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Physiological-Range Temperature Changes Modulate Cognate Antigen Processing and Presentation Mediated by Lipid Raft-Restricted Ubiquitinated B Cell Receptor Molecules

Bhuvana Katkere,* Sarah Rosa,* Adriana Caballero,* Elizabeth A. Repasky,† and James R. Drake*

BCR-mediated Ag processing and presentation is critical to the initiation and control of a humoral immune response. Trafficking of internalized Ag–BCR complexes to intracellular Ag processing compartments is driven by ubiquitination of the cytoplasmic domain of the BCR. Using a biochemical approach, it is here established that ubiquitinated Ag–BCR complexes are formed via a signaling-dependent mechanism and restricted to plasma membrane lipid rafts. Because the structure of lipid rafts is temperature sensitive, the impact of physiological-range temperature changes (PRTCs; 33–39˚C) on lipid raft-dependent and -independent BCR functions was investigated. Whereas the kinetics of lipid raft-independent BCR internalization is unaffected by temperature changes within this range, raft-dependent BCR signaling and ubiquitination as well as BCR-mediated Ag processing are significantly affected. The extent and duration of Ag–BCR ubiquitination is increased and prolonged at 37–39˚C (normal to febrile temperature) compared with that at 33˚C (peripheral body temperature). As might be expected, increased temperature also accelerates the overall kinetics of Ag–BCR degradation. Notably, at 33˚C the expression of peptide–MHC class II complexes derived from the BCR-mediated processing of cognate Ag is profoundly slowed, whereas the kinetics of expression of peptide–MHC class II complexes derived from fluid-phase Ag processing remains unchanged. These results establish the effect of PRTCs on multiple lipid raft-dependent BCR functions including the processing and presentation of cognate Ag, suggesting one mechanism by which PRTCs, such as fever, may impact the initiation and/or maturation of a humoral immune response. The Journal of Immunology, 2010, 185: 5032–5039.

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Abbreviations used in this paper: CTB, cholera toxin B; HEL, hen egg lysozyme; MHC, MHC class II-enriched compartment; MFI, mean fluorescence intensity; PC, phosphatidylcholine; PP1, 4-amino-5-(4-methylphenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine; PRTC, physiological-range temperature change; SA-Alexa 488, streptavidin Alexa 488; Spl, splenic B cells; TX-100, 0.1% Triton X-100; UBA, ubiquitin-associated domain; UQ1, ubiquitin-1.

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Materials and Methods

Animals

B10.BR/SgSnJ (B10.Br) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Albany Medical College Animal Facility. Mice were 8–12 weeks of age at the time of experimentation. Mice were housed in the Albany Medical College Animal Facility and received care in compliance with the Guide for the Care and Use of Laboratory Animals (Committee for the Purpose of Control and Supervision in Laboratory Animal Care, 1996, 8th ed., National Academy Press, Washington, D.C.).
Cells

The murine B cell line A20µWT expressing a wild-type human IgM BCR specific for phosphorylcholine (5) was maintained in α-modified Eagle’s medium with 5% FBS and 50 µM 2-ME in the presence of 500 µg/ml G418 (to maintain human IgM expression). For all the experiments, cells were grown to a density <0.1 x 10^6/ml at 37˚C, 5% CO2. Splenic B cells from B10.Br (expressing a non-hen egg lysozyme [HEL]-specific IgM b-PP1 (4-amino-5-(4-methylphenyl)-7-([154x482]9))2 (catalog no. 109-006-129; Jackson ImmunoResearch, West Grove, PA), FITC rat anti-mouse IgG (A9-1; catalog no. 553443; BD Pharmingen), FITC anti-mouse IgM (catalog no. 55515; BD Pharmingen), PE anti-CD45R/B220 (RA3-6B2, IgG2a; catalog no. 553500; BD Pharmingen), FITC anti-mouse IgM (DS-1, IgG1; catalog no. 553516; BD Pharmingen), PE anti-CD45R/B220, rabbit anti-human IgM-btm (catalog no. 309-065-095; Jackson ImmunoResearch), biotin anti-mouse IgM (catalog no. 55515; BD Pharmingen), anti-mouse IgM-btm (AF6-78; catalog no. 553519; BD Pharmingen), cholera toxin b (CTB; catalog no. 227039; Calbiochem, San Diego, CA), HRP conjugated CTB (HRP-CTB; catalog no. C3741; Sigma), PP1 (4-amino-5-(4-chlorophenyl)-7-([154x482]9))2 (catalog no. 0.115-006-006; Jackson ImmunoResearch), rabbit anti-human IgM-btm (catalog no. 309-065-095; Jackson ImmunoResearch), biotin anti-mouse IgM (catalog no. 55515; BD Pharmingen), mouse monoclonal 6C5 (catalog no. AM4300; Ambion, Austin, TX).

