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Dynamic Redistribution of the Activating 2B4/SAP Complex at the Cytotoxic NK Cell Immune Synapse

Pedro Roda-Navarro,* María Mittelbrunn,† Mara Ortega,* Duncan Howie,‡ Cox Terhorst,‡ Francisco Sánchez-Madrid,† and Elena Fernández-Ruiz2‡*

The 2B4 molecule (CD244) has been described as a coreceptor in human NK cell activation. However, the behavior of 2B4 during the cytotoxic NK cell immune synapse (NK-IS) formation remains undetermined. In this study, we demonstrate the redistribution of 2B4 and the signaling adaptor molecule, signaling lymphocyte activation molecule-associated protein (SAP), to the cytotoxic NK-IS upon formation of conjugates between resting NK cells and EBV-infected 721.221 human cells. Confocal microscopy showed that 2B4 localized at the central supramolecular activation cluster, surrounded by a peripheral supramolecular activation cluster containing talin within NK cell and ICAM-1 on target cells. Videomicroscopy studies with 2B4-GFP-transfected NK cells revealed that 2B4 redistributed to cytotoxic NK-IS as soon as the cell contact occurred. Simultaneously, a SAP-GFP also clustered at the contact site, where it remained during the interaction period. The 2B4 molecular clusters remained bound to the target cell even after NK cell detachment. These results underscore the function of 2B4 as an adhesion molecule and suggest a relevant role in the initial binding, scanning of target cells, and formation of cytotoxic NK-IS. Finally, these findings are indicative of an important role of the activating 2B4/signaling lymphocyte activation molecule-associated protein complex during the recognition of EBV-infected cells. The Journal of Immunology, 2004, 173: 3640–3646.

N atural killer cell-mediated cytotoxicity is initiated by specific interaction with virus-infected or neoplastic target cells. The cytolytic activity is tightly regulated by dominant inhibitory receptors for MHC class I molecules (1). Down-regulation of MHC class I on target cells during viral infection or tumor transformation enables the engagement of activating receptors triggering cytotoxicity and cytokine production. A wide repertoire of NK cell-activating receptors has been identified including the natural cytotoxicity receptors, Nkp46, Nkp30, and Nkp44; the Fc receptor CD16; and NKG2D. Moreover, 2B4 (CD244), NTB-A (Ly-108; signaling lymphocyte activation molecule F7 (SLAMF7)),3 and Nkp80 have been described as coreceptors, as their contribution to NK cell-mediated cytolysis depends on natural cytotoxicity receptor expression and function (2).

The glycoprotein 2B4 (3, 4), a member of the SLAM family of receptors (5), is expressed on NK cells, monocytes, basophils, TCRγδ T cells, and a subset of CD8+ T cells (6). Engagement of 2B4 leads to IFN-γ secretion, granule exocytosis, and stimulation of lytic activity by NK cells (7). The ligand of 2B4 is CD48, a GPI-anchored membrane protein also belonging to the SLAM family (5, 8). An activating role in NK cell-mediated lysis has been demonstrated for 2B4-CD48 interaction (9). Following cross-linking with specific mAb, 2B4 becomes tyrosine phosphorylated (10). In addition, pervanadate treatment also induces phosphorylation of 2B4 and recruits SLAM-associated protein (SAP) (11, 12). SAP is a signaling adaptor molecule encoded by the SH2D1A gene, which is mutated in patients with the inherited immunodeficiency X-linked lymphoproliferative disease. X-linked lymphoproliferative disease patients suffer from dysgammaglobulemia and abnormal immune responses to EBV infection (5, 13). Due to its high affinity interactions with the cytoplasmic tail of 2B4, it has been hypothesized that SAP could prevent binding of Src homology domain-containing phosphatase-2 (SHP-2) (11, 12, 14, 15). This possible role of SAP seems to be critical for 2B4 triggering activation (16). Interestingly, SHP-1 has also been shown to bind phosphorylated 2B4 (17). In addition, SAP is also an adaptor molecule that is prerequisite for binding of the src kinase Fyn to SLAM receptors (18, 19). A second adaptor molecule involved in 2B4-mediated activation is the linker for activation of T cells (LAT) (20).

