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Reduced Fluorescence versus Forward Scatter Time-of-Flight and Increased Peak versus Integral Fluorescence Ratios Indicate Receptor Clustering in Flow Cytometry

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Clustering of surface receptors is often required to initiate signal transduction, receptor internalization, and cellular activation. To study the kinetics of clustering, we developed an economic high-throughput method using flow cytometry. The quantification of receptor clustering by flow cytometry is based on the following two observations: first, the fluorescence signal length (FL time-of-flight [ToF]) decreases relative to the forward scatter signal length (FScToF), and second, the peak FL (FL-peak) increases relative to the integral FL (FL-integral) upon clustering of FL-labeled surface receptors. Receptor macromustering can therefore be quantified using the ratios FL-ToF/FSc-ToF (method ToF) or FL-peak/FL-integral (method Peak). We have used these methods to analyze clustering of two immune receptors known to undergo different conformational and oligomeric states: the BCR and the complement receptor 3 (CR3), on murine splenocytes, purified B cells, and human neutrophils. Engagement of both the BCR and CR3, on immortalized as well as primary murine B cells and human neutrophils, respectively, resulted in decreased FL-ToF/FSc-ToF and increased FL-peak/FL-integral ratios. Manipulation of the actin-myosin cytoskeleton altered BCR clustering which could be measured using the established parameters. To confirm clustering of CR3 on neutrophils, we applied imaging flow cytometry. Because receptor engagement is as a biological process dependent on cell viability, energy metabolism, and temperature, receptor clustering can only be quantified by gating on viable cells under physiological conditions. In summary, with this novel method, receptor clustering on nonadherent cells can easily be monitored by high-throughput conventional flow cytometry.

Membrane receptors enable intercellular dialogue and translation of external signals to the cellular interior. The ultimate requirement for efficient communication and subsequent activation is interaction of ligands with their receptors. Ligand binding frequently induces lateral assembly, spatial multimerization, and further aggregation of surface receptors. The resulting macromolecular clusters serve as bidirectional signal hubs and are a dynamic hallmark of immune cell activation and intracellular signal transduction (1). Receptor clustering into macromolecular clusters is mediated by cytoskeletal proteins (2), whose dysfunction can promote immunodeficiency (for review, see Ref. 3) and can recruit multiple kinases as well as phosphatases for further effector function (4–6). Quantification of receptor clustering therefore provides crucial information about the current cellular state of activation, which is particularly, but not uniquely, important in the immune system.

The naive BCR is composed of two μ H chains (μHC) and two L chains (LCs) covalently linked by disulfide bonds, and it can exist in monomeric or oligomeric conformations (7). The association of the BCR with the signal transducing transmembrane heterodimer Igα/Igβ (8) and cross-linking of the BCR, for instance, robustly activates B cells. After BCR stimulation, the depolymerization of F-actin precedes and modulates the increase in intracellular Ca2+ concentration, which subsequently modulates the F-actin cytoskeleton in B cells (9, 10).

Complement receptor 3 (CR3) is a heterodimeric transmembrane receptor of the integrin family, which is composed of two single-pass type I membrane proteins referred to as integrin-αM (CD11b) and β2 (CD18). CR3 mediates binding to a variety of ligands, including C3bi and ICAM-1 (CD54), and its expression marks several cell types, including macrophages, monocytes, neutrophils, and endothelial cells. The ligand-mediated conformational transition of CR3 is a dynamic hallmark of immune cell activation and intracellular signal transduction (1).

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and integrin-β2 (CD18) (11, 12). Structural and functional studies suggest that ligand binding modulates the dynamic equilibrium of low-, intermediate-, and high-affinity state of integrins (13). In low affinity, the extracellular domain of CR3 is in a flexed conformation, with the ligand binding I domain close to the cell membrane with the cytoplasmic tails of the α and β subunit in close proximity. In intermediate affinity, the headpiece structure is extended, but the ligand binding I domain of the α subunit is still in closed conformation. To reach the high-affinity state, the α7 helix of the α I domain shifts downward, which is accompanied by a swing-out of the β2 domain (14). Similar to the BCR, activation and clustering of CR3 requires cytoskeletal rearrangements, resulting in bidirectional essential “outside-in” and “inside-out” signaling processes for ligand binding and modulation of receptor affinity and avidity, both of which can be maximally increased in the presence of extracellular Ca2+ (15, 16). These signals initiate adhesive and migratory properties of immune cells and thereby critical early steps of inflammation.