Temperature control

Cells were cultured in bicarbonate-free RPMI 1640 media (R73388; Sigma) supplemented with 10% FBS and 50 µM 2-ME. Culture containers were sealed and fully immersed in a heated water bath set to the calibrated temperatures. A calibrated thermometer was used to set the temperature of all three baths (33, 37, and 39˚C). Unless otherwise noted, cells were preincubated at the indicated temperature for 2-6 h before analysis.

Isolation of lipid rafts and analysis of BCR distribution

Isolation of lipid rafts was done as previously described (5, 6) with the changes noted here. Lipid rafts were isolated by sucrose density gradient centrifugation from detergent lysates of A20µWT cells at the concentration of 10^6 viable cells/ml pulsed with biotinylated anti-hulgM (anti-BCR) for indicated times. In every experiment, to determine the lipid raft fraction, one tube had cells pulsed with HRP-labeled CTB (catalog no. C3741; Sigma) as a marker of GM1 bearing lipid rafts. After determining the raft and nonraft fractions, fractions were pooled to pull down all ubiquitinated proteins in the sample using either p62 ubiquitin-associated domain (UBA) or ubiquilin 1 (UQ1) beads. Total BCR was determined using the pooled fractions before pull-downs, and pooled fractions after pull-down were used to determine ubiquitinated BCR. The lysates were then run on SDS-PAGE/Western blot to determine the presence or absence of ubiquitinated BCR. For temperature experiments, cells were preincubated at 33 or 37˚C for 2 h.

BCR signaling

Intracellular calcium measurements were done as described previously (5, 6) with minor modifications for the temperature range changes in these experiments. Prior to preloading A20µWT cells with calcium-sensitive dyes Fluo-3 AM and Fura Red (catalog no. F1242 and no. F3021, respectively; Invitrogen, Eugene, OR), the cells were preincubated in their respective temperatures 33, 37, or 39˚C for 2 h. The cells were allowed to rest at their respective temperatures for 2 h before analysis began. In case of CTB-pretreatment experiment, after resting cells at room temperature for 2 h, unlabeled cells were pretreated with CTB at either 50 or 100 µg/ml for 5 min before starting acquisition. After acquiring baseline measurements for 30 s, the cells were stimulated with indicated BCR ligand, and data were acquired for 180 s. The relative intracellular calcium levels were analyzed using the FlowJo (Tree Star, Ashland, OR) program.

BCR internalization

Kinetics of Ag–BCR endocytosis was determined as described previously with modifications detailed below (5, 6, 13). A20µWT cells or splenic B cells from B10.Br mice were preincubated at either 33, 37, or 39˚C for 2 h with resuspension every half hour. After preincubation, the cells were pulsed with either biotinylated anti-hulgM (anti-BCR) at a final concentration of 10 µg/ml (A20µWT cells) or biotinylated anti-mouse IgM at a final concentration of 10 µg/ml (splenic B cells) and incubated at their respective temperatures for indicated times to allow for internalization of the ligand. The reaction was stopped by adding ice-cold buffer, and cells were stained with streptavidin Alexa 488 (catalog no. S32354; Invitrogen) to label ligand remaining on the surface, and all cells were stained with 0.1 µg/ml propidium iodide (catalog no. P3566; Invitrogen). To determine the level of fluorescence, a FacScan I (BD Biosciences) was used and data analyzed using the program Flow Jo (Tree Star). All analysis was performed on live cells (propidium iodide-negative). Fraction of Ab remaining at the cell surface was calculated by dividing the mean fluorescence intensity (MFI) at each time point by the MFI value at time 0.