Upon NK cell-target cell interaction, a highly organized complex of molecules is formed at the contact area of both cells, termed the NK cell immune synapse (NK-IS). The IS was first described on the interface of T cell-APC interaction and comprises a central supramolecular activation cluster (cSMAC) of receptor-ligand pairs and signaling proteins, surrounded by a peripheral region (pSMAC) containing LFA-1 and talin (21). The NK-IS displayed two distinct molecular distributions dependent upon the nature of the interaction, i.e., cytolytic or noncytolytic. Furthermore, specific killer cell Ig-like receptors induce clustering of...
HLA-C at the contacting surface of resistant target cells forming the so-called inhibitory NK-IS (22). In contrast, when NK cells interact with susceptible target cells, the redistribution of signaling molecules at the cSMAC, as well as LFA-1 and talin at the pSMAC resembles the T cell-APC IS organization (23). A differential distribution of actin, ezrin, CD43, and CD45 at activating and inhibitory NK-IS has also been described (24). In contrast to the inhibitory receptors, how activating receptors redistribute at the interface of NK/target cells remains ill understood, as only polarization of NGK2D and CD2 to the contact area of NK cell-target cell conjugates has been recently described (25, 26).

In this study, we report the results of studies of the dynamic behavior of 2B4 molecule during the resting NK cell interaction with the susceptible EBV-infected 721.221 human B cell line. Our data demonstrate the dynamic clustering of 2B4 and its associated molecule SAP at the NK-IS, which would explain the relevant role of this molecular complex during the NK cell-mediated control of EBV infection.

Materials and Methods

Cells, Abs, and reagents

The EBV-infected B cell line 721.221 was cultured in RPMI 1640 medium with Glutamax-1 (Invitrogen Life Technologies, Grand Island, NY), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (complete medium). PBMC were purified from healthy donors by Ficoll density centrifugation (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). After depleting monocytes by plastic adhesion, resting NK cells were purified by negative selection with immunomagnetic beads (Dynal Biotech, Oslo, Norway) coupled to anti-CD3, anti-HLA-DR, and anti-CD4 mAbs. After a second round of selection with immunomagnetic beads coupled to CD3 and CD19 mAbs, the obtained cell population, analyzed by flow cytometry, was >97% CD56⁺, CD3⁻.

T3b (anti-CD3), HP2/6 (anti-CD4), DR (anti-HLA-DR), MEM111/B10 (anti-ICAM-1), and D3/9 (anti-CD45) mAbs have been previously described (27); pp35 (anti-2B4), BAB281 (anti-NKp46), and MA152 (anti-ICAM-1), and D3/9 (anti-CD45) mAbs have been previously described (25, 26). To test the correct membrane expression of the 2B4-GFP, the Jurkat T cell line transfected with the plasmid was stained with pp35 mAb (data not shown).

Conjugate formation and immunofluorescence microscopy

Resting NK cells and 721.221 cell line were mixed at an E:T cell ratio of 2:1 and spun at 2500 rpm for 3 min at room temperature. After incubation at 37°C for 5 min (or 2 min in the case of conjugates stained with LAT), cells were gently resuspended and added to PCL-coated slides, briefly centrifuged, and fixed in 2% paraformaldehyde. In time course experiments, cells were mixed, spun 10 s at 2500 rpm, and added to PCL-coated slides for 30 s or 5 min, briefly centrifuged, gently resuspended, and fixed with 2% paraformaldehyde. For immunofluorescence assays, samples were permeabilized with 0.1% Triton X-100 for 5 min (SAP, LAT, and microtubule-organizing center (MTOC)) or as previously described for PYK-2 (28) and blocked with TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent; Boehringer Mannheim, Mannheim, Germany). FcR were blocked with human γ-globulin (100 μg/ml; Sigma-Aldrich). After staining with primary mAb, followed by an Alexa 488-labeled specific secondary Ab, samples were examined with a DMR microscope (Leica, Mannheim, Germany) using the Leica QFISH 1.0 software. For doubled staining, cells were incubated with pp35 mAb, followed by a goat anti-mouse Rhodamine Red X, saturated with mouse serum, and incubated with a biotinylated ICAM-1, followed by streptavidin-Alexa Fluor 488, or with talin or LAT rabbit anti-human polyclonal Ab, followed by a goat anti-rabbit Alexa 488.

