Bystander B cells rapidly acquire antigen receptors from activated B cells by membrane transfer: a novel mechanism for enhancing specific antigen presentation

Ben J. C. Quah¹, Vaughan P. Barlow¹, Virginia M^cPhun¹, Klaus I. Matthaei², Mark D. Hulett¹
& Christopher R. Parish¹

¹Divisions of Immunology and Genetics and ²Molecular Bioscience, John Curtin School of Medical Research, The Australian National University, Canberra ACT 2601, Australia

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Abbreviations used in this paper: APC, antigen presenting cell; B6, C57BL/6; BCR, B cell antigen receptor; CFSE, 5-(and 6-) carboxfluorescein diacetate succinimidyl ester; CSN, culture supernatant; DC, dendritic cell; HEL, hen egg lysozyme; Ig, immunoglobulin; MHC-II, MHC class II; OVA, ovalbumin; SEM, scanning electron microscopy; TCR, T cell receptor; TEM, transmission electron microscopy (TEM); Tg, transgenic.

Address correspondence to Chris R. Parish, Division of Immunology and Genetics, John Curtin School of Medical Research, The Australian National University, Canberra ACT 2601, Australia. Phone: 61-2-6125 2604; Fax 61-2-6125 2595; Email: christopher.parish@anu.edu.au

Abstract

The B cell antigen receptor (BCR) efficiently facilitates the capture and processing of a specific antigen for presentation on MHC class II molecules to antigen specific CD4⁺ T cells . Despite this, the majority of B cells are only thought to play a limited role in CD4⁺ T cell activation since BCRs are clonotypically expressed. Here we show, however, that activated B cells can, both *in vitro* and *in vivo*, rapidly donate their BCR to bystander B cells, a process that is mediated by direct membrane transfer between adjacent B cells and is amplified by the interaction of the BCR with specific antigen. This results in a dramatic expansion in the number of antigen-binding B cells *in vivo*, with the transferred BCR endowing recipient B cells with the ability to present specific antigen to antigen-specific CD4⁺ T cells.

Introduction

The activation of antigen-specific CD4⁺ T cells relies on the capacity of antigen presenting cells (APCs) to internalise and present antigen, as peptide fragments, on MHC class II (MHC-II) molecules. Dendritic cells (DCs) are regarded as the most potent APC but B cells expressing a BCR specific for a particular antigen are also extremely proficient at capturing and presenting antigen to antigen-specific CD4⁺ T cells . This arises from the remarkable efficiency of the BCR in capturing a specific antigen and facilitating antigen delivery to MHC-II-rich compartments which specialise in the generation of MHC-II-peptide complexes . However, since BCRs are clonotypically produced within a large repertoire of B cells, it has been generally accepted that the majority of B cells play only a minor role in presenting antigen to T cells .

Recent reports have demonstrated that a number of cell types, including pheochromocytoma cell lines , DCs , T cells , natural killer cells and B cell lymphomas have the capacity to transfer membrane and cellular components. In the case of B cell lymphomas, membrane exchange is thought to be due to transfer of membrane components during direct cell contact and possibly via membrane nanotubes (also referred to as cytonemes) , in processes that are enhanced by BCR crosslinking. Since the most unique difference between antigen-specific B cells is their BCR, membrane exchange may allow the exquisite antigen binding and processing capacity of one B cell to be transferred to another via BCR transfer. By this means it can be postulated that BCR transfer from antigen-specific B cells to bystander B cells during immune responses could enhance the capacity of the local B cell

pool to specifically bind and present antigen, a process which may greatly enhance the development of CD4⁺ T cell responses. In the present study we examined the potential of B cells to exchange their BCRs and how this affects their capacity to present specific antigen.

Results

B cells share BCRs following activation

The demonstration of membrane exchange between B cell lymphomas upon BCR cross-linking suggests that BCRs may be transferred between B cells upon cell activation. To assess this possibility, we cultured splenocytes from hens egg lysozyme (HEL)-specific BCR-transgenic (Tg) MD4 mice with splenocytes from C57BL/6 (B6).CD45.1 mice in the presence of the B cell mitogen, LPS. B cells from each of the two populations were discriminated on the basis of CD45 allotypic differences and BCR exchange determined by changes in the cell surface expression of immunoglobulin (Ig)M allotypes as B6.CD45.1 B cells express the 'b' allotype of IgM (IgM^b) whereas the MD4 B cells carry the 'a' allotype of IgM (IgM^a). Using this approach we observed substantial transfer of IgM^a to B6.CD45.1 B cells and a concomitant transfer of IgM^b to MD4 B cells (Figure 1A), with the acquisition of donor IgM steadily increasing over 3 days of culture. Negligible BCR transfer was observed in the absence of LPS but similar BCR transfer was observed when specific antigen in the presence of antigen-specific T helper cells or T helper cell signals, were used to activate B cells (Figure S1A and S1B in Supporting Information). BCR transfer was found to be

restricted to B cells as B220-negative cells acquired negligible amounts of donor IgM (Figure 1B).