BCR-mediated ligand degradation

Analysis of BCR degradation by SDS-PAGE/Western blot was done as previously described (14) with these noted modifications. Either A20µWT cells or splenic B cells used in the experiments were preincubated at their respective temperatures 33, 37, or 39˚C for 2 h. After preincubation, the cells were pulsed with their respective ligands, either biotinylated mAb Aw3.18 (detected with A85-1 rat anti-murine IgG2a, IgG2b, IgG3, and IgM), FITC rat anti-mouse IgG1 (A85-1; catalog no. 553443; BD Pharmingen, San Jose, CA), FITC anti-mouse IgM (catalog no. 55515; BD Pharmingen), mouse monoclonal 6C5 (catalog no. AM4300; Ambion, Austin, TX), FITC anti-GAPDH, and biotinylated anti-mouse IgM at a final concentration of 10 µg/ml (A20µWT cells) or biotinylated anti-mouse IgM at a final concentration of 10 µg/ml (splenic B cells) for indicated times. Cells were lysed at 1 x 10^5 viable cells/ml concentration in radio-immunoprecipitation assay buffer for 10 min on ice. After clearing the lysates by centrifugation for 15 min at 16,000 x g at 4˚C, the total BCR was analyzed by reducing SDS-PAGE/Western blotting with SA-HRP, and Super Signal West Dura ECL substrate (Pierce, Rockford, IL), using blue-sensitive x-ray film.

BCR ubiquitination

Analysis of BCR ubiquitination by SDS-PAGE/Western blot was done as previously described (14) with these noted modifications. Either A20µWT cells or splenic B cells used in the experiments were preincubated at their respective temperatures 33, 37, or 39˚C for 2 h. After preincubation, the cells were pulsed with their respective ligands, either biotinylated mAb Aw3.18 (detected with A85-1 rat anti-murine IgG2a, IgG2b, IgG3, and IgM), FITC rat anti-mouse IgG1 (A85-1; catalog no. 553443; BD Pharmingen, San Jose, CA), FITC anti-mouse IgM (catalog no. 55515; BD Pharmingen), mouse monoclonal 6C5 (catalog no. AM4300; Ambion, Austin, TX), FITC anti-GAPDH, and biotinylated anti-mouse IgM at a final concentration of 10 µg/ml (A20µWT cells) or biotinylated anti-mouse IgM at a final concentration of 10 µg/ml (splenic B cells) for indicated times. Cells were lysed at 1 x 10^5 viable cells/ml concentration in radio-immunoprecipitation assay buffer for 10 min on ice. After clearing the lysates by centrifugation for 15 min at 16,000 x g at 4˚C, the ubiquitinated proteins in the cleared lysate were purified by using either p62 UBA–agarose or UQ1–biotin–agarose beads (catalog no. UW9010 and no. UW9830, respectively; BioMol, Enzo, Plymouth Meeting, PA) overnight at 4˚C. After collecting the samples from ubiquitin pull-downs, they were analyzed by reducing SDS-PAGE/Western blotting with SA-HRP (catalog no. 21124; Pierce Biotechnology, Rockford, IL), and Super Signal West Dura ECL substrate, using blue-sensitive x-ray film. For experiments involving CTB pretreatment, samples were preincubated with the indicated concentration of CTB and incubated at 37˚C for 5 min before addition of anti–hulgM-btm and analysis of BCR ubiquitination as detailed earlier. For experiments involving the inhibition of Src family kinase activity, samples were pre-treated for 1 h at 37˚C with a mixture of 10 µM PP1 and 10 µM MP22 before addition of anti–hulgM-btm and analysis of BCR ubiquitination as detailed earlier.

Expression of HEL46–61–I-A^k peptide–MHC class II complexes

Splenocytes from either MD4.B10.Lr or B10.Br mice were pulsed with Ag (HEL; 100 nM for HEL–specific MD4.B10.Lr B Cells and 100 µM for non-HEL–specific B10.Lr B Cells), and the level of HEL46–61–I-A^k–specific mAb Aw3.18 (15) as previously reported (12, 13), with the exception of the substitution of Aw3.18 with A85-1 rat anti-murine IgG1–FITC (553443, Pharmingen) for C4H3. The MFI of Aw3.18 staining is reported for live (propidium iodide negative) B220^+ B cells falling within a “lymphocyte” forward and side scatter gate. Total I-A^k expression was monitored by staining parallel samples with 10-3.6–FITC (catalog no. 109901; BioLegend, San Diego, CA).
Results