Live-cell time-lapse fluorescence confocal microscopy

The 721.221 cells resuspended in HBSS with 2% FCS were allowed to adhere on FN-coated coverslips. The 2B4-GFP- or SAP-GFP-transfected NK cells were resuspended in HBSS with 2% FCS and added to the chamber. Cells were maintained at 37°C in 5% CO₂ and confocal images were acquired using the Leica TCS-SP confocal microscope. Confocal series of fluorescence and the differential interference contrast (DIC) images were simultaneously obtained. The maximal projection of the most representative sections of the green channel (GFP signal) and the corresponding DIC images was overlaid in a single image.

Results

Subcellular distribution of 2B4 and SAP in resting peripheral blood NK cells

Resting NK cells isolated from peripheral blood were phenotypically characterized by flow cytometry as CD3⁻, HLA-DR⁻, CD45⁺, CD56⁺, NKp46⁻, CD57⁻, and CD69⁻ (data not shown). Resting NK cells and 721.221 cell line were mixed in the vertical axis) were assembled into a single image.

Immunooprcipitation and Western blotting

Immunoprecipitation experiments were performed with Jurkat T cell lysates and the 10C4.2 anti-iSAP mAb or Ig isotype control. SAP peptide (200 μg/ml) was used to elute bound proteins. Eluates were separated on a 4–15% SDS-polyacrylamide gel, followed by Western blotting with anti-iSAP polyclonal Ab. NK cells were lysed in 0.1% Nonidet P-40, and lysates were resolved in 12% polyacrylamide gel transferred to nitrocellulose membranes and analyzed by Western blot with the anti-iSAP 10C4.2 mAb.
these cells express high levels of CD48, the cellular ligand of 2B4. When NK-721.221 cell conjugates were stained with the anti-2B4 mAb, the receptor was clustered at cell-to-cell contact area (Fig. 2, A and B). The adhesion molecule ICAM-1 expressed on target cells was also accumulated at the interface of both cells (Fig. 2A), whereas the phosphatase CD45 was homogeneously distributed (Fig. 2, A and B), as previously described (24). CD48 on the surface of the target cells was also localized at NK-IS (Fig. 2C, see arrowheads). The signaling adaptor molecule SAP, which is known to bind to 2B4 (11), was also localized at cell-to-cell contact areas (Fig. 2, A and B). As controls, the signaling molecule PYK-2, as well as the MTOC and talin (data not shown), were also localized at NK cell-target cell contact areas, as reported for activated NK cells (Fig. 2A, see arrowheads) (28). LAT was homogeneously distributed on the plasma membrane, but a patch was observed at the central zone of NK cell-target cell contact, colocalizing with the 2B4 cluster (Fig. 2A). SAP also colocalized with 2B4 at the NK...

FIGURE 2. The 2B4 and SAP localize at the NK-IS. A, NK-721.221 cell conjugates were allowed to adhere to PLL, fixed and stained for 2B4, ICAM-1, CD45, SAP, PYK-2, α-tubulin (MTOC), and LAT/2B4 or SAP/2B4. DIC images are shown. The localization of the MTOC is indicated by arrowheads. B, Quantification (%) of cell conjugates in which 2B4, SAP, or CD45 was localized at the NK cell-721.221 cell contact area. More than 100 conjugates were analyzed in three independent experiments. Histogram bars represent the arithmetic mean ± SD. C, Expression of CD48 in NK cell-target cell conjugates. Accumulation of CD48 at different NK-721.221 cell synapses is indicated by arrowheads. D, Comparison of NK cell-target cell conjugate formation at two time points. Immunofluorescence and quantification (%) of 2B4 and MTOC clustering at 30 s and 5 min after conjugate formation. One hundred conjugates from three independent experiments were analyzed. Histogram bars represent the arithmetic mean ± SD. NK cells are identified by their smaller size.
cell-target cell contact (Fig. 2A). These data demonstrate that 2B4 and SAP are localized to the cytotoxic NK-IS and support the relevance of 2B4-triggered signaling during the control of EBV infection by NK cells.

