Integrins Influence the Size and Dynamics of Signaling Microclusters in a Pyk2-dependent Manner*‡

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Background: The mechanism by which integrin signaling regulates lymphocyte cytolysis activity is poorly understood.

Results: The extent of integrin ligation influences the size and dynamics of signaling microclusters.

Conclusion: Integrons regulate the magnitude and duration of proximal signaling that affect lymphocyte degranulation kinetics and the rate of target cell lysis.

Significance: Integrin engagement ensures more rapid cytolitic granule release and faster target cell destruction.

Integrin engagement on lymphocytes initiates “outside-in” signaling that is required for cytoskeleton remodeling and the formation of the synaptic interface. However, the mechanism by which the “outside-in” signal contributes to receptor-mediated intracellular signaling that regulates the kinetics of granule delivery and efficiency of cytolysis activity is not well understood. We have found that variations in ICAM-1 expression on tumor cells influence killing kinetics of these cells by CD16.NK-92 cytolytic effectors suggesting that changes in integrin ligation on the effector cells regulate the kinetics of cytolysis activity by the effector cells. To understand how variations of the integrin receptor ligation may alter cytolysis activity of CD16.NK-92 cells, we analyzed molecular events at the contact area of these cells exposed to planar lipid bilayers that display integrin ligands at different densities and activating CD16-specific antibodies. Changes in the extent of integrin ligation on CD16.NK-92 cells at the cell/bilayer interface revealed that the integrin signal influences the size and the dynamics of activating receptor microclusters in a Pyk2-dependent manner. Integrin-mediated changes of the intracellular signaling significantly affected the kinetics of degranulation of CD16.NK-92 cells providing evidence that integrins regulate the rate of target cell destruction in antibody-dependent cell cytolysis (ADCC).

A great deal of evidence demonstrates that adhesion molecules function as a molecular glue to mediate contact between immune cells and target cells. However, they also mediate “outside-in” signaling regulating functions of immune cells. Particularly, ligation of lymphocyte function-associated antigen-1 (LFA-1 saturation or αβ2 integrin) by intercellular adhesion molecule 1 (ICAM-1) is necessary to induce cytoskeleton remodeling and formation of a well-organized immune cell/target cell interface (1–3). The formation of such interface is especially important for cytotoxic lymphocytes to exercise efficient cytolysis activity against target cells via delivery of cytolytic granules (4–6). However, how specific engagement of the integrin receptors regulates kinetics of granule delivery and efficiency of cytolysis activity is not well understood.

To unravel the mechanism by which integrins influence activating receptor-mediated signaling in cytotoxic lymphocytes and facilitates cytolytic granule delivery to target cells, we took advantage of well-characterized CD16.NK-92 cytolytic effectors that express several integrin receptors, whose binding to ICAM-1 and/or other ligands influence cytolysis activity of these cells (7). We exploited tumor target cells with varying levels of ICAM-1 expression and have found that changes in the ICAM-1 level significantly influence the kinetics of CD16.NK-92 cell degranulation and the rate of target cell lysis in ADCC. This suggested that the difference in extent of β2-integrin ligation regulates the kinetics of the target cell killing by CD16.NK-92 effectors. Exposure of CD16.NK-92 cells to planar lipid bilayers that contain a ligand for CD16 receptor and ICAM-1 ligand at different densities enabled us demonstrate that integrin receptors control the size and dynamics of signaling microclusters as well as the duration of the activating receptor-mediated proximal signaling, thereby ensuring more rapid granule release and faster target cell destruction.

The abbreviations used are: LFA-1, lymphocyte function-associated antigen-1; ICAM-1, intercellular adhesion molecule 1; ADCC, antibody-dependent cellular cytotoxicity; NINTA-DG5S, 1,2-di-(9Z-octadecenoyl)-sn-glycerol-3-(5-amino-1-carboxypentyl)iminodiacetic acid(succinyl) [nickel salt]; DOPC, 1,2-dioleyl-sn-glycerol-3-phosphocholine; PFA, paraformaldehyde; IRM, interference reflection microscopy; TIFRMI, total internal reflection fluorescent microscopy; LAMP-1, lysosomal-associated membrane protein 1; IFN-γ, interferon γ; SLP-76, SH2 domain containing leukocyte protein of 76 kDa; Zap-70, Zeta-chain-associated protein kinase 70; HS1, hematopoietic lineage cell-specific protein 1; ILK, integrin-linked kinase; Pyk2, protein tyrosine kinase 2; Src, Src family kinases; ADAP, adhesion and degranulation-promoting adapter protein; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocytes; NK cells, natural killer cells.

