CR1-mediated ATP Release by Human Red Blood Cells Promotes CR1 Clustering and Modulates the Immune Transfer Process*

Mark I. Melhorn1, Abigail S. Brodsky2, Jessica Estanislaũ, Joseph A. Khoory3, Ben Illigens3, Itaru Hamachi1, Yasutaka Kurishita1, Andrew D. Fraser1, Anne Nicholson-Weller4, Elena Dolmatova*, Heather S. Duffy*, and Ionia C. Ghiran1,2.

From the1 Division of Allergy and Inflammation, the2 Division of Infectious Diseases, the3 Division of Cardiology, the Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02115 and the4 Department of Synthetic Chemistry & Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan

Background: CR1 on human red blood cells (RBC) capture immune complexes and deliver them to phagocytes.

Results: RBC CR1-mediated ATP release increases RBC lipid mobility, CR1 avidity, and neutrophil phagocytosis.

Conclusion: ATP release following CR1 ligation alters both RBC and neutrophil function.

Significance: A new role for ATP from human RBC in modulating immune complex transfer.

Humans and other higher primates are unique among mammals in using complement receptor 1 (CR1, CD35) on red blood cells (RBC) to ligate complement-tagged inflammatory particles (immune complexes, apoptotic/necrotic debris, and microbes) in the circulation for quiet transport to the sinusoids of spleen and liver where resident macrophages remove the particles, but allow the RBC to return unharmed to the circulation. This process is called immune-adherence clearance. In this study we found using luminometric- and fluorescence-based methods that ligation of CR1 on human RBC promotes ATP release. Our data show that CR1-mediated ATP release does not depend on Ca2+ or enzymes previously shown to mediate an increase in membrane deformability promoted by CR1 ligation. Furthermore, ATP release following CR1 ligation increases the mobility of the lipid fraction of RBC membranes, which in turn facilitates CR1 clustering, and thereby enhances the binding avidity of complement-opsonized particles to the RBC CR1. Finally, we have found that RBC-derived ATP has a stimulatory effect on phagocytosis of immune-adherent immune complexes.

All vertebrates have a system for clearing the circulation of inflammatory particles, which depends on complement recognizing and tagging the particles with opsonins and then ligating the opsonized particle to a cell-bound complement receptor for quiet disposal by the sinusoidal macrophages of the liver and spleen. This process, known as immune-adherence clearance (1), is critical for host defense and for continuous protection of the vasculature from inflammation. For the vast majority of vertebrates, including mice and rats, platelets are responsible for binding and transporting circulating immune complexes using adherent factor H-like protein as complement receptor. Unlike all other mammals, higher primates including humans use red blood cells (RBC) as transporting cells due to a unique expression pattern of complement receptor 1 (CR1, CD35)3 on their membranes, a receptor that interacts with all the opsonic complement fragments: C1q, mannose binding lectin, C3b, and C4b (2–4).

We have previously shown that CR1 is constitutively dispersed on the surface of circulating RBC and upon its ligation by complement-opsonized microbeads, CR1 actively clusters due to the interaction between the two PDZ motifs present in its cytoplasmic domain with the PDZ domain containing protein FAP-1 (5). Functionally, binding of complement-opsonized microbeads to CR1 triggers a significant Ca2+ influx, the magnitude of which depends directly on the genetically determined expression levels of CR1 on RBC membranes. Subsequent activation of PKA and protein kinase CK2 (formerly known as casein kinase II) triggers a signaling cascade that results in increased phosphorylation of β-spectrin and increased RBC membrane deformability (6). Upon binding complement-opsonized particles, RBC themselves become “opsonized” by IgG and complement fragments. Remarkably, during the immune transfer process, RBC are spared, and only the cargo is removed by resident macrophages via receptor-mediated endocytosis. The lack of erythro-phagocytosis during immune transfer has been attributed to the inhibitory signaling of CD47 on RBC to SIRPα on macrophages (7, 8), as well as to the distinctive CR1 clustering on RBC that prevents phagocytosis by inhibiting the efficient “zippering” of macrophage plasma membrane around

3 The abbreviations used are: CR1, complement receptor 1/CD35; AU, arbitrary units; CK2, protein kinase casein kinase 2; COZY, complement-opsonized zymosan; HBSS, Hanks’ balanced salt solution; Hct, hematocrit; MRP, multidrug resistance-associated protein; PKA, protein kinase A; PMN, polymorphonuclear neutrophils; TBB, 4,5,6,7-tetramethylbenzotriazole; TRITC, tetramethylrhodamine isothiocyanate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

**This work was supported, in whole or in part, by National Institutes of Health Grant HL096795 (to I. C. G.) and without Ca2+/Mg2+, respectively; Hct, hematocrit; MRP, multidrug resistance-associated protein; PKA, protein kinase A; PMN, polymorphonuclear neutrophils; TBB, 4,5,6,7-tetramethylbenzotriazole; TRITC, tetramethylrhodamine isothiocyanate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
Role of ATP from RBC in Phagocytes

the RBC. This process requires a continuous, uniform and high-density deposition of IgG and complement fragments on the surface of target cells (9). During the early phases of phagocytosis, a key event that promotes the recruitment of phagocytic cells and the enhancement of target internalization, the “find me and remove me” signal, is the release of ATP as it was shown in apoptotic Jurkat T-cells through pannexin-1 (10). In a similar fashion, during the immune-transfer process, RBC-adherent immune complexes are identified, ligated, and then removed from circulating RBC by tissue phagocytic cells. Therefore, we asked whether ligation of CR1 on RBC by complement-opsonized particles would promote ATP release in a similar fashion and could modulate the immune capture and transfer processes.

In this study, using a luciferase/luciferin assay, fluorescence-based microscopy and flow cytometry, we identified that ligation of CR1 on human RBC by either antibody or complement-opsonized zymosan (COZY) promotes ATP release. Using pharmacological inhibitors, we found that CR1-mediated ATP release is mediated by multidrug resistance-associated proteins (MRPs) and does not depend on CK2 or PKA activity, enzymes previously shown to mediate the increase in membrane deformability triggered by CR1 ligation (6). By using fluorescence recovery after photobleaching (FRAP) and fluorescence microscopy we demonstrated that extracellular ATP increases the mobility of the lipid fraction of RBC membranes, which in turn facilitates CR1 clustering, enhancing the binding avidity of complement-opsonized particles to the RBC CR1. In addition, RBC-derived ATP is likely promoted by CD47 ligation, which could modulate the immune capture and transfer processes.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies were obtained as follows: mouse anti-CR1 mlgG, YZ1 (11) and 3C10 (gifts of Dr. Lloyd Klickstein, formerly Harvard Medical School, now Novartis); affinity-purified custom made extra- and intracellular chicken anti-human pannexin-1 pIgY (4512, 4515) (gift of Dr. Gerhard Dahl, University of Miami, Miami, FL, and Dr. Eliana Scemes, Albert Einstein College of Medicine, New York) (12); AffiniPure goat anti-mouse IgG and ChromPure mouse IgG (115-005-003 and 115-001-003, Jackson ImmunoResearch, West Grove, PA); mouse anti-CD47 (556044, BD Biosciences, Franklin Lakes, NJ); LEAF purified mouse IgG (401404, Biolegend, San Diego, CA); Alexa Fluor® 488 goat anti-mouse IgG, Alexa Fluor® 488 goat anti-chicken IgG, Alexa Fluor 488 F(ab)2 fragment of goat anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG, rabbit anti-BSA pAb, rabbit anti-pannexin 1 pAb (N-term), Zenon® Alexa Fluor 488 mouse IgG, labeling kit, (A11029, A11039, A11070, A11032, A11133, 487900, and Z25002, Invitrogen); 2-2Zn(II) probe (Dr. Itaru Hamachi, R&D Systems, Minneapolis, MN); apyrase from potato with 0.2% NaCl for 45 s, followed by addition of an equal volume of balanced salt solution with (HBSS2+Cl) and without (HBSS–Cl) CaCl2 and MgCl2, 1 μM FluoSpheres® (λexcitation/emission 505/515) and 0.2-μm non-fluorescent sulfate latex microspheres (microbeads), bovine serum albumin (BSA) Alexa Fluor 647 conjugate (D-275, 14025, 14175, F-8852, S37491, A34785, Invitrogen); ENLITEN® rLuciferase/Luciferin ATP assay reagent and 10 mM rATP standard (EF2021, P1132, Promega, Madison, WI); Ficol and Percoll (GE Healthcare, Pittsburgh, PA); and HEK293T cells (gift of Dr. Gina Sosinsky, University of California, San Diego).