BCR transfer is rapid, is not due to secreted Ig and is enhanced by specific antigen

The rate of BCR transfer between B cells was assessed by mixing whole cultures of LPS-activated MD4 and B6.CD45.1 splenocytes and determining IgM^a transfer to B6.CD45.1 B cells over short periods of co-culture (Figure 2A). At 37 °C substantial BCR transfer occurred within 10-20 min and by 60 min B6.CD45.1 B cells had gained ~60% of the IgM^a levels that they had acquired after 3 days of continuous culture with MD4 splenocytes (Figure 2A). Similar IgM^a transfer was observed with washed activated MD4 splenocytes, whereas low IgM^a transfer was observed with the MD4 culture supernatant (CSN), this residual activity being completely depleted when CSN was passed through an 800nm filter (Figure 2A). Intriguingly, rapid BCR transfer also occurred after 1 hr incubation at 4 °C, conditions which normally inhibit endocytic and exocytic processes, including Ig secretion (Figure 2A). Indeed, we have found that activated B cells from μ_s ^{-/-} mice, which express surface IgM^a but have essentially no capacity to secrete IgM^a, have a similar capacity to transfer their BCRs relative to wild type IgM^a secretors (Figure 2B). These results indicate that IgM transfer is not due to IgM secretion.

Freshly isolated B cells were unable to transfer their BCRs, whereas LPS-activated B cells could readily donate BCRs to both non-activated and activated B cells (Figure 2C), although only viable B cells mediated transfer (Figure S2). Furthermore, the presence of

specific antigen (HEL), but not an unrelated antigen (ovalbumin, (OVA)), substantially increased the transfer of HEL-specific IgM^a to both LPS-activated and freshly isolated bystander B cells (Figure 2D). BCR transfer, therefore, appears to be dependent on donor B cell activation, does not require recipient B cell activation and can be further enhanced by BCR-specific antigen.

BCR donation to bystander B cells involves membrane transfer

It appears likely that BCR transfer is mediated by membrane donation. Consistent with this we observed that CD45.2 molecules and cell surface molecules covalently labelled with fluorescein were also transferred from MD4 cells to B6.CD45.1 B cells and those B cells that had acquired higher levels of these surface molecules were also the same B cells that gained higher levels of IgM^a, suggesting co-transfer of the molecules (Figure 3A). Furthermore, by labelling the activated MD4 B cells with the membrane intercalating dye, PKH-26, we could directly show membrane transfer in parallel with transfer of IgM^a (Figure 3B). This was further enhanced by the addition of BCR-specific antigen over a wide concentration range (Figure 3B). These results suggest that membrane donation between B cells is responsible for BCR transfer.

Confocal microscopy was used to directly visualise membrane exchange between PKH-26-labelled activated MD4 B cells and activated EGFP⁺ B cells (Figure 3C). PKH-26⁺ and EGFP⁺ B cells rapidly formed cell aggregates (Figure 3Ci and 3Cii) and diffusion of PKH-26 into the membranes of recipient EGFP⁺ B cells was evident, suggesting PKH-26

labelled membranes were redistributing into the membranes of adjacent EGFP⁺ B cells. Analysis of the interaction between LPS-activated lymphocytes and bystander resting lymphocytes by transmission (TEM) and scanning electron microscopy (SEM) revealed close association between plasma membranes after cell mixing (Figure S3), with membranous nanotube-like extensions also connecting the cells. Intriguingly, attempts to interfere with most of the known molecular processes involved in membrane fusion and exchange (recently reviewed in) had no inhibitory effect on BCR transfer (Figure S4).

Bystander B cells that acquire an antigen-specific BCR gain the ability to present antigen to CD4⁺ T cells

To test if the donated BCR could enhance antigen presentation we assessed the capacity of bystander B cells, after they had acquired the BCR from antigen (HEL)-specific B cells, to stimulate CD4⁺ T cells specific for the same antigen. Purified B cells from CBA/H (*H-2^k*) and EGFP⁺ MD4 mice were co-cultured, under activating conditions, to allow transfer of the HEL-specific BCR of the MD4 B cells to the bystander CBA/H B cells. The different B cell populations were separated by flow cytometry and after pulsing with HEL assessed for their ability to stimulate CFSE-labelled HEL-peptide/I-A^k complex-specific CD4⁺ T cells from TCR-Tg 3A9 mice . This revealed that CBA/H B cells that had acquired the HEL-specific BCR stimulated, over a 1000-fold antigen dose range, a substantial proportion (up to 70%) of the HEL-specific CD4⁺ T cells to up-regulate CD69 and proliferate based on CFSE dilution (Figure 4). In contrast, the CBA/H B cells that were not co-cultured with the MD4 B

cells induced only a low proportion of the HEL-specific T cells to up-regulate CD69 and proliferate. The enhanced T cell responses induced by CBA/H B cells could not be attributed to contaminating MD4 B cells since the sorted CBA/H B cell population was of high purity (<1% EGFP⁺ cells, data not shown) and MD4 B cells lacked the appropriate I-A^k MHC-II for effective antigen presentation.