Kinetic of 2B4 redistribution was analyzed in comparison with that of MTOC. The 2B4 accumulated at NK-IS in 48% ± 0.3 of 30-s conjugates and 62% ± 3.2 of 5-min conjugates. By contrast, MTOC was concentrated in only 10% ± 1.5 of 30-s conjugates and 54% ± 1.2 of 5-min conjugates (Fig. 2D). These data suggest that 2B4 clustering occurs very rapidly after the initial cell-to-cell contact and that this molecule could be involved in early adhesive events.

Segregation of 2B4 at the cSMAC

To topographically map the 2B4 localization within the NK-IS, NK-721.221 cell conjugates were also analyzed by confocal microscopy. As shown in Fig. 3A, clusters of 2B4 were localized at the central zone of the contact area. Next, conjugates were double stained with 2B4 and talin, a component of the cortical cytoskeleton localized at the peripheral zone of NK-IS (23). A ring of talin at the pSMAC surrounded 2B4 clusters at the cSMAC. Likewise, when conjugates were double stained with 2B4 and ICAM-1, the cellular ligand of LFA-1 expressed on target cells, ICAM-1 was also detected at the pSMAC surrounding 2B4 clusters (Fig. 3B).

The 2B4 distribution pattern was observed in 70% of a total of 20 conjugates scored from three different donors showing a peripheral localization of talin. Finally, NK-target cell conjugates were stained with both LAT and 2B4, and a partial colocalization of 2B4 clusters with LAT at the NK-IS was found (Fig. 3C, see arrowhead). These data show that, like other costimulatory receptors such as CD2 or CD28 in T cell-specific IS, the activating coreceptor 2B4 is localized at the cSMAC of cytotoxic NK-IS, and that the colocalization of 2B4 and LAT only occurs at the interface of resting NK cells and 721.221 target cells.

**FIGURE 3.** The 2B4 segregates at the cSMAC of the NK-IS. NK-721.221 cell conjugates were allowed to adhere to PLL, fixed and stained for 2B4 (red) ([A]), and double stained for 2B4 (red) and talin, ICAM-1, and LAT (green) ([B] and [C]). One representative confocal section, DIC images, and three-dimensional reconstructions ([A] and [B]) or three-dimensional high magnification ([B], right panels) are shown. The colocalization of 2B4 and LAT is shown by an arrowhead.

**Dynamic redistribution of 2B4 and SAP**

To study the dynamic distribution of these molecules during the resting NK cell-target cell interaction, NK cells were transiently transfected with 2B4- or SAP-GFP constructs. The localization of these molecules was tracked by live-cell time-lapse fluorescence confocal microscopy (Figs. 4 and 5). The 2B4 clusters appeared at the cell-to-cell contact as early as 30 s after the interaction between NK cells and target cells (Fig. 4A, see arrowhead and Supplemental Movie 1). The 2B4 clusters became well established after 3 min (Fig. 4A). When NK cells simultaneously contacted with two susceptible target cells, 2B4 was clustered at both sites of cell-to-cell interactions as soon as the contact occurred (Fig. 4B; 0.30, 2.30, and 7.00 min; see arrowheads and Supplemental Movie 2). Interestingly, the cluster of 2B4 was stable during the whole interaction period (Fig. 4B; 20.30 min). Likewise, SAP-GFP also clustered at the contact site, and these clusters were maintained during the whole interaction, similar to that observed with 2B4-GFP (Fig. 4C and Supplemental Movie 3). In addition, the staining of SAP-GFP-transfected NK-target cell conjugates with 2B4 revealed a clear-cut colocalization of this molecule with SAP-GFP at the contact zone of cell conjugates, as it was observed by confocal microscopy (Fig. 4D).

Remarkably, the interaction of NK cells with 721.221 cells was associated with the formation in NK cells of very thin and elongated cytoplasmic protrusive structures, which connect both cells. This connective structure was formed and maintained after NK cell detachment (Fig. 5). A very high concentration of 2B4 and SAP was detected in this structure (Fig. 5, see arrowheads and Supplemental Movies 4 and 5). When connective structures were broken, a portion of 2B4-containing membrane remained attached to the target cells (Fig. 5A; 7.45 min). The significance of this phenomenon is currently unclear, but it could be due to the high affinity of 2B4 with its ligand CD48 (8, 9).