Fresh RBC and Polymorphonuclear Neutrophils (PMN) Preparation—Blood was obtained from healthy adult volunteers in accordance with the guidelines of the Institutional Review Board (IRB) of Beth Israel Deaconess Medical Center and after informed consent was obtained in accordance with the Declaration of Helsinki. Unless indicated otherwise, blood (3 to 40 ml) was obtained by venipuncture and drawn into vacutainers (3–10-ml volumes, containing K2 EDTA; BD Biosciences) with subsequent centrifugation at 500 × g (5 min). Plasma, buffy coat, and the top layer of RBC were aspirated and 1 ml of RBC were washed twice in HBSS2+Cl containing 0.05% IgG-free BSA at 500 × g (5 min). PMN were isolated from a starting volume of 40 ml of blood drawn into a 60-ml syringe containing 14 ml of citrate solution and 6 ml of dextran T500. The buffy coat was layered on 15 ml of Ficoll and centrifuged at 350 × g (15 min). The PMN pellet was resuspended in 25 ml of 0.2% NaCl for 45 s, followed by addition of an equal volume of 1.6% NaCl for RBC lysis. PMN were washed and resuspended in 100 μl of HBSS2+Cl.

Complement-opsonized Microbeads—Complement-opsonized microbeads were prepared with minor modifications from the protocol previously described (6). In brief, 0.2-μm microbeads or 1-μm FluoSpheres were briefly sonicated and reacted with BSA or Alexa Fluor 647-conjugated BSA. Beads were vortexed, briefly sonicated, and centrifuged at 14,000 × g (10 min) followed by washing, which was repeated four times. Beads were then reacted at room temperature with 500 μl of rabbit anti-BSA pAb (10 min), followed by washing. Beads were then incubated with an equal volume of human serum at room temperature for 8 min, washed, and resuspended in 100 μl. Beads were then centrifuged at 14,000 × g (10 min) and only the supernatant containing single beads was used for experiments. Control beads did not receive serum. RBC were incubated with complement-opsonized microbeads (20 min), washed, and then fixed with 0.05% acrolein (5 min). RBC were then blocked in 0.05% cold water fish gelatin (15 min) and incubated with 1 μg/ml of Zenon-labeled anti-CR1 mAb YZ1 (10 min). Cells were washed and imaged using a 60 × 1.42 UPlan Apo objective on an Olympus BX62 fluorescence microscope fitted with an Retiga Em® Q imaging- cooled CCD camera using the FITC and TRITC channels. The microscope, camera, and shutters (Ludl, Hawthorne, NY) were controlled by Slidebook 5.0/5.5 (Intelligent Imaging Innovations, Denver, CO).

ATP-release Measurements—Hematocrit (Hct) was adjusted to 2 or 10% in HBSS2+Cl containing 0.05% IgG-free BSA. Cells were incubated at 37 °C (20 min) with anti-CR1 monoclonal antibody (3C10, 5 μg/ml) followed by centrifugation at 300 × g.
(2 min) and then washed in the same medium. Cell suspension (55 μl) were pipetted into a black 96-well plate and secondary antibody (goat anti-mouse, Jackson, 115-005-003) was added in the same concentration (volume: 5 μl), followed by incubation at room temperature (70 min). A kinetic experiment determined this to be an effective time point (data not shown). ATP release was measured using the GloMax®-Multi+ Detection System (Promega) and ENLITEN® (luciferase/luciferin) reagent, which was injected in equal volumes to the sample into each well and bioluminescence was read. Values were recorded in relative light units. Ligation of RBC with anti-CD47 was performed according to the protocol for RBC/zymosan ligation at 2% Hct (see below). Each sample was processed in triplicates unless mentioned otherwise. An ATP standard using rATP (Promega) was prepared each day in the following concentrations: 0.5, 1, 5, 10, 50, 100, and 200 nM.

Hemolysis Evaluation—Hemolysis was assessed by spectrophotometric analysis at 415 nm after the cells were centrifuged at 2,000 × g (60 s). RBC samples (2% Hct, 60 μl) showing absorbance at 415 nm >0.016 or >0.27% when compared with a lysis standard, were excluded from the study. Maximal absorption was determined by lysing RBC hypotonically (1:100 dilution in H2O). Absorption values of the lysis standard were linear for dilutions between 1:100 and 1:5,000. The total ATP concentration of the lysed RBC was determined using a 1:100 dilution showing an A415 nm of 0.006. We detected 28.9 ± 1.8 nm ATP, which corresponds to 8,000 RBC and translates to an ATP concentration of 1.7 mM per RBC, almost identical to the 1.65 mM reported by Miseta et al. (14). To minimize the ATP contamination from lysed RBC, the cells were washed prior to ATP measurements. In addition, we plotted hemolysis values over the ATP concentration, and removed any data whose correlation coefficient (r^2) exceeded 0.4.

We did notice that the use of certain 1.5-ml reaction tubes increased hemolysis, whereas a polypropylene copolymer tube (1415–2500, USA Scientific, Ocala, FL), reduced this risk. In some cases, a pink coating would remain on the polypropylene surface of these other tubes after centrifugation. As further precaution, we used ultra low retention pipette tips (USA Scientific) in order to avoid the risk of increased hemolysis, whereas a polypropylene copolymer tube was also used. We also performed experiments using an ATP kit (1415–2500, USA Scientific, Ocala, FL), to confirm this result. In addition, we performed experiments using an ATP kit (1415–2500, USA Scientific, Ocala, FL), to confirm this result.

Complement-opsonized Zymosan—One hundred microliters of zymosan suspension (Sigma) was washed twice in half-ionic strength dextrose GVB consisting of a 1:1 solution of GVB and 0.15 mM CaCl2, 5 mM MgCl2 and 5% dextrose in H2O (15) and resuspended in 100 μl of dextrose GVB. COZY, was prepared by incubating 100 μl of human serum or the same volume of dextrose GVB for control (zymosan) at 37°C (20 min). Samples were then washed twice at 5,600 × g (10 s) and resuspended in 100 μl of HBSS2− containing 0.05% IgG and protease-free BSA. Five microliters of COZY or zymosan suspension were added to 250 μl of RBC (2% Hct) and incubated at 37°C (15 min). Because zymosan particles interfered negatively with ATP detection methods the RBC were washed (300 × g, 30 s) prior to ATP measurement, a step that significantly decreased the total ATP concentration in the samples. Forty μl of RBC suspension were then added to a black 96-well plate and ATP was measured after incubation at room temperature (5 min).

Direct Imaging of ATP Release Using 2-2Zn(II)—RBC (10⁶/ml), resuspended in phosphate-free buffer containing 107 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 11.5 mM glucose, and 20 mM HEPES, pH 7.4 (HBS buffer, were preincubated with 1 μM 2-2Zn(II) (13) (10 min) followed by ligation of either control or YZ1 anti-CR1 mAb (5 min). Cells were then washed once and seeded on a clean microscope slide. Series time lapse images consisting of 60 frames recorded at 3-s intervals were acquired using the FITC channel and a 60 × 1.42 UPlan Apo objective. Cross-linking of the anti-CR1 mAb was done by slowly adding 8 μl of goat anti-mouse Alexa Fluor 594 secondary antibody under the coverslip, 10 frames into image acquisition. At the end of the experiment, cells were imaged using the TRITC channel to identify CR1 clusters. Signal intensity, as well as the spatial distribution of both probes associated with ATP (FITC channel) or CR1 clusters (TRITC channel) were quantified using iVision 4.5 (BioVision, Exton, PA).