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Integrins Control Activating Microcluster Size and Dynamics

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Chemicals—The human NK-92 cell line transduced to express FcγRIIIa receptor (176V allele of CD16 that binds to the antibody Fc fragment with high affinity) has been described in detail elsewhere (7, 8). The human breast cancer cell line SKBR3 was supplied by ATCC. In some experiments, SKBR3 cells were treated with 100 units/ml IFN-γ for 48 h prior to the assay.

Herceptin, humanized antibody against human HER2/neu used in this study have been previously described (7, 9). Hybridomas producing mouse blocking antibody TS1/18 specific for human CD18 (β2 integrin chain), mouse non-blocking TS2/4 antibody against human CD11a (αL integrin chain), mouse blocking antibody R6.5 specific for human ICAM-1, and mouse 3G8 antibody specific for human CD16 were purchased from AdipoGen. The human breast cancer cell line SKBR3 breast cancer cells were plated in 96-well round bottom plate. In some samples, mouse TS1/18 washed, and labeled with 51Cr. 5.0 μCi 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids and 0.001 mol% biotinyl-CAP lipids (Avanti Polar Lipids) at 4 mol% were used to prepare bilayers. The final concentrations of biotin-CAP and NiNTA-DOGS in the bilayers were 0.01 mol% and 17.5 mol%, respectively.

Streptavidin (2 μg/ml) and monobiotinylated anti-CD16 (3G8) monoclonal antibody labeled with Alexa Fluor 488 (2 μg/ml) were reacted sequentially with the biotinylated bilayers to produce the antibody density of 50 molecules/μm², unless indicated otherwise. In some experiments the density of anti-CD16 on bilayer was varied from 5 to 500 molecules/μm² by changing the concentration of biotinyl-CAP lipids in the bilayer from 0.001 mol% to 0.3 mol%, respectively. Where indicated, Cy5-ICAM-1-His₆ molecules were incorporated into the bilayers at the density 300 molecules/μm², a physiological density of this molecule on the surface of APC (4, 10, 11, 13). Densities of Cy5-ICAM-1 and anti-CD16 antibody on the bilayers were determined as described elsewhere (4). In one set of experiments, the density of Cy5-ICAM-1 was varied while keeping the density of the anti-CD16 unchanged.

Imaging—CD16.NK-92 cells were injected into pre-warmed to 37 °C flow cell. In some experiments the cells were treated with Pyk2 inhibitor. The inhibitor was present in extracellular medium at concentrations ranging from 1 to 10 μM. IRM images of the cells that formed tight contact with the bilayers were obtained using a confocal fluorescence microscope (Zeiss LSM510 Meta confocal Laser Scanning Microscope). Images of CD16 clusters bound to fluorescent anti-CD16 antibodies were acquired with a TIRF microscope (Andor Revolution XD system equipped with Nikon TIRF-E illuminator and 100X/1.49 NA objective, Yokogawa CSU-X1 spinning disk confocal, and Andor iXon X3 EM-CCD camera). To image activated Src kinases, CD16.NK-92 cells attached to the bilayers were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained for phospho-Src (pSsrc) kinases with anti-Tyr416 rabbit polyclonal antibody following by Alexa Fluor 568-labeled secondary goat anti-rabbit antibody. Fixed and stained cells were then imaged by TIRF microscopy.

To ensure accurate comparison, the measurements were performed in a series of parallel experiments on the same day using the same reagents and freshly prepared planar bilayers. The images were acquired using the identical microscope.
Integrins Control Activating Microcluster Size and Dynamics

settings. The same batches of the cells were utilized in all experiments.

Events Timing and Degranulation Kinetics at CD16.NK92/Bilayers Interface—The timing of ICAM-1 accumulation, signaling microcluster formation and the onset of granule release have been measured in individual CD16.NK-92 cells interacting with the bilayers. Mean timing for each of the events was calculated by averaging the observed timings for more than 20 cells. For measuring the degranulation kinetics, percentage of degranulating cells at various time points in several imaging fields was determined.

