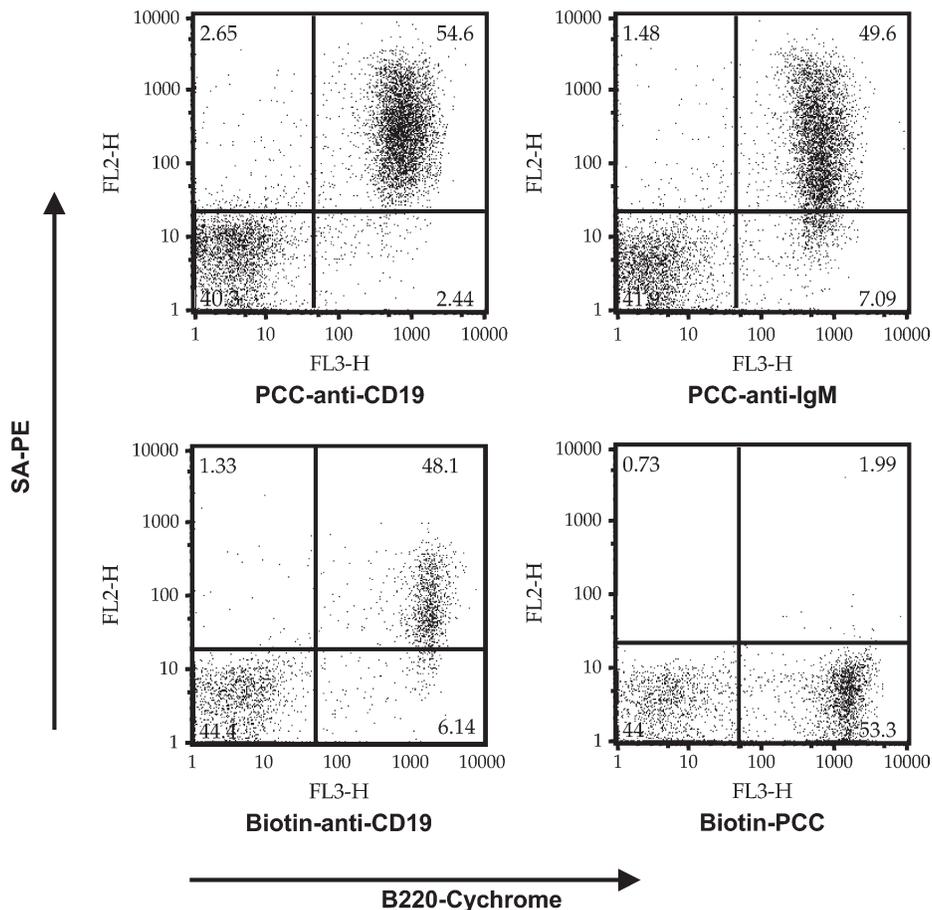
Purified AND transgenic CD4 T cells were labeled with 5( and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Inc.). CFSE-labeled transgenic T cells were washed, counted and re-suspended in HBSS for injection. Cell suspensions of  $10 \times 10^6$  purified CD4 T cells were injected i.v. After 1 h, 10 µg of PCC–anti-CD19, PCC–anti-mouse IgM or biotin–PCC was i.v. administered to mice. Recipient mice were sacrificed 72 h after transfer. The spleens

were collected and cell suspensions were prepared for flow cytometry analysis.

## Results

*PCC, as a model antigen is efficiently targeted to B cells*

We first constructed antigen linked to anti-CD19 or anti-IgM as a means to target the model antigen, PCC, to B lymphocytes for antigen presentation. The PCC antigen–anti-B cell antibodies are not conventional immune complexes in that their uptake into B cells was not mediated via FcR, but instead targeted to either cell-surface CD19 or IgM. We first examined the ability of B cells to bind antigen linked to anti-CD19 or anti-IgM *in vitro*. Not surprisingly, both biotin-labeled anti-CD19 as well as PCC conjugated to anti-CD19 or anti-IgM readily binds to B lymphocytes upon incubation together *in vitro* (Fig. 1). The specificity of the PCC–anti-CD19 complex binding to B cells through CD19 was confirmed with purified anti-CD19 antibody cold-competition assays (data not shown). As another specificity control, biotinylated PCC alone fails to significantly bind to B220+ cells (Fig. 1, lower right panel). The biotin–PCC was the same as that utilized in the making of PCC–anti-CD19 immune conjugates. All of these