Fluorescence Recovery after Photobleaching—RBC were labeled with lipid dye DiO at room temperature for 30 min, washed once, then treated with ATP (0.1 μM or 1 μM) or incubated with either control or anti-CR1 mAb in the presence or absence of 5 units/ml of apyrase. A 1 × 1-μm² region of the cell bleached by a 488-nm laser beam used at 0.21% intensity for 35 ms, using a Vector photomanipulation system (Intelligent Imaging Innovations (3i), Denver CO) coupled to an Olympus BX62 fluorescence microscope using a dedicated FRAP filter cube that allowed simultaneous acquisition and bleaching using the 488 nm as excitation/bleaching wavelength. The laser was calibrated before each experiment and the intensity of fluorescence post-bleaching was between 35 and 45% of the original pre-bleach value, to minimize RBC membrane damage. Each recording was done at a rate of 8 frames/s. For FRAP analysis, the photobleaching rate during fluorescence imaging was recorded and quantified on a nearby cell of similar fluorescence emission intensity, and subsequently subtracted during FRAP analysis using a dedicated FRAP analysis module in Slidebook 5.0.

Measurement of RBC Membrane Deformability Using Two-dimensional Filters—The ability of RBC to undergo capillary-like deformations was measured using a two-dimensional microchannel array as previously described (6). The device uses hydrostatic pressure to drive the RBC through the microchannel array. RBC (8 μl, 20% Hct) were loaded into the inlet reservoir of the microchannel and driven into the capillary-like area by lowering the waste reservoir tubing. Once RBC entered the capillary-like area, the waste reservoir tubing was raised to a height that allowed control RBC from healthy donors to pass through the 25-μm length of the capillary in 2–3 s. The cells were recorded using a 40 × 0.75 Ph2 Plan Fluorite objective on a TE300 Nikon-inverted microscope (Nikon Inc., Melville, NY), using a Retiga EXi (QImaging, Surrey, Canada) CCD camera controlled by iVision 4.7 software (BioVision) at a rate of 10 frames/s. Movies representing 600 frames then were analyzed off-line frame by frame, and the results were expressed as the number of frames from entry to egress (16). RBC that displayed unusual shapes (echinocytes, acanthocytes) or overlapped, whereas in the capillary-like area were excluded from measure-
Role of ATP from RBC in Phagocytes

FIGURE 1. Ligation of CR1 on RBC stimulates ATP release. A, primary and secondary antibody cross-linking of RBC CR1 released ATP. RBC (2% Hct) were reacted with anti-CR1 mAb (3C10) or control mAb, followed by secondary goat anti-mouse IgG. The bars are the mean of triplicates ± S.D. Data show one of three experiments using different donors all with similar results. B, COZY stimulates ATP release from RBC. RBC (2% Hct) were incubated with zymosan or COZY as outlined under "Experimental Procedures" and washed prior to reading the ATP levels. The bars represent the average of five experiments ± S.E. using four different donors. C, ARL 67156, an ectonucleotidase inhibitor, has no effect on ATP released. 10% Hct were reacted with anti-CR1 mAb YZ1 or control Ab with or without ARL 67156 and incubated at 37 °C for 1 h. Bars represent the difference between IgG and anti-CR1-treated cells in triplicate readings ± S.D. D, CR1 ligation of donors that are genetically determined to express low or high levels of CR1. RBC (2% Hct) were ligated with anti-CR1 mAb. Experiments of one low and one high CR1 expresser were performed in parallel. Graph represents three independent experiments with one low CR1 expresser and two different high CR1 expressers.

ments. For each experimental condition at least 30 “usable” RBC were counted.

Immunoblotting—RBC were analyzed as previously described (5). Briefly, RBC (2 μl) were lysed in 100 μl of 1× NuPAGE® LDS Sample Buffer (Invitrogen) and kept at 37 °C (5 min). Samples were run on 4–12% BisTris gels (Invitrogen), transferred to nitrocellulose paper, and blocked with blotting-grade blocker (Bio-Rad) in Tris buffer with 0.1% Tween 20 at room temperature (1 h). Membranes were then incubated with primary and secondary antibodies according to the manufacturer’s instructions and blots were developed using the LAS 4,000 imaging system (Fujifilm, Tokyo, Japan). SeeBlue® Plus2 Pre-Stained Standard (4–250 kDa, Invitrogen) was used to determine the relative molecular mass of the bands.

RBC Ghosting—RBC ghosts were prepared for immunoblotting by lysing 6 μl of packed RBC in 1 ml of ice-cold ghosting buffer containing 5 mM sodium phosphate (pH 8.2), 2 mM MgCl2, 5 mM EGTA supplemented with an equimolar concentration of MgCl2 (Mg2+-EGTA), and protease inhibitor mixture cOmplete (Roche Diagnostics) at 4 °C (20 min) while continuously rocking. RBC ghosts were centrifuged at 10,000 × g at 4 °C (10 min), washed free of hemoglobin, and centrifuged again in ghosting buffer. The resulting pellet was treated as the RBC sample.

Flow Cytometry—RBC were incubated at room temperature (15 min) with primary Abs (0.5–1 μg/ml as noted in the figure legends), washed three times with 0.5% IgG-free BSA in HBSS+ +, and incubated with Alexa Fluor 488- or Alexa Fluor 594-labeled secondary Ab specific to the primary Ab and diluted according to the manufacturer’s recommendations. Samples were analyzed using a LSRII or FACScan (BD Biosciences), and ≥10,000 events were acquired and analyzed by FlowJo Version 9.2 (Treestar, Ashland, OR). Mean fluorescence intensity in the text refers to the mean as generated by FlowJo.

Statistical Analysis—Tests were performed using Prism version 4.0 (GraphPad software) and detailed for each experiment. p values were determined with a two-tailed, parametric t test or the respective analysis of variance analysis and values of p < 0.05 were considered significant. Error bars represent S.D. unless noted otherwise; figures are representatives of one experiment of one donor and experiments were independently repeated three times with a total of three donors, unless noted otherwise.

RESULTS

RBC CR1 Ligation Promotes ATP Release—Ligation of RBC CR1 with the anti-CR1 mAb 3C10 followed by secondary antibody ligation (Fig. 1A) increased the ATP concentration in the supernatant from 10.8 ± 0.7 to 14.9 ± 1 nM (p < 0.01). When experiments were repeated using COZY as a physiologic CR1 ligand, a more robust ATP efflux was observed, increasing the concentration of ATP in the supernatant from 3.3 ± 0.6 to 5.9 ± 1.2 nM (p < 0.05, n = 5). Although we noticed that the basal amount of ATP released by RBC varied significantly among various healthy donors, ligation of CR1 generated the same type of response. To verify that the measured ATP was not an effect of RBC lysis, we plotted the obtained ATP values over the hemolysis values and did not find a correlation (r2 = 0.09, data not shown). Extracellular ATP, which is the result of
either a constitutive or an active secretory process, is degraded by ectonucleotidases present on plasma membranes on blood cells (17). To assess if RBC ectonucleotidases might affect ATP measurements, we added the nonspecific ecto-ATPase inhibitor ARL 67156. The presence of ARL 67156 (100 μm) did not significantly (p = 0.9) affect the amount of ATP measured upon RBC CR1 ligation (Fig. 1C). These results are in full agreement with a recent finding that human RBC do not express detectable levels of the ecto-ATPase CD39/NTDase2 (18). Therefore, ARL 67156 was not used in any subsequent experiments. Previously, we have shown that the effect of CR1 ligation on RBC membrane deformability depends significantly on the genetically determined levels of CR1, with low CR1 expressers promoting lesser increase in membrane deformability compared with high CR1 expressers. Therefore, we next tested the effect of CR1 levels on ATP release. Our cumulative results (Fig. 1D) obtained from two high CR1 expressers and one CR1 expresser have shown that the pre-existing levels of CR1 have no significant effect on CR1-mediated ATP levels (p = 0.1, n = 3).

