A Balance between Positive and Negative Signals in Cytotoxic Lymphocytes Regulates the Polarization of Lipid Rafts during the Development of Cell-mediated Killing

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Abstract

Plasma membrane microdomains containing sphingolipids and cholesterol (lipid rafts) are enriched in signaling molecules. The cross-linking of certain types of cell surface receptors initiates the redistribution of these lipid rafts, resulting in the formation of signaling complexes. However, little is known about the regulation of the initial raft redistribution and whether negative regulatory signaling pathways target this phase of cellular activation. We used natural killer (NK) cells as a model to investigate the regulation of raft redistribution, as both positive and negative signals have been implicated in the development of their cellular function. Here we show that after NK cells form conjugates with sensitive tumor cells, rafts become polarized to the site of target recognition. This redistribution of lipid rafts requires the activation of both Src and Syk family protein tyrosine kinases. In contrast, engagement of major histocompatibility complex (MHC)-recognizing killer cell inhibitory receptors (KIRs) on NK cells by resistant, MHC-bearing tumor targets blocks raft redistribution. This inhibition is dependent on the catalytic activity of KIR-associated SHP-1, a Src homology 2 (SH2) domain containing tyrosine phosphatase. These results suggest that the influence of integrated positive and negative signals on raft redistribution critically influences the development of cell-mediated cytotoxicity.

Key words: natural killer cell • signal transduction • cytotoxicity, immunologic • tyrosine kinase • tyrosine phosphatase

Introduction

A major portion of eukaryotic membranes is in a fluid, unordered state due to the low melting temperatures of glycerolipids. However, within the glycerolipid environment there are "islands" of sphingolipids that, unlike glycerolipids, have head groups capable of being both hydrogen bond donors and acceptors. This results in extensive lateral hydrogen bonding among sphingolipid head groups and much higher melting temperatures (40–80°C), which make "sphingolipid islands" an ordered population at physiological temperatures (1). These ordered fractions, known as lipid rafts, are relatively detergent resistant and, besides sphingolipids, contain cholesterol and a number of membrane-associated glycophosphatidylinositol-linked and fatty-acylated proteins (2, 3). Lipid rafts have been implicated in immune cell activation (4–10). Key signaling molecules associate with lipid rafts, including protein tyrosine kinases (PTKs),[1] heterotrimeric and small G proteins, adaptor proteins, and phosphoinositides (2–5). Cross-linking of surface receptors in hematopoietic cells results in the enrichment of these receptors in the rafts along with other downstream signaling molecules, such as phospholipase C (PLC)γ1, Vav, Zap70, and Syk (4, 6–9). Furthermore, rafts redistribute to and cluster at the site of TCR engagement when T cells are experimentally stimulated with beads coated with anti-CD3 and anti-CD28 antibodies (10). Based on these results, raft reorganization is proposed to be associated with T cell costimulation, presumably by recruiting signaling molecules and excluding phosphatases.

However, it remains unclear if raft redistribution is simply caused by a passive aggregation of cross-linked receptors or if there is another level of regulation involved in raft

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[1]*Abbreviations used in this paper:* CTx, cholera toxin B subunit; KIRs, killer cell inhibitory receptors; MCD, methyl-β-cyclodextrin; PTKs, protein tyrosine kinases.

redistribution. In this study, we evaluated the regulation of raft redistribution during the development of cell-mediated cytotoxicity. We used NK cells as a model because it has been well characterized in NK cells that both positive and negative signals critically regulate the development of cell-mediated cytotoxicity. NK cells are $CD16^+CD56^+TCR^-sIg^-$ lymphocytes capable of killing certain tumor cells or virus-infected cells ("natural cytotoxicity"). Although receptors involved in the activation of NK cells during natural cytotoxicity are still poorly defined, it is known that downstream signaling molecules include PTKs, as well as Rho family low-molecular-mass G proteins, adaptor proteins, and calcium (11–16).