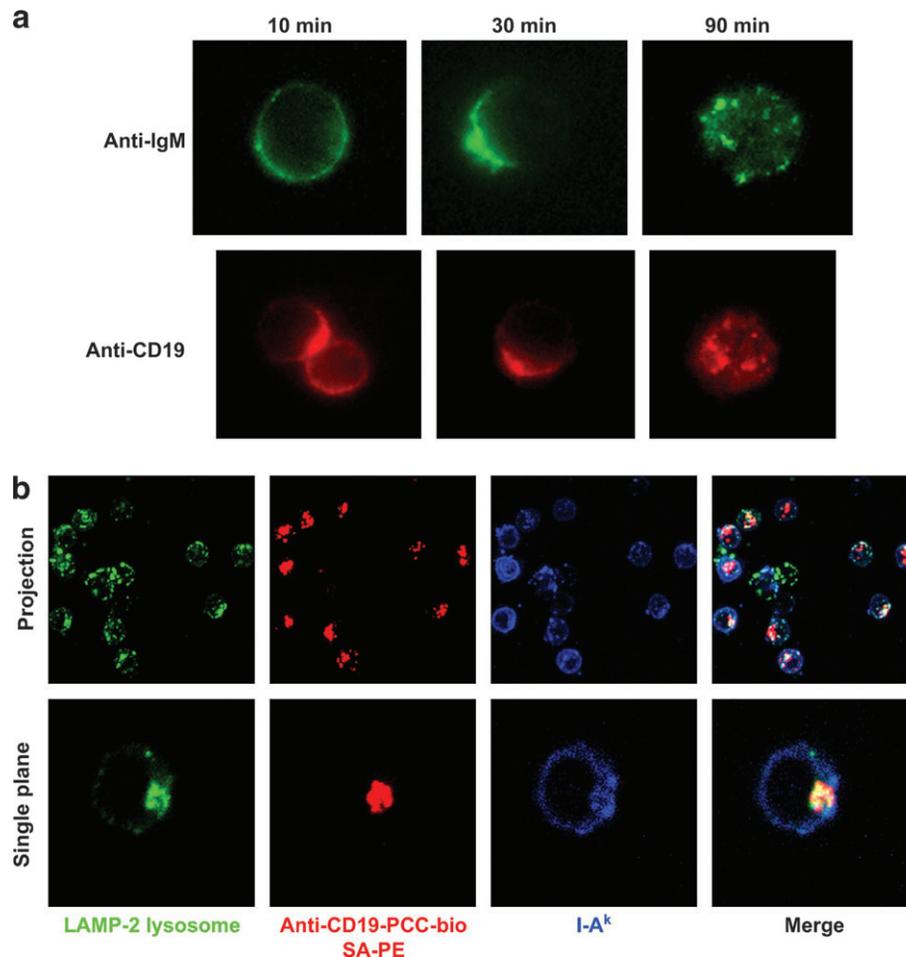
**Fig. 1.** PCC conjugated to anti-CD19 or anti-IgM readily binds to B220+ B cells *in vitro*. Spleen cells were incubated with biotin–PCC conjugated to anti-CD19 or anti-IgM antibody. The binding of PCC antigen was detected in the presence of avidin–PE on B220+ B cell populations. As positive and negative controls, spleen cells were also incubated with biotin–anti-CD19 and biotin–PCC, respectively.

studies were performed in the presence of FcR-blocking reagents (anti-CD16/32) to focus antigen uptake through CD19 as intended. Moreover, the inability of control IgG conjugated to PCC to be found on purified B cells suggests that uptake is not mediated via FcRs.