**Visualization of CR1-mediated ATP Release Using a Fluorescent ATP-sensing Probe**—To directly image ATP release promoted by RBC CR1 ligation, we employed a novel fluorescent nucleotide-sensing probe, 2-2Zn(II), which binds the extracellular side of the plasma membrane, and upon interaction with ATP, fluoresces in an ATP concentration-dependent manner (13, 19, 20). First, to test the specificity of the probe for ATP, 2-2Zn(II)-treated RBC were incubated at room temperature (5 min) in the presence or absence of ATP diphosphohydrolase (apyrase) and subsequently analyzed by flow cytometry. Our data show that the presence of apyrase (Fig. 2A, filled histogram) significantly decreased the fluorescence-associated with RBC from 22 to 2.3 mean fluorescence intensity confirming that the probe was specific for ATP. Next, RBC pretreated with 2-2Zn(II) were incubated either with control or anti-CR1 mAb (YZ1) and then analyzed by both, flow cytometry (Fig. 2B) and microscopy (Fig. 2, C–H). Ligation of RBC CR1 induced an increase in 2-2Zn(II) fluorescence from control levels of 78 to 159 mean fluorescence intensity on anti-CR1-treated RBC. We used fluorescence microscopy to directly image the ATP release from RBC following CR1 ligation. Incubation of RBC with control IgG (Fig. 2C) shows the baseline ATP release at 350 arbitrary units (AU) for the membrane bound probe and 100 AU for the unbound probe in the buffer several micrometers away from the cell (histogram of fluorescence intensity, Fig. 2D). Following ligation of RBC with anti-CR1 mAb, the fluorescence associated with 2-2Zn(II) (Fig. 2E) increased to 1100 AU for the membrane-bound probe and 400 AU for the unbound probe in the buffer (histogram of fluorescence intensity, Fig. 2F). Of note, the probe when used at the recommended concentration induced significant RBC aggregation, which can be seen in the flow cytometry histograms as bi-modal peaks, both in control and anti-CR1-treated cells and in microscopy images as clusters of RBC. Next, we asked whether the release of ATP is associated with distinct areas in the plasma membrane where CR1 clusters are present. Similarly, RBC were prepared with anti-CR1 mAb (YZ1) followed by incubation with Alexa Fluor 594 secondary goat anti-mouse Ab (Fig. 2G) and the intensity profiles of both CR1 clusters (red) and ATP (green) around the plasma membrane were plotted 3 min after CR1 cross-linking (Fig. 2H). No distinct pattern linking the areas of CR1 clustering (red peaks, arrows) and increased ATP release was found, suggesting that either the CR1 clusters and ATP channel(s) are not in direct vicinity, or the diffusion rate of ATP is significantly faster than the acquisition rate of our imaging system (one frame every 1.5 s for an acceptable signal to noise ratio). Of note, the lower values on the y axis representing the fluorescence emission intensity of both CR1 and ATP probes in Fig. 2H are due to the fact that the final image has only 8-bit/channel (256 levels of gray) instead of a 12-bit/channel (4095 levels of gray) as the original CR1 and ATP images.

CR1-mediated ATP Release Does Not Require Extracellular Ca\(^2+\), PKA, or CK2 Activity—We previously reported that CR1 ligation increased RBC membrane deformability and depended critically on Ca\(^2+\) influx, PKA, and CK2 activity (6). Therefore, we tested whether the observed CR1-mediated ATP release also required the presence of extracellular Ca\(^2+\) and the activity of these enzymes. Incubation of RBC with COZY in the presence of 5 mM Mg\(^2+\)-EGTA increased ATP release when compared with control from 9.0 ± 1.4 to 13.5 ± 1.0 nM, p < 0.05 (n = 3) (Fig. 3A, right bars). We noticed that the overall ATP release of Mg\(^2+\)-EGTA-treated cells was greater than that of HBSS\(^+\)-treated cells (10.0 ± 0.9 and 4.7 ± 0.4 nM, n = 3, p < 0.01) (Fig. 3A, left bars), suggesting a negative regulatory role of extracellular Ca\(^2+\). Next, we investigated whether PKA is required for CR1-dependent ATP release by ligating CR1 in the presence of the selective PKA inhibitor, KT 5720. We found that inhibition of PKA prior to CR1 ligation did not significantly alter the amount of ATP released from RBC compared with CR1 ligation (1.39 ± 0.19 to 1.4 ± 0.3 AU; p = 0.98; n = 4) (Fig. 3B). Next we investigated the involvement of CK2, another enzyme we have found to be critical for CR1-mediated increase in membrane deformability. Our data show that ligation of CR1 in the presence of 10 μM TBB, a selective ATP/GTP competitive inhibitor of CK2 (21), did not significantly decrease ATP release from RBC (1.53 ± 0.16 to 1.80 ± 0.23 AU; p = 0.4, n = 3) (Fig. 3C).

Extracellular ATP and Ligation of CR1 Increase RBC Lipid Mobility—We have previously shown that ligation of CR1 with either antibody or complement-opsinized cargo significantly increases human RBC membrane deformability (6). Our results have also shown that although the spectrin phosphorylation promoted by CR1 ligation lasted less than 15 min, the effect on membrane deformability persisted for several hours suggesting that other mechanisms have critical roles in membrane deformability (6). New data demonstrate that the interaction between spectrin and plasma membrane lipids as well as rearrangement of lipid subdomains in RBC membranes play a considerable role in RBC membrane stability and deformability (22). Therefore, we next tested the effect of extracellular ATP and that of CR1 ligation on plasma membrane lipid mobility using FRAP. Fig. 4A shows serial snapshots of a representative RBC during a FRAP experiment. The arrow in the second image points to the area bleached by the laser beams. When RBC were pretreated with 0.1 μM ATP, no discernible effect on either the fluorescence τ<sub>0</sub>, recovery time (p > 0.17), nor on the percent mobile fraction of RBC lipids (p > 0.2) was observed. Increasing the concentration of extracellular ATP to 1 μM significantly
decreased the $t_{1/2}$ recovery time from 10.84 ± 0.73 s to 8.98 ± 0.53 s ($p < 0.05$) (Fig. 4B) and increased the lipid mobile fraction from 85.63 ± 0.96 to 90.62 ± 0.97%, $p < 0.01$ (Fig. 4C). Unlike pre-treatment of RBC with ATP, ligation of CR1 did not significantly alter the $t_{1/2}$ recovery time of fluorescently labeled lipids ($p = 0.181$). However, ligation of RBC CR1 using mAb YZ1...
followed by secondary antibody ligation increased the mobile fraction from 82.57 ± 1.01% in IgG control treated cells to 90.35 ± 1.13%, p < 0.05 (Fig. 4E). The presence of 5 units/ml of apyrase during ligation of CR1 partially reversed the observed decrease in the mobile fraction to 84.56 ± 0.8%, p < 0.05. A representative recovery rate is shown in Fig. 4F. Next, we asked whether extracellular ATP is important for the effect of CR1 ligation on RBC membrane deformability as measured using microfiltration through an array of two-dimensional microchannels as we have previously described (6). Our results (Fig. 4G) show that removal of extracellular ATP with apyrase had no detectable effect on basal RBC deformability when compared with buffer-treated cells (average transit times, 2.34 ± 0.3 and 2.41 ± 0.25 s, p = 0.1). Similarly, the increase in membrane deformability promoted by CR1 ligation (average transit times recorded at 10 frames/s, 1.72 ± 0.3 s, p < 0.01) did not depend on the presence of extracellular ATP, as pre-treatment of CR1-ligated RBC with 5 units/ml of apyrase did not reverse the increase in RBC membrane deformability promoted by CR1 ligation (average transit times, 1.62 ± 0.3 s, Fig. 4G). These results suggest that the ATP released by RBC does not have a permissive autocrine effect on the CR1-mediated increase in membrane deformability.

