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Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158)

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Summary: The killer cell immunoglobulin-like receptor (KIR) family includes receptors that bind to HLA class I molecules on target cells and inhibit natural killer (NK)-cell cytotoxicity, and receptors such as KIR3DL7 with no known ligand and function. Inhibitory KIR recruit the tyrosine phosphatase SHP-1 to block signals transduced by any one of a number of activation receptors. Inhibition of overall protein tyrosine phosphorylation by SHP-1 during binding of KIR to MHC class I on target cells is selective, suggesting that a limited number of substrates are dephosphorylated by SHP-1. We have chosen to study KIR inhibition as it occurs during binding of KIR to MHC class I on target cells, despite the technical limitations inherent to studies of processes regulated by cell contact. KIR binding to MHC class I on target cells inhibits tyrosine phosphorylation of the activation receptor 2B4 (CD244) and disrupts adhesion of NK cells to target cells. Inhibition of proximal events in NK activation may increase the availability of NK cells by liberating them from non-productive interactions with resistant target cells. As the receptors and the signaling pathways that induce NK cytotoxicity are not fully characterized, elucidation of the inhibitory mechanism employed by KIR may provide insight into NK activation.

Introduction

An important function of inhibitory killer cell immunoglobulin-like receptors (KIR) is to provide tolerance to self (1–3). Hematopoietic cells are particularly sensitive to lysis by natural killer (NK) cells and require recognition of their own MHC class I by NK inhibitory receptors in order to be spared. Target cells that are eliminated by NK cells *in vivo* may include cells infected by viruses that interfere with MHC class I expression, and tumor cells that have lost expression of MHC class I. However, the role of inhibitory KIR has yet to be fully evaluated *in vivo*. The presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) in the cytoplasmic tail of inhibitory KIR and the recruitment of tyrosine phosphatases by phosphorylated ITIM, leading to inhibition of NK-cell activation, has become a paradigm for the negative control of cellular responses by an increasingly large number of receptors (4–6). Therefore, the mechanism of NK inhibition by

***Table 1.* KIR nomenclature**

KIR[a]	CD[b]	Signal	HLA ligand
2DL1	158a	Inhibitory	C group 2
2DS1		Activating	C?
2DL2	158b	Inhibitory	C group 1
2DL3	158b	Inhibitory	C group 1
2DS2		Activating	C?
2DL4		Potential for both	G
2DL5		Potential for inhibition	?
2DS4		Activating	?
2DS5		Potential for activation	?
3DL1		Inhibitory	Bw4
3DS1		Potential for activation	?
3DL2		Inhibitory	A3, A11
3DL7		Potential for inhibition	?

[a] 2D and 3D designate KIR with two or three Ig domains, respectively. L and S refer to long and short cytoplasmic tails, respectively. The centromeric to telomeric arrangement of genes is *KIRC1* (*3DL7*) – *2DL2* or *2DL3* – *2DL1* – *2DL4* – *3DL1* – [*2DL5* – *2DS5* – *2DS1*] – *2DS4* – [*2DS2*] – *3DL2*. Genes in square brackets are absent in some KIR haplotypes (8).

[b] A complete CD158 nomenclature is under development.

KIR is likely to provide insights into negative regulatory controls in diverse biological systems. This review attempts to summarize the current state of knowledge on the mechanism by which KIR inhibits NK cytotoxicity.

The group of receptors encoded in a gene cluster at the human chromosomal location 19q13.4 (7, 8) was originally called killer cell inhibitory receptors, since it included receptors such as p58 (9) and NKB1 (10), which block activation upon recognition of HLA-C and HLA-B molecules on target cells, respectively. The acronym KIR was proposed to identify this receptor family (11). Killer cell Ig-like receptors has become a more suitable spelling out of KIR, since it accounts for activating members of the KIR family (12) and distinguishes KIR from lectin-like receptors with similar inhibitory function, such as CD94/NKG2 and mouse Ly49. CD158 was proposed at the last Human Leukocyte Differentiation Antigen Meeting as a designation for the entire KIR family. *Table 1* lists the KIR family members and the corresponding CD158 subsets.

The KIR family

Inhibitory members of the KIR family include receptors with two Ig domains (KIR2D, domains D1 and D2) and a long (L) cytoplasmic tail. KIR2DL1 (CD158a) is specific for group 2 HLA-C allotypes defined by asparagine at position 77 and lysine at position 80. KIR2DL2 and 2DL3 (grouped together as CD158b) are specific for group 1 HLA-C allotypes with a serine at 77 and asparagine at 80. The specificity of 2DL2 and 2DL3 for HLA-C group 1 and of 2DL1 for HLA-C group 2 is not completely tight, as recognition of the opposite HLA-C group has also been observed in functional assays and by direct binding (13, 14). HLA-C has an obvious important role in providing protection from NK cells because every HLA-C allotype is recognized by an inhibitory KIR. KIR3DL1 has three Ig domains (KIR3D, domains D0, D1, and D2) and a long cytoplasmic tail. 3DL1 is specific for B allotypes that carry the Bw4 serological determinant. All of these inhibitory receptors contribute to NK tolerance to self through the recognition of their specific MHC class I ligands on target cells (3). The receptor KIR3DL2 has binding affinity for HLA-A3 (15). Inhibition of $3DL2^+$ NK clones by HLA-A3 and HLA-A11 on target cells has been observed (16) but a contribution of 3DL2 in tolerance to self has not been readily apparent (3). Expression of inhibitory KIR on NK cells is not dictated by the HLA haplotype of the individual. All *KIR* genes are expressed in NK cells regardless of whether the individual has corresponding MHC class I ligands or not. Each NK cell expresses a small repertoire of about two to six KIR (3).