We next wanted to examine the kinetics of binding and uptake of antigen into B cells through the CD19 or IgM receptors *in vitro*. For these studies, purified B cells were incubated with biotin-anti-CD19 or anti-IgM ranging from 10 to 90 min in culture. The cells were then fixed and fluorescence microscopy was then performed at the indicated time points (Fig. 2). Within 10 min in culture, both anti-CD19 and anti-IgM were found to cover the vast majority of the cell surface. Within 20–30 min, the anti-CD19 bound to antigen at the surface begins to form a cap at one side of the cell surface. A similar capping response was found after IgM cross-linking within approximately the same period of time, 20–30 min. The intensity of staining was slightly greater for IgM capping as compared with the capping of the CD19 molecules. Within 90 min, the cell-surface staining had disappeared and virtually

all of the antibodies had become internalized into intracellular vesicles. In general, the kinetics of binding and uptake into B cells was similar between anti-CD19 and anti-IgM cell-surface targets by microscopy (Figs 2A and 5A) and by flow cytometry (data not shown).

To determine whether PCC entering the B cell by CD19 was targeted to the lysosome, we purified B cells and co-stained with biotinylated PCC-anti-CD19 conjugates and the lysosome marker LAMP-2 (Fig. 2B). Two sets of images are illustrated, one that shows a projection of all z-sections (Fig. 2B, upper panels) and another that defines marker locations through a single optical plane (Fig. 2B, lower panels). As illustrated earlier, antigen linked to anti-CD19 rapidly finds its way into the cell. Analysis of the merged profiles for antigen, Class II and the lysosomal marker shows some co-localization, indicating that some intracellular vesicles are likely to contain all three. The data suggest that antigen entering the B cell through CD19 was delivered to the lysosomal compartment as a means by which to gain entry to the MHC class II processing pathway.



**Fig. 2.** Antibody binding to either sIgM or surface CD19 readily forms a polarized cap and is internalized into lysosomes in the B cell within 90 min (A). Fluorescent microscopy was performed after *in vitro* incubation of purified B cells with either anti-IgM or anti-CD19 and FcR-blocking antibodies. Global surface staining is noted within 10 min of incubation. Polarized cell capping is found by 30 min while internalization from the cell surface occurs by 90 min of incubation with either anti-IgM or anti-CD19 binding. (B). For co-localization studies, purified B cells were incubated with biotinylated PCC-anti-CD19 followed by avidin-PE. Cells were fixed, permeabilized and labeled with anti-LAMP-2 mAb and anti-Class II mAb as indicated in *Methods*.

### B cells efficiently present antigen taken up through the CD19 receptor

Although antigen could be taken up into B cells through the CD19 surface protein, it was not clear if this pathway would lead to antigen presentation in the context of MHC class II molecules, subsequently activating antigen-specific T cells. To test this possibility, AND TCR transgenic T cells were used as a marker of PCC peptide presentation. For AND T cells to respond, PCC protein would need to be internalized and processed to present p88–104 in the context of I-E<sup>k</sup>. Indeed, PCC conjugated to anti-CD19 stimulated AND responder T cells nearly 100-fold more efficiently as compared with PCC protein alone or control IgG linked to PCC (Fig. 3A). T cell responses were proportional to the concentration of PCC conjugated to anti-CD19. B cells could present unconjugated PCC for AND T cell stimulation only if PCC was present in vast excess ( $> 10 \mu\text{g ml}^{-1}$ ). By comparison, PCC linked to irrelevant IgG (Fig. 3A, solid squares) or PCC added to cultures along with anti-CD19 as an uncomplexed combination (Fig. 3A, solid triangles) stimulated T cells in a manner similar to PCC alone and also 100-fold less efficiently than antigen linked to anti-CD19. Similarly, the degree of T cell stimulation was linearly related to the number of B cells presenting antigen (Fig. 3B). As controls, haplotype-mismatched C57Bl/6 B cells pulsed with PCC–anti-CD19 failed to stimulate AND T cells. As controls, B cells alone, of any haplotype, fail to stimulate AND T cells.