Currently, common techniques to visualize spatial accumulation or receptor clustering are fluorescence (FL) microscopy or imaging flow cytometry for macroclusters or total internal reflection microscopy for microclusters (1, 17, 18). Other sophisticated techniques that address close molecular interactions include the use of engineering expression constructs (7). To accelerate and simplify measurement of the kinetics of receptor macroclustering, also referred to as spatial accumulation or cap formation (capping), we developed a versatile, user-friendly, and economical real-time assay using conventional flow cytometry, which represents a widely available high-throughput technique.

### Materials and Methods

#### Mouse B cells

Mice (C57BL/6) were sacrificed according to institutional guidelines, spleens were homogenized, erythrocytes were lysed, and cells were counted and labeled with CD43 (Ly-48) MicroBeads (Miltenyi Biotec) for 20 min at 4˚C. Cells were purified by negative selection using the autoMACS Separator (Miltenyi Biotec) and counted again, and purity was checked by staining with FITC-conjugated B220 (BD Biosciences), and Cy5-conjugated anti-IgM (Southern Biotechnology Associates)–specific Abs. Purity was typically $\geq 95\%$. Splenocytes, primary murine B cells, and murine WEHI231 B cells (20) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 $\mu$g/ml penicillin-streptomycin, and 50 $\mu$M 2-ME (Life Technologies) at 37˚C and 5% CO2 (R10 medium). B cells were infected with the pCru retrovirus encoding GFP as described previously (21).

#### Isolation of human neutrophils

Healthy volunteers were recruited with the approval from the ethics committee of the Friedrich Alexander University of Erlangen-Nuremberg. Venous blood was drawn into sodium citrate–containing tubes for cell isolation. Twenty milliliters of fresh blood was mixed with an equal volume of PBS and loaded onto a density gradient following centrifugation for 20 min, room temperature, and 800 × g (Lymphoflot; Biotest). The obtained red cell pellet was washed once with HBSS, and granulocytes were enriched by sedimentation in 3% dextran-500 in HBSS for 18 min. After centrifugation,
erythrocytes were lysed with sterile dH2O for 28 s. Lysis was stopped with an equal volume of 1.8% sodium chloride solution. Granulocytes were washed, counted, and resuspended in 100 μl HBSS containing 2% BSA (HBSS–2% BSA) and kept on ice until measurement.

**Labeling of B cells for flow cytometry**

B cells were labeled with Cy5-conjugated goat anti-μ H chain (μHC) Fab fragment under nonsaturating conditions as previously described (21) and were in some experiments pretreated with cytochalasin D (Sigma-Aldrich) or Blebbistatin (Calbiochem). Cells were stimulated with indicated concentrations of goat anti-μHC F(ab')2 (Jackson ImmunoResearch Laboratories), goat anti–mouse μHC Ab coupled to FITC (10 μg/ml; Southern Biotechnology Associates), or rat anti-μHC–specific mAb b.7.6 (22).

**FL microscopy**

B cells were stained with FITC-labeled anti-μHC–specific Ab (Southern Biotechnology Associates) or Alexa 647–conjugated goat anti-μHC Fab fragment (Jackson ImmunoResearch Laboratories) under nonsaturating conditions and attached to glass slides as described previously (21). In some experiments, cells were pretreated with cytochalasin D (Sigma-Aldrich) or blebbistatin (Calbiochem). Cells were fixed, in some experiments stained with DAPI (Carl Roth), and pictures were taken with a Zeiss Axiophot 2 FL microscope equipped with a monochromatic charge-coupled device camera (Hamamatsu Photonics) coupled to Zeiss Axiovision 4.6 software.

**Flow cytometry**

Mouse. B cells or splenocytes were labeled on ice and resuspended in prewarmed (25–37˚C) R10 medium (see above) at concentrations of 4 × 10^3–4 × 10^6 cells/ml. Cells were treated as indicated and analyzed in a Beckman Coulter Galios flow cytometer with adjusted settings for 2 min before the addition of agonistic Ab. Cells were then monitored continuously for additional 18 min or as indicated. Data were analyzed with Kaluza software.

Human. Neutrophils (10^6) were resuspended in 100 μl HBSS–2% BSA and kept on ice until acquisition. Immediately prior to each measurement, 300 μl prewarmed (room temperature) Ab solution was added, containing either HBSS–2% BSA or HBSS–2% BSA supplemented with 1 mM MgCl2 and 1 mM CaCl2 or 200 nM PMA (Sigma-Aldrich). The following Abs were used: allophycocyanin-conjugated Ab to CD11b (clone CBRM1/5), FITC-conjugated Ab to CD18 (clone 6.7), mouse monoclonal allophycocyanin- or FITC-conjugated IgG1 (all Abs from eBioscience). Acquisition was carried out for a period of 1000 s in a Beckman Coulter Galios flow cytometer with adjusted settings. Data were analyzed with Kaluza software.