Several KIR with two Ig domains and short (S) cytoplasmic tails, such as 2DS1 and 2DS2, have activating function (12). KIR2DS associate with DAP12 subunits via a charge interaction in the transmembrane region (17). DAP12, containing an immunoreceptor tyrosine-based activation motif (ITAM), initiates a src-kinase and syk/ZAP70 kinase-dependent activation cascade. Although direct binding of 2DS1, 2DS2, and 2DS4 to HLA-C is almost undetectable (13, 14, 18–20), functional experiments with NK clones and T-cell clones suggest that an activation signal can be delivered through recognition of HLA-C on target cells (21, 22). A proposed function for such receptors is to promote killing of tumor cells or virus-infected cells that have selectively lost expression of a protective MHC class I. Sensitivity to lysis by NK cells may actually be increased if the remaining class I allotypes are recognized by an activating KIR2DS (6, 23).

KIR2DL4 has the potential for both inhibitory and activating function, as it contains an ITIM in the cytoplasmic tail and a charged amino acid residue (arginine) in the transmembrane region, indicative of association with another molecule. Soluble recombinant 2DL4 binds to cells expressing HLA-G, suggesting a role of 2DL4 in pregnancy (24, 25). HLA-G is expressed primarily on trophoblast cells that invade the decidua at the maternal–fetal interface during early pregnancy. Abundant maternal NK cells in the decidua encounter invading trophoblast cells (26). The unique D0–D2 domain

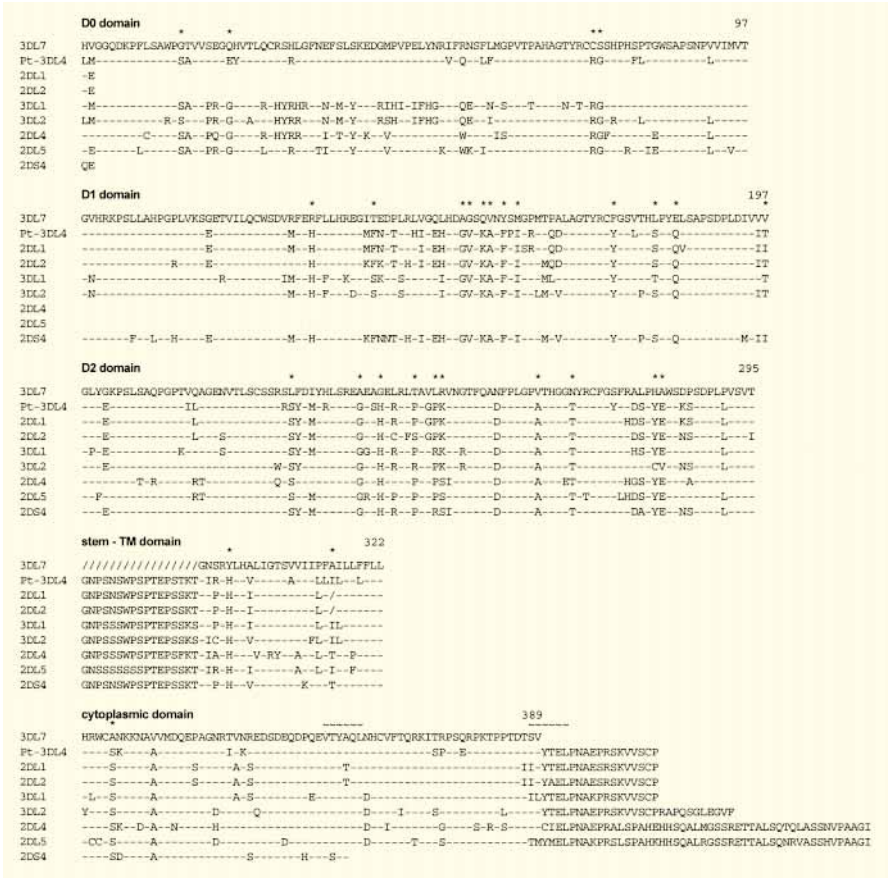

***Fig. 1.* Comparison of the predicted KIR3DL7 amino acid sequence to those of other KIR.** Identity to 3DL7 is indicated by −, deletion by /. The cytoplasmic ITIM are indicated by ∼∼. Amino acids unique to 3DL7, not found in any human, chimpanzee (29), or pygmy chimpanzee (30) KIR, are marked by *. Pt-3DL4 is a chimpanzee KIR. The numbering corresponds to the 3DL7 sequence.

configuration of 2DL4 is also found in 2DL5, a KIR whose ligand and function are unknown (27).

KIR3DL7

KIRC1, the first gene at the centromeric end of the KIR complex, encodes a putative inhibitory receptor with three Ig domains (8, 28). Transcription of *KIRC1* has not been detected at the RNA level. Using nested PCR with RNA from peripheral blood, we have isolated full-length cDNA clones corresponding to the deduced mRNA of *KIRC1* (N. Wagtmann, V. Renard, M. Sandusky, M. Peterson, E. O. Long, unpublished; GenBank accession AF352324). Expression of *KIRC1* may be very low because amplified cDNA was not easily detected except after using internal primers in a second round of PCR. As predicted from the gene sequence (28), the cDNA encodes a KIR with three Ig domains but without the stem that connects the D2 domain with the transmembrane domain of all other KIRs. The cytoplasmic tail carries the first ITIM and stops at the level of the second ITIM. In accordance with a unified human and chimpanzee KIR nomenclature proposed by Parham (29), the *KIRC1* product should be called 3DL7. (The names 3DL3 to 3DL6 have been assigned to chimpanzee KIR that have no human orthologs.) The 3DL7 sequences of three individuals, deduced from the *KIRC1* gene sequenced by Torkar et al. (28) (accession AF072407 and AF072410) and by the Lawrence Livermore National Laboratories (http://www-bio.llnl.gov/bbrp/genome/genome.html) (accession AC006293), and from the cDNA described here, differ from each other by two to five amino acids, indicating some polymorphism in this gene.