Evaluation of CD16 Microcluster Size—To characterize moving microclusters we analyzed multiple images in each field during the period of 90 min. The images were taken every 10 or 30 s. We defined microcluster as a coherent group of bright pixels. Moving microclusters were defined as those observed on several consequent imaging planes with little or no significant change in shape, and the position of its center at least once over the period of observation is located outside of the original position.

For analysis of the size of individual CD16 microclusters, we examined the series of images taken every 30 s. To single out individual microclusters, intensity profiles of each of the moving microclusters were examined. Microclusters that demonstrated unimodal intensity distribution (see supplemental Information) were considered as individual microclusters. We then calculated average background fluorescent level for each of the analyzed cells. Each coherent pixel group with all pixels’ intensities equal or exceeding 1.3 times the fluorescent background level was considered having a positive signal and included in the analysis. Regions were drawn around each selected individual moving microcluster at image planes corresponding to 5, 10, or 30 min after the initial cell-bilayer contact. MetaMorph Region Measurements function was utilized to measure sizes and intensities of these microclusters. Average values for each of these parameters were calculated and expressed in μm² or arbitrary units, respectively.

Analysis of CD16 Microclusters Movement—Parameters of microcluster movement were studied using the series of sequential images collected every 10 s. For each cell we determined the time difference between initiation and termination of centripetal microcluster movement. This time difference was termed mobility. We then examined the two planes corresponding to the beginning and the end of the microcluster movement and measured the distance that each microcluster traveled. The distance was termed microcluster displacement. We calculated average mobility and displacements for at least 20 cells at each experimental condition.

Quantification of Activated Src Kinases Microclusters—The average size of activated Src kinases (pSrc) clusters and average integrated fluorescence intensities of the microclusters in each cell were calculated and expressed in μm² and arbitrary units, respectively. We then evaluated total amount of the fluorescent molecules recruited to the cell/bilayer interface) and the area were determined for these regions using the Region measurement function of MetaMorph Software suite. The integrated fluorescence of pSrc molecules and pSrc density at the contact area of each cell were calculated, and average values are expressed in arbitrary units and arbitrary units per μm², respectively.

RESULTS

Ligation of β₂ Integrins Accelerates the Kinetics of Target Cell Killing by CD16.NK-92 Cells—We examined the kinetics of Herceptin-induced killing of SKBR3 breast cancer cells mediated by CD16.NK-92 effectors. SKBR3 cells have a low level of ICAM-1 that serves as a ligand for β₂ integrins on CD16.NK-92 cells (7). We have shown that treatment of SKBR3 with IFN-γ for 48 h results in significant up-regulation of cell-surface ICAM-1 molecules but not Her2/neu receptor (7). We compared the extent of Herceptin-induced specific lysis of IFN-γ treated or untreated SKBR3 cells by CD16.NK-92 effectors at various time-points. The killing rate was determined as the initial slope of the plots (Fig. 1A). The up-regulation of ICAM-1 resulted in ~2-fold increase of the rate of target cell killing by CD16.NK-92 (Fig. 1B). Because IFN-γ treatment did not change Her2/neu receptor expression on SKBR3 cells, the observed increase in ADCC rate cannot be attributed to an elevated level of the epitope recognizable by the antibody. However, IFN-γ treatment could also up-regulate expression of HLA-E, which is a ligand for CD94/NKG2A receptor on NK-92 cells, and modulates expression of other activating receptor ligands (16). To test the role of ICAM-1 in the observed increase of the ADCC, we utilized TS1/18 antibody that blocks specific interactions of all β₂ integrins with their ligands (17), TS2/4 antibody that also bind to the integrins but are not inhibitory (18), and R6.5 blocking antibody against ICAM-1 (19). The presence of TS1/18 antibodies in the extracellular medium completely abrogated the lysis of IFN-γ-treated SKBR3 cells (Fig. 1A), whereas TS2/4 antibodies had no effect on the rate of the killing, and anti-ICAM-1 antibody significantly reduced the kinetics and magnitude of target cell lysis (supplemental Fig. S1). The killing of IFN-γ-treated SKBR3 cells was also diminished after lowering expression of ICAM-1 with ICAM-1 siRNA (supplemental Fig. S2). These data provide evidence that increasing the extent of β₂ integrin ligation by ICAM-1 results in significant acceleration of the killing kinetics of tumor cell destruction by CD16.NK-92 cytolytic effectors in ADCC responses.