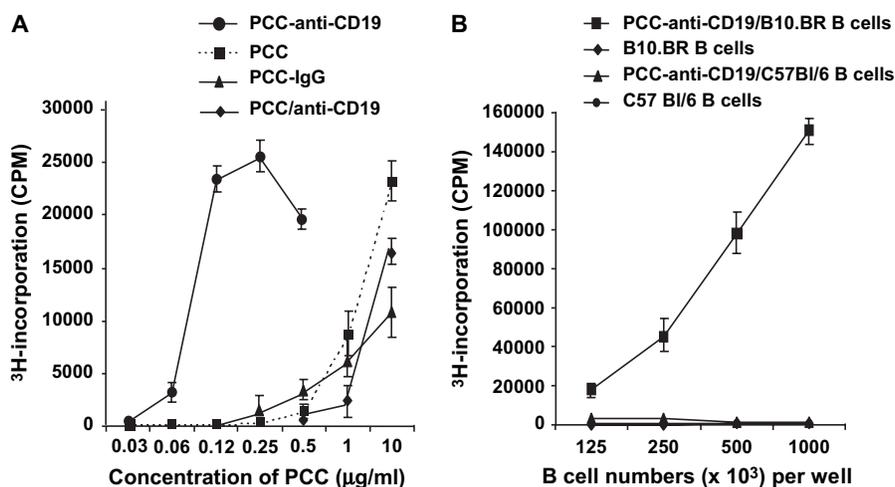
In a second group of parallel studies, purified B cells were pulsed with PCC linked to either anti-CD19 or anti-IgM. While antigen targeted to either CD19 or IgM on B cells will stimulate AND T cells, uptake through CD19 appears at least as efficient a route of presentation as directly compared with uptake of complexes through sIgM (Fig. 4). As described earlier, PCC alone or control IgG linked to PCC is relatively inefficiently presented for T cell activation. Both types of immune conjugates were constructed on a similar basis of avidin–biotin association.

### The uptake of antigen through CD19 stimulates the expression of surface B7-1/B7-2 co-stimulatory proteins

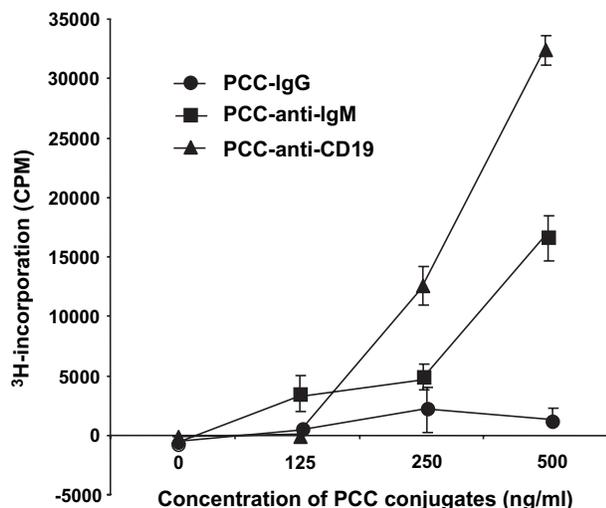
The efficient activation of naive T lymphocyte responder cells requires the presentation of antigen along with co-stimulatory second signals from the APC. Upon the targeting of PCC to CD19, the expression of B7-1 and B7-2 were examined by flow cytometry. As found in Table 1, B cells significantly up-regulate the expression of B7-2 while the expression of B7-1 was only slightly enhanced after CD19 targeted antigen binding. The increase in B7-2 was similar to that found after sIg cross-linking (data not shown), although not as dramatic as the increase observed after LPS stimulation of B cell populations. These observations suggest that B cells may become activated and efficient APCs after the uptake of antigen via CD19. Antigen alone (PCC) or control IgG linked to PCC failed to initiate significant increases in B7 expression.

### Antigen targeted to CD19 *in vivo* stimulates antigen-specific T cells

We next tested the ability of B cells to acquire antigen *in vivo* through either the CD19 or IgM receptors. Mice were given i.v. injections consisting of 10  $\mu\text{g}$  of PCC–anti-CD19 or PCC–anti-IgM conjugates in PBS. After 2 h, mice were sacrificed and the uptake of antigen was measured in splenocytes by flow cytometry in the presence of streptavidin–PE (Fig. 5A). Remarkably, 38% of splenocytes acquired PCC–anti-CD19 after only 2 h *in vivo* (Fig. 5A, upper left panel), only slightly less than the uptake of biotin–anti-CD19 in the absence of PCC antigen (Fig. 5A, lower left panel). This observation suggests that the presence of PCC on the anti-CD19 antibody does not significantly interfere with its binding to B cells or its uptake. Virtually, no detectable biotinylated PCC was taken up in a non-specific manner (Fig. 5A, lower right panel). PCC conjugated to anti-IgM found its way onto approximately half of the splenocyte population after 30 min *in vivo* (Fig. 5A, upper right panel). A similar frequency of uptake by B cells