These data show the formation of 2B4- and SAP-containing connective structures, the dynamic redistribution of 2B4 and SAP, and the association of these molecules at NK-IS throughout the interaction between resting NK cells and EBV-infected cells.

**Discussion**

In this study, we report the subcellular localization of activating coreceptor 2B4 and signaling adaptor molecule SAP during the recognition of susceptible EBV-infected 721.221 cells by human NK cells. In this model, a cytotoxic NK-IS was established due to the absence of MHC class I molecules on target cells. We have analyzed this NK-IS by confocal microscopy and live-cell time-lapse fluorescence confocal microscopy. First, 2B4 molecule was shown to be homogeneously distributed on the surface of resting peripheral blood NK cells adhered to FN and PLL, but localized at the cytotoxic NK-IS when conjugates between NK cells and target cells were formed, which suggest that 2B4 is translocated to the NK-IS after cell contact occurs. In these experiments, we showed how SAP and PYK-2 NK cell molecules and ICAM-1 target cell molecule also localized at the NK-IS. Previous studies have demonstrated accumulation of ICAM-1 at the contact site of APCs in T cell IS (30). Moreover, the interaction of ICAM-1 with LFA-1 expressed on resting NK cells mediates the adhesion to target cells (31). The localization of LFA-1 and talin at the pSMAC and multimolecular signaling complex at the cSMAC of the cytotoxic NK-IS has recently been reported (23). Our data show the localization of the activating coreceptor 2B4 at the cSMAC and the

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adhesion molecule ICAM-1, expressed on target cells, at the pS-MAC. These data suggest that the rearrangement of surface proteins at the cytotoxic NK-IS resembles the one described for CD4 T cells and CTLs, which consists of localization of receptors involved in activating signaling pathways in a cSMAC, surrounded by a pSMAC containing the pair LFA-1/ICAM-1 and talin (21, 32).

It has been previously demonstrated that phosphorylated 2B4 is localized in lipid rafts upon activation of NK cells by specific mAbs or CD48+ target cells (33), and that lipid rafts are accumulated at cytotoxic NK-IS (34). In contrast, it has been described that LAT is constitutively associated with rafts in T lymphocytes (35), and with 2B4 in the NK cell line YT (7). As we found that a fraction of LAT and 2B4 molecules colocalized at cytotoxic NK-IS, we can suggest that 2B4 and LAT are in part associated within lipid rafts concentrated at the NK-IS. Accordingly, the localization of mouse 2B4 in lipid rafts was shown to be required for the association of this molecule with LAT (36). In addition, our data show that a fraction of LAT is not translocated to NK-IS and does not colocalize with 2B4.

The dynamic redistribution of activating receptors or signaling molecules during the NK cell-target cell interaction has not been described to date. Then we developed chimeric 2B4- and SAP-GFP and transfected resting NK cells to study this issue by time-lapse videomicroscopy. Our data show a stable clustering of 2B4 and SAP, and demonstrate that 2B4 clusters can be simultaneously formed when one NK cell interacts with two target cells. Simultaneous clusters have also been described for talin, but not for the signaling molecules SLP-76 and protein kinase C-θ, which were polarized in the NK cell toward only one of the two targets (23). Likewise, ICAM-1 clusters were detected at the contact site of both

FIGURE 4. Dynamic redistribution of 2B4 and SAP at the NK-IS. The 721.221 target cells were seeded onto FN-coated coverslips, and then resting NK cells transiently transfected with 2B4-GFP (A and B) or SAP-GFP (C) were added to the chamber. Cells were monitored by time-lapse confocal microscopy at 30-s intervals, and representative images are shown. Each time point shows the DIC image overlaid with the fluorescence images of NK cells. D, Conjugates were formed with SAP-GFP-transfected NK cells and target cells, adhered to FN, and fixed and stained with 2B4. Confocal sections, DIC, and merged images are shown. Clustering of different GFP and colocalization of 2B4 and SAP-GFP are shown by arrowheads.