NK cell activation is blocked when killer cell inhibitory receptors (KIRs) on the surfaces of NK cells engage MHC class I molecules on resistant target cells (11, 17–19). This inhibition is mediated by KIR-associated SHP-1, a Src homology 2 (SH2) domain containing tyrosine phosphatase, that dephosphorylates and inactivates signaling molecules involved in NK cell activation (20–24). This well characterized signaling model of NK cell activation enabled us to evaluate a potential regulatory role for specific signaling molecules in lipid raft reorganization. Furthermore, studies of NK cell–mediated cytotoxicity allowed us to evaluate lipid raft redistribution in a more physiological system in which NK cells are activated by direct contact with target cells. Using this experimental model, we have found that raft reorganization during the development of cell-mediated cytotoxicity depends on a balance between the positive and negative signals through opposing activities of proximal PTKs and phosphatases. Our results imply that raft polarization is a critical event during the development of cell-mediated cytotoxicity, and inhibiting raft redistribution is a novel mechanism of negative regulation mediated by inhibitory receptors.

Materials and Methods

Reagents, Cells, and Antibodies. Unless otherwise indicated, all chemicals were from Sigma Chemical Co. Human NK cells were cloned and passaged as previously described (25). $DX9^+$ NK cell clones were identified as previously described (15). The P815 murine mastocytoma cell line and the K562 human erythroid leukemia cell line were obtained from American Type Culture Collection. HLA class I–deficient 721 cells and HLA-B58 transfected 721 cells were provided by Peter Parham (Stanford University, Palo Alto, CA). Anti-p70 KIR mAb DX9 was provided by Lewis Lanier (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Piceatannol was obtained from Boehringer Mannheim. PP1 was obtained from BIOMOL Research Labs., Inc. Herbimycin was obtained from GIBCO BRL.

Vaccinia Viruses. Catalytically inactive SHP-C453S as well as pSC65 vector control have been described (20). KIR3DL was provided by Marcus Colonna (Basel Institute of Technology, Basel, Switzerland). Wild-type Syk and SykT were provided by Jean-Pierre Kinet and Andrew M. Scharenberg (Harvard Medical School, Boston, MA) (26).

Cytotoxicity Assays. The ^{51}Cr-release assays measuring direct NK cell–mediated cytotoxicity were performed as previously described (25). Lytic units were calculated based on 20% cytotoxicity (27).

Raft Redistribution Assay. NK cells were stained for 30–45 min on ice with FITC–CTx (cholera toxin B subunit; 8 μg/ml). The labeled cells were washed twice in PBS containing 0.2% BSA and resuspended at a final concentration of 10^7 cells/ml for NK cells and 5×10^6 cells/ml for target cells. Equal volumes (50 μl) of NK cells and target cells were mixed, briefly pelleted, and then incubated for 5 min at 37°C. The cells were then fixed and transferred to glass slides by cytospin. NK cells that had formed conjugates were assessed for raft redistribution using a fluoromicroscope (Carl Zeiss, Inc.). A total of 50–100 conjugates was evaluated per slide, and the evaluation was performed by an individual blinded to the sample identities.

Results

Rafts Become Polarized during the Development of NK Cell–mediated Cytotoxicity. Natural cytotoxicity is initiated after conjugate formation between an NK cell and a susceptible target. We examined lipid raft reorganization that rapidly ensues after this conjugate formation. As cholera toxin B subunit (CTx) binds GM1 ganglioside (28), a marker of glycolipid-enriched rafts, human NK cells were stained with FITC–CTx. After washing, NK cells were incubated with hydroethidine-labeled target cells at 37°C, fixed, and spun onto glass slides. Fig. 1 shows representative photographs of NK cell–target cell conjugates. When incubated with the NK-resistant cell line P815 (red), NK cell rafts (green) are dispersed throughout the plasma membrane (Fig. 1 A). In contrast, incubation of NK cells with the NK-sensitive cell line K562 results in reorganization of lipid rafts into "macrorafts" polarized at the area of contact with the sensitive target (Fig. 1 B).