**Fig. 3.** B cells pulsed with PCC–anti-CD19 stimulated T cells *in vitro*. Purified B cells were incubated with titrations of PCC conjugated to anti-CD19 (circles), with PCC alone (squares), with PCC linked to non-specific IgG (triangles) or with PCC mixed with unconjugated anti-CD19 (diamonds). AND transgenic, PCC-responsive T cells were used as the read-out for antigen presentation by B cells after 3 days of co-culture. In panel B, B cells pulsed with PCC–anti-CD19 or PCC alone were titrated in co-cultures with AND transgenic T cells. As indicated, both haplotype-compatible B10.BR and haplotype-mismatched C57Bl/6 B cells were utilized as APCs. All data represent the average counts per minute for triplicate cultures.



**Fig. 4.** Antigen presentation by B cells through CD19 is more effective than through sIgM. In parallel studies, purified B cells were incubated with PCC conjugated to either anti-IgM or anti-CD19 and co-cultured with AND transgenic responder T cells. As illustrated, delivery of PCC antigen through CD19 is approximately twice as efficient as PCC taken up through sIgM. Data represent the average of triplicate wells.

was also observed in the lymph nodes of these recipient mice (data not shown).

We also assessed the *in vivo* uptake of antigen-anti-CD19 in PBMCs (Fig. 5B). Individual mice were *i.v.* administered PCC-anti-CD19 and were serially bled at the indicated time points. Antigen binding was measured with streptavidin-PE in the B220 fraction of PBMCs. Nearly 85% of the peripheral blood B220+ cells have acquired antigen after only 30 min of exposure to soluble antigen conjugates *in vivo*. This level of antigen binding remains relatively stable during 2 h *in vivo* and then begins to decrease after 4 h, most likely due to the turnover of antigen conjugates at the cell surface. A second possibility is that there was a dramatic efflux of B cells that have acquired antigen out of the circulation and into peripheral tissues. Our data support the former hypothesis of B cells that are actively metabolizing antigen taken up through the CD19 receptor. This is supported by parallel studies from individual mice in which peripheral B cells, splenic B cells and lymph nodes were assessed by fluorescence for antigen turnover after 4 h. We found that turnover of fluorescence, as a probe for antigen, was identical between these three sources of B cells (data not shown). Moreover, we also did not find increased numbers of B cells with fluorescence in the peripheral lymphoid tissues over the time points assessed in Fig. 5.

#### *T cell activation in vivo after targeting antigen to the CD19 complex of B lymphocytes*

Upon directing specific antigen to B cells through surface CD19, we tested whether antigen-specific T lymphocytes could be activated *in vivo*. Naive AND T cells were labeled with CFSE and administered to recipient mice on the same day as receiving *i.v.* administration of PCC conjugated to either anti-CD19 or anti-IgM. Splenic cells or lymph node cells were obtained 3 days later. As found in Fig. 6, T cells undergo at least three divisions within the first 3 days of exposure to antigen

**Table 1.** The percentage of B7-1- and B7-2-positive B cells in the presence of different stimulators<sup>a</sup>

	Medium	PCC-anti-CD19	PCC-anti-IgM	PCC	PCC-IgG	LPS
B7-1	5	10	12	3	4	20
B7-2	5	29	36	5	8	54

<sup>a</sup>Purified B cells were incubated with medium alone or with 10  $\mu\text{g ml}^{-1}$  PCC-anti-CD19, PCC-anti-IgM or LPS, at 37°C for 24 h. The cells harvested and stained with anti-B7-1-FITC, anti-B7-2-PE and B220 Cy. Cells were gated on B220+ populations.