**Image stream**

Neutrophils (10^6) were left untreated or stimulated with 200 nM PMA for 10–20 min. Subsequently, cells were fixed, washed with HBSS–2% BSA and stained with a PE-labeled Ab to CD11b (clone ICRF44) in HBSS–2% BSA. After washing, cells were resuspended in 80 μl HBSS–2% BSA and analyzed by an Amnis ImageStream X flow cytometer. The brightfield and the CD11b-PE signals of 10,000 neutrophils were acquired by the INSPIRE software, and overlay images were generated. Data were analyzed by the IDEAS software package with gating first on single cells; second, on focused cells; and third, with criteria on CD11b-positive cells. The feature Δ Centroid XY indicates clustering of CD11b on the surface of neutrophils. The same analysis template file was used for each sample for gating and to calculate Δ Centroid XY.

**FIGURE 2.** Reproducibility of the methods ToF and Peak for BCR stimulation. Murine splenocytes (4 × 10^6/ml) that had been rested overnight were stained with anti-μHC-Fab-Alexa 647. Cells were measured by flow cytometry for 2 min. Cross-linking of Alexa 647–labeled μHC-Fab was carried by the addition of goat anti–mouse-μHC Fab fragments (10 μg/ml; see arrows) at ∼25˚C, and then, cells were analyzed for another 18 min at ∼25˚C. At various time points, the FL-ToF and FSc-ToF of viable (A) and apoptotic/necrotic B cells (B) or the FL-peak/FL-integral signals of viable (C) or apoptotic/necrotic (D) μHC-positive cells were analyzed, and ratios were calculated. Data represent the mean ± SD of median values of four stimulations pooled from two experiments. Data were analyzed with a two-tailed nonpaired Student t test. The p values were considered significant when marked with *, **, and *** as p < 0.05, 0.01, and 0.002, respectively.
Statistics

Data were analyzed with a two-tailed nonpaired Student t test using Microsoft Excel and GraphPad Prism. The p values were considered significant when marked with *, ** and *** as p < 0.05, 0.01, and 0.002, respectively.

Results

In flow cytometry, the time-of-flight (ToF) signal is defined by the width and length of a pulse when a cell passes the laser beam. From fluorescently labeled cells flow cytometry reads two independent FL signals: 1) the peak (height; FL-peak) and 2) the integrated area/ integral (width; FL-integral) signal. These parameters provide additional information and extend the data provided by just recording FL intensity. Our method is based on the hypothesis that the FL-ToF versus the forward scatter (FSc)-ToF signal will decrease, and simultaneously, FL-peak will increase relative to FL-integral over time when receptor clustering occurs on the surfaces of cells (Fig. 1A). To test this hypothesis, we examined two types of immune receptors, namely the BCR and CR3, of nonadherent murine B cells and human neutrophils, respectively.

Analysis of BCR clustering by methods ToF and Peak

To study BCR clustering, primary splenic B cells were isolated by negative selection and either attached on a glass slide for FL microscopy or kept in solution for flow cytometric analysis. Attached B cells were stained on ice with FITC-labeled anti-μHC-specific Ab and incubated then at 37°C for the time points indicated and fixed (Fig. 1B). Soluble cells were stained with a Cy5-labeled monovalent goat anti-mouse μHC Fab fragment at a nonsaturating concentration (21). This interaction is known not to induce clustering as revealed by the absence of the increase of intracellular Ca²⁺ following monomeric BCR binding (our unpublished data). Clustering was then induced by the addition of polyclonal goat anti-μHC F(ab')2 fragments. At consecutive time points, the anti-BCR Cy5 (FL6)-ToF/FSc-ToF ratio and the FL6-peak/FL6-integral ratio were calculated from viable primary B cells defined by FSc/SSc gating (Fig. 1C, 1D). Full receptor activation or complete BCR clustering was observed after 40–50 min. Evaluation of the experimental data revealed that the FL6-ToF/FSc-ToF ratio and the FL6-peak/FL6-integral ratios decrease or increase, respectively, after receptor cross-linking (Fig. 1C, 1D). In accordance with our hypothesis, the FSc-ToF signal remained rather constant because the overall shape of the cells does not change during receptor activation. In contrast, the FL-ToF signal decreased upon BCR capping.