The Level of ICAM-1 on Target Cells Influences Conjugate Formation and the Kinetics of Cytolytic Granule Release by CD16.NK-92 Cells—Variations in the ICAM-1 level on target cells could affect the killing kinetics in two principle ways. First, a higher extent of β₂ integrin engagement by ICAM-1 could merely enhance effector/target cell conjugate formation resulting in more efficient killing. Second, increasing the β₂ integrin ligation could potentiate the integrin-mediated signaling, accelerating recruitment and release of cytolytic granules. The latter is consistent with the increase of the killing rate of SKBR3 cells after ICAM-1 up-regulation (Fig. 1B).
Integrins Control Activating Microcluster Size and Dynamics

We did indeed find that a lower level of ICAM-1 expression on SKBR3 cells impaired the ability of CD16.NK-92 effectors to form conjugates with these cells (supplemental Fig. S3). But we also examined the kinetics of granule release by CD16.NK-92 cells exposed to monolayers of SKBR-3 cells in the presence of Herceptin. The target cells were either untreated or treated with IFN-γ as above to vary the level of cell surface ICAM-1. Degranulation was measured by the appearance of CD107 (LAMP-1) molecules on the surface of the effector cells exposed to monolayers of the target cells at different time points. Fig. 1C shows that the percentage of degranulating CD16.NK-92 cells and average amount of granules released by individual effector cells responding to SKBR3 with elevated levels of ICAM-1 was substantially higher at every time point. The observed difference suggested that β2 integrin mediated signaling enhances the kinetics of granule release (Fig. 1D). Consistent with this, inhibition of Pyk2, a proximal signaling kinase in the integrin-mediated signaling pathway (20), resulted in a decrease of the specific lysis of SKBR3 targets by CD16.NK-92 cells (supplemental Fig. S4A). The inhibitory effect was dose-dependent and was more profound for killing of IFN-γ-treated SKBR3 cells (supplemental Fig. S4B).

β2-Integrins Facilitate Cellular Adhesion and the Kinetics of Cytolytic Granule Release at the CD16.NK-92/Bilayer Interface—To dissect the influence of β2 integrins on adhesion and kinetics of granule delivery at the single cell level, we analyzed interactions of CD16.NK-92 cells to planar lipid bilayers containing ligand for the CD16 receptor in the presence or absence of ICAM-1 and visualized degranulation of individual cells, essentially as we have previously described in cytotoxic T cells (4, 10, 11).

Although CD16.NK-92 cells formed contacts with the bilayers regardless of the presence of ICAM-1, both the percentage of cells contacting the bilayers (Fig. 2A) and the adhesion area (Fig. 2, B and C) were significantly larger on ICAM-1-containing bilayers. Importantly, 90% of the cells that established contact with ICAM-1-containing bilayers degranulated, while only 60% of the cells contacting bilayers without ICAM-1 did so (Fig. 2A). These data suggest that in addition to promoting cellular adhesion, β2 integrins induce a signal facilitating granule delivery to the contact surface.

We then examined the kinetics of granule release at the CD16.NK-92/bilayer interface by TIRF microscopy (Fig. 3A). Granule release was observed at distinct locations that were preserved during the time of observation (Fig. 3A and supplemental Fig. S5). These locations were adjacent to, but did not overlap with the clusters of CD16 receptors (Fig. 3A). On average, the granule release by CD16.NK-92 cells on ICAM-1-containing bilayers was observed 5 min after the cells were brought in contact with the bilayers, while granule release by CD16.NK-92 cells interacting with bilayers that do not present ICAM-1 was delayed until 10 min (Fig. 3A and supplemental Fig. S6). The kinetics of granule release were assessed by measuring the fraction of degranulating cells as a function of time followed by the appearance of the CD16 microclusters. The amount of time between formation of CD16 microclusters and the release of the granules in the presence of ICAM-1 was 3.3 times shorter (Fig. 3B). These data reiterate findings produced with live target cells (see Fig. 1, C and D) and provide evidence that integrin-induced signaling regulates the kinetics of cytoltyc granule release by CD16.NK-92 effectors.

Analysis of the Dynamics of Activating Microclusters—It is well established that proximal signaling mediated by antigen-specific receptors in T and B lymphocytes is compartmentalized and occurs in signaling microclusters containing activating receptors (21–26). To understand mechanism by which β2 integrins influence intracellular signaling from activating receptors that regulates the kinetics of granule delivery and release, we analyzed the dynamics of CD16-containing microclusters at the CD16.NK-92/lipid bilayer interface in the presence and absence of ICAM-1.