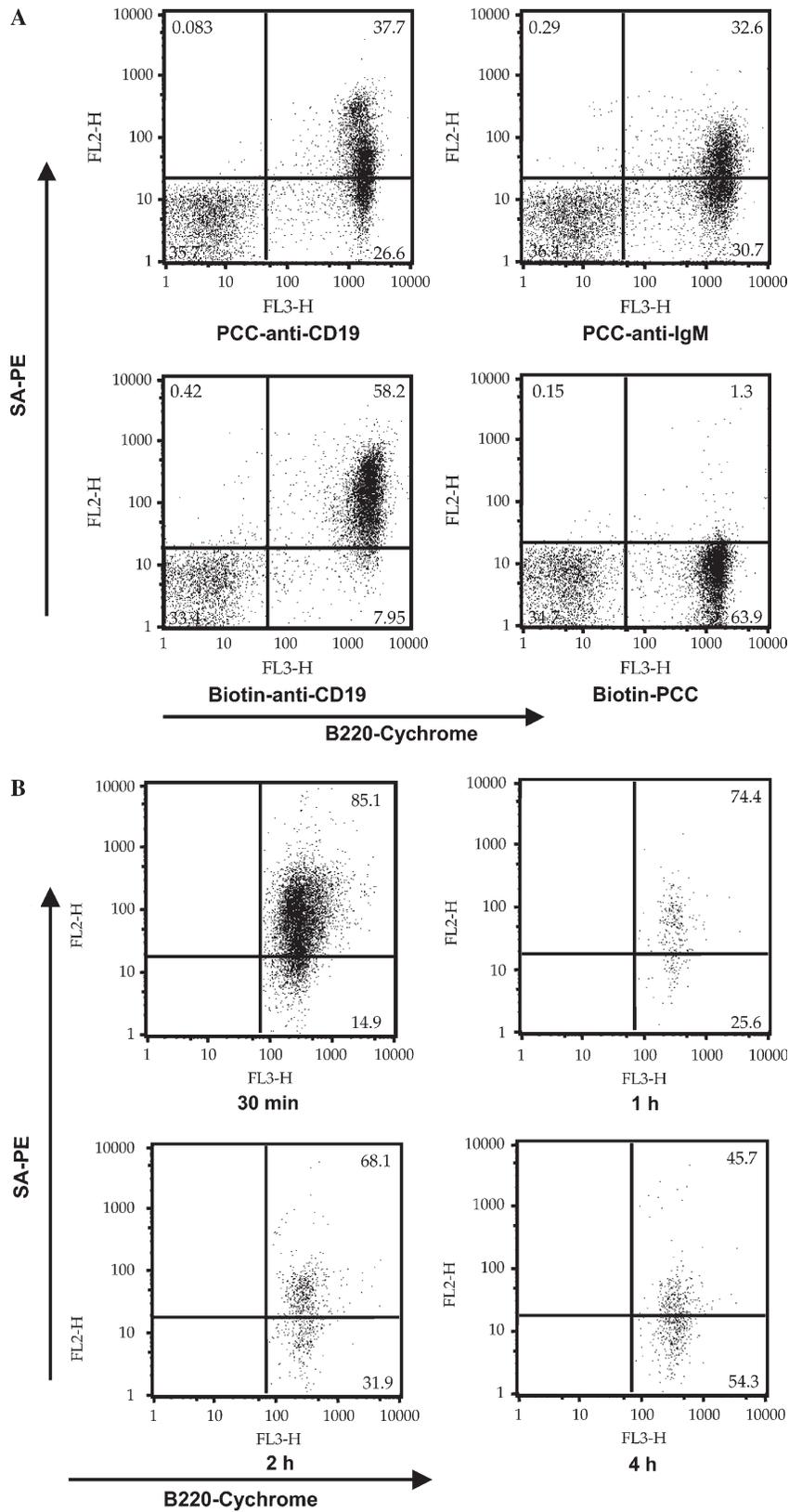
*in vivo*. By comparison, AND T cells do not respond to soluble unconjugated PCC administered *in vivo* (Fig. 6, left panel).