Gating strategy to measure BCR clustering

Repeated experiments with continuous monitoring of labeled splenocytes over 20 min revealed statistically significant decreases or increases in ToF and peak/integral ratios, respectively, upon BCR activation (Fig. 2A, 2C). A minimal density of 4 x 10⁴ cells/ml is sufficient to obtain fully reproducible results, and data were identical.
in ranges of $4 \times 10^4$–$10^6$ cells/ml (Supplemental Fig. 1). Results obtained using low to medium flow speed (50–600 event/s) were also comparable (data not shown). Pilot experiments revealed that the methods ToF and Peak worked hardly with apoptotic/necrotic cells defined by FSc/SSc gating (data not shown). To validate this hypothesis, we used aged (24 h) splenocytes and defined FSc and SSc of viable, apoptotic, and necrotic cells by Annexin V/propidium iodide (PI) staining. Back-gating on viable (Annexin V$^-$/PI$^-$; gate A), apoptotic (Annexin V$^+$/PI$^-$; gate D), and secondary necrotic (Annexin V$^+$/PI$^+$; gate E) cells was performed for FSc/SSc (Supplemental Fig. 2). These gates were then applied to distinguish viable and apoptotic/necrotic cells (for an example of the full gating strategy see Fig. 3). In contrast to viable cells, B cells in the apoptotic/necrotic cell gate (Supplemental Fig. 2) did neither show decreases in FL-ToF/FSc-ToF ratios nor increases in FL-peak/FL-integral ratios (Fig. 2B, 2D).

We further reproduced these results using bivalent monoclonal μHC-specific Ab b.7.6. on WEHI 231 B cells (22), with the polyclonal μHC-specific F(ab')$_2$ fragment expectedly being more effective (data not shown). Hence, both the polyclonal and the monoclonal agonistic anti-BCR Abs can be used to monitor BCR clustering by flow cytometry on primary B cells and the transformed B cell line WEHI231. An additional parameter that could influence the FL readout is clusters of cells themselves. To address this question, we compared single versus aggregated cells. Interestingly, aggregated cells (see gate in Supplemental Fig. 2C) exhibited BCR clustering as measured by methods ToF and Peak, but it was less pronounced compared with viable cells (Supplemental Fig. 3B). We suspect that this effect is most likely due to limited accessibility of the BCRs for the agonistic Abs when cells have formed aggregates. This notion suggested moreover that there is a dose dependency in Ab receptor clustering that should be measurable. As hypothesized, we did observe an acceleration of BCR clustering using increasing concentrations of the anti-μHC F(ab')$_2$ fragment. This effect was again less potent on aggregated cells (Supplemental Fig. 3A, 3B). Given that receptor clustering is an energy-dependent, enzymatic process and not unspecific protein clustering elicited by high concentrations of agonistic Abs, we hypothesized that receptor clustering would be markedly reduced in the cold, either because of low enzymatic activity or decreased lateral mobility in the plasma membrane. To test this hypothesis, we incubated WEHI cells with anti-μHC Fab-Cy5 at room temperature. After addition of anti-μHC F(ab')$_2$ fragment to induce receptor clustering, samples were

FIGURE 4. Clustering of CR3 on the surface of neutrophils analyzed by methods calculating ToF, peak, and integral signals. Neutrophils were isolated from human blood of four different donors, labeled with allophycocyanin-labeled isotype matched control Ab (A) or allophycocyanin-labeled isotype matched control anti-CD11b–specific Abs (B). Cells were stimulated with PMA and CD11b-allophycocyanin (FL6)-ToF/FSc-ToF or FL6-peak/FL6-integral were recorded for 1000 s. Uniform gates set every 100 s were applied to calculate ratios of FL6- versus FSc-ToF signals as well as that of FL6-peak versus FL6-integral signals. Md, median.
incubated at 26°C or 4°C for indicated time points. BCR clustering was prominent in single WEHI231 B cells of the viable cell population only after incubation at room temperature, whereas no clustering was observed on cells incubated at 4°C (Supplemental Fig. 3C–F).