Ubiquitinated Ag–BCR complexes are restricted to lipid raft membrane domains

Previous studies have established that the cytoplasmic tail of a relatively small fraction of Ag–BCR complexes is ubiquitinated, directing those Ag–BCR complexes to MHC class II-enriched compartment (MHC)-like late endosomes for processing to peptide–MHC class II complexes (14, 16). Other studies have also established that a relatively small fraction of Ag–BCR complexes are found in plasma membrane domains termed lipid raft, which are known platforms for receptor signaling (2–5). To establish the relationship between these two subpopulations of Ag–BCR complexes, the ubiquitination state of the Ag–BCR complexes in and outside of lipid rafts was determined. As previously reported by many groups, Ag binding results in the formation of Ag–BCR complexes that can be found both in and outside of lipid rafts (Fig. 1). However, when an ubiquitin pull-down strategy is used to focus the analysis on the subset of ubiquitinated Ag–BCR complexes, only complexes within lipid rafts were found to be ubiquitinated (Fig. 1). Although these results do not establish whether all raft resident Ag–BCR complexes are ubiquitinated or, in contrast, whether there also exists a fraction of raft resident complexes that are not ubiquitinated, the results do establish that the subset of ubiquitinated Ag–BCR complexes is restricted to membrane domains with the biochemical properties of lipid rafts.

BCR ubiquitination occurs via a Src family kinase signaling-dependent mechanism

Because it is well known that lipid rafts are critical BCR signaling platforms (3, 5, 6) and ubiquitinated Ag–BCR complexes were found restricted to these membrane domains (Fig. 1), it was of interest to evaluate the role of BCR signaling in the formation of ubiquitinated Ag–BCR complexes. Because the initial steps of BCR signaling center on phosphorylation of BCR ITAM tyrosine residues by Src family kinases (17), the impact of blocking this early step of BCR signaling on the formation of ubiquitinated Ag–BCR complexes was determined. Pretreatment of A20μWT B cells with a mixture of the two well-characterized Src family kinase inhibitors PP1 and PP2, which blocks BCR signaling but does not alter the level of BCR expression nor the kinetics of BCR internalization (6), results in a profound block in the formation of ubiquitinated Ag–BCR complexes (Fig. 2). These results establish that BCR ubiquitination occurs via a mechanism involving Src family kinase-mediated BCR signaling and are consistent with the possibility that the molecules directly involved in the process of BCR ubiquitination are present in or readily recruited to plasma membrane lipid rafts.

PRTCs selectively impact lipid raft-dependent BCR functions

Lipid rafts are composed of a subset of membrane lipids enriched in long-chain highly saturated fatty acid tails that under appropriate conditions form lipid “ordered” membrane microdomains, the structure of which is highly sensitive to temperature changes over a physiological range (10, 11, 18). Therefore, the effect of PRTCs, between 33˚C (peripheral body temperature) and 39˚C (mild fever), on both lipid raft-dependent as well as lipid raft-independent BCR functions was investigated.

Previous work has established that whereas BCR signaling is highly dependent on plasma membrane lipid rafts (2–5), Ag–BCR internalization occurs predominately via a lipid raft-independent mechanism (5). Therefore the impact of PRTC on BCR signaling and internalization was investigated. As might be expected, the kinetics of lipid raft-independent Ag–BCR internalization was unaffected within the relatively narrow temperature range 33–39˚C (Fig. 3A; similar results were observed using splenic B cells, data not shown). These results show that temperature changes between 33 and 39˚C do not have a profound overall effect on cell physiology (i.e., increasing temperature to 39˚C does not dramatically increase the overall kinetics of cellular metabolism), suggesting that any impact that PRTC may have on lipid raft-dependent BCR functions will likely occur via the direct impact of temperature on membrane structure. Consistent with this scenario, analysis of lipid raft-dependent BCR-induced intracellular calcium signaling at 33 versus 39˚C revealed a significant decrease in BCR signaling at 39˚C (Fig. 3B). These results suggest that the effect of PRTC on BCR signaling is due to the direct effect of temperature on lipid raft structure/function.