**Extracellular ATP Is Required for Efficient Binding of Complement-opsonized Cargo to CR1**—The increase in membrane lipid mobility by extracellular ATP, as seen in Fig. 4, could also influence the ability of CR1 to diffuse and partition across the RBC membrane, and consequently impact the avidity of CR1 complement-opsonized particle interaction. Therefore, we tested whether ATP alters the binding avidity of CR1 by incubating RBC ligated with complement-opsonized microbeads in the presence or absence of 5 units/ml of apyrase. The fluorescence associated with RBC was then quantified by flow cytometry. Removal of extracellular ATP with 5 units/ml of apyrase decreased the binding efficiency of complement-opsonized microbeads to RBC from 34.5% (Fig. 5A, middle panel) to 1.55%, respectively (Fig. 5A, right panel), indicating that either binding or maintaining the binding of complement-opsonized microbeads to CR1 on RBC requires extracellular ATP. In a separate experiment, inhibiting CR1-mediated increases in membrane deformability by blocking CK2 activity using TBB also decreased the percentage of beads bound to RBC from 31.1% (Fig. 5B, first panel) to 17.4% (Fig. 5B, second panel). To test whether extracellular ATP could overcome the decrease in binding efficiency induced by TBB, during incubation with complement-opsonized microbeads, we added 20 μM ATP to the TBB-treated RBC. Addition of extracellular ATP effectively restored CR1 binding efficacy to the initial levels of 31.0% (Fig. 5B, third panel).

**Extracellular ATP Is Required for Both CR1 Clustering and Enhanced PMN Phagocytosis of RBC-adherent Complement-opsonized Microbeads**—We have previously shown that CR1 on RBC clusters following its ligation with complement-opsonized microbeads or anti-CR1 antibody (6). A possible expla-
nation for the decreased binding efficiency we reported in Fig. 4F is a requirement of extracellular ATP for CR1 clustering. To test this hypothesis, RBC were incubated with complement-opsonized 0.2-μm microbeads labeled with Alexa Fluor 647-conjugated BSA in the presence or absence of 5 units/ml of apyrase followed by CR1 staining using Alexa Fluor 488-direcly labeled anti-CR1 mAb (YZ1). When the intensity profiles of CR1 in buffer-treated and apyrase-treated RBC were analyzed and plotted against the intensity profile of complement-opsonized microbeads, we did not detect a difference between
the CR1 molecule profiles directly involved in binding of the cargo (Fig. 6A, top right) and those of by-standing CR1 molecules (Fig. 6A, bottom right) suggesting that apyrase prevented the ligand-induced clustering of CR1. This lack of CR1 clustering after apyrase ATP hydrolysis could also explain the lower binding avidity of CR1 for complement-opsonized microbeads, which we have previously shown using fixed RBC that have permanently diffused CR1 (5). Although ATP was critical for CR1 clustering, we asked if RBC-derived ATP could also modulate the uptake of RBC-adherent complement-opsonized cargo by PMN. Freshly isolated PMN were incubated with complement-opsonized FluoSpheres (λ_{excitation/emission} = 505/515) adherent to RBC, in the absence or presence of 10 μM ATP. We found that addition of ATP during complement-mediated phagocytosis increased the percentage of PMN phagocytosis from 17.4% (Fig. 6B, middle panel) to 28.1% (Fig. 6B, right panel), suggesting that RBC-derived ATP could be important not just for CR1 clustering, but could also stimulate phagocytosis of RBC cargo by PMN. In addition the same increase mediated by ATP was noted during complement-independent phagocytosis (Fig. 6B, lower row) where, in the absence of RBC, ATP increased phagocytosis of IgG-opsonized microbeads from 13.7 to 19.3%. Because CR1-mediated ATP release does not take place in the proximity of tissue resident macrophages, which are involved in the immune transfer, it is less likely that this ATP, which is released during CR1-mediated capture of the immune complexes, would directly influence the macrophages. To test if RBC possess a second physiologic mechanism to increase ATP release during direct interaction with tissue macrophages, we ligated CD47, the RBC surface molecule that serves as the ligand to SIRPα on macrophages, a molecule that deliver the “do not ingest me” signal during the immune transfer process (7, 8). Ligating CD47 with anti-CD47 mAb, without secondary Ab cross-linking, resulted in a robust increase of
FIGURE 6. Extracellular ATP is required for the clustering of RBC CR1 and stimulated PMN phagocytosis of RBC-adherent complement-opsonized microbeads. A, apyrase prevents clustering of CR1-bound complement-opsonized microbeads to human RBC. RBC in the absence (upper row) or presence (lower row) of 5 units/ml of apyrase were incubated with complement-opsonized microbeads labeled with Alexa Fluor 647-conjugated BSA (30 min), washed, and then incubated with directly labeled Alexa Fluor 488 anti-CR1 mAb. Arrowheads in merged images indicate complement-opsonized microbeads (red), arrowheads in CR1 panels indicate microbead-binding site on RBC (CR1 clusters in buffer and does not cluster in apyrase-treated cells). Fluorescence intensity graphs for both channels (red, complement-opsonized microbeads; green anti-CR1) recorded across RBC membranes were plotted for buffer-treated (right panel, top) and apyrase-treated RBC (right panel, bottom). Graphs show representative histograms that were plotted from 6 buffer-treated and 8 apyrase-treated RBC.

B, extracellular ATP prevents phagocytosis of RBC-attached complement-opsonized microbeads. Freshly isolated PMN were incubated with buffer alone (top left) or with complement-opsonized microbeads labeled with Alexa Fluor 647-conjugated BSA, adherent to RBC in the absence (top middle) or presence (top right) of 10 μM ATP (20 min) at a 5:1 PMN:RBC ratio. Lower row shows PMN incubated with RBC that were incubated with microbeads that did not receive serum (labeled IgG beads) in the absence (middle) or presence (right) of 10 μM ATP. Cells were analyzed using flow cytometry. The results are representative of four independent experiments using RBC and PMN from three different donors.

C, ligating RBC CD47 leads to ATP release. RBC (250 μl, 2% Hct) were ligated with anti-CD47 mAb and incubated at 37 °C (15 min). RBC were then centrifuged at 300 × g (60 s), 210 μl of supernatant were exchanged for fresh buffer and RBC were washed once more. 40 μl of RBC suspension were plated on a black 96-well plate and ATP was measured using the ENLITEN assay as described under “Experimental Procedures.” Average ATP values of five independent experiments using five different donors are shown ± S.E.
ATP release from 3.4 ± 0.5 to 9.4 ± 1.9 nm (n = 5, p < 0.05) (Fig. 6C), indicating that the ligation of CD47 on RBC with its physiologic ligand SIRPα on macrophages could be such a second physiologic response.

**Inhibition of Pannexin-1 Does Not Affect CR1-mediated ATP Release**—Pannexin-1 was reported to be involved in RBC ATP release following several different types of stimuli such as low oxygen tension and mechanical deformation (23). Therefore, we investigated the role of pannexin-1 in CR1-mediated RBC ATP release by either using 10Panx, a deca-peptide inhibitor of pannexin-1, or scrambled 10Panx, a control deca-peptide. We found that pretreatment of RBC with 50 μM 10Panx had no significant inhibitory effect on CR1-mediated ATP release, despite a decrease in measured ATP (Fig. 7A). The same concentration (50 μM) of scrambled 10Panx also did not significantly alter CR1-mediated ATP release. Of note, when used in our system, both compounds caused RBC lysis (8%) at 200 μM, the concentration previously shown to inhibit RBC ATP release (24). Of note, the greater ATP values in Fig. 7A are derived from a cell suspension with 10% Hct and differing incubation requirements for 10Panx as described in the figure legend. We next assessed the presence of pannexin-1 on human RBC, by using immunoblotting of lysed-ghosted and intact RBC and pannexin-1-transfected HEK cells as positive control. Our method using rabbit anti-pannexin 1 pAb failed to detect pannexin-1 antigen in human RBC (Fig. 7B) unlike a previous report (12). We next used flow cytometry to further investigate the presence of pannexin-1 on human RBC using custom made chicken anti-pannexin-1 pAb directed against either the extracellular (4512 pAb) or intracellular (4515 pAb) domain of pannexin-1 (12). We were unable to confirm the presence of pannexin-1 in either human or mouse RBC (data not shown). To test the anti-pannexin-1 pAb, we used isolated PMN, which are known to express pannexin-1 (25). Although PMN were positive, RBC were again negative for pannexin-1 (data not shown).