FIGURE 5. Formation of connective structures during the interaction of resting NK cells and target cells. The 721.221 target cells were seeded onto FN-coated coverslips, and then resting NK cells transiently transfected with 2B4-GFP (A) or SAP-GFP (B) were added to the chamber. Cells were monitored by time-lapse confocal microscopy at 30-s intervals, and representative images are shown. Each time point shows the DIC image overlaid with the fluorescence images of NK cells. The accumulation of GFP at the connective structure is indicated by arrowheads.
APCs in T cell-APC triple-cell conjugates (30). Kinetic and dynamic studies show that 2B4 clusters occur very rapidly, which suggests that it could be involved in the initial binding of resting NK cells to target cells, before the killing process. In this regard, it has been described recently that coexpression of CD48 with ICAM-1 on insect cells enhances the adhesion of resting NK cells (31). In addition, the signaling adaptor molecule SAP has been suggested to prevent the binding of SHP-2 to the cytoplasmic tail of 2B4 (11). Moreover, it is conceivable that SAP might couple the src kinase fyn to 2B4, as it has been described for SLAM (18, 19). Our data show the association of SAP with 2B4 at cytotoxic NK-IS and strongly suggest that the permanency of activating 2B4/SAP complex is necessary to block the recruitment of phosphatases during the interaction between resting NK cells and EBV-infected cells. In this regard, the stable localization of SAP at NK-IS during the interaction period would prevent the blockade of lipid raft polarization to NK-IS, exerted by SHP-1 associated to inhibitory killer cell Ig-like receptors (34). Then this fact would permit the stable assembly of the multimolecular signaling complex associated to glycolipid-enriched microdomains at the cSMAC (37). This signaling complex includes PTKs as Lck, Fyn, and the transmembrane LAT. Finally, these data would also explain the exclusion of phosphatases, as SHP-1 from the NK-IS very early after cytokotoxic interactions is formed (38).

We found a very thin membrane structure, called connective structure, associated to the interaction formed between resting NK cells and 721.221 target cells. This structure is formed in unstable conjugates when NK cells are detached from target cells. Similar unstable conjugates and connective structures have been previously reported in cytotoxic contacts formed between mouse NK cells and target cells, where they have been proposed to be involved in inducing physical damage to susceptible targets during NK cell-mediated cytotoxicity (39). Interestingly, we find a high concentration of 2B4 and SAP in this connective structure. In addition, the transmission of proteins and membranes fragments from target cells or APCs to NK cells or T cells has been reported recently (40, 41). Our data show the opposite phenomenon and demonstrate that target cells can acquire 2B4-containing membrane fragments.

This study reports a temporal distribution of activating 2B4/SAP complex to the cytotoxic NK-IS and demonstrates for the first time the segregation of an NK cell-activating receptor (2B4) at the cSMAC and the LFA-1 ligand ICAM-1 at the pSMAC. This distribution of molecules at cytotoxic NK-IS is in agreement with previously reported data in T cells and NK cells (21, 23, 32). Recently, it has been reported that CD2 is localized at the NK-IS in the pSMAC (26). This observation has been done after 30 min of conjugate formation. In contrast, the rapid accumulation of 2B4 at early contacts as well as the behavior of this molecule forming clusters in two contact sites in triple-cell conjugates has been previously described for adhesion molecules such as ICAM-1 and ICAM-3 (30, 42). These facts, together with the formation of such 2B4-containing connective structures when primary resting NK cells are detached from target cells, underscore the role of 2B4 in cell-to-cell adhesion. In addition, our data also report for the first time the transmission of an activating NK cell receptor to susceptible target cells, extending the processes of protein and membrane fragment transmission to a bidirectional phenomenon. Studies on the dynamic distribution of other NK cell-activating receptors and signaling molecules will provide further insights into the molecular mechanisms responsible for NK cell activation during virus-infected or tumor cell recognition.

Finally, our data also suggest that 2B4 could be present at other types of cellular IS, as T cells or monocytes. The expression of CD48 in T and B cells as well as in DC might imply that the binding of 2B4 to CD48 could participate in a variety of cell-to-cell interactions that are important for immune responses.

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References


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molecules in the NK cell immune synapses during MHC class I-regulated non-