Antigen-specific B cells can donate their BCR to bystander B cells during antigen specific immune responses *in vivo*

To assess whether BCR transfer occurs *in vivo*, an antigen-specific immune response was initiated in mice. Freshly isolated CFSE-labelled MD4 spleen cells, mixed with a HEL-OVA antigen conjugate and LPS, were injected i.v. into B6.CD45.1 recipient mice containing adoptively transferred CFSE-labelled OVA-peptide/I-A^b complex-specific TCR-Tg OT-II lymphocytes to provide cognate T cell help. The IgM^a expression on all CD45.2⁺ (donor) and CD45.2⁻ (host) leukocytes was monitored over a 7 day period (Figure 5Ai). This showed that at day 3 after adoptive transfer, the MD4 B cells had begun to donate their BCR to bystander B cells, with ~28% of the host B cell pool expressing IgM^a at this time point. This correlated with extensive MD4 B cells proliferation (Figure 5Aii). The MD4 B cells did not proliferate much further over the next 4 days but the number of host B cells acquiring IgM^a increased to 39% on day 4 and was maintained at approximately one third of the B cell pool over the next 3 days (Figure 5Ai and 5Aii). As observed *in vitro*, *in vivo* BCR sharing was essentially limited to B220⁺ cells (Figure 5Ai). BCR transfer was also considerably enhanced 1 hr after

challenging the animals with the BCR-specific antigen (i.e., 63% of the bystander B cells acquired the donor BCR, Figure 5B). CD45.2 transfer to host B6.CD45.1 B cells was also evident *in vivo*, a finding consistent with membrane transfer (Figure 5C). Indeed, co-staining for both IgM^a and CD45.2 revealed that host B cells appeared to have acquired both markers in parallel and this coincidental transfer increased not only after *in vivo* challenge with HEL but also after *ex vivo* exposure of B cells for 1 hr at 4 °C to a wide range of HEL concentrations. Recipient B cells that had acquired the HEL-specific BCR also gained the ability to present antigen to CD4⁺ T cells (Figure S5).

Discussion

In this report we describe a novel mechanism by which bystander B cells can acquire an antigen-specific BCRs from activated B cells and gain the ability to capture and present specific-foreign antigen, thereby increasing the effective APC pool. BCR transfer is mediated by direct membrane donation, demonstrating an important role for membrane transfer between antigen-specific and non-antigen-specific B cells during immune responses. Recently, there have been several reports indicating that many cell types have the capacity to transfer membrane and cellular components as a form of intercellular communication , although the functional significance of this phenomenon is unclear. It should be noted, however, that DCs and B cells have been reported to acquire antigen tethered to cell surfaces and efficiently present these to antigen-specific T cells. In these cases, antigen could be acquired from multiple cell types but uptake appeared to be restricted to DCs or B cells

bearing an antigen-specific BCR and hence appeared to be recipient, but not donor, driven as we have reported here. As with these reports, and despite our own extensive studies (Figure S4), the molecular basis of membrane exchange is uncertain. However, our confocal and electron microscopy studies suggest that plasma membranes from activated and bystander B cells may coalesce via short membranous extensions resembling short membrane nanotubes. In this regard it should be noted that membrane nanotubes can transfer material between cells at temperatures as low as 0.7 °C and similarly BCR transfer occurs quite efficiently at 4 °C. Furthermore, it has been reported that the formation of membrane nanotube-like extensions, referred to as cytonemes, are increased upon BCR stimulation, which in our study significantly enhances BCR transfer.

An intriguing aspect of this study is the speed and magnitude of BCR transfer between B cells, even at 4 °C. Once appropriately activated B cells can within minutes share their BCR with adjacent B cells, this process being substantially enhanced following BCR engagement by specific antigen. Furthermore, *in vivo* studies revealed that up to two thirds of the splenic B cells in recipient animals gained the HEL-specific BCR of the transferred Tg B cells once the Tg B cells had been specifically activated by antigen and CD4⁺ T helper cells. This represents at least a 9-16 fold expansion in the number of B cells that can bind significant levels of specific antigen. Additional studies revealed that the bystander B cells that acquired the HEL-specific BCR could very efficiently present HEL to HEL-specific TCR Tg T cells, these B cells being able to stimulate antigen-specific CD4⁺ T cell responses with >1000 times less antigen than bystander B cells that have not acquired specific BCRs. Thus, based on these

data, BCR sharing results in a rapid expansion in the number of B cells that can present specific antigen to T cells. Significantly, a number of studies have identified an important role for B cells and, in particular, B cells bearing antigen-specific BCRs, in CD4⁺ T cell responses. Therefore, we postulate that BCR transfer is an important mechanism by which B cells can help facilitate the amplification and development of antigen-specific CD4⁺ T cells during an immune response.

Methods

Animals

Mice were obtained from the Animal Services Division, Australian National University and from the Australian Phenomics Facility and were bred under specific pathogen-free conditions. Mouse strains used were B6, CBA/H, and B6.CD45.1 (B6 congenic for CD45.1). Tg mouse strains were MD4 (BCR-Tg expressing HEL-specific-IgM^a and IgD^a on a B6 background), OT-II (TCR-Tg specific for I-A^b-OVA₃₂₃₋₃₃₉ peptide on a B6 background) and 3A9 (TCR-Tg specific for I-A^k-HEL₄₆₋₆₁ peptide on a B10.BR background). The Rosa-EGFP Tg (EGFP-Tg) mice were generated by crossing a Rosa26 stop/flox-EGFP mouse (kindly provided by Professor Martyn Goulding, Department of Neurobiology, Salk Institute, University of California, San Diego) with a generalised Cre recombinase-expressing mouse TNAP Cre, to activate expression of EGFP. Double Tg (MD4/EGFP-Tg) mice were also used and were generated by crossing MD4 mice with EGFP-Tg mice. Secretory IgM^a deficient B6 mice ($\mu_s^{-/-}$) and 129sv (IgM^a) mice were generously provided by Dr. Michael

R. Ehrenstein (Department of Rheumatology, University College, London, UK) and tested for IgM secretion by ELISA. Mice were used at 4-20 weeks of age and were housed and handled according to the guidelines of the ANU Animal Experimentation Ethics Committee.