**MRP1 Mediates CR1-promoted ATP Release**—We next investigated the role of MRPI (ABCC1) in CR1-mediated ATP release by using MK-571, a specific MRP inhibitor. When RBC were treated with MK-571 (either 10 or 100 μM) a dose-dependent response in ATP release was noted (Fig. 7C) in accordance with a previous report (26). However, when MK-571 (10 and 100 μM) was added to the ATP standard, a significant decrease of the ATP readout was detected (Fig. 7D) (at 50 nm ATP, 10 μM MK-571 reduced the ATP readout by 43% and 100 μM MK-571 by 87%, n = 3), showing that the decrease in ATP release noted in Fig. 7C was likely due to interference of MK-571 with the luciferase/luciferin assay, and not because of a biological process. After compensating for the inhibitory effects of MK-571 seen in the ATP standard, our results indicate that MK-571 induces a slight increase of basal ATP release by RBC from 5.0 ± 0.5 to 7.0 ± 0.6 nm, when RBC were treated with 10 μM MK-571, and to 9.3 ± 0.6 nm when treated with 100 μM MK-571. Next we ligated RBC on CR1 using COZY in the presence or absence of 10 μM MK-571 (Fig. 7F). Our data show that inhibition of RBC MRP reduces the amount of ATP released from RBC following CR1 ligation from 9.8 ± 1.0 to 8.3 ± 0.4 nm (p < 0.05, suggesting that MRP1 is partially involved in CR1-mediated ATP release.

**DISCUSSION**

In this study we investigated the effect of CR1 ligation on RBC ATP release using antibodies and complement-opsonized particles, as physiological ligands for RBC CR1. Although ligation of CR1 by both methods elicited a robust ATP release, we did notice that not all anti-CR1 antibodies elicited the same response in regard to an increase in ATP release. Although 3C10 was able to induce ATP release either directly or upon cross-linking. The use of YZ1 was less efficient in the ENLITEN assay system, but did elicit reliable signals using the 2-2Zn(II) probe. One possible explanation is that different antibodies bind to distinct regions on the CR1 molecule (27) and therefore induce different biological responses, similar to the effect of various anti-glycophorin A antibodies on RBC membrane deformability, as reviewed in Ref. 28, or that YZ1 does not cross-link CR1 as effectively as 3C10, COZY, or complement-opsonized microbeads.  

Measuring ATP release from RBC possesses several unique and significant challenges, such as: absorption of the hemoglobin in the emission spectra of oxyluciferin (550–600 nm) (29), obligatory requirements of Mg2+ for the luciferase/luciferin reaction, relatively high intracellular ATP concentration (1.7 mM), fragility of RBC membranes, which when ruptured releases ATP in a channel-independent manner, and interference of free hemoglobin. For this reason, we reduced the risk of hemolysis by using a specific 1.5-mL copolymer reaction tube as noted under “Experimental Procedures” and by washing RBC prior to reading ATP release. In addition to these technical aspects, several key purinergic inhibitors such as suramin and Gd3+ interfere with the standard luciferase/luciferin detection method (30), as well as the MRP inhibitor MK-571, as described in the present study. We verified our results obtained using the luminometric approach by employing an alternative detection method, based on a novel membrane-bound fluorescence probe that directly detects the RBC-mediated ATP release with few if any of the aforementioned challenges (Fig. 2). Using the 2-2Zn(II) probe, we detected the same increase in ATP concentration following CR1 ligation with a halo-like pericellular distribution, reminiscent of the ATP distribution around migrating neutrophils (31). Interestingly, using the 2-2Zn(II) probe and fluorescence microscopy or flow cytometry, we were able to detect ATP release even when using anti-CR1 mAb YZ1. This could be because the spatial resolution and/or signal to noise ratio of both methods surpasses that of the luminometer. However, the obligatory requirement of the probe for a phosphate-free buffer, unlike the one commonly used by flow cytometers during hydrodynamic focusing of the sample, may somehow favor its use in microscopy. We have also noticed that the time between blood drawing, the temperature of incubation, as well as consumption of some over the counter medications significantly altered the basal ATP levels.

The role of extracellular ATP was studied extensively especially when involved in neutrophil and platelet functions (32, 33), whereas ATP released by apoptotic cells was shown to be critical in their identification and phagocytosis (“find me and...
Role of ATP from RBC in Phagocytes

FIGURE 7. Effect of ATP channel inhibitors on RBC. A, inhibition of Pannexin-1 does not affect CR1-mediated ATP release. RBC (10% Hct) were treated with the either 10Panx or the scrambled 10Panx control peptide at room temperature prior to incubation with primary and secondary antibodies at 37 °C (10 min). The bars are the mean of triplicates ± S.E. Data depicts one of three experiments all with similar results. B, immunoblot analysis of pannexin-1 levels in RBC. HEK293T cells were transfected with anti-human pannexin-1 and used as a positive control (lane 1). Non-transfected HEK293T cells were used as a negative control (lane 2). Lysates from RBC show no bands at 50 kDa, the anticipated size for glycosylated pannexin-1, or at 37 kDa, the size of non-glycosylated pannexin-1 (lane 3). RBC ghost control is shown in lane 4. The blot is representative of three replicates. C, the effect of MRP inhibitor MK-571 on RBC ATP detection using the ENLITEN assay system. RBC (2% Hct) were incubated with 10 or 100 nM MK-571 at 37 °C (20 min) and 40 μl of unwashed cell suspension was analyzed for ATP release. The bars represent the combined values of two experiments in triplicates with similar values ± S.E. D, the effect of MK-571 on the luciferase/luciferin ATP assay. 10 or 100 nM MK-571 were added to the ATP standard (0, 1, 5, 10, 20, and 50 nM ATP) and the results were obtained using the ENLITEN assay system. The graph represents the average of three independent experiments. Diamond, ATP standard in HBSS buffer with 0.05% IgG-free BSA; triangle, ATP standard with the addition of 10 μM MK-571; square, ATP standard with the addition of 100 μM MK-571; RLU, relative light units. E, Zymosan or COZY were added and samples were incubated at 37 °C (20 min). Cells were then washed with HBSS (including 0.05% IgG-free BSA) and ATP release was measured as described under “Experimental Procedures.” The bars represent one experiment in triplicates ± S.D. F, CR1-mediated ATP release is regulated by MRPs. RBC (2% Hct) were pretreated with 10 μM MK-571 at room temperature (10 min). Then zymosan or COZY were added and samples were incubated at 37 °C (20 min). Cells were then washed with HBSS (including 0.05% IgG-free BSA), and ATP release was measured as described under “Experimental Procedures.” The bars show one representative experiment in triplicates ± S.D.

remove me” signal) by macrophages and monocytes, in vitro (10). Here we hypothesized that ligation of CR1 by immune complex-loaded RBC would promote RBC ATP release, possibly as a first step in priming liver and spleen macrophages to remove CR1-adherent complement-opsonized microbeads or preparing the RBC membrane for the immune-transfer process. We showed that RBC-derived ATP is critical for effective binding of CR1 to complement-opsonized targets, affecting membrane lipid mobility but not membrane deformability, as measured by the two-dimensional microchannel array. Increased lipid mobility following CR1 ligation could suggest that the immune-transfer process may involve
formation of membrane-derived microvesicles containing CR1 together with the complement-opsonized cargo. Formation of microparticles is triggered by Ca\(^{2+}\) influx, localized qualitative and quantitative changes in membrane lipids, and it is followed by loss of specific classes of membrane lipids and proteins (34), conditions that are present in old circulating RBC and accelerated in diseases associated with the increased immune-clearance process such as systemic lupus erythematosus (16).