## Discussion

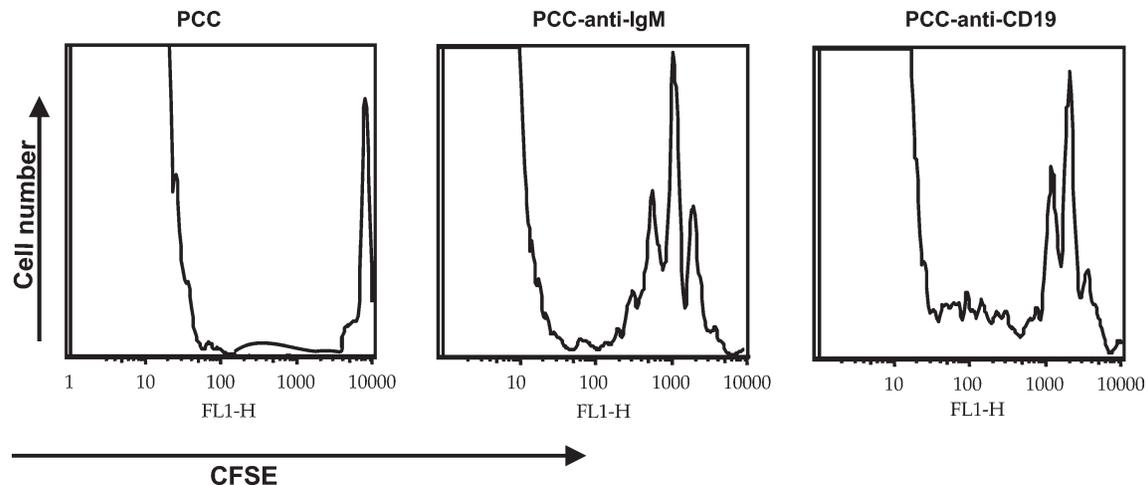
The basis for the present study is founded in the observations that the CD19 surface protein both lowers the threshold of activation of B cells through the BCR and also participates in the presentation of C3d-coated antigen during the course of normal immune responses. We reasoned that the CD19 protein may be an excellent candidate by which to target foreign antigen for presentation to the immune system.

The CD19 molecule, yet another member of the Ig supergene family, is present on pre-B lymphocytes until the plasma cell stage of differentiation, making it an ideal marker for B cells throughout both primary and secondary lymphoid tissues (12, 22). The CD19 protein may be found alone at the cell surface but also forms complexes with CD21 (also known as complement receptor 2) and with CD81 (TAPA-1). CD19 contributes to the activation of B cells by its direct ligation to BCR, independent of CD21 or, alternatively, by its formation of complexes with CD21/CD81/BCR. In a manner similar to CD4 or CD8 on T cells, the functions of CD19 have been regarded as a 'co-receptor' or as 'co-stimulatory' for B cells since the signals delivered through the BCR are enhanced from 1000- to 10 000-fold by the presence of CD19 (11, 12).

The associated protein, CD21, is capable of binding C3d-coated antigens for uptake and presentation by B lymphocytes in concert with CD19 and BCR co-ligation (12). The cytoplasmic tail of CD19 possesses critically conserved tyrosine residues that function as SH2-binding domains leading to increased calcium mobilization and the subsequent activation of the JNK, ERK and p38 MAP kinase activation pathways (21, 26–28). Signaling through CD19, and two tyrosine residues in particular, is essential for virtually all aspects of B cell development, including germinal center formation and the maturation of both T-dependent and T-independent antibody responses (26). With a better understanding of these B cell co-stimulatory mechanisms, it is now clear how earlier studies have found that CD19-deficient B cells fail to respond to conventional BCR signaling, make little or no antibody and fail to form normal germinal centers (12, 14, 15). In contrast, an over-expression of CD19 is linked with B cell hyperactivity and autoimmunity, including auto-antibody production and pathology (29, 30). Recent studies have identified both the over-expression and specific polymorphisms of human CD19 with autoimmune syndromes



**Fig. 5.** Soluble antigen conjugated to anti-CD19 is rapidly bound by B cells *in vivo*. PCC conjugated to anti-CD19 or anti-IgM was administered by i.v. injections in recipient mice. After 2 h, animals were sacrificed and the binding of antigen was assayed with avidin-PE on B220+ splenic B cells (panel A). As controls, biotin-anti-CD19 and biotin-PCC were administered in an identical manner. In panel B, mice were injected with soluble PCC-anti-CD19 conjugates and bled at the indicated time points. The binding of antigen in PBMCs is noted with avidin-PE.