Cell preparation and purification

Leukocytes were obtained from spleen and/or lymph nodes as previously described . Leukocyte subsets were purified by magnetic cell separation in LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) using streptavidin-conjugated MicroBeads (Miltenyi Biotec) to target biotin-conjugated mAb-labelled cells. CD4⁺ T cells were enriched from pooled lymph nodes as well as spleen, and B cells were enriched from spleen. The cells were incubated with biotin-conjugated mAbs (Pharmingen) specific for unwanted cell populations with mAbs used for CD4⁺ T cell enrichment being specific for CD8 (53-6.7), CD11b (M1/70), CD11c (HL3) and B220 (RA3-6B2) and with mAbs used for B cell enrichment being specific for CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3) and CD90.2 (53-2.1). Negatively selected B cell and T cell populations were found to be 90-98% pure, as assessed by flow cytometry.

Fluorescent dye and covalent labelling of cells

Lymphocytes were labelled with the intracellular dye, 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and were cell surface labelled with LC-N-hydroxysuccinimidyl-fluorescein (Pierce Rockford, IL), as previously

described for CFSE labelling . PKH-26 (Sigma) labelling was performed according to the manufacturers instructions. The UV-excitable dye Hoechst 33258 (Calbiochem, La Jolla, CA) was used to discriminate between viable, dead and apoptotic cells . Cells ($1-5 \times 10^6/\text{ml}$) were labelled with $1 \mu\text{g}/\text{ml}$ of Hoechst 33258 for 7 min at 37°C prior to flow cytometry.

Preparation of HEL-OVA conjugates

Maleimide-activated OVA (Sigma) was conjugated to sulfhydrylated-HEL (Sigma) to generate stable HEL-OVA conjugates. OVA was maleimide activated with a 25-fold molar excess of succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Pierce Rockford, IL). HEL was incubated with a 7-fold molar excess of *N*-succinimidyl *S*-acetylthioacetate (SATA; Pierce) and conjugated SATA was de-acetylated in 0.5 M hydroxylamine/25 mM EDTA in PBS (pH 7.4). Activated proteins were then conjugated with a 4-fold molar excess of HEL to OVA, before being dialysed against PBS in a 10 kDa cut off dialysis bag.

Cell culture, cell culture fractionation and antigen presentation assays

Activated B cells were generated by culturing purified B cells or RBC-depleted splenocytes ($1 \times 10^6/\text{ml}$) in supplemented DMEM (sDMEM, Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS) as previously described in 6 well plates (Nunc, Wiesbaden, Germany) in the presence of $10 \mu\text{g}/\text{ml}$ LPS (Sigma) for up to 3 days at 37°C in 5% CO_2 . In some experiments, cultures were harvested and cells readjusted to a final

concentration of 1×10^6 cells/ml for re-incubation in 96 well U-bottomed plates (Nunc) either at 37 °C in 5% CO₂ or at 4 °C (on ice) for various times. To remove CSN components cells were washed at least 3 times with 10 ml of PBS. CSN was harvested by pelleting cells at 300xg for 5 min and aspirating off 3/4 of the uppermost supernatant. Full removal of cell debris was accomplished by filtering CSN through 800 nm cut-off cellulose filters (Millipore, Bedford, MA).

For antigen presentation assays, purified CFSE-labelled 3A9 TCR-Tg CD4⁺ T cells (1×10^5) were cultured with or without 1.5×10^5 purified B cells, in a total of 200 µl of sDMEM/10%FCS in 96 well U-bottomed plates (Nunc). B cells were pulsed with HEL on ice for 20 min, washed and cultured with CD4⁺ T cells. Cultures were incubated for 15-18 hr or for 3 days, at which time cells were analysed by flow cytometry for CD69 expression and CFSE content.

***In vivo* experimentation**

RBC-depleted MD4 splenocytes (3×10^7), with 10 µg of HEL-OVA and 10 µg of LPS, were adoptively transferred via the lateral tail vein into B6.CD45.1 mice in 200 µl of PBS. B6.CD45.1 hosts had received i.v. 3×10^7 RBC-depleted CFSE-labelled lymphocytes from OT-II lymph nodes and spleen 2 hr prior to MD4 adoptive transfer, to provide cognate T cell help. Spleen cells from host mice were harvested at various times, depleted of RBC by lysis and adjusted to $1-4 \times 10^7$ cell/ml, ready for antibody staining and flow cytometry. In some experiments animals were challenged i.v. with an additional bolus 10 µg of HEL 1 hr prior to

spleen harvesting.

Antibody staining

Cells for flow cytometry analysis were stained on ice with specific mAbs and secondary fluorochrome conjugates as previously described . MAbs specific for CD4 (GK1.5), CD16/CD32 (2.4G2), CD45.1 (A20), CD45.2 (104), B220/CD45R (RA3-6B2), CD69 (H1.2F3), CD90.2 (53-2.1), K^k (36.7-5), K^b (AF6-88.5), IgM^a (DS-1) and IgM^b (AF6-78) were purchased from Pharmingen (Burlingame, CA). The specificity of antibody binding and secondary reagents was monitored through the use of isotype matched control antibodies (eBioscience, San Diego, CA).