3DL7 contains some unique amino acids not found in any other KIR of humans and chimpanzees (*Fig. 1*). Otherwise, 3DL7 resembles a patchwork of sequences found in other KIR. The D0 domain is very similar to that of chimpanzee (Pt) KIR3D receptors, in particular Pt-3DL4, which shares specificity for group 2 HLA-C with KIR2DL1 (29). The D0 domain shares sequences with 2DL4, Pt-3DL4, and 3DL1, and the D1 domain has sequences found predominantly in 3DL2 and 3DL1. The D2 domain is more mixed and includes stretches of sequences identical to 2DS4 and 2DL4. Rajalingam et al. (30) have proposed that *KIRC1* may correspond to an ancestral gene from which several other KIR genes were derived during evolution. Despite the presence of a conserved

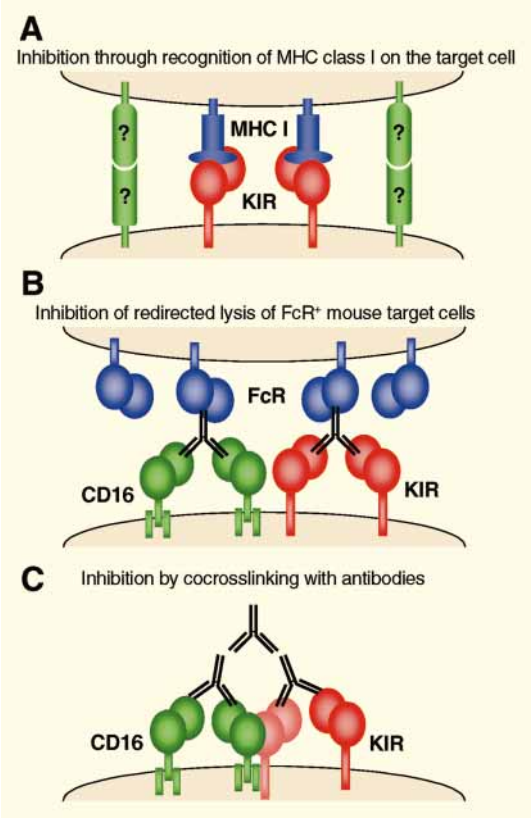

Fig. 2. Experimental systems for the study of signal inhibition by KIR.

ITIM in the cytoplasmic tail, it is possible that the absence of a stem would preclude an inhibitory function for 3DL7. However, the strong sequence conservation in *2DL4*, *2DL5*, and *KIRC1* genes among humans, chimpanzees, and pygmy chimpanzees suggests that these genes serve a function (30).

Inhibition of NK-cell activation signals by KIR

A fundamental question on the mode of inhibition by KIR is whether a central step in the NK activation pathway is specifically blocked or whether KIR can target multiple signals for inhibition. The complete block of cytotoxicity, lymphokine production, and of Ca^{2+} flux exerted by KIR is compatible with both hypotheses. At the cellular interface of the NK–target cell conjugate, the clustering of KIR may lead to its tyrosine phosphorylation by an src kinase within the activation pathway. However, the specific requirements for KIR clustering upon binding to MHC class I and the resulting KIR phosphorylation are not known. Furthermore, phosphorylation of KIR by a kinase that is involved in the activation pathway seems to put KIR at a disadvantage. How can KIR inhibit a tyrosine kinase-dependent signal when its own tyrosine phosphorylation is a prerequisite for inhibition? Placing KIR downstream of the initial signaling event in NK activation inevitably delays its ability to turn off the signal. Such a delay could be overcome if the critical site of KIR inhibition is further downstream in the signaling pathway for activation. However, existing evidence maps KIR function at a fairly proximal step of the activation signal (31–34).

Experimental approaches to study inhibition by KIR

The ideal setting to study how KIR exerts its inhibitory effect would be with normal NK cells receiving physiological signals from soluble mediators (e.g. cytokines, chemokines) while they contact other cells that express a ligand for KIR. Relevant target cells would be those that are sensitive to lysis by NK cells unless protection by MHC class I is provided. Such a setting is difficult to reproduce and would reduce the range of feasible experiments. Nevertheless, normal NK cells are easy to obtain, and they can be expanded and cloned *in vitro*. Biochemical analysis of the negative signal delivered by KIR during NK–target cell contacts is complicated by the signals and proteins contributed by target cells and by the lack of synchrony in the interaction between cells. To reconstitute inhibition by MHC class I on target cells, the class I-negative mutant cell line 721.221 transfected with specific class I genes is often used. It is therefore possible to observe under controlled conditions the outcome of KIR engagement by MHC class I on target cells (*Fig. 2A*). Early experiments using such a system established that the block exerted by KIR lies upstream of Ca^{2+} flux (31, 32).

Much progress in the characterization of NK receptors was achieved by use of a redirected lysis assay, also referred to as reverse antibody-dependent cellular cytotoxicity (*Fig. 2B*). FcR^+ target cells (e.g. mouse P815 cells) are used to cross-link receptors on NK cells with mAbs that bind NK receptors. mAbs specific for activation receptors induce lysis of P815 by NK cells, whereas mAbs specific for inhibitory receptors can inhibit the lysis triggered by mAbs specific for activation receptors. However, some limitations also apply to this system. The presence of mouse target cells complicates biochemical analysis of NK signaling components, which are often conserved among distinct species. Furthermore, the use of Abs to cross-link receptors may not reproduce important parameters of receptor–ligand interactions during cell contact, such as density, topology, and specific localization in the membrane.

Antibodies can be used to bypass the complications inherent to cell mixing experiments. A strong activation signal is delivered to NK cells simply by cross-linking CD16. This

signal is inhibited by co-cross-linking an inhibitory receptor (*Fig. 2C*). This approach has been applied to study the effect of inhibitory KIR on CD16 signaling. However, results obtained by this approach can differ from those of NK–target cell mixing experiments.

Phosphorylation and clustering of KIR

The inhibition by KIR acts only when the receptor is engaged in close proximity to the activation signal. Independent cross-linking of KIR and of the FcεRI receptor on a transfected rat basophil cell line did not impair the activation induced by IgE, whereas co-cross-linking the two receptors did (35). Free src homology 2 (SH2)-containing tyrosine phosphatase (SHP)-1 has a low catalytic activity because its own SH2 domain blocks the catalytic site (36). Therefore, binding of the SH2 domains of SHP-1 to phosphorylated KIR releases the inhibition and induces dephosphorylation of substrates that are in close proximity to KIR. The need to co-ligate KIR with an activation receptor in order to achieve inhibition may serve two purposes. One, as mentioned, is to bring active SHP-1 to the site where activation signals are transmitted. The second is to move KIR to the site of activation where active src kinases will phosphorylate the ITIM.