To quantify the difference in raft reorganization between NK cells stimulated with sensitive (K562) versus resistant (P815) cells, we evaluated NK–K562 and NK–P815 conjugates in three separate experiments (Fig. 2). For each experiment, 100 conjugates were analyzed. The results are expressed as the percentage of conjugates that have reorganized rafts. As shown in Fig. 2, there is a marked difference between sensitive and resistant cells in their ability to induce the formation of macrorafts polarized toward the target. Less than 10% of NK cells in contact with resistant targets polarize their rafts, whereas polarization is observed in close to 50% of NK cells in contact with the sensitive target cells. Similar results were obtained when another NK-sensitive cell line, 721, was used (data not shown).

Depletion of Cholesterol Inhibits Raft Polarization and NK Cell Activation. Cholesterol critically influences the stability of lipid microdomains. Methyl-β-cyclodextrin (MCD) depletes cholesterol from cell membranes and disrupts rafts (29), which can then lead to inhibition of certain forms of cellular activation (7, 29–31). To investigate the importance of raft polarization on NK cell activation, NK cells were pretreated with MCD and then analyzed by confocal microscopy and cellular cytotoxicity assays. MCD treatment resulted in the loss of evenly distributed rafts on unbound NK cells, and polarized macrorafts could no longer be detected when

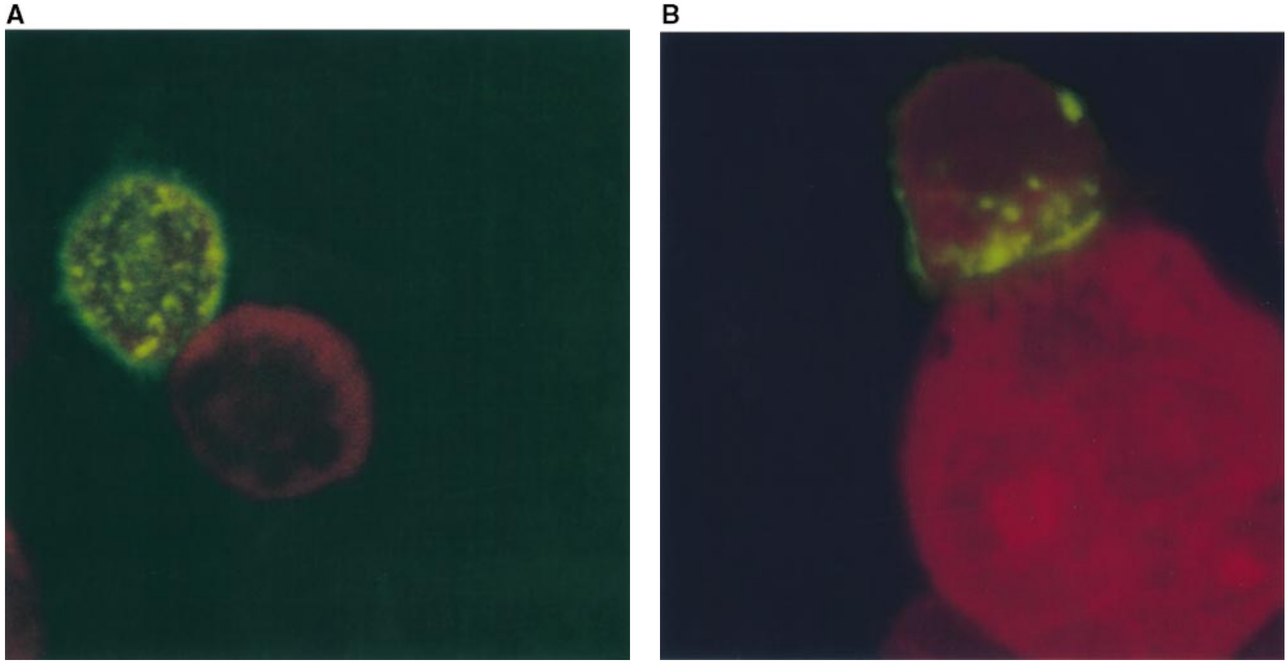

Figure 1. Rafts polarize at the site of NK–target engagement upon stimulation with NK-sensitive target cells. FITC–CTx-labeled NK cells were incubated at 37°C for 5 min with hydroethidine-labeled P815 cells (A) or K562 cells (B), fixed, and spun onto glass slides. Images were recorded during confocal microscopy.