Upon initial contact of CD16.NK-92 cells with the bilayers, several undersized CD16-containing activating microclusters...
The observed difference started to move centripetally (supplemental Fig. S9). Then the microclusters began to grow in size and remained stationary over the entire area of cell/bilayer interface. Within this period, the newly formed microclusters were small enlarged during the first 1.5–2 min after the initial contact. The movement of microclusters continued for about 10–15 min (supplemental Movies S1 and S2). The moving microclusters then coalesced into distinct, well-structured donut-shaped structures (Fig. 3A and supplemental Fig. S7). As pointed out above, the microclusters were adjacent to but did not overlap with the sites of granule release, and granules were released within the donut-shaped aggregates (Fig. 3A). The observed pattern of microclusters and adjacent granule release sites at the interface resembled multifocal synapses (6, 27).

The degree of integrin receptor engagement influences the kinetics of cytolytic granule release at CD16.NK-92/bilayer interface. CD16.NK-92 cells were exposed to bilayers containing anti-CD16 antibodies (50 mol/μm²) in the presence or absence ICAM-1 molecules. A, representative TIRFM images of the CD16.NK-92 contact area in the presence (upper row) or absence (lower row) of ICAM-1 at indicated time points. CD16 staining, green; granule staining, red. B, percent of adherent CD16.NK-92 cells releasing cytotoxic granules in response to stimulation with the bilayers that do (filled circles) or do not (open circles) present ICAM-1 as a function of time. Zero time was determined by the appearance of CD16 signaling microclusters. Arrows indicate time required for half of the adherent cells to degranulate under each of the conditions. Results are representative of four independent experiments with at least 20 cells in each group of experiments.

To characterize CD16-containing signaling microclusters in CD16.NK-92 cytolytic effectors, we evaluated the size of the microclusters by either directly measuring their linear dimensions (supplemental Fig. S8) or from the integrated fluorescence intensity of the observed microclusters (supplemental Fig. S9). By either measure, we found that the size of microclusters on ICAM-1-containing bilayers was almost twice as large as the area of microclusters on bilayers without ICAM-1 (Fig. 4A and supplemental Fig. S9). The observed difference remained the same for up to 30 min (Fig. 4B). Importantly, the

 Integrins Control Activating Microcluster Size and Dynamics
size of individual microclusters did not depend on the changes of the density of anti-CD16 antibodies incorporated into the bilayers over a 100-fold range, but was linked to the presence or absence of ICAM-1 (Fig. 4, C and D). These data suggest that the ligation of \( \beta_2 \)-integrins triggers outside-in signaling that influence the size of CD16-activating microclusters. To provide evidence for the role of integrin-mediated signaling in controlling the size of microclusters, we first varied the extent of integrin ligation on CD16.NK-92 cells by changing the density of ICAM-1 on planar bilayers and found that lowering the density below 60 mol/\( \mu \)m\(^2\) led to a statistically significant decrease in size of the activating microclusters (Fig. 4E). It has to be noted that regardless of ICAM-1 density, we observed accumulation of ICAM-1 molecules at the cell/bilayer interface indicative of the productive engagement of integrins on the surface of the effector cells. We then examined the effect of the Pyk2 inhibitor that diminishes proximal integrin-mediated signaling. Fig. 4F shows that treatment of CD16-NK-92 cells with the inhibitor caused a decrease of the size of signaling microclusters at the CD16-NK-92/bilayers interface. Because the size of CD16 signaling microclusters correlates with the number of activating receptors recruited to each microcluster, the data provide evidence that \( \beta_2 \)-integrin-mediated signaling could effectively modulate the proximal signaling from activating receptor, which is linked to the kinetics of cytolytic granule release and the efficiency of NK cell cytolytic activity (6, 11, 28).

**\( \beta_2 \)-Integrins Influence the Microcluster Displacement and Mobility—**Because microclusters signal when they are on the move (23), those that move long distances are expected to contribute more to the magnitude and kinetics of proximal signaling. This prompted us to investigate parameters that are associated with the observed movement of signaling microclusters (Fig. 5A and supplemental Movie S3). The first parameter analyzed is the shortest distance from the initial to the end point of microcluster travel at the synaptic interface, which was termed microcluster displacement. Thus, microcluster displacement is the length of an imaginary straight path, which is typically distinct from the path that microclusters actually travel (Fig. 5A and supplemental Fig. S10). The second parameter analyzed is the average time period within which individual microclusters are moving. This parameter was called microcluster mobility (supplemental Fig. S10).