Several models can account for the ability of KIR to inhibit an src kinase-dependent signaling cascade even though it requires an src kinase to phosphorylate its own cytoplasmic tail. First, inhibitory KIR may be constitutively phosphorylated. In that case, inhibition would still depend on KIR clustering at the site of activation upon binding to MHC class I on the target cell. Second, KIR may be associated with a tyrosine kinase. KIR phosphorylation would be induced after activation of the kinase upon KIR clustering, much like ITAM-dependent activation receptors. The third model is that KIR phosphorylation is executed by those kinases that are initiating the NK activation signal. In that case, inhibition by KIR would have to either be extremely fast in order to stop a signaling cascade that has already started, or target a downstream effector of the activation pathway. None of these models has been excluded so far, although the lack of detectable KIR phosphorylation in the absence of co-ligation with activation receptors supports the third model. Furthermore, KIR binding to HLA-C on target cells results in extensive clustering at the cell interface, a property that may accelerate KIR's ability to stop the activation signals (37). Unexpectedly, out of several inhibitors tested, only Zn^{2+} chelators, such as 1,10-phenanthroline, reduced the extent of KIR clustering. The propensity of KIR to cluster may contribute to its inhibitory function by concentrating KIR near activating receptors before full induction of triggering signals.

Earlier work had established that Zn^{2+} binding to KIR through histidine residues plays a role in the inhibitory function of KIR (38, 39). More recently, biochemical studies with soluble 2DL1, purified after expression in bacteria, revealed a dimerization of 2DL1 induced by Zn^{2+} and by the related metals Cu^{2+} and Co^{2+} (14, 40). Metal-induced dimers of KIR bound to HLA-C with greater affinity or avidity than did KIR monomers. Surface plasmon resonance experiments showed that the presence of Zn^{2+} but not other divalent cations altered the kinetics of the KIR–HLA-C association and dissociation, pointing to a direct role for Zn^{2+} in KIR multimerization (14). Zn^{2+}-dependent dimerization of KIR may contribute to KIR clustering and to the inhibitory function of KIR. However, KIR dimers have not been detected at the surface of NK cells, even after chemical cross-linking (M. Peterson, unpublished). It is possible that KIR dimerization on NK cells is induced only transiently upon interaction with HLA-C. Additional KIR–KIR or KIR–class I interactions would be necessary to achieve clustering beyond KIR dimerization. A form of KIR–KIR clustering (handshake) was observed in a crystal of 2DL2 in the absence of the HLA-C ligand (41).

Crystal structures of KIR2D–HLA-C complexes have the potential to hold clues about KIR clustering upon binding to HLA-C on opposing target cells. The stoichiometry of 2DL2 bound to HLA-Cw3 was 1:1 in the crystal of the complex (42). The same 1:1 stoichiometry had been observed for 2DL1 bound to HLA-Cw4 in solution (43). A lattice array formed by a second contact of KIR to another HLA-C molecule was revealed by the packing in the 2DL2–HLA-Cw3 crystal (42). If this array occurs at the cell surface, it would result in polymerization of KIR on the NK cell and of HLA-C on the target cell, even though no direct KIR–KIR or HLA-C–HLA-C contacts are formed. Functional experiments with mutated KIR and HLA-C molecules are needed to test these models for KIR clustering.

SHP-1 binding to phospho-ITIM

The importance of a hydrophobic amino acid two residues upstream of the ITIM tyrosine for binding of SHP-1 (44) has been confirmed by *in vitro* binding and activation studies and by functional expression of mutated KIR (45, 46). The presence of an ITIM (V/IxYxxL/V) in the cytoplasmic tail of receptors, such as gp49B, paired immunoglobulin-like receptor B (PIR-B), and PD-1, has been a reliable predictor of the inhibitory function of those receptors (47–50).

The weight of the evidence clearly favors a phospho-ITIM-

dependent recruitment of SHP-1 as a key step in the inhibitory signal. Recruitment of SHP-1 by KIR upon binding MHC class I on target cells has been detected (D. N. Burshtyn, unpublished). In that experimental context (*Fig. 2A*), tyrosine phosphorylation of SHP-1 was more easily detected than that of KIR (34). Overexpression of a catalytically inactive SHP-1 in NK cells prevented the inhibition mediated by KIR (44, 51). The inhibitory function of ITIM-containing Ly49A receptors in mouse NK cells was partially impaired in SHP-1-deficient moth-eaten mice, which express normal SHP-2, a SHP-1-related phosphatase (52). Complete impairment of an ITIM-mediated inhibition was observed in the DT40-cell line only after inactivation of both *SHP-1* and *SHP-2* genes (53). Phosphorylated KIR ITIM also bind SHP-2 (54, 55), which could compensate for the lack of SHP-1 in mutant cells. A mutant KIR in which only the first ITIM was preserved bound SHP-2 more efficiently than SHP-1 after cross-linking with activation receptors (55). This finding is relevant to the function of the KIR family members that have only the first ITIM, such as 2DL4 and 3DL2. Finally, chimeric KIR containing SHP-1 in place of its cytoplasmic tail inhibited NK cells, showing that SHP-1 is sufficient for the inhibitory signal (34, 56).