NK cells formed conjugates with sensitive tumor targets (e.g., K562; data not shown). The MCD treatment subsequently blocked the generation of natural cytotoxicity (Fig. 3), suggesting that raft polarization plays an important role in the development of cell-mediated cytotoxicity.

Raft Polarization Requires Signaling by PTKs. Several lines of evidence suggest that rafts are important for lymphocyte activation: most tyrosine-phosphorylated proteins and other important second messengers have been found in lipid rafts (4, 6–7), and pharmacological disruption of lipid rafts impairs cellular activation (6, 7, 29–31). However, the mechanisms regulating raft redistribution have remained unclear. We wanted to determine if raft polarization is dependent on the activity of proximal tyrosine Src and Syk family tyrosine kinases. NK cells were preincubated with the Src kinase inhibitors herbimycin A (32–34) or PP1 (35), or with the Syk kinase inhibitor piceatannol (36). The IC_{50} for each drug on Src or Syk PTK activities was first determined to identify the appropriate concentration for each subsequent experiment. Washed NK cells were then incubated with NK-sensitive K562 cells. Raft polarization was quantified, and, in parallel, the cytotoxic activity of NK cells treated with the pharmacological inhibitors was measured. Fig. 4 shows that preincubation of NK cells with either a Src or Syk family PTK inhibitor blocks both raft polarization and cytotoxicity induced by K562 cells.

SykT is a catalytically inactive truncated mutant of Syk kinase that can inhibit cytotoxicity when expressed in NK cells (14). We transiently expressed SykT in NK cells using recombinant vaccinia virus. The expression levels of the virus-encoded Syk T and wild-type Syk were equivalent to the levels of endogenous Syk (data not shown). As shown in Fig. 5, expression of SykT inhibits raft polarization and

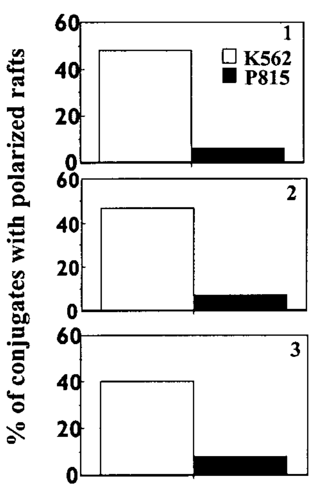

Figure 2. Comparison of raft polarization of NK cells after stimulation with NK-sensitive versus NK-resistant target cells. FITC–CTx-labeled NK cells were incubated at 37°C for 5 min with hydroethidine-labeled K562 cells or P815 cells. NK cell–target cell conjugates were scored for raft polarization by fluorescence microscopy. 100 conjugates were scored per blinded sample. The three results shown are representative of eight total experiments.

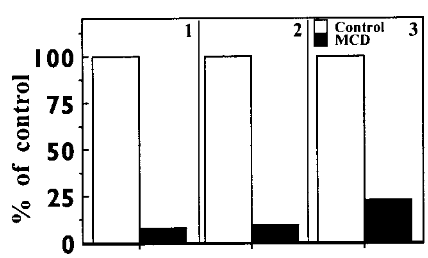

Figure 3. Depletion of cholesterol blocks NK cell activation. NK cells were pretreated with 10 mM MCD at 37°C for 1 h, washed, and then incubated for 4 h with ^{51}Cr-labeled K562 cells. Results are expressed as lytic units per 10^6 cells.