We found that the microcluster displacement correlated with the size of the interface and the location of initial microcluster formation within the synaptic interface. CD16.NK-92 exposed to ICAM-1-containing bilayers formed a more sizable adhesion area that increases the potential for a microcluster to travel a longer distance. Indeed, an average displacement of microclusters at the contact area of CD16.NK-92 cells exposed to bilayers containing ICAM-1 at 200–300 mol/\( \mu \)m\(^2\) was 1.5 times longer than the microcluster displacement at the contact area of CD16.NK-92 exposed to bilayers without ICAM-1, i.e. 7 \( \mu \)m and 5 \( \mu \)m, respectively (Fig. 5B). The mobility of microclusters on ICAM-1-containing bilayers was 1.7-times greater than for the cells exposed to bilayers free of ICAM-1, namely, 17 and 10 min, respectively (Fig. 5D). ICAM-1 at a density significantly below 300 mol/\( \mu \)m\(^2\) did not enhanced microcluster displacement and mobility (Fig. 5, B and D) despite productive integrin engagement as was evident from ICAM-1 accumulation at the cell/bilayer interface. Importantly, inhibition of Pyk2 signaling kinase had more profound effect on both displacement and mobility of microclusters (Fig. 5, C and E) then on their size (Fig. 4F).

**Recruitment of Activated Src Kinases to the Contact Surface—**To provide additional evidence that ICAM-1 interactions with \( \beta_2 \)-integrins influence the magnitude of proximal activating signaling in CD16.NK-92 cells, we visualized accumulation of activated (phosphorylated) Src kinases (pSrc) at the synaptic
Integrins Control Activating Microcluster Size and Dynamics

EFFIGURE 6. Influence of integrin engagement on recruitment of pSrc to the CD16.NK-92 cell/bilayer interface. CD16.NK-92 cells were exposed to bilayers with or without ICAM-1 for 5 or 10 min. The bilayers were then fixed with paraformaldehyde, permeabilized, stained with antibodies against pSrc followed by staining with fluorescent secondary antibody. A, representative TIRFM images of contact area of CD16.NK-92 cells exposed to bilayers in the presence (left panel) or absence (right panel) of ICAM-1. Average areas (B) and intensities (C) of pSrc-containing microclusters recruited to the contact surface of CD16.NK-92 in the presence (filled bars) or absence (empty bars) of ICAM-1, 5 or 10 min after the initial contact formation. At least 100 microclusters from 3 independent experiments were analyzed for each group, and the results are shown as mean ± S.D., *p < 0.05 by Student’s t test. Total average intensity (D) and density (E) of pSrc recruited to the cell/bilayer interface in individual cells exposed to bilayers that do (filled bars) or do not (empty bars) display ICAM-1, 5, or 10 min after the initial contact formation. At least 20 cells were analyzed in each group. Results are representative of three independent experiments and are shown as mean ± S.D., *p < 0.05 by Student’s t test.

interface in response to CD16 receptor ligation in the presence or absence of ICAM-1 on the bilayers. Regardless of the presence of ICAM-1, we observed the formation of pSrc clusters at the contact area of CD16.NK-92 cells exposed to the bilayers containing anti-CD16 antibody (50 mol/μm²) and ICAM-1 at indicated density, and CD16 microcluster mobility and displacement were determined 10 min after the initial cell/bilayer contact. A, representative image of CD16 microclusters and the microcluster tracks are shown. White lines indicate the microcluster displacement. See supplemental Fig. S10 and “Experimental Procedures” for details. B, average displacements of CD16 microclusters at the CD16.NK-92/bilayer interface in the presence of ICAM-1 at indicated density. C, average displacements of CD16 microclusters at the CD16.NK-92/bilayer interface in the presence of indicated concentrations of Pyk2 inhibitor. D, average mobility of CD16 microclusters at the contact area of CD16.NK-92 cells exposed to the bilayers containing anti-CD16 antibody and various densities of ICAM-1 molecules. E, average mobility of CD16 microclusters at the contact area of CD16.NK-92 cells exposed to the bilayers containing anti-CD16 antibody (50 mol/μm²) and ICAM-1 (300 mol/μm²). CD16.NK-92 cells were treated with Pyk2 inhibitor at indicated concentrations. At least 100 microclusters (n=100) from at least 20 cells were analyzed at each condition in three independent experiments. Results are shown as mean ± S.D.; ***, p < 0.001; ns, not significant.