**Receptor clustering measured by methods ToF and Peak occurs on the cell surface**

To assess whether our method describes clustering of receptors on the cell surface, we attempted to quench surface FL of FITC-marked Abs by trypan blue (TB) (23). To demonstrate that TB quenches only surface FL, we infected primary splenic B cells with a retrovirus encoding GFP (21). Infected cells were then stained with FITC-marked anti-BCR mHC Ab (blue histograms). Compared with infected cells expressing GFP (red histogram), the peak of intracellular FL caused by GFP expression is maintained. We conclude that TB expressing GFP (red histogram), the peak of intracellular FL specifically quenches FITC FL elicited by FITC-labeled anti-BCR Abs by trypan blue (TB) (23). To demonstrate that TB quenches only surface FL, we infected primary splenic B cells with a retrovirus encoding GFP (21). Infected cells were then stained with FITC-marked anti-BCR mHC Ab (0.5 μg/ml), accompanied by a low dose of PE-marked anti-BCR κ LC Ab (0.5 μg/ml). FL1 (FITC-marked anti-μHC Ab) as well as FL2 (PE-marked anti-BCR κ LC Ab; data not shown) ToF, peak, and intensities were recorded (Fig. 3D, 3E), and ratios were calculated (Fig. 3F, 3G). Note, the FL appears because of the addition of FL-marked agonistic Abs only after 2 min (Fig. 3D, 3E). In the presence of TB, the PE (FL2)-ToF ratio is completely unchanged, whereas there is a low but significant decrease of the FL2-peak ratio (Fig. 3F) (1.2 ± 0.2%). This occurs already at very early time points, presumably because of a direct or indirect mild quenching effect of TB on PE. Notably, both curves (± TB) exhibit a similar constant slope. However, in the presence of TB, the decline of the FITC (FL1)-ToF ratio is significantly reduced when receptor clustering starts (after 6–8 min; compare Fig. 2) as well as after 20 min. Concomitantly, the FL1-peak ratio is significantly reduced (Fig. 3G). We therefore conclude that TB does not interfere with the process of BCR clustering per se (as shown by unchanged PE anti-κ LC (FL2)-ToF and unchanged PE anti-κ LC (FL2)-peak ratios elicited by clustering of PE anti-κ LC-labeled BCRs), but quenches surface FL. Hence, the methods ToF and Peak are useful to monitor surface receptor clustering. However, we were only able to block clustering to ~40%. Thus, in this case, we also measure clustering of internalized BCRs. To corroborate this result, we reasoned that alterations in trafficking and internalization should alter the accumulation of the BCR on the cell surface, which in turn should be measurable by the methods ToF and Peak. We thus inhibited de novo actin polymerization with cytochalasin D, which perturbs BCR internalization and capping (24), and Myosin IIa with Blebbistatin, which prevents internalization of multimeric high affine BCRs extracting Ag from APCs (25). Indeed, whereas cytochalasin D appeared to reduce visible surface BCR capping, Blebbistatin appeared to induce distinct BCR surface clusters (Supplemental Fig. 4A–D). In accordance, cytochalasin D slowed the decrease in BCR/FSc-ToF ratios and the increase in the BCR peak ratio, and Blebbistatin enhanced the decrease in BCR/FSc-ToF ratios and the increase in the BCR peak ratio (Supplemental Fig. 4E, 4F). Hence, manipulation of the actin-myoinskeleton altered BCR clustering, which could be measured by flow cytometry with methods ToF and Peak using the established parameters.

**Analysis of CR3 clustering by methods ToF and Peak**

To analyze clustering of CR3 (CD11b/CD18), freshly isolated neutrophils were either kept on ice or activated with PMA or bivalent cations. Clustering of CR3 is a rather rapid process, which starts almost immediately after neutrophil activation (see below). Therefore, the FSc- and CD11b-alkophycocyanin (FL6)-ToF as well as the CD11b-alkophycocyanin (FL6)-peak and CD11b-alkophycocyanin (FL6)-integral signals were continuously recorded for a period of 1000 s (compare Figs. 2 and 3), starting immediately after activation and addition of fluorescently labeled Abs. Consecutive time gates were used to determine the kinetics of CD11b-alkophycocyanin-ToF/FSc-ToF and CD11b-alkophycocyanin-peak/CD11b-alkophycocyanin-integral ratios (Fig. 4A, 4B). In full accordance with the results obtained for BCR clustering, these measurements revealed a progressive decrease of the FL-ToF/FSc-ToF in the presence of Ca2+ and PMA. This effect was only visible when CD11b (the α-chain of CR3) was stained with specific Abs that recognizes the activated epitope in CD11b but not with isotype control Abs (Fig. 5). To exclude the possibility that the CD11b-alkophycocyanin/FSc-ToF ratio and the CD11b-alkophycocyanin-peak/CD11b-alkophycocyanin-integral ratios merely changed because of morphological alterations known to occur after neutrophil activation, we plotted the single parameters...
(CD11b-allophycocyanin-ToF, FSC-ToF, CD11b-allophycocyanin–integral, and CD11b-allophycocyanin–peak) separately (Supplemental Fig. 5). These plots clearly show that the changes of the ratios CD11b-allophycocyanin–ToF/FSC-ToF and CD11b-allophycocyanin–peak/CD11b-allophycocyanin–integral are not entirely due to activation-induced morphological alterations influencing the FSC-ToF values. Interestingly, basal anti-CD11b–induced clustering of CR3 was observed on cells even under nonstimulating buffer conditions, which is most likely induced by the divalent nature of the anti-CD11b Ab and only partially because of some spontaneous activation of CD11b even on cooled and resting neutrophils. Because this Ab recognizes specifically the activated state of CD11b (26, 27), it will lock spontaneously activated CR3 in its active configuration.