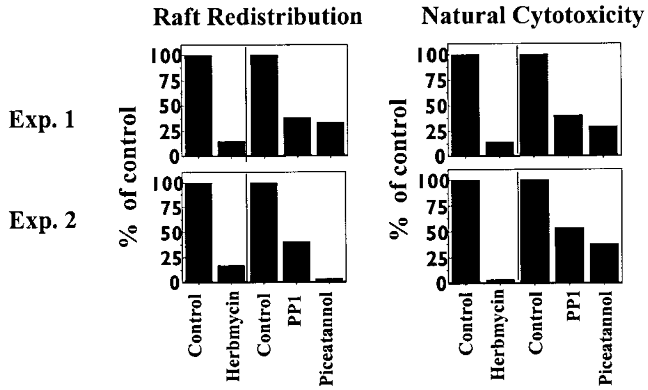

Figure 4. Inhibition of Src and Syk family kinases blocks raft redistribution. Left panels: NK cells were pretreated with 2 μM herbimycin A or DMSO at 37°C for 16 h or with DMSO, 10 μM PP1, or 25 μg/ml piceatannol at 37°C for 20 min, labeled with FITC–CTx, and incubated with hydroethidine-labeled K562 cells. NK–target conjugates were scored for raft polarization. The results are expressed as percentage of control. Right panels: NK cells pretreated with drugs as above were incubated with ^{51}Cr-labeled K562 for 4 h. Lytic units per 10^6 cells were measured, and the results are expressed as percentage of control. The two results shown are representative of five total experiments.

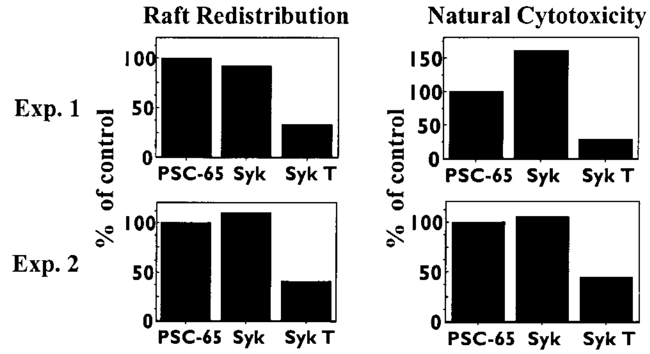

Figure 5. Expression of dominant negative, catalytically inactive Syk inhibits raft redistribution. Left panels: NK cells were infected for 4 h at a multiplicity of infection of 20 with control recombinant vaccinia viruses (PSC-65) or recombinant vaccinia encoding either wild-type Syk or catalytically inactive, truncated Syk (Syk T). Infected NK cells were stained with FITC–CTx and incubated with hydroethidine-labeled K562. NK–target conjugates were scored for raft redistribution. Right panels: infected NK cells were incubated with ^{51}Cr-labeled K562 cells for 4 h. The two results shown are representative of five total experiments.

NK cell–mediated cytotoxicity when compared with expression of wild-type Syk or vector alone (PSC-65). Therefore, experiments using either pharmacologic or genetic approaches suggest that raft polarization requires the activity of proximal PTKs.

KIR Engagement Blocks Raft Reorganization. NK activation is negatively regulated by MHC-recognizing KIRs. However, the exact mechanism by which KIRs exert their effect is still unclear. Negative signals mediated by KIRs might block raft redistribution before polarized macrorafts are formed. In this case, the blockade of raft redistribution would prevent accumulation of downstream signaling molecules and inhibit signal amplification. Alternatively, negative regulators might accumulate in the macrorafts along with key positive signaling molecules. To distinguish between these two possibilities, we investigated the relationship between raft redistribution and KIR engagement. KIR3DL is a KIR that is recognized by the mAb DX9 and recognizes the serologically defined HLA-BW4 allotype (e.g., HLA-B58) (37–39). DX9$^+$ and DX9$^-$ NK clones were isolated and then incubated with either 721 cells or HLA-B58–transfected 721 cells (721-B58). The inhibitory effect of KIR engagement on both raft redistribution and natural cytotoxicity is illustrated in Fig. 6. Incubation of DX9$^+$ NK cells with 721-B58 cells, but not 721 cells, results in inhibition of raft redistribution and natural cytotoxicity. The raft redistribution and natural cytotoxicity were not inhibited when DX9$^-$ cells were incubated with 721-B58 cells, indicating that KIR engagement is responsible for the inhibition of raft redistribution.