The initial release of ATP at the time of CR1 ligation is unlikely to have an effect at the time when the transporting RBC reaches the sinuses of the liver and spleen. However, our data indicate that when RBC CD47 is ligated, experimentally by anti-CD47 mAb ex vivo, there is a robust release of ATP from the RBC. In vivo, the CD47 ligand SRIRα is located on resident tissue macrophages and its ligation could provide a site-specific ATP signal. This second CD47-mediated burst of pro-phagocytic ATP, in conjunction with the clustered CR1 distribution and inhibitory effect of SRIRα on phagocytosis could be responsible for the effective but spatially limited removal of immune adherent particles that spare the carrier RBC (9, 36). We have previously shown that human PMN ingested faster and killed more effectively RBC-adherent Salmonella montevideo than free-floating and complement-opsonized individuals of this species (37). Interestingly, several bacteria display cell-wall-associated ecto-ATPase activity that was shown to increase their virulence and promote platelet aggregation (38, 39). Although the binding of C3b/C4b fragments to CR1 is not ATP dependent, CR1 clustering is, therefore it is conceivable that circulating bacteria could use their 5-ectonucleotidase activity in two distinct ways: 1) to limit the ability of CR1 to cluster and effectively immobilize bacteria to the RBC surface and 2) to prevent phagocytes from ingesting and killing the bacteria. ATP release by human RBC has been described for over 15 years, but the identity of the ATP channels on circulating RBC has been elusive. Several proteins have been proposed to be either directly involved, such as: 1) pannexin-1 (40, 41), 2) MRP1 (42), 3) P2X7 (43), or 4) indirectly, regulating of the activity of ATP channels such as cystic fibrosis transmembrane conductance regulator (44).

Our initial approach to find the ATP channel opened by ligation of CR1 on RBC started with pannexin-1 and involved several methods such as flow cytometry, microscopy, and immunoblotting (Fig. 2, C and D), as well as functional studies using the pharmacological inhibitor, \(^{10}\)Panx (Fig. 2A). When using custom made chicken anti-pannexin-1 pAbs (4212, 4215), which were previously described to bind to human RBC (18), our method failed to confirm the presence of pannexin-1 on circulating human RBC using both flow cytometry and fluorescence microscopy. One conceivable explanation for this discrepancy could be that the pAb (4512) was raised against the peptide “VQQKSLQSESIGN” found in the extracellular pannexin-1 loop of the mouse, which differs from that of the human extracellular loop of pannexin-1 (bold and underlined amino acid), “VQQKNSLQSESIGN,” thus potentially limiting its efficacy against human pannexin-1. Taken together, our data show that using the existing anti-pannexin-1 Abs, both custom made and commercially available, we could not detect pannexin-1 in human RBC with the methods and instruments available in our laboratory.

Our next target was MRP1, a protein putatively involved in ATP release, which was identified initially in a study using a mouse model of cystic fibrosis. It is also found to be up-regulated and over-functional in circulating RBC from cystic fibrosis patients (42). Because the inhibitor of MRP, MK-571, interfered with the detection system, washing of the cells to remove MK-571 prior to the ATP detection assay, was required. Despite this manipulation, MK-571 significantly decreased the CR1-mediated ATP release, suggesting that MRP1 plays a role in mediating, either directly or indirectly the CR1-mediated ATP release in human RBC. In addition to its inhibitory effect following CR1 ligation, MK-571 consistently enhanced the basal release of ATP from RBC. This result is in contrast to a study using human astrocytes (26), where MK-571 inhibited the basal ATP release. It is possible that astrocytes respond differently to MK-571 compared with human RBC, or that the direct effect of MK-571 was not accounted for on luciferase/luciferin reaction efficiency.

In conclusion, our study shows that ATP release by human RBC following CR1 ligation has two major roles: 1) increases RBC lipid mobile fraction and promotes CR1 avidity for complement-opsonized particles by enhancing CR1 clustering and 2) increases the efficiency of the immune-transfer process from RBC to the effector phagocytic cells. The current findings on the functional consequence of RBC CR1 ligation in the immune-transfer process are important especially now when unexpected and non-redundant roles for human RBC CR1 in pathologies ranging from malaria (45) and sepsis (46) to Alzheimer disease (35, 47–49) are beginning to be unraveled.

Acknowledgments—We thank Dr. Gina Sosinsky (University of California, San Diego) for the gift of HEK293T cells; Drs. Gerhard Dahl (University of Miami) and Eliana Sencos (Albert Einstein College of Medicine, New York) for gifts of the custom made chicken anti-pannexin-1 pAbs and Dr. Lloyd Klickstein (Novartis) for the gift of the YZ1 and 3C10 anti-CR1 antibodies.

REFERENCES

1. Nelson, R. A., Jr. (1953) The immune adherence phenomenon. An immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis. Science 118, 733–737

2. Fearon, D. T. (1980) Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte and monocyte. J. Exp. Med. 152, 20–30

3. Klickstein, L. B., Barbashov, S. F., Liu, T., Jack, R. M., and Nicholson-Weller, A. (1997) Complement receptor type 1 (CR1, CD35) is a receptor for Clq. Immunity 7, 345–355

4. Ghiran, I., Babashov, S. F., Klickstein, L. B., Tas, S. W., Jensensius, J. C., and Nicholson-Weller, A. (2000) Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J. Exp. Med. 192, 1797–1808

5. Ghiran, I., Godek, A. M., Weaver, G., Klickstein, L. B., and Nicholson-Weller, A. (2008) Ligation of erythrocyte CR1 induces its clustering in complex with scaffolding protein FAP-1. Blood 112, 3465–3473

6. Godek, A. M., Mirchev, R., Golan, D. E., Khoory, J. A., Burns, J. M., Shevkoplyas, S. S., Nicholson-Weller, A., and Ghiran, I. C. (2010) Ligation of complement receptor 1 increases erythrocyte membrane deformability. Blood 116, 6063–6071

7. Oldenborg, P. A. (2004) Role of CD47 in erythroid cells and in autoimmunity. Leuk. Lymphoma 45, 1319–1327

8. Tsai, R. K., and Discher, D. E. (2008) Inhibition of “self” engulfment through deactivation of myosin-II at the phagocytic synapse between hu-
Role of ATP from RBC in Phagocytes