Flow cytometry

Analytical flow cytometry was performed in a LSR (Becton Dickinson, Mountain View, CA), modified with a yellow He-Ne laser emitting a spectral line at 594nm (Research Electro-Optics, Inc. Boulder, CO) and cell sorting to purify B cell populations was performed using a FACStar plus (Becton Dickinson) or a FACSVantage with the DiVa option (Becton Dickinson). Unless otherwise indicated, statistical analysis of the proportion of a cell population expressing a marker was based on histogram profiling using Overton subtraction as applied by FlowJo software (Tree Star, OR).

Confocal Microscopy

Confocal microscopy was performed with a Radiance Confocal Microscope (BioRad) using the Argon 488 nm laser as the source of excitation. For live cell imaging, 1×10^5 LPS activated MD4 splenic B cells, pre-labelled with PKH-26 and a Cy-Chrome-conjugated B220-specific mAb, were added to 5×10^5 Cy-Chrome-conjugated B220-specific mAb-labelled LPS-activated EGFP-Tg splenic B cells on a cooling stage set at 4 °C and images taken for 60 min.

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References

Figure 1. B cells exchange BCRs following prolonged activation by LPS

(A) B6.CD45.1 and MD4 spleen cells were co-cultured in the presence of LPS. Dot plots show expression of IgM^a and IgM^b on live (Hoechst 33258^{low}) CD45.1⁺ (red) and CD45.1⁻ (black) B220⁺ cells over 3 days. Values are percentage of B6.CD45.1 B cells expressing IgM^a (red) or MD4 B cells expressing IgM^b (black) relative to background expression on B cells cultured independently (represented by quadrant gates). (B) Transfer of IgM^a to B6.CD45.1 B220⁺ cells and B220⁻ cells after 3 days culture with MD4 cells (red open histograms). Grey filled histogram represents background IgM^a staining of B6.CD45.1 splenocytes cultured alone and black open histogram depicts IgM^a expression on MD4 B cells.

Figure 2. Activated B cells rapidly transfer BCRs to both resting and activated B cells through non-soluble factors in a process enhanced by specific antigen

(A) Fractions of day 3 LPS-activated MD4 spleen cell cultures were added to 3 day LPS-activated B6.CD45.1 spleen cells and incubated at 37 °C for up to 2 hr or at 4 °C for 1 hr. Percentage B6.CD45.1 B cells expressing IgM^a was assessed as in Fig. 1A and IgM^a transfer represented as a proportion (%) of IgM^a transfer over 3 days continuous co-culture. MD4 cell culture fractions included whole culture, MD4 cells washed to remove soluble factors, MD4 culture supernatant (CSN) depleted of cells by centrifugation and the cell-depleted CSN passed through a 800 nm filter. (B) The capacity of B cells from $\mu_s^{-/-}$ mice to transfer IgM^a to B6 B cells after continuous co-culture for 3 days at 37 °C or after brief (3hr) co-incubation at 4 °C. For 3 day incubations, B6 spleen cells (IgM^b) were co-cultured with spleen cells from $\mu_s^{-/-}$ mice (IgM^a) or 129sv ($\mu_s^{+/+}$) mice (the founder strain for the IgM^a expressed in $\mu_s^{-/-}$ mice but which can secrete IgM^a) in the presence of LPS. For brief incubations, 3 day LPS activated spleen cells were incubated together for ~3 hr. B6 B cells were then assessed for

IgM^a surface expression. Numbers are the percentage of B6 B cells expressing IgM^a after co-culture with donor B cells relative to IgM^a expression by B6 B cells cultured alone. The histogram depicts the concentration of IgM in the culture supernatants of either $\mu_s^{-/-}$ or $\mu_s^{+/+}$ splenocytes cultured for 3 day with LPS. (C) Day 3 LPS-activated MD4 spleen cells were incubated with either 3 day LPS-activated or freshly isolated B6.CD45.1 spleen cells for 1 hr at 4 °C. B cells were then assessed for IgM^a and IgM^b surface expression. Numbers are the percentage of B cells expressing non-endogeneous IgM. (D) Day 3 LPS-activated MD4 spleen cells were incubated with LPS-activated B6.CD45.1 spleen cells in the presence of HEL or OVA for 1 hr at 4 °C. B6.CD45.1 B cells were then assessed for IgM^a surface expression represented as a proportion (%) of IgM^a transfer over 3 days continuous co-culture. Data in (A), (C) and (D) are representative of 3 independent experiments.

Figure 3. BCR transfer occurs concomitantly with transfer of other membrane components