the density did not change significantly from 5 to 10 min after initial contact of the cells with the bilayers (Fig. 6, B and C). In contrast, without ICAM-1 on the bilayers, the average size and density of pSrc microclusters decreased approximately by 20% from 5 to 10 min after initial cell exposure to the bilayers (Fig. 6, B and C). Thus, the proximal activating signaling at the synaptic interface of CD16.NK92 cells exposed to the bilayers containing ICAM-1 appeared to be more robust and was maintained for a longer time as opposed to that in CD16.NK-92 cells on ICAM-1-free bilayers.

We then measured the kinetics of pSrc recruitment to the interface and the average density of the recruited pSrc (Fig. 6, D and E). There was no difference in the total amount or the density of pSrc recruited to the contact area in the presence or absence of ICAM-1 within 5 min after the initial contact of
Integrins Control Activating Microcluster Size and Dynamics

CD16.NK92 cells with the bilayers. The difference became apparent later, i.e. 10 min after the contact was established, and the total amount and the density of the recruited pSrc were significantly higher in the presence of ICAM-1.

DISCUSSION

It is thought that an efficient response by cytolytic effectors requires the formation of a highly specialized interface between target and effector cell called the cytolytic synapse (4–6, 10, 11, 13). The formation of this structure usually depends on the ligation of both activating and integrin receptors. Signaling initiated by each receptor is required for cytoskeleton remodeling, formation of activating microclusters and the adhesion ring junction, polarization of the effector cell, and cytolytic granules release. While major signaling and adaptor proteins that are involved in the development of full blown signaling in cytotoxic lymphocytes are known, how each signaling pathway contributes to the initiation of the cytolytic response is not well understood. The principal finding of this study is that engagement of β2-integrins but not CD16-activating receptors influences the size and the dynamics of signaling microclusters in CD16.NK-92 cells. This result is consistent with previous findings showing that variation of the extent of TCR ligation in T cells exposed to ICAM-1-containing lipid bilayers presenting stimulatory peptide MHC ligands at various densities did not significantly affect the size of TCR signaling microclusters (23). In addition, it has also been shown that in the absence of the integrin ligation, the contact interface is profoundly diminished between CTL and a lipid bilayer that displays only cognate pMHC ligands (4).

To reveal the critical role of β2-integrins in regulating the proximal signaling and the formation of contact interface, we utilized CD16.NK-92 cytolytic effectors that could still kill target cells and establish contacts with planar lipid bilayers in the absence of ICAM-1 ligand for β2−integrins. This may be due to the presence of other β2-integrins, such as αMβ2, and α2β1 on the surface of NK-92 cells, which could recognize other protein and non-protein ligands (29). For instance, αMβ2 integrin has been shown to interact with casein and serum albumin presumably via recognition of RGD sites on denatured proteins (32). These proteins were used to block lipid bilayers and could serve as altered β2-integrin ligands. In fact, blocking β2-integrins with TS1/18 antibodies precludes CD16-mediated cytolysis activity of CD16.NK-92 cells (7) and prevents these cells from establishing contact with lipid bilayers (data are not shown). Similarly, the ability to augment the naturally low level of ICAM-1 on SKBR3 target cells by IFN-γ treatment, allowing us to study how differential expression of ICAM-1 by live target cells influences CD16.NK-92-mediated ADCC.

Thus, variations of ICAM-1 ligand density on planar lipid bilayers and the surface of live target cells allowed us to vary the extent of β2-integrin ligation on CD16.NK-92 effectors and to analyze systematically how variations in β2-integrin ligation affect the killing kinetics of the target cells, the area of the contact surface, the size and dynamics of activating CD16 signaling microclusters, and the kinetics and topography of cytolytic granule release.