To confirm that the PRTC-induced change in BCR signaling is a direct effect of temperature on lipid rafts (as opposed to a temperature-induced change in the kinetics of BCR signaling “enzymology”), the impact of the lipid raft ligand CTB (a lipid raft-modifying agent) on BCR signaling and internalization was investigated. Previous studies have established that binding of CTB to model membranes increases line tension at lipid raft boundaries (19) and elicits a redistribution of the liquid ordered (i.e., raft) and liquid-disordered (i.e., nonraft) phases of the bilayer (19). Consistent with these previous findings, prebinding of 50–
100 μg/ml CTB to B cells before ligation of the BCR profoundly impacts lipid raft-dependent BCR signaling (Fig. 4A) but has little or no effect on lipid raft-independent BCR internalization (Fig. 4B). Notably, the impact of CTB prebinding on raft-dependent BCR signaling can be either positive or negative, depending on the ligand used to engage the BCR. Specifically, prebinding of CTB inhibits BCR signaling initiated by haptenated protein [i.e., phosphorylcholine–BSA (Fig. 4A, lower plot)], which will only interact with the BCR via its two Ag binding sites, whereas CTB prebinding augments BCR signaling elicited by a polyclonal anti-BCR Ab (Fig. 4A, upper plot), which will bind the BCR at multiple epitopes distributed across the constant region of the molecules. Although the precise reason for the differential impact of CTB on BCR signaling elicited by different BCR ligands is unclear, the results nevertheless establish that binding of the known raft “modulator” CTB can act like PRTC to selectively modulate lipid raft-dependent BCR functions. Taken together, these results establish that whereas PRTCs between 33 and 39˚C do not profoundly change overall B cell physiology and do not significantly impact lipid raft-independent BCR functions such as internalization, PRTC significantly impacts lipid raft-dependent BCR functions such as BCR signaling.

Having demonstrated that the subset of ubiquitinated Ag–BCR complexes that are the source of Ag for rapidly generated peptide–MHC class II complexes (14, 16) are restricted to lipid raft membrane domains and that the function of these membrane domains is sensitive to PRTC, it was of interest to determine the impact of PRTC on the BCR-mediated processing and presentation of cognate Ag. One of the first steps of BCR-mediated Ag presentation is the proteolytic processing of BCR internalized ligand. As illustrated by the results presented in Fig. 5, changing
the experimental temperature from 33 to 39˚C significantly accelerates the kinetics of BCR-mediated Ag processing in both the well-characterized A20\(\mu\)WT model B cell line (Fig. 5A, 5C) and primary murine splenic B cells (Fig. 5B–D). However, because previous work has established that it is not the bulk Ag–BCR complexes that are rapidly converted to peptide–MHC class II complexes, but rather the subset of ubiquitinated Ag–BCR complexes (14, 16), the impact of PRTC on BCR ubiquitination was investigated.

Whereas BCR ubiquitination was detectable both at 33 and 39˚C, BCR ubiquitination was prolonged and more extensive in both transformed and normal B cells at the higher temperatures of 37–39˚C (Fig. 6). Nevertheless, the ubiquitinated ligand–BCR complexes were found to remain restricted to membrane lipid rafts at all temperatures (Supplementary Fig. 1). Moreover, pretreatment of A20\(\mu\)WT B cells with different concentrations of the lipid raft-modifying agent CTB (which selectively enhances anti-IgM–induced lipid raft-dependent BCR functions; Fig. 4) results in

FIGURE 5. Fever range temperature accelerates the kinetics of BCR-mediated Ag processing. A, A20\(\mu\)WT B cells were preincubated at the indicated temperature for 2 h, pulsed with anti–BCR-btn at the same temperature, whole cell lysates prepared, and the kinetics of anti–BCR-btn degradation determined by SDS-PAGE and blotting with SA-HRP. GAPDH was used as a loading control. Shown are representative results from one of three independent experiments. B, B10.Br splenic B cells were preincubated at the indicated temperature for 2 h, and the kinetics of BCR-mediated Ag processing of anti–BCR-btn was determined by staining with SA-HRP and reducing SDS-PAGE/Western blot. Shown are representative results from one of three independent experiments. C, Densitometric analysis of the average degradation (over three independent experiments) of total Ag–BCR by A20\(\mu\)WT cells and splenic B cells (Spl). Bars = ±1 SD. Statistical analysis: A20\(\mu\)WT, 33 versus 39˚C at 60 min, \(p = 0.005\); splenocytes, 33 versus 39˚C at 2 h, \(p = 0.006\). D, Longer exposure of a blot from an experiment similar to that shown in (B). The arrows labeled HC and LC indicate the intact H and L chains of the bound anti–BCR-btn Ab. The arrow labeled DB indicates a degradation band (derived from the proteolytic degradation of the intact anti–BCR-btn H chain) that is generated more rapidly at 39˚C. Shown are representative results from one of three independent experiments.