SHP-1 substrate selection during inhibition by KIR

Identification of the direct substrates of SHP-1 targeted for dephosphorylation during inhibition by KIR would clarify the mechanism of inhibition and help define pathways of NK activation. Although substrates that are directly dephosphorylated by SHP-1 have not been identified yet, it is not for a lack of candidates. Inhibition upon recognition of MHC class I on target cells (*Fig. 2A*) results in a selective reduction of tyrosine phosphorylation of a few proteins in NK cells (31, 32, 57). In one such study, total phosphorylated proteins were compared after mixing NK cells with sensitive or resistant target cells. Engagement of 3DL1 by HLA-Bw4-bearing targets resulted in a specific loss of proteins with an apparent molecular weight of 50, 135, and 158 (32). The phosphorylation state of specific proteins was monitored upon target cell mixing, and a decreased phosphorylation of syk and a Grb2-associated 36 kDa protein (presumably linker for activation of T cells (LAT)) were noted after mixing with resistant target cells (32, 57). A similar result was obtained with a γ/δ T-cell clone expressing inhibitory CD94/NKG2A whose engagement resulted in reduced tyrosine phosphorylation of an unknown 130 kDa protein and of the kinase ZAP70, but not the T-cell receptor ζ chain (33).

The system of redirected lysis of P815 cells (*Fig. 2B*) has been used extensively in functional studies of inhibitory receptors, but not for biochemical studies. In this system, activation through cross-linking of a chimeric CD16/syk receptor expressed in a T-cell line was inhibited by co-ligation of 2DL2/3, indicating that KIR can inhibit at the level, or downstream of, the tyrosine kinase syk (58).

The effect of KIR on the CD16 signal has been studied by mAb-mediated cross-linking (*Fig. 2C*). Inhibition through cross-linking of KIR and CD16 in NK-cell clones resulted in the loss of a prominent tyrosine phosphorylated band at 36 kDa as well as several bands in the 70–150 kDa range, in addition to a global reduction in protein tyrosine phosphorylation. Specifically, decreases in the CD16-induced tyrosine phosphorylation of the ζ chain, ZAP70, phospholipase Cγ1 (PLCγ1) and PLCγ2, and SLP-76 were noted (51, 58). A similar study evaluating the impact of CD94/NKG2A-mediated inhibition of CD16 signals reported a decrease in the tyrosine phosphorylation of syk, ζ chain, and shc (59). It is not known whether any of these molecules represent the direct substrate of SHP-1 or whether their reduced tyrosine phosphorylation is caused by dephosphorylation of an upstream substrate by SHP-1. Some of these molecules may not be necessary for NK cytotoxicity, as mice deficient in LAT, SLP-76, or Syk display normal NK function (60–62).

An approach that would directly identify SHP-1 substrates is the expression of SHP-1 trapping mutants (i.e. a mutant that binds but does not cleave phosphotyrosine) in NK cells. Such an approach was used in the macrophage cell line BAC1.2F5 and in primary bone marrow-derived macrophage cells to identify the ITIM-containing signal regulatory protein (SIRP) as a SHP-1 substrate (63). SIRP is a receptor that also binds to the SH2 domains of SHP-1. Similar trapping experiments have identified substrates in other cell types. In B cells, the B-cell linker protein (BLNK) was trapped following IgM cross-linking(64), and in mast cells two proteins of 25 and 30 kDa were detected following stimulation by antigen/IgE complexes (65). However, SHP-1 bound to these proteins in the absence of a specific inhibitory signal. To identify substrates of SHP-1 during KIR-mediated inhibition we have transfected an NK-cell line with a chimeric KIR receptor that contains SHP-1 in place of the KIR cytoplasmic tail. This chimeric receptor inhibits NK-cell function when it binds to HLA-C on target cells (34, 56). Expression of a KIR–SHP-1 chimeric receptor carrying a trapping mutation in the catalytic domain of SHP-1 is currently in use to identify substrates of SHP-1 that are involved in NK activation signals.

Further information on potential SHP-1 substrates can be derived from the crystal structure of the SHP-1 catalytic do-