(A) Upper Panels: Simultaneous expression of IgM^a and CD45.2 on B6.CD45.1 B cells cultured with MD4 B cells for 3 days with LPS. The percentage of B6.CD45.1 B cells positive for IgM^a and CD45.2 after co-culture with MD4 B cells is shown adjacent to the respective axis for each marker. The boxed area represents background staining for CD45.2 and IgM^a of B6.CD45.1 spleen cells cultured alone. Lower Panels: Simultaneous transfer of IgM^a and pre-labelled MD4 surface molecules to resting B6.CD45.1 B cells. Day 3 LPS-activated MD4 spleen cells were covalently cell surface labelled with fluorescein and pre-labelled with antibodies to IgM^a and CD45.2. After extensive washing, cells were incubated for 1 hr at 37 °C with freshly isolated B6.CD45.1 spleen cells pre-labelled with antibodies to B220 and CD45.1, and immediately analysed by flow cytometry. The percentage of B6.CD45.1 B cells positive for IgM^a and fluorescein is shown as in the upper panels. The

boxed area represents background staining for IgM^a and background fluorescein fluorescence of freshly isolated B6.CD45.1 spleen cells. (B) Day 3 LPS-activated MD4 spleen cells, labelled with PKH-26, were incubated with day 3 LPS-activated B6.CD45.1 spleen cells for 1 hr at 4 °C in the presence of various concentrations of HEL. B cells were then assessed for expression of PKH-26 and IgM^a (lower panels). Numbers are the percentage of B6.CD45.1 B cell expressing each marker. (C) Day 3 LPS-activated MD4 spleen cells were stained with PKH-26 and an anti-B220 mAb and incubated on a cooling stage set at 4 °C with anti-B220 mAb labelled day 3 LPS-activated EGFP-Tg spleen cells and analysed by confocal microscopy. Red = PKH-26, green = EGFP and white bar = 5µm. (i-ii) Green and red channel merging of simultaneous confocal images of B cells showing many membrane adhesions between the two B cell populations within 60 min of co-incubation (i = 59 min and ii = 17 min). Arrows show overlap of EGFP and PKH-26 fluorescence within the same focal plane. Data in (A) – (C) are representative of 3 independent experiments.

Figure 4. Bystander B cells that acquire an antigen-specific BCR gain the ability to present antigen to CD4⁺ T cells

B cells purified from CBA/H (*H-2^k*) spleen were cultured with LPS in either the absence or presence of purified splenic B cells from MD4/EGFP-Tg (*H-2^b*) mice. After 3 days culture EGFP⁺ MD4 B cells were depleted from CBA/H-MD4 B cell co-cultures by flow cytometry. CBA/H B cells purified from the co-cultures (CBA/H +/- MD4), as well as CBA/H and MD4 B cells cultured alone, were then pulsed with HEL for 20 min at 4 °C, washed and equal numbers (1.5×10^5) cultured with 1×10^5 purified CFSE-labelled CD4⁺ 3A9 TCR-Tg T cells specific for a HEL-peptide presented by I-A^k. After 0.5 days CD4⁺ T cells were assessed for CD69 expression. After 3 days CD4⁺ T cells were also assessed for CFSE expression, with

the numbers in each histogram referring to the percentage of T cells that have entered one or more divisions based on CFSE dilution. Data is representative of 3 independent experiments.

Figure 5. Antigen-specific B cells transfer their BCR to bystander B cells *in vivo* during antigen specific immune reactions.

A) CFSE-labelled MD4 spleen cells, together with 10 µg of HEL-OVA and 10 µg of LPS, were injected i.v. into B6.CD45.1 mice 2 hr after the i.v. injection of CFSE-labelled OT-II lymphocytes. Spleen cells from mice were analysed over the next 7 days by flow cytometry.

(i) Dot plots show B220 and IgM^a expression on host (CD45.2⁻; black events) and donor (CD45.2⁺; red events) cell populations, with numbers referring to the percentage of splenocytes (boxed region) that are MD4 B cells (B220⁺, CD45.2⁺, IgM^a^{high}). Histograms show IgM^a expression on host B cells (B220⁺, CD45.2⁻, CFSE⁻) and MD4 B cells (B220⁺, CD45.2⁺, IgM^a^{high}), and isotype control mAb binding to host B cells, with numbers showing the percentage of host cells expressing IgM^a relative to the isotype control. (ii) Viable MD4, OT-II and recipient (host) B cells were assessed for CFSE expression. (B) An identical experiment to that described in (A) except that on day 3 after commencement of the experiment one animal was challenged iv with a 10 µg bolus of HEL 1 hr prior to spleen cell harvest and marker analysis. (C) As in (A) except that on day 4 after commencement of the experiment one animal was challenged iv with a 10 µg bolus of HEL 1 hr prior to spleen harvest. In addition spleen cells from a non-HEL injected animal were incubated at 4 °C for 1 hr in the presence of various amounts of HEL prior to measurement of marker expression. Analysis involved assessing expression of IgM^a and CD45.2 on B6.CD45.1 host B cells compared with autofluorescence and isotype control mAb binding (boxed region). Numbers are the percentage of B6.CD45.1 B cells expressing CD45.2 or IgM^a above background

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Figure 2

Figure 3

Figure 4

Figure 5

Supporting Information

Bystander B cells Rapidly Acquire Antigen Receptors from Activated B cells by Membrane Transfer: A Novel Mechanism for Enhancing Specific Antigen Presentation

Ben J. C. Quah, Vaughan P. Barlow, Virginia M^cPhun, Klaus I. Matthaei, Mark D. Hulett and Christopher R. Parish

Figure S1A. CD40L stimulation induces BCR transfer to bystander B cells

B6.CD45.1 splenocytes (expressing both IgM^b and CD45.1) were labelled with CFSE (5 μ M) and then co-cultured with MD4 splenocytes (expressing both a HEL-specific IgM^a, IgD^a transgene on a C57BL/6 background and CD45.2) for 3 days with CD40L (prepared from CD40L-baculovirus-infected Sf9 cell membranes, as described in Supplemental Methods, and used at amounts generating maximal B cell proliferation), IL-4 (50 ng/ml, Peprotech Inc., Rocky Hill) and HEL (1 μ g/ml). Both B6.CD45.1 and MD4 splenocytes were also cultured alone as controls. B cell blasts were then analysed by flow cytometry for IgM^a, with regions gated on the basis of single colour control samples as shown. Note the substantial increase in IgM^a expression on B6.CD45.1 (CFSE⁺) B cells after co-culture with MD4 B cells.