man cells. J. Cell Biol. 180, 989–1003
9. Swanson, J. A., and Baer, S. C. (1995) Phagocytosis by zippers and triggers. Trends Cell Biol. 5, 89–93
10. Cheleni, F. B., Elliott, M. R., Sandilos, J. K., Walk, S. F., Kinchen, J. M., Lazarowski, E. R., Armstrong, A. J., Penuela, S., Laird, D. W., Salvesen, G. S., Isaksen, B. E., Bayliss, D. A., and Ravichandran, K. S. (2010) Pannexin 1 channels mediate "find-me" signal release and membrane permeability during apoptosis. Nature 467, 863–867
11. Changelian, P. S., Jack, R. M., Collins, L. A., and Fearon, D. T. (1985) PMA and ATP in human erythrocytes. FEBS Lett. 187, 97–100
12. Locovei, S., Doh, L., and Dahl, G. (2006) Pannexin 1 in erythrocytes. Function without a gap. Proc. Natl. Acad. Sci. U.S.A. 103, 7655–7659
13. Kurishita, Y., Kohira, T., Ojida, A., and Hamachi, I. (2012) Organelle-localizable fluorescent chemosensors for site-specific multicolor imaging of nucleoside phosphate dynamics in living cells. J. Am. Chem. Soc. 134, 18779–18789
14. Miseta, A., Bogner, P., Berényi, E., Kellermayer, M., Galambos, C., Wheatley, D. N., and Cameron, I. L. (1993) Relationship between cellular ATP, potassium, sodium and magnesium concentrations in mammalian and avian erythrocytes. Biochim. Biophys. Acta 1175, 133–139
15. Scharfstein, J., Correa, E. B., Gallo, G. R., and Nussenzweig, V. (1979) Human C4-binding protein. Association with immune complexes in vitro and in vivo. J. Clin. Invest. 63, 437–442
16. Ghiran, I. C., Zeidel, M. L., Shevkoplyas, S. S., Burns, J. M., Tsokos, G. C., and Kytaras, V. C. (2010) Systemic lupus erythematosus serum deposits C4d on red blood cells, decreases red blood cell membrane deformability, and promotes nitric oxide production. Arthritis Rheum. 63, 503–512
17. Regateiro, F. S., Cobbold, S. P., and Waldmann, H. (2013) CD73 and adenosine generation in the creation of regulatory microenvironments. Clin. Exp. Immunol. 171, 1–7
18. Bönner, F., Borg, N., Burghoff, S., and Schrader, J. (2012) Resident cardiac immune cells and expression of the ectonucleotidase enzymes CD39 and CD73 after ischemic injury. PLoS One 7, e4370
19. Ojida, A., Takashima, I., Kohira, T., Nonaka, H., and Hamachi, I. (2008) Turn-on fluorescence sensing of nucleoside polyphosphates using a xanthene-based Zn(II) complex chemosensor. J. Am. Chem. Soc. 130, 12095–12101
20. Kurishita, Y., Kohira, T., Ojida, A., and Hamachi, I. (2010) Rational design of FRET-based ratiometric chemosensors for in vitro and in cell fluorescence analyses of nucleoside polyphosphates. J. Am. Chem. Soc. 132, 13290–13292
21. Bain, J., Plater, L., Elliott, M., Shipiro, N., Hastie, M., Seldon, M. (2008) Microparticles in health and disease. Semin. Thromb. Hemost. 34, 683–691
22. Gandy, S., Haroutunian, V., DeKosky, S. T., Sano, M., and Schadt, E. (2013) CR1 and the “vanishing amyloid” hypothesis of Alzheimer’s disease. Biol. Psychiatry 73, 393–395
23. Brown, E. (1991) Phagocytosis. BioEssays 17, 109–117
24. Pilczez, F. H., Nicholson-Weller, A., and Ghiran, I. (2005) Phagocytosis of Salmonella montevideo by human neutrophils. Immune adherence increases phagocytosis, whereas the bacterial surface determines the route of intracellular processing. J. Infect. Dis. 192, 200–209
25. Fan, J., Zhang, Y., Chuang-Smith, O. N., Frank, K. L., Guenther, B. D., Kern, M., Slievel, P. M., and Herzberg, M. C. (2012) Ecto-5’-nucleotidase. A candidate virulence factor in Streptococcus sanguinis experimental endocarditis. PLoS One 7, e38059
26. MacFarlane, G. D., Sampson, D. E., Clawson, D. J., Clawson, C. C., Kelly, K. L., and Herzberg, M. C. (1994) Evidence for an ecto-ATPase on the cell wall of Streptococcus sanguis. Oral Microbiol. Immunol. 9, 180–185
27. Sridharam, M., Adderley, S. P., Bowles, E. A., Egan, T. M., Stephenson, A. H., Ellsworth, M. L., and Sprague, R. S. (2010) Pannexin 1 is the conduit for low oxygen tension-induced ATP release from human erythrocytes. Am. J. Physiol. Heart Circ. Physiol. 299, H1146–H1152
28. Sridharam, M., Bowles, E. A., Richards, J. P., Krantic, M., Davis, K. L., Dietrich, K. A., Stephenson, A. H., Ellsworth, M. L., and Sprague, R. S. (2012) Pannexin receptor-mediated ATP release from erythrocytes requires the voltage-sensitive anion channel. Am. J. Physiol. Heart Circ. Physiol. 302, H533–559
29. Abraham, E. H., Sterling, K. M., Kim, R. J., Salikova, A. Y., Huffman, H. B., Crockett, M. A., Johnston, N., Parker, H. W., Boyle, W. E., Jr., Hartov, A., Demidenko, E., Eifrid, J., Kahn, J., Grubman, S. A., Jefferson, D. M., Robson, S. C., Thakar, J. H., Lorico, A., Rappa, G., Sartorelli, A. C., and Okunieff, P. (2001) Erythrocyte membrane ATP binding cassette (ABC) proteins. MRP1 and CFTR as well as CD39 (ecto-apyrase) involved in RBC ATP transport and elevated blood plasma ATP of cystic fibrosis. Blood Cells Mol. Dis. 27, 165–180
30. Suadicani, S. O., Brosnan, C. F., and Scemes, E. (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J. Neurosci. 26, 1378–1385
31. Sprague, R. S., Ellsworth, M. L., Stephenson, A. H., Kleinhenz, M. E., and Lonigro, A. J. (1998) Deformation-induced ATP release from red blood cells requires CFTR activity. Am. J. Physiol. 275, H1726–H1732
32. Odihambo, C. O., Orteno, W., Adhiambio, C., Odera, M. M., and Stoute, J. A. (2008) Increased deposition of C3b on red cells with low CR1 and CD55 in a malaria-endemic region of western Kenya. Implications for the development of severe anemia. BMC Med. 6, 23
33. Ohl, H., Tamano, M., and Okada, N. (2008) Low CR1 (C3b receptor) level on erythrocytes is associated with poor prognosis in hemodialysis patients. Nephron Clin. Pract. 108, 23–27
34. Lambert, J. C., Heath, S., Even, G., Campion, D., Sleeper, K., Hiltunen, M., Combarros, O., Zelenika, D., Bulloid, M. J., Tavernier, B., Letenneur, L., Bettens, K., Ber, C., Pasquier, F., Fievet, N., Barberie-Gateau, P., Engelbohrs, S., De Peyn, M., Mateo, I., Franck, A., Heilsaral, S., Porcellini, E., Hanon, E., European Alzheimer’s Disease Initiative Investigators, de Pancorbo, M. M., Lendon, C., Dufouil, C., Jaillard, C., Leveillard, T., Alva-
rez, V., Bosco, P., Mancuso, M., Panza, F., Nacmias, B., Bossù, P., Piccardi, P., Annoni, G., Seripa, D., Galimberti, D., Hannequin, D., Licastro, F., Soininen, H., Ritchie, K., Blanché, H., Dartigues, J. F., Tzourio, C., Gut, I., Van Broeckhoven, C., Alpérovitch, A., Lathrop, M., and Amouyel, P. (2009) Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer’s disease. *Nat. Genet.* 41, 1094–1099

48. Corneveaux, J. J., Myers, A. J., Allen, A. N., Pruzin, J. J., Ramirez, M., Engel, A., Nalls, M. A., Chen, K., Lee, W., Chewning, K., Villa, S. E., Meechoovet, H. B., Gerber, J. D., Frost, D., Benson, H. L., O’Reilly, S., Chibnik, L. B., Shulman, J. M., Singleton, A. B., Craig, D. W., Van Keuren-Jensen, K. R., Dunckley, T., Bennett, D. A., De Jager, P. L., Heward, C., Hardy, J., Reiman, E. M., and Huentelman, M. J. (2010) Association of CR1, CLU and PICALM with Alzheimer’s disease in a cohort of clinically characterized and neuropathologically verified individuals. *Hum. Mol. Genet.* 19, 3295–3301

49. Crehan, H., Holton, P., Wray, S., Pocock, J., Guerreiro, R., and Hardy, J. (2012) Complement receptor 1 (CR1) and Alzheimer’s disease. *Immunobiology* 217, 244–250