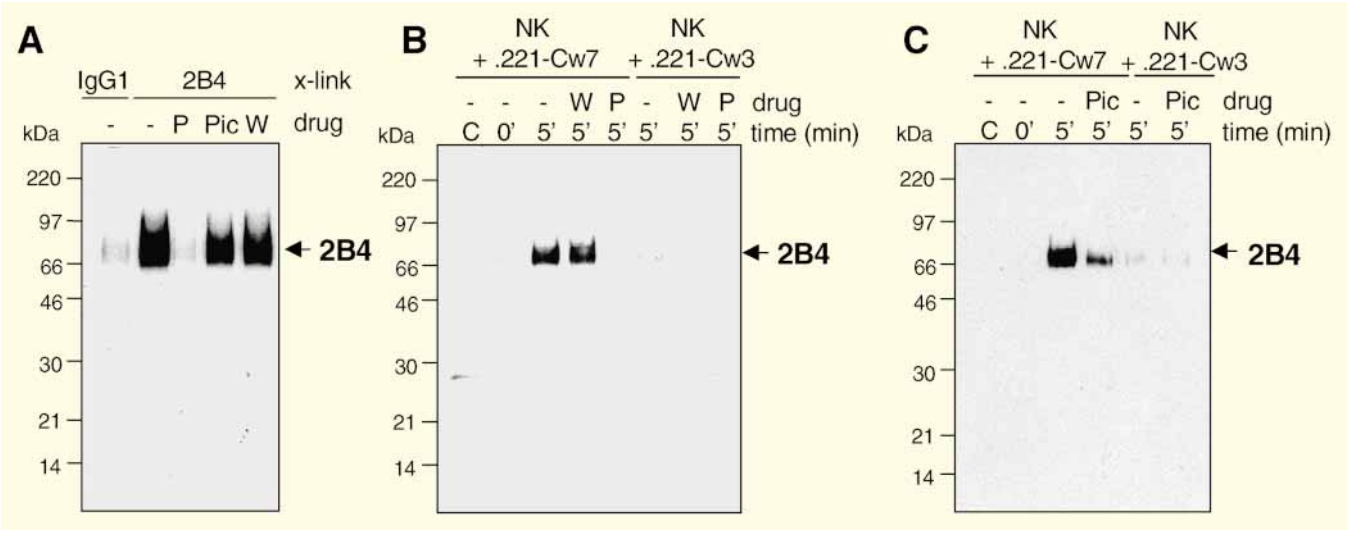

***Fig.* 3. Inhibition of 2B4 tyrosine phosphorylation. A.** Purified human NK cells were incubated in DMSO alone (−), 10 μM PP1 (P), 25 μg/ml piceatannol (Pic), or 100 nM wortmannin (W) for 30 min at 37 °C. NK cells were then treated with a control IgG1 or anti-2B4 antibody, cross-linked with goat anti-mouse antibody and incubated for 5 min at 37 °C as described (34). 2B4 was immunoprecipitated and analyzed by 10–20% SDS-PAGE followed by anti-phosphotyrosine Western blot using biotinylated 4G10. **B, C.** Purified human NK cells were preincubated with inhibitors as described in A, mixed with the target 221-Cw7 or 221-Cw3 at an E/T ratio of 2 and incubated at 37 °C for the indicated amount of time. Note that the HLA-E-negative 221-Cw7 cells were sensitive to killing by the NK cells whereas the HLA-E-expressing 221-Cw3 cells were protected through the engagement of the inhibitory receptor CD94/NKG2A on NK cells (34). Subsequently, cells were lysed and immunoprecipitated with a control IgG1 (C) or anti-2B4. Immunoprecipitates were analyzed as described in A.

main bound to a phosphopeptide (66). Amino acids at positions P−4, P−2, and P+3 relative to the phosphotyrosine contributed to substrate binding. Together with measurements of dephosphorylation of synthetic peptides, these results suggested the sequence motif D/ExL/I/Vx$_n$pYxxL/I/V (n=1 or 2) as a preferred substrate for the catalytic domain of SHP-1. This motif conforms to the ITIM sequence, consistent with the ITIM-containing receptor SIRP serving as docking site and as substrate for the SHP-1 SH2 domains and catalytic domain, respectively (63). However, the precise timing of SHP-1 recruitment by KIR and the exact localization of active SHP-1 are additional parameters that may be critical for substrate selection.

KIR inhibits tyrosine phosphorylation of the activating receptor 2B4 (CD244)

During contact with sensitive target cells, NK cells are activated by the engagement of various surface receptors (67). Although this activation process is poorly understood, it is clear that inhibitory KIR can block several different signaling pathways. To explore how KIR may be interfering with the activation pathway, we studied the influence of KIR on 2B4-mediated NK activation. 2B4 contains four tyrosine motifs (TxYxxI/V) in its cytoplasmic tail, similar to those found in CD84, Ly9, and the T-cell stimulatory receptor CD150. The 2B4 signal can synergize with other ITAM-based activation receptors, such as NKp46 and CD16 (68). Tyrosine-phosphorylated 2B4 binds the small SH2 domain-containing molecule SAP (also called SH2D1A) or the phosphatase SHP-2, but not both at the same time (69). Co-immunoprecipitation suggests a constitutive association of 2B4 with the scaffold protein LAT (70). Patients with the X-linked lymphoproliferative disease who lack a functional SAP molecule have defective NK activation through the 2B4 receptor, implying an essential role of SAP in 2B4 signaling (71–74). However, further insight into the mechanism of NK activation by 2B4 is still missing. Tyrosine phosphorylation of 2B4 can be directly induced by antibody cross-linking, demonstrating that this phosphorylation does not depend on other activating receptors (34). Similarly, in cell mixing experiments, engagement of 2B4 by its ligand CD48 expressed on sensitive target cells resulted in rapid 2B4 phosphorylation. In contrast, 2B4 phosphorylation was inhibited by the binding of either KIR or CD94/NKG2A to their respective MHC class I ligands on target cells (*Fig.* 3) (34). Therefore, inhibitory receptors can interfere with a step as proximal as phosphorylation of an activation receptor.

Although it is possible that SHP-1 dephosphorylated 2B4

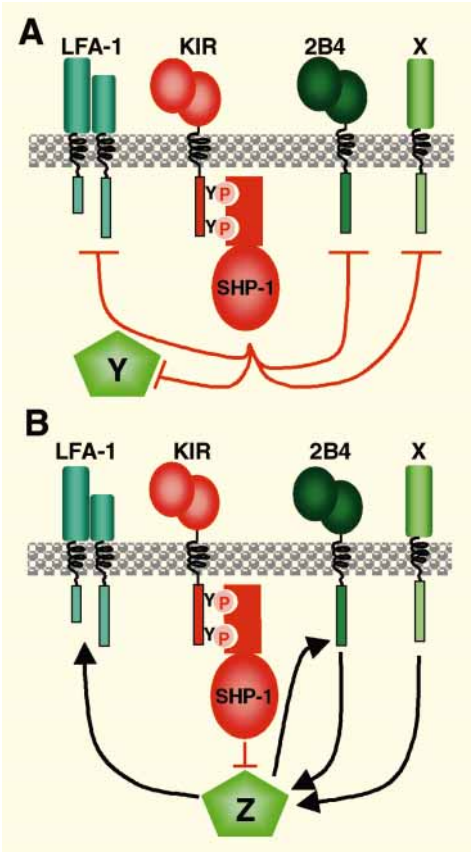

Fig. 4. Models of inhibition by KIR. A. KIR inhibits through the SHP-1-mediated dephosphorylation of multiple targets, such as 2B4, other activation receptors (X), and downstream signaling molecules (Y). **B.** KIR targets a central signaling component (Z) for dephosphorylation that is downstream of 2B4 and other activating receptors (X), and is essential for full 2B4 phosphorylation, target cell adhesion through LFA-1, and NK activation.

directly, even signals that are upstream of 2B4 phosphorylation may be targets for SHP-1 during inhibition. 2B4 phosphorylation depends on the activity of an src-family kinase: overexpression of the src kinase fyn induces 2B4 phosphorylation, and pervanadate-induced 2B4 phosphorylation is greatly reduced in 2B4-transfected JCam cells that lack the src kinase lck (71, 75). Similarly, we found that 2B4 phosphorylation induced by cross-linking with antibodies in normal NK cells was blocked by the src kinase inhibitor PP1 (*Fig. 3A*). In contrast, whereas the syk inhibitor piceatannol did not inhibit 2B4 phosphorylation induced by cross-linking with antibodies, it partially blocked 2B4 phosphorylation induced by target cell mixing (*Fig.* 3). This difference underscores the importance of interpreting results in the context of the experimental system. 2B4 phosphorylation after cross-linking with antibody may depend only on src kinases, such that cross-linked KIR may inhibit by direct dephosphorylation of 2B4 or the relevant src kinase (*Fig. 4A*). In contrast, phosphorylation of 2B4 during mixing with target cells requires more complex signals, making it possible that KIR inhibits by targeting another signaling component of NK-cell activation for dephosphorylation by SHP-1 (*Fig. 4B*).