**Confirmation of CR3 clustering by imaging flow cytometry**

To confirm and visualize CR3 clustering on neutrophils, we used imaging flow cytometry. Human neutrophils were isolated as described above and incubated in the presence or absence of PMA for indicated time points. After fixation, cells were stained with PE-labeled Abs raised against CD11b, and data of each sample were acquired for 10,000 cells. Using the IDEAS software package, gating was performed on single, focused, and CD11b-positive cells as is exemplarily depicted for the time point 20 min after incubation of unstimulated in comparison with PMA-activated cells (Fig. 6A). Images of surface stained human neutrophils for CR3 including brightfield, fluorescent, and overlay images are displayed for unstimulated and PMA-activated cells at time points 10 and 20 min (Fig. 6B). Ten minutes after activation with PMA, an increase in surface expression of CR3 was observed. This rapid upregulation of CR3 surface expression on granulocytes results most probably from translocation of CR3 from an intracellular pool, which subsequently starts to focally accumulate in the cellular membrane. With the help of the Δ Centroid XY feature, clustering of CR3 was quantified (Fig. 6C). Briefly, this feature compares the distance of the centroids of the fluorescent with equivalent brightfield image. If the FL starts to spatially accumulate, the value for Δ Centroid XY increases compared with uniform, global FL distribution. To further confirm our results, we used an Ab to CD18 to study the capping behavior of the second chain of the heterodimeric CR3. In contrast to the CD11b-specific Ab that exclusively binds to the activated CD11b chain, the FITC-conjugated Ab to CD18 recognizes CD18 regardless of its conformational state. Similar to CD11b, FSC-ToF and FL1 (CD18-FITC)-ToF as well as the FL1-peak and FL1-integral signals were continuously recorded for a period of 1000 s. During the first 240 s of the analysis, the FL1/FSC-ToF ratio increased as an effect of Ab binding, which does not affect FSC-ToF. After the initial phase of Ab binding, the PMA-induced clustering of CD18 could as well be monitored as a decrease of the FL1/FSC-ToF ratio. The effect of Ab binding cannot be monitored by the ratio of peak versus integral FL, because within the first 4 min, during Ab binding, both peak as well as integral FL values increase in parallel (Supplemental Fig. 6).

**FIGURE 6.** Analysis of CR3 clustering by ImageStream. Human neutrophils were left untreated or treated with PMA for 10 and 20 min. After fixation cells were stained with PE-labeled Ab to CD11b and analyzed by an ImageStream cytometer. Using the IDEAS software package, (A) doublets or aggregated cells were excluded by gating on single cells. Afterward, focused and CD11b-positive cells were selected. (B) Images of single cells of the brightfield and fluorescent channel were acquired, and overlays were generated. Original magnification ×40. (C) The Δ Centroid XY feature was calculated after gating on single, focused, and CD11b-positive cells.
Discussion

We describe in this study a reliable high-throughput technique to monitor and quantify receptor macroclustering (capping) on viable cells by conventional flow cytometry. The principle behind this method is that the FL-ToF versus FSc-ToF ratio and the FL-peak versus FL-integral ratio decrease or increase, respectively, when receptors cluster. Importantly, we used six different polynomial as well as monoclonal, labeled, or unlabeled Abs, murine and human cells, primary and transformed cells, B cells, and neutrophils; hence, this method is broadly applicable. We would like to mention that the ratios we calculated are relative parameters and that the same process (BCR clustering) can elicit different changes in the ratios depending on the type of Ab and fluorochrome. In addition to assessing receptor cluster formation, the method described in this paper may also allow monitoring of cellular polarization, given that the latter phenomenon results in the redistribution of bright fluorescent matter, such as GFP fusion proteins, or fluorescently labeled organelles.