To further confirm that the inhibition of raft reorganization is mediated by KIRs, we expressed KIR3DL by infecting DX9$^-$ NK cells with recombinant vaccinia virus encoding KIR3DL. Nearly half of the NK cells exposed to infectious recombinant virus expressed high levels of KIR3DL as detected by flow cytometry (data not shown). As shown in Fig. 7, expression of KIR3DL in DX9$^-$ NK cells confers the inhibitory effect on both raft redistribution and natural cytotoxicity when these NK cells encounter 721-B58 cells. NK cells infected with control vaccinia virus (WR) were still able to undergo raft redistribution and mediate the killing of 721-B58 cells. These results are consistent with the notion that the inhibitory effect of KIRs on cytotoxicity is mediated by the blockade of raft redistribution.

Interrupting KIR–MHC Interactions Reverses the KIR-mediated Blockade of Raft Redistribution. KIR-mediated inhibition requires interaction between KIRs and specific MHC class I molecules. Preincubation of KIR-expressing NK cells with specific anti-KIR antibody reverses KIR-mediated inhibition of natural cytotoxicity by interrupting the interaction between KIRs and their ligands (38, 39). To test

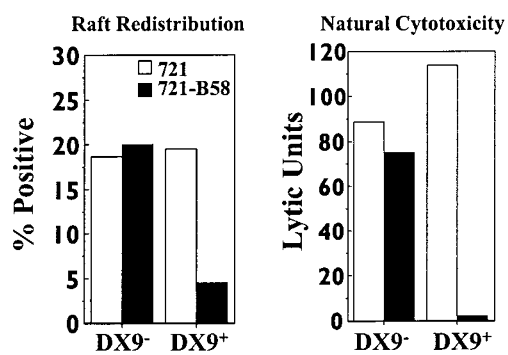

Figure 6. KIR engagement blocks raft redistribution. Left panel: FITC–CTx-stained DX9$^+$ or DX9$^-$ NK cells were incubated with 721 cells or with HLA-B58–transfected 721 cells (721-B58) and scored for raft redistribution. The results are expressed as the percentage of conjugates with polarized rafts. Right panel: DX9$^+$ or DX9$^-$ NK clones were incubated for 4 h with ^{51}Cr-labeled K562 cells. The results were expressed as lytic units per 10^6 cells. The results shown are representative of three different experiments.

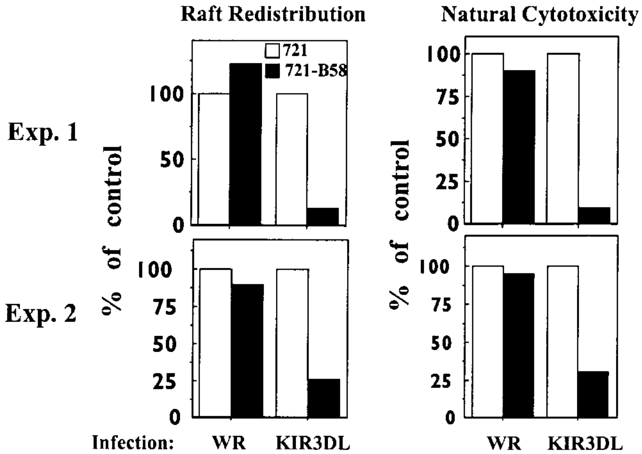

Figure 7. Expression of KIR3DL in DX9⁻ NK clones confers an inhibitory effect on raft redistribution. DX9⁻ NK cells were infected for 4 h at a multiplicity of infection of 20 with either recombinant vaccinia virus encoding KIR3DL or wild-type vaccinia virus (WR). Left panels: infected NK cells were stained with FITC–CTx and incubated with 721-B58 cells or parental 721 cells and scored for raft redistribution. Right panels: infected NK cells were incubated with ^{51}Cr-labeled 721-B58 cells or 721 cells. Lytic units per 10^6 cells were measured. The two results shown are representative of six total experiments.

whether interrupting the KIR3DL–HLA-B58 interaction would also reverse the blockade of raft redistribution, we preincubated DX9$^+$ or DX9$^-$ NK cells with DX9 mAb on ice for 5 min before incubating them with 721 or 721-B58 cells. Preincubation of DX9$^+$ cells with DX9 mAb completely reversed the inhibitory effect of KIR engagement on both raft reorganization (Fig. 8) and natural cytotoxicity (data not shown), whereas there was little effect on DX9$^-$ cells. These results confirm that KIR–MHC interaction is necessary for the inhibitory effect on raft redistribution.