KIR inhibits NK-cell adhesion to target cells

Target cell killing is the outcome of multiple steps in the NK-cell activation process, with formation of a tight interface between NK and target cells occurring early, and with polarized degranulation occurring at a late step. The β2 integrin leukocyte function-associated antigen (LFA)-1 (CD11a/CD18) plays an important role in adhesion of NK cells by binding to intercellular adhesion molecule-1 (ICAM-1) on target cells. Tight adhesion through LFA-1 is itself dependent on inside-out signals that increase the affinity of LFA-1 and induce cytoskeletal rearrangements (76). Anti-CD11a antibodies blocked the formation of conjugates between the NK-cell line YTS and target cells, underscoring the central role of LFA-1 in the adhesion process (56). The src kinase inhibitors PP1 and herbimycin A reduced conjugate formation and blocked target cell lysis, suggesting a role for specific signals in the tight adhesion between YTS and target cells. The dependence of adhesion on intracellular signals provides potential targets for inhibition by KIR. YTS–target cell adhesion was interrupted rapidly by recognition of MHC class I on target cells by inhibitory KIR (56). The activity of SHP-1 is sufficient for this inhibition, as the same effect was observed using NK cells expressing a KIR/SHP-1 chimera in which the cytoplasmic tail of KIR was replaced with SHP-1. Therefore, KIR can interfere through the activity of SHP-1 with signals that lead to tight adhesion to target cells. The block in adhesion may contribute to the protection of normal cells from NK killing and may allow NK cells to scan potential targets more efficiently by accelerating the release from cells expressing the right MHC class I ligand.

Conclusions

Two models can explain the inhibitory effect of KIR on different elements of the NK activation pathway. Activation of SHP-1 by KIR could result in the dephosphorylation of multiple targets, including candidates such as 2B4 and signaling components of the inside-out signal for LFA-1 (*Fig. 4A*). Alternatively, KIR may target only one or very few central signaling components that are essential for adhesion to target cells and NK-cell cytotoxicity (*Fig. 4B*). The fact that different

tyrosine kinases contribute to 2B4 phosphorylation during target cell contact may reflect a dependence on a central signaling component that controls adhesion and 2B4 phosphorylation. Identification of SHP-1 substrates using a trapping mutant of SHP-1 during inhibition by KIR will put these models to a direct test.

Progress in unraveling the KIR inhibitory pathway is hindered by an incomplete picture of NK activation signals. To dissect the contribution of signals from individual receptors to the various steps in the activation pathway, we are developing systems to reconstitute individual steps such as recognition of ligands by adhesion molecules and activating receptors. Beads coated with either ligands or mAbs to receptors have been used successfully to elicit polarized responses by T cells (77). However, cell-based assays are preferable; for instance, beads coated with chimeric 2DL1-Fc failed to induce clustering of HLA-Cw4 on target cells, indicating that lateral mobility of KIR within the membrane is necessary for clustering (37). Other systems such as planar lipid membranes or transfected cell lines that do not express ligands for NK receptors will approximate the NK–target cell interaction with greater fidelity. Expression of ligands for NK-activating receptors and co-expression of MHC class I molecules in such systems should extend our knowledge of the mechanisms underlying NK activation and the steps in the activation process that are directly inhibited by KIR.

References

1. Ciccone E, et al. Self class I molecules protect normal cells from lysis mediated by autologous natural killer cells. Eur J Immunol 1994;**24**:1003–1006.
2. Cambiaggi A, et al. Natural killer cell acceptance of H2 mismatch bone marrow grafts in transgenic mice expressing HLA-Cw3 specific killer cell inhibitory receptor (CD158b). Proc Natl Acad Sci USA 1997;**94**:8088–8092.
3. Valiante NM, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity 1997;**7**:739–751.
4. Burshtyn DN, Long EO. Regulation through inhibitory receptors: lessons from natural killer cells. Trends Cell Biol 1997;**7**:473–479.
5. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 1999;**17**:875–904.
6. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science 2000;**290**:84–89.
7. Wagtmann N, Rojo S, Eichler E, Mohrenweiser H, Long EO. A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors. Curr Biol 1997;**7**:615–618.
8. Wilson MJ, et al. Plasticity in the organization and sequences of human *KIR/ILT* gene families. Proc Natl Acad Sci USA 2000;**97**:4778–4783.
9. Moretta A, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. J Exp Med 1993;**178**:597–604.
10. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. J Exp Med 1994;**180**:537–543.
11. Long EO, Colonna M, Lanier LL. Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. Immunol Today 1996;**17**:100.
12. Biassoni R, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med 1996;**183**:645–650.
13. Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. J Immunol 1998;**161**:571–577.
14. Valés-Gómez M, Erskine RA, Deacon MP, Strominger JL, Reyburn HT. The role of zinc in the binding of killer cell Ig-like receptors to class I MHC proteins. Proc Natl Acad Sci USA 2001;**98**:1734–1739.
15. Döhring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. J Immunol 1996;**156**:3098–3101.
16. Pende D, et al. The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. J Exp Med 1996;**184**:505–518.
17. Wu J, Cherwinski H, Spies T, Phillips JH, Lanier LL. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. J Exp Med 2000;**192**:1059–1067.
18. Biassoni R, et al. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. Eur J Immunol 1997;**27**:3095–3099.
19. Kim J, Chwae YJ, Kim MY, Choi IH, Park JH, Kim SJ. Molecular basis of HLA-C recognition by p58 natural killer cell inhibitory receptors. J Immunol 1997;**159**:3875–3882.
20. Valés-Gómez M, Reyburn HT, Erskine RA, Strominger JL. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. Proc Natl Acad Sci USA 1998;**95**:14326–14331.
21. Moretta A, et al. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 1995;**182**:875–884.
22. Mandelboim O, et al. Enhancement of class II-restricted T cell responses by costimulatory NK receptors for class I MHC proteins. Science 1996;**274**:2097–2100.