Using both primary splenic B cells as well as immortalized B cells (WEHI231) we could quantify kinetics of BCR clustering and demonstrate that our method follows the basic principles of receptor clustering, because it is dependent on cellular viability and temperature. Programmed cell death or apoptosis is known to be an ATP-dependent process (28, 29). Cells undergoing apoptosis are still equipped with limited levels of intracellular ATP, which could account for the residual receptor clustering. On the other hand, in contrast to viable cells, apoptotic/necrotic cells were found to be incapable of BCR capping. This is probably due to cleavage of cytoskeletal elements occurring early during the process of programmed cell death (30). In conclusion, careful gating on viable cells is essential to perform the methods described in this paper.

It is highlighted by the influence of temperature that capping is an active process. B cells incubated on ice during BCR stimulation were not capable of achieving full receptor activation when compared with cells incubated at room temperature. This is likely due to decreased membrane fluidity. These crucial technical issues will have direct impact on reproducibility, making strict gating and temperature controls mandatory.

Concerning experiments with neutrophils to investigate CR3 capping kinetics, we found that the CD11b-specific Ab was able to induce capping by itself even without CR3 activating stimuli, such as divalent cations or PMA. This effect is primarily due to the nature of the divalent CD11b-specific Ab, which is prone to induce clustering of the CR3 on the surface of neutrophils by cross-linking adjacent CR3 molecules with its two Fab entities. Second, this Ab recognizes specifically the activated state of CD11b (26, 27) and therefore will lock spontaneously activated CR3 in its active configuration. From our results, we deduce that both PMA and Ca2+ accelerate basal Ab induced CR3 clustering and that CR3 was not activated on neutrophils before measurement. For the latter issue, further evidence is provided by the usage of a pan-CD18 Ab. In contrast to the anti-CD11b Ab, which specifically recognizes an activated epitope in CD11b exposed only after conformational changes of CR3, the CD18 Ab recognizes ligand molecules already expressed in heterodimeric complexes not only with CD11b but also with CD11a and CD11c on the cellular surface. When analyzing β2-integrin capping by pan anti-CD18 Ab starting from the first to the sixth minute, an increase by method ToF was observed, which is due to Ab binding to surface expressed CD18. Only then after saturated binding of the Ab does a decrease in FL-ToF/FSc-ToF ratio indicate capping of β2-integrin. In contrast, this change of FL intensity induced by Ab binding is not observed by method Peak because the change in FL is contained in both FL-peak and FL-integral and is canceled down by generating the ratio of these parameters in contrast to method ToF, where only one parameter measures altered FL.

We established that active receptor aggregation on live and nonfixed cells can be quantified in real time by conventional flow cytometry and want to note that chemical fixation with paraformaldehyde might interfere with the calculation of FL-ToF/FSc-ToF ratios by flow cytometry, albeit capping can be observed by conventional immunofluorescence microscopy. Surface receptor clustering or capping of internalized receptors are simultaneous processes (2). There are two pools of BCRs that respond differentially to BCR stimulation: one that becomes phosphorylated and not internalized and serves as signaling platform and one that becomes internalized. Both events appear to be mutually exclusive (31). Therefore, it is likely that our method also describes BCR clustering on the cell surface. To address this issue, we performed experiments where we quenched surface FL, showing that we analyze clustering of surface BCR, but likely also internalized BCRs. Still, we recommend that internalization dynamics should be performed for each individual cell type and receptor. In the case of primary B cells in our hands, 60–70% of BCR surface expression is maintained during the first 20 min of BCR stimulation (data not shown). In summary, we have developed a novel technique to successfully quantify kinetics of immune receptor capping using the BCR as target on primary human B cells and CR3 on neutrophils, also in heterogeneous cell populations such as splenocytes. Thus, this high-throughput method would allow the screening of peripheral blood cells from individuals with immunological dysfunction related to defects in immune receptor clustering.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental FIGURE 1. Titration of cell numbers.
Murine spleen cells (4 x 10⁵ - 4 x 10³ /ml) that had been rested overnight were stained with anti-µHCFab-Cy5. Cells were measured in the flow cytometer for 2 min. Cross-linking of Cy5-labeled anti-µHCFab was carried by the addition of goat anti-µHC F(ab)₂ fragments (10μg/ml; see arrows) at ~25°C and then cells were analyzed for another 18 min at ~25°C. At various time-points the fluorescence time-of-flight (FL-ToF) and forward scatter time-of-flight (FSc-ToF) signals were calculated.
Supplemental FIGURE 2. Determination of the viable and apoptotic/necrotic cell gates of primary splenocytes by Annexin V / propidium iodide staining.