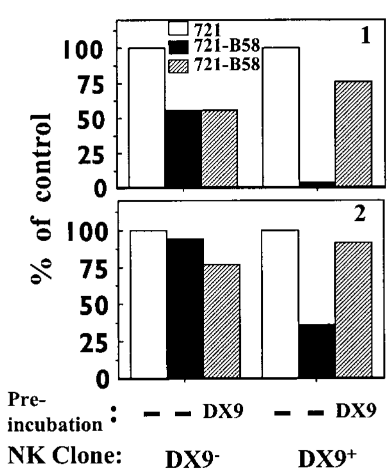

Figure 8. Interruption of KIR–MHC interactions reverses KIR-mediated blockade of raft redistribution. (A) DX9$^+$ or DX9$^-$ NK cells stained with FITC–CTx were preincubated with 10 μg/ml DX9 antibody on ice for 5 min or left untreated. The cells were then incubated with hydroethidine-labeled 721-B58 cells or 721 cells and scored for raft redistribution. The two results shown are representative of three different experiments.

SHP-1 Activity Is Necessary for the KIR-mediated Blockade of Raft Redistribution. KIR-mediated inhibition depends on the protein tyrosine phosphatase SHP-1. Previous studies have demonstrated that overexpression of the dominant negative SHP-1 (SHPC453S), which is catalytically inactive, reverses the KIR-mediated inhibitory effect on natural cytotoxicity and antibody-dependent cellular cytotoxicity (20, 21). To test if the blockade of raft reorganization also depends on SHP-1, we expressed SHPC453S or the wild-type SHP-1 in DX9$^+$ cells. In these experiments, the expression levels of the virus-encoded SHP-C453S and SHP-1 were equivalent to that of endogenous SHP-1 (data not shown). Expression of the dominant negative (Fig. 9) but not the wild-type SHP-1 (data not shown) reversed the KIR-mediated blockade of raft redistribution, indicating that KIR-mediated inhibition of raft redistribution depends on SHP-1.

Discussion

Cellular activation is a result of multiple enzyme–substrate reactions that amplify, diversify, and regulate signals initiated from surface receptors. Most of these reactions occur at the plasma membrane, where signaling molecules cluster in a multimolecular complex around surface receptors. This clustering enables compartmentalization of key second messengers and results in the amplification of signal transduction cascades. Rafts can act as platforms holding signaling molecules together (4, 6, 7). This was recently highlighted by the demonstration that rafts redistribute to and cluster at the site of TCR engagement when T cells are stimulated with bead-coupled antibodies (10). The redistribution and clustering of rafts leads to higher and more sta-

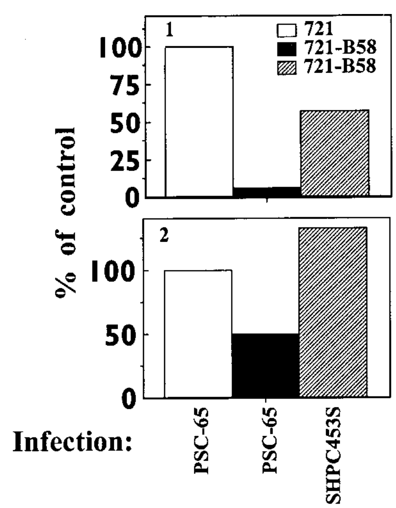

Figure 9. Expression of dominant negative SHP-1 reverses KIR-mediated inhibition of raft redistribution. DX9$^+$ or DX9$^-$ NK cells were infected for 4 h at a multiplicity of infection of 20 with control recombinant vaccinia virus (PSC-65) or dominant negative, catalytically inactive SHP-1 (SHPC453S). Infected NK cells were stained with FITC–CTx, incubated with hydroethidine-labeled 721-B58 cells or 721 cells, and then scored for raft redistribution. The two results shown are representative of four different experiments.