Figure S1B. Activation of B cells by T helper cells can induce BCR transfer to bystander B cells

EGFP⁺ spleen cells from HEL-specific BCR-Tg (MD4/EGFP-Tg) mice (2x10⁶/ml) were cultured with spleen cells from OVA-specific TCR-Tg OT-II mice (2x10⁶/ml) in the presence of the conjugated antigen HEL-OVA. B cells from each population were then monitored for

MD4 (Donor)-derived IgM^a expression after 3 days of co-culture (expressed as a % relative to fluorescent controls). B cells from OT-II spleen cells (Recipient B cells) cultured alone were used as a control to monitor background levels of IgM^a expression. These cultures showed that in the presence of increasing concentrations of specific antigen, which resulted in increasing activation of the OT-II T cells and MD4 B cells (data not shown), there was a dramatic increase in transfer of IgM^a from the MD4 (Donor) B cells to the OT-II bystander (Recipient) B cells.

Figure S2. Viable cells but not cell debris, apoptotic cells or dead cells are responsible for BCR transfer

Day 3 LPS-activated MD4 spleen cell cultures were separated by flow cytometry into viable, dead and apoptotic cells as well as a cell debris fraction based on Hoechst 33258 staining and

forward scatter properties (dot plots). These fractions, as well as the total MD4 spleen cell culture, were then incubated with day 3 LPS-activated B6.CD45.1 spleen cells for 1 hr at 4 °C. B6.CD45.1 B cells were then assessed for IgM^a expression after incubation either in the presence of the various MD4 spleen cell culture fractions (black open histogram) or in the absence of the fractions (grey filled histogram), with IgM^a expression on the different MD4 B220⁺ fractions also assessed (grey open histogram). The percentage of IgM^{a+} B6.CD45.1 B cells, after incubation with the various MD4 fractions, is indicated in each histogram. Data is representative of 4 independent experiments.

Figure S3. Electron Microscopy of Cell-Cell Interactions

Day 3 LPS-activated MD4 spleen cells were mixed with freshly isolated B6.CD45.1 spleen cells for 40 min on 0.1% poly-L-lysine-coated plastic coverslips and then prepared for EM analysis as described in the Experimental Procedures. Activated lymphocytes (MD4 B cells) in contact with resting lymphocytes (B6.CD45.1 cells) were chosen for analysis by SEM (i-iv) and TEM (v and vi). Panels ii, iv and vi are magnifications of membrane contacts between cells depicted in panels i, iii and v respectively. The larger cell in each panel, which exhibits extensive membrane ruffling by SEM, is a LPS-activated B cell whereas the smaller cell represents a resting B lymphocyte. Note the close association between the plasma membranes of the interacting lymphocytes and contact between the cells by short nanotube-like structures.

Figure S4. Enhancement and Inhibition of BCR Transfer by Various Reagents.

Day 3 LPS-activated MD4 spleen cells were incubated with freshly isolated B6.CD45.1 spleen cells for 1-2 hr in the continuous presence (C) of various reagents, and surface expression of IgM^a (i.e. MD4-derived IgM) was then assessed on B220⁺ B6.CD45.1 cells by flow cytometry. In some cases MD4 cells were pre-treated (P) with the various reagents as indicated and washed twice before incubation with B6.CD45.1 cells. The %IgM^{a+} B6.CD45.1 B220⁺ cells resulting from the treatment incubations was then divided by the %IgM^{a+} B6.CD45.1 B220⁺ cells from untreated incubations and multiplied by 100 to give % of normal IgM^a transfer. The shaded region indicates the 95% confidence interval around the mean IgM^a transfer observed in control cultures (based on controls from untreated incubations from 12 samples across 4 independent experiments). Each data value the mean of 1-10 experiments.

References in Figure relevant to cell membrane exchange:

% of normal IgM^a transfer

0 50 100 150 200 250

Comments on Figure S4. Additional data on the molecular basis of BCR transfer between B cells

In an attempt to define the molecular basis of BCR transfer a large number of inhibitors of cell adhesion, membrane integrity and membrane fusion were assessed for their ability to inhibit BCR donation to bystander cells. The culture system entailed mixing LPS-activated transgenic (MD4) B cells with freshly isolated splenic B cells and assessing BCR/membrane transfer after co-incubation for 2 hr at 37°C. This system most closely mimics the *in vivo* situation where there is rapid unidirectional transfer of BCR from activated B cells to bystander naïve B cells.