Murine spleen cells that had been rested overnight were stained with Annexin V-FITC and propidium iodide (PI). Single cells (A) were classified as (B) viable cells (Annexin V negative, PI negative; gate A), apoptotic cells (Annexin V positive, PI negative; gate D) and necrotic cells (Annexin V positive, PI positive; gate E). These gates were then used to define the FSCc/SSc parameters of the different populations (C). (D) reveals the final gates used to distinguish cell populations that are mostly viable (black gate) or mostly apoptotic or necrotic (blue gate).
Supplemental FIGURE 3 Dependence of BCR clustering on antibody concentration and temperature. WEHI231 B cells were stained with anti-μHCFab-Cy5 (FL6) on ice for 10 min. To induce clustering of the BCR the cells were subsequently incubated with various concentrations of goat anti-μHCF(ab)_2 antibodies ranging from 0.1–10 µg/ml for the time-points indicated. The ratio of FL6/FSc-ToF was calculated for (A) viable single cells and (B) aggregated cells. After staining of viable WEHI231 B cells with anti-μHCFab-Cy5 at room temperature, cells were incubated with goat anti-μHCF(ab)_2 (10µg/ml) either at (C) 4°C or at (D) 26°C. Comparison of BCR clustering at 4 and 26°C by method (E) ToF and (F) Peak.
Murine splenocytes (4 x 10⁶/ml) that had been rested overnight were incubated with Dimethylsulfoxide (DMSO), Cytochalasin D (10 μM) or Blebbistatin (100 μM) for 30 min at 37°C and attached to glass slides (A-D) or left in solution (E, F). Cells were then stained with anti-µHC F(ab)²-Alexa647 and left unstimulated or were stimulated with goat anti-mouse-µHC F(ab)² fragments (10 μg/ml) for 15 min, fixed, stained with DAPI and analyzed by fluorescence microscopy (A-D). In (E and F), cells were measured by flow cytometry for 2 min. Cross-linking of Alexa647-labeled µHC was carried by the addition of goat anti-mouse-µHC F(ab)² fragments (10 μg/ml; see arrows) at ~25°C and then cells were analyzed for another 18 min at ~25°C. At various time-points the fluorescence time-of-flight (FL-ToF) and forward scatter time-of-flight (FSc-ToF) of viable µHC-positive cells, or the fluorescence peak and integral signals of viable µHC-positive cells were analyzed and ratios were calculated. Data represent the mean ± SD of median (Md) values of 4 stimulations pooled from 2 experiments. Data from Cytochalasin D and Blebbistatin-treated samples were compared with a two-tailed non-paired student's t-test. P-Values were considered significant when marked with *, ** and *** as p < 0.05, 0.01 and 0.002, respectively. Data from DMSO vs. Cytochalasin D, or from DMSO vs. Blebbistatin treated samples also differed significantly at some time points (DMSO vs. Cytochalasin D, ToF ratio, 16 min, p = 0.03; DMSO vs. Blebbistatin, ToF ratio, 12 min, p = 0.02; DMSO vs. Cytochalasin D, Peak ratio, 12/14/16 min, p = 0.03/0.01/0.02; DMSO vs. Blebbistatin, Peak ratio, 8/12/14/16/18 min, p = 0.04/0.002/0.006/0.02/0.01).
Supplemental Figure 5. FSc ToF, CD11b-APC ToF, CD11b-APC Peak and CD11b-APC Integral

Neutrophils from four healthy volunteers were isolated. Cells were resuspended in buffer containing Ca²⁺ or PMA in the presence of the APC-conjugated antibody to CD11b. APC-labelled IgG1k served as control. (A) FSc and CD11b-APC ToF as well as (B) CD11b-APC ToF peak and integral were recorded over 1000 s and are displayed as Mean (Mn) or Median (Md) signals ± SD.
Supplemental FIGURE 6. CD18-FITC/FSc ToF and CD18-FITC Peak/Integral.
Neutrophils from four healthy volunteers were isolated. Cells were resuspended in buffer containing Ca²⁺ or PMA in the presence of freshly added FITC-labelled monoclonal antibody to CD18 or a FITC-conjugated isotype matched control antibody. (A) CD18-FITC and FSc ToF were recorded and are displayed as ratios of the Mean (Mn) or Median (Md) ± SD. (B) CD18-FITC peak and integral were recorded and are displayed as ratios of the Mean (Mn) or Median (Md) ± SD.