**Fig. 6.** B cells administered soluble, i.v. PCC-anti-CD19 stimulate T cells *in vivo*. Mice were injected with soluble PCC conjugated to anti-CD19 or anti-IgM or unconjugated PCC antigen. On the same day, mice also received CFSE-labeled AND transgenic T cells. Recipient mice were sacrificed after 3 days and the turnover of T cells from the splenic cells or lymph nodes cells were examined by flow cytometry.

(17, 31). CD19, as a co-receptor for BCR, could possibly lower the threshold of B cell responses to self-proteins and contribute to auto-antibody production in clinical syndromes such as systemic lupus erythematosus (SLE).

We and others have defined an important role for B cells as APCs in the activation of T cells and subsequent antibody responses (1–7). BCR-mediated uptake of foreign antigens as well as auto-antigens can activate CD4 T cell responses nearly 10 000-fold more efficiently as compared with the passive uptake of antigen by APCs (6). Moreover, BCR-mediated uptake of self-antigen may also be important in the maintenance of peripheral T cell tolerance depending on many factors including the genetic predisposition of the host to autoimmunity (32, 33). Studies from the Shlomchik laboratory have demonstrated that B cell presentation of auto-antigens is critical for the spontaneous activation of T cells and subsequent autoimmune pathology in the MRL model of human SLE (10).

In this study, we investigated the kinetics of binding, processing and presentation of foreign antigen through the CD19 protein of B cells. We hypothesized that this B cell-surface protein would be ideal to target antigen for uptake, given its essential role for B cell activation and by its association with the processing of C3d-antigen complexes through CD21. We have found that B cells readily bind antigens linked to anti-CD19 antibody both *in vivo* and *in vitro* with kinetics similar to that found with antigen targeted to sIgM. Again, in a similar manner to IgM binding, cross-linked CD19 polarizes to a cap at the cell surface within 30 min before it is internalized into intracellular vesicles for processing and presentation by MHC class II. This is further confirmed by antigen co-localization with the lysosomal compartment. IgM capping has been linked to capping with the CD19 complex (13, 34). As found previously, we have demonstrated that CD19 ligation up-regulates the expression of B7-1 and B7-2 co-stimulatory molecules thought to be critical in the activation of responding CD4 T cells (35). The up-regulation of B7-1/B7-2 did not require the use of adjuvants for B cell stimulation through Toll receptor signaling. Simple cross-linking of CD19

with soluble antigen-anti-CD19 *in vivo* or *in vitro* enhances the expression of surface co-stimulatory markers.

Antigen taken up through CD19 activates T cells ~100-fold more efficiently as compared with passive uptake of PCC antigen by APCs. Moreover, CD19-mediated presentation of antigen was also as efficient as uptake of the same antigen by the BCR. Remarkably, antigen administered in soluble form by the i.v. route is found on the majority of peripheral B cells, both spleen cells and PBMCs, within 30 min. The rapid binding of CD19 may make this protein an excellent target to clear B cells or B cell lymphomas from the circulation with toxin-labeled anti-CD19 (23, 24). This may be an important feature of regulating autoimmune responses marked by auto-antibody production, such as in SLE or rheumatoid arthritis. Antigen taken up via the i.v. route also efficiently stimulates resident PCC-responsive AND T cells *in vivo*. We have no evidence for the induction of T cell tolerance by the administration of soluble antigen as described in these studies.

The targeting of antigen to B cell CD19 protein may be an efficient means by which T cell activation may be enhanced, in particular to antigens that may be only weakly immunogenic on their own. Indeed, weakly immunogenic tumor antigens targeted to the CD19 protein can activate detectable anti-tumor CD4 and CD8 T cell responses (preliminary data of J. Yan, *et al.*). This approach may also be ideal since virtually all B cells express the CD19 protein. We do not yet know how the ligation of CD19 may affect BCR functions on the same cell or how CD21 signaling may be affected. As described earlier, CD19 can amplify BCR signaling independent of CD21 or other co-stimulatory molecules. However, our work demonstrates a novel and fundamentally relevant mechanism by which antigen may be targeted for the activation of CD4 T cell immunity.

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## Abbreviations

APC	antigen-presenting cell
CFSE	carboxyfluorescein diacetate succinimidyl ester
i.v.	intravenously
NIH	National Institutes of Health
PCC	pigeon cytochrome c
slg	surface Ig
SLE	systemic lupus erythematosus

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