FIGURE 6. Fever range temperature increases the level of Ag–BCR ubiquitination. A, A20\(\mu\)WT B cells were preincubated for 2 h at the indicated temperature. Cells were pulsed with anti–BCR-btn for the indicated times at that same temperature and ubiquitinated ligand–BCR complexes isolated by UQ1 pull-down. The level of ubiquitinated ligand–BCR complexes was determined by SDS-PAGE and blotting with SA-HRP. B, Splenocytes from B10.Br mice were preincubated for 2 h at the indicated temperature. The cells were then pulsed with anti-IgM-btn at those same temperatures for the indicated times. The cells were then lysed, and ubiquitinated ligand–BCR complexes were detected as in (A). Shown are representative results from one of three independent experiments. For (A) and (B), the ubiquitin pull-down was done from the same samples as shown in Fig. 5A and 5B, which serves as a “loading” control for this figure. C, Densitometric analysis of the average level (across three independent experiments) of ubiquitinated Ag–BCR complexes detected at 5 min with A20\(\mu\)WT cells (A). Error bars = ±1 SD.
mediated by a ubiquitinated subset of Ag–BCR complexes that are mediated processing and presentation of cognate Ag, which is Figs. 5–8 establishes the selective impact of PRTC on the BCR-mediated Ag presentation. Taken together, the results presented in Aultimated to the same level as that observed at the higher temperature. Nevertheless, after 24 h at 33˚C, BCR-mediated Ag presentation is essentially blocked for the first 8–10 h of Ag exposure (Fig. 8A). Fig. 7). Taken together, these results suggest that elevated temperature, such as would be encountered during a fever, affects the structure and function of lipid raft membrane domains in such a way as to augment the ubiquitination of internalized Ag–BCR complexes, which would be predicted to enhance the efficiency of BCR-mediated Ag processing and presentation.

To characterize further the immunological impact of PRTC on the BCR-mediated processing and presentation of cognate Ag, the impact of temperature on the kinetics of cell surface expression of peptide–MHC class II complexes derived from ubiquitinated Ag–BCR complexes was investigated. At all three temperatures tested (i.e., 33, 37, and 39˚C), the kinetics of the BCR-independent processing and presentation of noncognate Ag internalized by fluid-phase endocytosis was essentially unaffected (Fig. 8A), establishing that (as observed for lipid raft-independent BCR internalization; Fig. 3A) these PRTCs have a minor (if any) impact on lipid raft-independent B cell functions. In stark contrast, the kinetics of BCR-mediated Ag processing is much more sensitive to PRTC. Specifically, at 39˚C the kinetics of BCR-mediated Ag presentation is slightly accelerated compared with the kinetics observed at 37˚C, whereas at 33˚C, BCR-mediated Ag processing is essentially blocked for the first 8–10 h of Ag exposure (Fig. 8B). Nevertheless, after 24 h at 33˚C, BCR-mediated Ag presentation ultimately recovers to the same level as that observed at the higher temperatures (Fig. 8B). Taken together, the results presented in Figs. 5–8 establishes the selective impact of PRTC on the BCR-mediated processing and presentation of cognate Ag, which is mediated by a ubiquitinated subset of Ag–BCR complexes that are restricted to temperature-sensitive lipid raft membrane domains.

Discussion

BCR-mediated Ag processing entails Ag–BCR endocytosis, delivery of ubiquitinated Ag–BCR complexes to MIIC where Ag is converted to antigenic peptide–MHC class II complexes and finally delivery of these peptide–MHC class II complexes to the surface of the cell. Previous work by this laboratory and others has established that whereas some aspects of BCR-mediated Ag processing are highly dependent on lipid rafts [e.g., BCR signaling (2–5)], other BCR functions such as endocytosis are predominately lipid raft-independent events (5). Because the structure and function of lipid raft membrane microdomains are known to be temperature sensitive (10, 18), the current study was undertaken to determine the effect of PRTCs on both lipid raft-dependent and -independent BCR functions.

The results presented in this report establish two important features of B cell immunobiology. First, biochemical analysis established that ubiquitination of Ag–BCR complexes occurs via a signaling-dependent mechanism and that the subset of ubiquitinated Ag–BCR complexes that mediates the relatively rapid processing of Ag and conversion to peptide–MHC class II complexes (14) is completely contained within the subset of Ag–BCR complexes found in plasma membrane lipid rafts that are known to be intimately involved in BCR signaling. Second, physiologically relevant changes in temperature, which have previously been shown to impact the structure and function of membrane lipid rafts (10, 18), have profound yet selective effects on lipid raft-dependent BCR functions such as signaling (2–5) and receptor ubiquitination but have limited or no effect on lipid raft-independent BCR functions such as clathrin-coated pit-mediated internalization (5, 6). These findings significantly extend our understanding of B lymphocyte immunobiology and provide insight into how B cell responses may be tailored by environmental cues.