We have found that in the presence of ICAM-1, the average size of moving microclusters was uniform and was characterized by a tight unimodal distribution (Fig. 4A). The average microcluster size remained steady for over 30 min (Fig. 4B). When CD16.NK-92 cells were stimulated via CD16 receptor in the absence of ICAM-1 on bilayers, the smaller moving microclusters still maintained their size and uniformity (Fig. 4, A and B). We postulate that RGD-containing denatured proteins present on bilayers engaged the NK cell integrins, albeit to a lower extent, resulting in a smaller average size of the microclusters. Importantly, variations in the extent of CD16 ligation within a wide range did not affect either the size or temporal uniformity of the signaling microclusters regardless of the presence of ICAM-1 (Fig. 4, C and D). This suggested that the size of the microclusters in CD16.NK-92 cells is regulated by the integrin signaling, but not by the CD16-mediated signaling. Indeed, inhibiting Pyk2 kinase, a key signaling protein in the β2-mediated signaling pathway (Fig. 7), led to a smaller size of signaling microclusters (Fig. 4F) and a significant decrease of microcluster mobility and displacement (Fig. 5, C and E). Importantly, the inhibition of microcluster dynamics (Fig. 5, C and E) was more profound than the decrease of microcluster size (Fig. 4F). This suggests that integrin-mediated signaling has a greater impact on microcluster dynamics than on their size.
Analysis of the recruitment of pSrc to the CD16.NK-92/bilayer interface showed that the development of proximal activation signaling in the NK cell cytolytic effectors takes a relatively long period of time, i.e. about 10 min (Fig. 6, D and E). This is significantly longer as compared with cytolytic T cells, which could reach maximal signaling within 2 min (11, 33). This difference is in accord with the delay in cytolytic granule release by the NK cells (Fig. 3A and supplemental Fig. S6) as opposed to that by cytotoxic T cells, which release granules within 1–2 min after the contact with the bilayer (11).

Our data are consistent with previous findings that “outside-in” integrin-mediated signaling contributes to activating signal longevity through the regulation of persistence and mobility of SLP-76 microclusters and their interaction with ZAP-70 (34, 35). In accord with this, recent findings have shown that effective integrin signaling in T cells is linked to ZAP-70 phosphorylation, while the catalytic activity of ZAP-70 contributes to TCR signal amplification (36).

Our previous findings showing that the kinetics of proximal signaling regulates the kinetics of granule release and target cell lysis by CTL (6, 11, 28). Consistent with this, the size and dynamics of signaling microclusters appears to regulate magnitude and kinetics of proximal signaling, which influence the kinetics of granule release (Figs. 1C and 3B). The latter control the kinetics of target cell lysis (Fig. 1, A and B).

Cytolytic granules travel along the microtubules from the periphery toward the synaptic interface (37, 38) and are released in multiple locations (Fig. 3A, supplemental Movies S4 and S5). These locations are likely determined by the points of microtubule attachment to the actin cytoskeleton mesh at the interface through Adhesion and Degranulation-Promoting Adapter Protein (ADAP), as has been previously described for T cells (37). The granule release sites had characteristically lower density of ICAM-1 molecules (supplemental Fig. S5), which is consistent with previous observations showing that cytolytic granules are released in the locations devoid of actin cytoskeleton (39). Here we show that the sites of granule release are surrounded by clusters of activating CD16 receptors (Fig. 3A, supplemental Movies S4 and S5). Such structures resemble multifocal synapses in T cells (6, 27). Consistent with previous observations (40), we have shown here that the locations of granule release at the NK cell interface remain unchanged over a long period of time (supplemental Fig. S5), ensuring uninterrupted delivery of cytolytic granules to the same locations on the target cell membrane. This increases the probability of successful granule penetration into a target cell and the induction of target cell death. Thus, even though a mature cytolytic synapse is not required for cytolytic activity (41, 42), the formation of a very well structured synaptic interface is essential for efficient cytolytic granule delivery and target cell destruction (6).

In conclusion, integrin signaling mediates the formation of a large synaptic interface and controls the size, dynamics, and stability of signaling microclusters, thereby ensuring the triggering of robust, rapid, and sustained signaling that is necessary to exercise effective cytolytic activity by cytolytic lymphocytes against target cells. CTL mostly rely on $\alpha_\lambda \beta_2$ (LFA-1) integrin and, therefore, are more effective during inflammation, a condition resulting in up-regulation of the LFA-1 ligand, ICAM-1, on the target cell surface. Because NK cells express and could effectively utilize other integrin receptors such as $\alpha_\lambda \beta_2$, $\alpha_\kappa \beta_2$, and $\alpha_\phi \beta_2$, their functioning appeared to be less dependent on inflammation and they play an important role in the earlier detection and destruction of tumor cells, virus-infected cells, or other aberrant cells prior to the development of inflammation and full blown acquired immune response.

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Integrins Control Activating Microcluster Size and Dynamics

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