A total of 26 mAbs or mAb combinations were examined for their ability to modify BCR transfer, mAbs being chosen that recognise cell surface molecules on B cells. Particular attention was given to investigating molecules that have been shown to be involved in cell adhesion and cell fusion (reviewed in Chen, E.H. & Olsen E.N., *Science* 308, 369-373, 2005), with the same mAb clones being used that have been reported previously to block function. Also all mAbs were used at saturating concentrations. None of the mAbs tested inhibited BCR transfer, although a few mAbs significantly enhanced BCR transfer, namely anti-CD2, CD19, CD44, CD48 and CD62L. The effect of the CD19-specific mAb is not surprising as CD19 is a signalling molecule that can associate with the BCR and BCR engagement by antigen (HEL) also enhances transfer. CD2 and CD48 represent a receptor-ligand pair that may facilitate transfer, CD44 has been implicated in cell fusion whereas the role of CD62L requires further investigation. Metabolic inhibitors (sodium azide, 4 °C), protein kinase C inhibitors (Rottlerin, Gu6976), modifiers of plasma membrane lipid organization (annexin V, apolipoprotein-E, methyl β -cyclodextrin, CBZ-D-FFG, polyethyleneglycol-lipid), cytoskeleton/microtubule disrupters (cytochalasin-B, latrunculin-B, colchicine), broad spectrum ion channel blockers (hexamethylamiloride, amantadine), gap junction blockers (1-octanol) and inhibition of protein secretion (Brefeldin A) had little or no effect on transfer. Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, that facilitate intracellular membrane fusion, appeared to be not involved as N-ethylmaleimide slightly enhanced rather than inhibited transfer. Fixation of the donor MD4 B cells with glutaraldehyde or paraformaldehyde prevented transfer. Schiff base formation has been reported to stabilise cell adhesion (Rhodes, J., *Immunology Today* 17, 436-39, 1996) but the continual presence of high concentrations (170 mM) of lysine, an inhibitor of Schiff base formation, actually enhanced BCR transfer. Furthermore, reduction of cell surface aldehydes by pre-treatment of donor MD4 B cells with sodium borohydride enhanced the rate of BCR transfer (data not shown), a result consistent with Schiff base formation interfering with BCR/membrane exchange between B cells.

Figure S5. Antigen-specific B cells transfer their BCR to bystander B cells *in vivo* during antigen specific immune reactions and convert bystander B cells to antigen specific APCs.

Freshly isolated CFSE-labelled MD4 spleen cells, together with 10 μ g of HEL-OVA and 10 μ g of LPS, were injected i.v. into B6.CD45.1 recipient mice 2 hr after the i.v. injection of CFSE-labelled OT-II lymphocytes. On day 4, animals were challenged iv with a 30 μ g bolus of either HEL or HEL-OVA 1 hr prior to spleen harvest. Spleen cells were then labelled with IgM^a, B220 and CD45.2 specific mAbs and B6.CD45.1 B cells (B220⁺, CD45.2⁻) sorted into populations expressing low levels (Low) and medium levels (Med) of IgM^a by flow cytometry as indicated in the dot plot profile. Graded numbers of the different B cell populations, from both HEL and HEL-OVA challenged mice, were then assessed for their capacity to activate, (CD69⁺ and/or divided based on CFSE dilution), a constant number (1.5×10^5) of CFSE-labelled CD4⁺ OT-II lymphocytes after 3 days of co-incubation. Data are representative of 3 independent experiments.

Supporting Methods

Electron microscopy

Activated MD4 spleen cells (3 day LPS-activated) were mixed with freshly isolated B6.CD45.1 RBC-depleted spleen cells at a concentration of 4×10^7 cells/ml each in 0.3 ml sDMEM and left to attach to plastic coverslips coated with 0.1 % poly-L-lysine for 40 min at 37 °C. Cells were then immediately prepared for transmission electron microscopy (TEM) or scanning EM (SEM). For TEM, samples were fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 hr, washed 3 times in 0.1 M sodium cacodylate buffer and post fixed in 1 % OsO₄ in 0.1 M sodium cacodylate for 20 min. After 2 washes in buffer, the samples were dehydrated in a graded ethanol series to 100 % ethanol and embedded in Spurr's resin at 70 °C. Samples were photographed using a Hitachi H7000 electron microscope at 75 kV. For SEM, samples were prepared using the same method as for TEM until the 100 % ethanol step. They were then transferred into 100 % amyl acetate (3 washes) and critical point dried. The samples were coated with gold-palladium and photographed using a Hitachi S4500 FESEM at 10 kV.

CD40L preparation

SF9 cells were infected with 10 % v/v recombinant CD40L baculovirus stock (Kindly provided by Dr Phil Hodgkin, Walter and Eliza Hall Institute, Melbourne). After 5 days at 27 °C in air, infected cells were harvested and sedimented at 300g for 5 min. Cells from the equivalent of 1000 ml of 5-day culture were then resuspended in 7 ml of homogenisation buffer (20 mM Tris-HCL pH 7.4, 10 mM NaCl, 0.1 mM MgCl₂, 0.5 mM CaCl₂, 0.1 mM PMSF) and homogenised with a Polytron homogeniser (Kinematica GmbH, Lucern, Switzerland). Membranes containing CD40L were then enriched from the cell homogenate by density centrifugation using a 3 ml 41 % sucrose underlay and sedimentation at 96,000g for 1 hr at 4 °C. The membrane-containing interface was then collected and washed twice, by diluting the membranes up to 10 ml in PBS and centrifugation at 100,000g for 1 hr at 4 °C and removing the supernatant. The final membrane pellet was resuspended in PBS (10 ml/1000 ml of infected SF9 cells originally harvested), passed through an 18 gauge needle 15 times, aliquoted and stored at -70 °C until use.