Two previous reports have established that whereas ubiquitination of the cytoplasmic tail of the BCR (i.e., IgH/CD79A/B) is necessary for the trafficking of Ag–BCR complexes to HLA-DM/H-2M MIIC Ag processing compartments and subsequent conversion to peptide–MHC class II complexes, BCR ubiquitination is not necessary for Ag–BCR internalization (14, 16). Because BCR signaling and internalization are mutually exclusive events (1) [with BCR signaling being highly dependent on membrane lipid raft domains (2–5), whereas the bulk of BCR internalization occurs via a lipid raft/BCR signaling-independent
mechanism (5–7)], it was of great interest to establish the role of lipid rafts and signaling in BCR ubiquitination. The results presented in this report establish the restriction of ubiquitinated Ag–BCR complexes to lipid raft membrane domains and a role for Src family kinase-based BCR signaling in the mechanism of BCR ubiquitination. This finding is consistent with the published observation that blocking BCR ubiquitination has no effect on BCR internalization [which occurs predominately via a lipid raft- (14) and signaling- (6) independent mechanism] and supports the published suggestion that BCR ubiquitination may occur via a BCR signaling/lipid raft-dependent pathway (14). Studies to establish the molecular mechanism of BCR ubiquitination are currently underway.

As lymphocytes circulate throughout the body, they can be exposed to temperatures ranging from a peripheral body temperature of 33°C (91.4°F) or less to a core body temperature of 37°C (98.6°F). Moreover, during infection or exercise, core body temperature can rise to 39°C (102.2°F) or higher. Although temperature changes across the range 33–39°C are below the level that induces a “heat shock” response (e.g., 42°C), such changes will have an impact on cellular structures and functions. One cellular structure that is exquisitely sensitive to temperature is the lipid bilayer of cellular membranes.

Lipid rafts are subdomains of the lipid bilayer enriched in lipids possessing long-chain, fully saturated fatty acid tails, making this region of the bilayer thicker than the nonraft region of the bilayer. The height mismatch between the lipid raft and nonraft regions of the plasma membrane generates a “line tension” that prevents smaller raft regions from fusing into one large lipid raft (11). However, changes in temperature across the range 33–39°C (i.e., PRTCs) can overcome this energy barrier, allowing reorganizations of the raft and nonraft regions of the membrane (18). It was therefore of interest to determine the effect of PRTC on both lipid raft-dependent as well as lipid raft-independent BCR functions. The prototypical lipid raft-dependent BCR function is signaling (2–5), which was found to be highly sensitive to PRTC. Notably, calcium-based BCR signaling is greatest at lower temperature (i.e., 33°C) where lipid rafts would be expected to exist as small independent structures (as opposed to a single large raft), suggesting that the lipid raft organization that prevails at 33°C is the most conducive to this aspect of BCR signaling. Moreover, the observation that BCR signaling is greatest at lower temperature argues that the effect of temperature on signaling is not simply due to a temperature-induced acceleration of the rate of enzyme-based signaling (because this scenario would predict greater BCR signaling at 37–39°C), but rather supports the notion that the change in signaling is due to a temperature-induced change in raft structure/function. Consistent with this scenario is the observation that prebinding of CTB [which, like temperature, is known to be a raft-modifying agent (19)] also selectively impacts lipid raft-dependent BCR signaling. In contrast with BCR signaling, the bulk of internalization of Ag–BCR complexes has been shown to occur independent of both BCR signaling (6) as well as plasma membrane lipid rafts (5). Accordingly, modification of lipid rafts by either PRTC or CTB prebinding has no significant effect on the kinetics of Ag–BCR internalization. Taken together, these results establish that PRTCs, which are known to have significant effect on the structure and function of lipid rafts (18), have a selective effect on lipid raft-dependent BCR functions such as BCR signaling but leave lipid raft-independent BCR functions such as Ag–BCR internalization relatively unchanged.