ble tyrosine phosphorylation of signaling molecules, presumably by recruiting key signaling molecules and excluding phosphatases. However, due to the high concentration of antibodies bound on the beads, the redistribution of rafts could be caused by physical forces, so it remains unanswered whether or not raft redistribution is a regulated process. In this paper, we have studied raft redistribution using NK cells that are directly stimulated by viable target cells. We show that rafts become aggregated and polarized at the site of contact between NK cells and sensitive target cells. The detection of raft redistribution in this physiological system implies that raft redistribution is a regulated process.

The functional role of rafts during lymphocyte activation has been evaluated in several studies. The absence of detectable ζ and PLCγ1 tyrosine phosphorylation and diminished calcium signals upon pharmacological disruption of rafts was interpreted as evidence for temporal order of events in which raft formation and polarization precedes activation of signal transduction cascade (6, 7, 29–31, 40). This view is supported by the observation that even cross-linking of glycophosphatidylinositol-linked peripheral membrane proteins can stimulate T cells, presumably through an increased local concentration of key signal transducing molecules (40–43). However, our data suggest that the polarization of lipid rafts is signaling dependent. Inhibition of the activity of Src or Syk family PTKs blocks reorganization of lipid rafts. Moreover, inhibitory signals initiated by KIR engagement also block raft polarization in an SHP-1–dependent manner. Because regulatory second messengers accumulate in rafts, and, at the same time, raft polarization depends on activation signals, it is likely that there is a positive feedback loop between raft aggregation and positive signal propagation. Instead of a unidirectional sequence of events (receptor cross-linking→raft aggregation→signal transduction), we believe that these three processes are interdependent. Lateral bonding between sphingolipids within rafts increases spatial rigidity of the receptor–ligand interactions. These rigid receptor–ligand interactions might then keep the signaling complex together for a longer time, which in turn might aggregate more sphingolipid-associated proteins into the cellular interphase and increase the probability of a complete cellular activation. Consistent with this notion, the interaction between cytoskeleton and lipid rafts has been proposed to increase raft stability (40), and a recent study suggests that the interaction between cytoskeleton and lipid rafts depends on tyrosine kinase activity (44). Furthermore, rafts are proposed to act as transporters of surface receptors and key signaling molecules during the formation of immunological synapses (10, 45–47). This process has been shown to be driven by the movement of cytoskeleton and dependent on signal transduction (48).

KIRs block the activation of NK cells through SHP-1–mediated dephosphorylation of molecules involved in the activation cascade (20–23). Several direct targets of SHP-1 have been proposed (15, 24), but none of the current models have been able to fully explain the sequence of events during inhibitory signaling. Our data suggest that the negative signals mediated by KIRs arise during the earliest phase of NK activation, specifically before the formation of "macrorafts." Presumably, KIR-associated SHP-1 dephosphorylates and inactivates key signaling molecules that are required for the signal cascade leading to raft aggregation. On the other hand, aggregation of lipid rafts helps exclude phosphatase activity from the sites of positive signal propagation (10, 49). In our biochemical assays, neither KIRs nor SHP-1 could be detected in rafts (Jevermovic, D., Z. Lou, and P.J. Leibson, unpublished observation). Overall, the efficacy of raft redistribution and aggregation would ultimately be a function of the balance between positive and negative signals generated after interaction with specific target cells.

Our study provides new insights as to the regulatory events that critically influence the development of cell-mediated cytotoxicity. Lipid raft redistribution and polarization is regulated by positive and negative signals from the membrane receptors, and the integration of these signals can ultimately determine the commitment of cytotoxic lymphocytes to cellular cytotoxicity. This study also discloses a novel inhibitory mechanism that has the potential to be employed by certain homologous inhibitory receptors expressed on cells of both hematopoietic and nonhematopoietic lineages.

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