Trafficficking of internalized Ag–BCR complexes to HLA-DM/H-2M* MIIC where they are converted from Ag–BCR complexes to antigenic peptide–MHC class II complexes is a critical aspect of BCR-mediated Ag processing that has been shown to be dependent on BCR ubiquitination (14, 16), whereas fluid-phase processing and presentation of noncognate Ag occurs independent of BCR signaling and/or ubiquitination (14, 20). In light of the observation reported herein that ubiquitinated Ag–BCR complexes are restricted to lipid raft membrane domains, it was of interest to determine the relative impact of PRTC on BCR-mediated versus fluid-phase Ag processing. Consistent with the high-level partitioning of ubiquitinated Ag–BCR complexes into temperature-sensitive lipid rafts, changes across the physiological temperature range profoundly alter the kinetics of BCR-mediated Ag processing. Contrastingly, PRTC has essentially no effect on the kinetics of the lipid raft-independent fluid-phase processing of noncognate Ag. These results extend the observation that ubiquitinated Ag–BCR complexes are restricted to temperature-sensitive lipid raft domains and establishes that the BCR-mediated processing of cognate Ag, which results in the formation of peptide–MHC class II complexes with unique biological properties (12), occurs via a lipid raft-dependent pathway that is highly sensitive to PRTC.

Two questions raised by the results presented in this report are the relative roles of ubiquitinated versus nonubiquitinated Ag–BCR complexes in the formation of antigenic peptide–MHC class II complexes as well as the mechanism of internalization of these distinct subsets of Ag–BCR complexes. Previous work from this laboratory has established that blocking BCR ubiquitination profoundly inhibits the rapid formation of Ag–class II complexes (14) but has little impact on the intracellular persistence of internalized Ag–BCR complexes that are slowly processed to peptide–MHC class II complexes (13). This suggests that ubiquitinated Ag–BCR complexes are efficiently recognized by the ubiquitin-dependent endosomal sorting complex required for transport sorting machinery upon internalization and thus efficiently sorted to or within multivesicular body-like MIIC for the rapid conversion into antigenic peptide–MHC class II complexes, whereas nonubiquitinated Ag–BCR complexes are initially sequestered within a distinct endocytic compartment and then slowly delivered to or trafficked within MIIC for processing to peptide–MHC class II complexes. This scenario is consistent with the finding reported herein that incubation of B cells at 33°C, which results in the lowest level of Ag–BCR ubiquitination, results in a delay in the cell surface expression of derivative antigenic peptide–MHC class II complexes but has no effect on the level of peptide–MHC class II complexes ultimately expressed on the surface of the cell (Fig. 8).

Internalization of the nonubiquitinated Ag–BCR complexes [which have been estimated to compose greater than 90% of all cell surface Ag–BCR complexes (14)] occurs via plasma membrane clathrin-coated pits (6–9), likely using an adapter protein such as the well-characterized endocytic adaptor AP-2. Ubiquitinated Ag–BCR complexes [which are estimated to compose between 1 and 10% of all cell surface Ag–BCR complexes (14)] are likely also internalized via plasma membrane clathrin-coated pits [possibly via clathrin recruited to lipid rafts (21)]. Internalization of these complexes likely occurs either via direct interaction of the ubiquitinated Ag–BCR complexes with the adaptor AP-2 or possibly via the endocytic adaptor EPS15, which binds AP-2 and possesses a ubiquitin interacting motif. The existence of these two pathways of Ag–BCR internalization and trafficking would allow B cells to both rapidly express cell surface of peptide–MHC class II complexes (which would be beneficial under conditions such as those in a sensitized animal where T cell help is preexisting) and to express these peptide–MHC class II complexes for a prolonged period of time (which would be beneficial under conditions such as those in a naive animal where CD4 T cell help may not be available for 3–4 d). The precise molecular mechanisms controlling the internal-
ization and intracellular trafficking of these distinct Ag–BCR complexes are currently under study.

Taken in total, the results presented in this report establish the selective impact of PRTCs across the range 33–39°C on lipid raft-dependent BCR functions such as BCR signaling and BCR-mediated processing and presentation. Somewhat surprisingly, lipid raft-dependent BCR signaling was actually augmented at the low-range physiological temperature of 33°C, suggesting that the earliest aspects of B cell activation may have evolved to occur at locations in the body that are normally maintained at this temperature (i.e., peripheral locations such as the skin, hands, or feet). In contrast, the kinetics of BCR-mediated Ag processing and presentation are more rapid at the upper range of physiological temperatures (i.e., 37–39°C), suggesting that the latter aspects of BCR activation such as Ag presentation to CD4 T cells may have evolved to be optimal at locations in the body normally maintained at this higher temperature, such as lymph nodes within the core of the body or during fever. Together, these results highlight the impact of PRTCs on the cell biology of BCR-mediated Ag processing and suggest at least one mechanism by which physiologically relevant changes in body temperature such as fever can fine-tune the immune response.

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Disclosures

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References