23. Colonna M. Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. Immunol Rev 1997;**155**:127–133.
24. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells [published erratum appears in J Exp Med 2000;**191**:2027]. J Exp Med 1999;**189**:1093–1100.
25. Ponte M, et al. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. Proc Natl Acad Sci USA 1999;**96**:5674–5679.
26. Loke YW, King A. Immunology of human placental implantation: clinical implications of our current understanding. Mol Med Today 1997;**3**:153–159.
27. Vilches C, Rajalingam R, Uhrberg M, Gardiner CM, Young NT, Parham P. KIR2DL5, a novel killer-cell receptor with a D0-D2 configuration of Ig-like domains. J Immunol 2000;**164**:5797–5804.
28. Torkar M, Norgate Z, Colonna M, Trowsdale J, Wilson MJ. Isotypic variation of novel immunoglobulin-like transcript/killer cell inhibitory receptor loci in the leukocyte receptor complex. Eur J Immunol 1998;**28**:3959–3967.
29. Khakoo SI, et al. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. Immunity 2000;**12**:687–698.
30. Rajalingam R, Hong M, Adams EJ, Shum BP, Guethlein LA, Parham P. Short *KIR* haplotypes in pygmy chimpanzee (*Bonobo*) resemble the conserved framework of diverse human *KIR* haplotypes. J Exp Med 2001;**193**:135–146.
31. Kaufman DS, Schoon RA, Robertson MJ, Leibson PJ. Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I. Proc Natl Acad Sci USA 1995;**92**:6484–6488.
32. Valiante NM, Phillips JH, Lanier LL, Parham P. Killer cell inhibitory receptor recognition of human leukocyte antigen (HLA) class I blocks formation of a pp36/PLC-γ signaling complex in human natural killer (NK) cells. J Exp Med 1996;**184**:2243–2250.
33. Carena I, Shamshiev A, Donda A, Colonna M, Libero GD. Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-γ/δ stimulated by nonpeptidic ligands. J Exp Med 1997;**186**:1769–1774.
34. Watzl C, Stebbins CC, Long EO. NK cell inhibitory receptors prevent tyrosine phosphorylation of the activation receptor 2B4 (CD244). J Immunol 2000;**165**:3545–3548.
35. Bléry M, et al. Reconstituted killer cell inhibitory receptors for major histocompatibility complex class I molecules control mast cell activation induced via immunoreceptor tyrosine-based activation motifs. J Biol Chem 1997;**272**:8989–8996.
36. Hof P, Pluskey S, Dhe-Paganon S, Eck MJ, Shoelson SE. Crystal structure of the tyrosine phosphatase SHP-2. Cell 1998;**92**:441–450.
37. Davis DM, Chiu I, Fassett M, Cohen GB, Mandelboim O, Strominger JL. The human natural killer cell immune synapse. Proc Natl Acad Sci USA 1999;**96**:15062–15067.
38. Rajagopalan S, Winter CC, Wagtmann N, Long EO. The Ig-related killer cell inhibitory receptor binds zinc and requires zinc for recognition of HLA-C on target cells. J Immunol 1995;**155**:4143–4146.
39. Rajagopalan S, Long EO. Zinc bound to the killer cell-inhibitory receptor modulates the negative signal in human NK cells. J Immunol 1998;**161**:1299–1305.
40. Fan QR, Long EO, Wiley DC. Cobalt-mediated dimerization of the human natural killer cell inhibitory receptor. J Biol Chem 2000;**275**:23700–23706.
41. Snyder GA, Brooks AG, Sun PD. Crystal structure of the HLA-Cw3 allotype-specific killer cell inhibitory receptor KIR2DL2. Proc Natl Acad Sci USA 1999;**96**:3864–3869.
42. Boyington JC, Motyka SA, Schuck P, Brooks AG, Sun PD. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. Nature 2000;**405**:537–543.
43. Fan QR, Garboczi DN, Winter CC, Wagtmann N, Long EO, Wiley DC. Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. Proc Natl Acad Sci USA 1996;**93**:7178–7183.
44. Burshtyn DN, et al. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. Immunity 1996;**4**:77–85.
45. Burshtyn DN, Yang WT, Yi TL, Long EO. A novel phosphotyrosine motif with a critical amino acid at position-2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. J Biol Chem 1997;**272**:13066–13072.
46. Burshtyn DN, Lam AS, Weston M, Gupta N, Warmerdam PAM, Long EO. Conserved residues amino-terminal of cytoplasmic tyrosines contribute to the SHP-1-mediated inhibitory function of killer cell Ig-like receptors. J Immunol 1999;**162**:897–902.
47. Rojo S, Burshtyn DN, Long EO, Wagtmann N. Type I transmembrane receptor with inhibitory function in mouse mast cells and NK cells. J Immunol 1997;**158**:9–12.
48. Lu-Kuo JM, Joyal DM, Austen KF, Katz HR. gp49B1 inhibits IgE-initiated mast cell activation through both immunoreceptor tyrosine-based inhibitory motifs, recruitment of *src* homology 2 domain-containing phosphatase-1, and suppression of early and late calcium mobilization. J Biol Chem 1999;**274**:5791–5796.
49. Bléry M, Kubagawa H, Chen CC, Vély F, Cooper MD, Vivier E. The paired Ig-like receptor PIR-B is an inhibitory receptor that recruits the protein-tyrosine phosphatase SHP-1. Proc Natl Acad Sci USA 1998;**95**:2446–2451.
50. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of Lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;**11**:141–151.
51. Binstadt BA, et al. Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. Immunity 1996;**5**:629–638.
52. Nakamura MC, Niemi EC, Fisher MJ, Shultz LD, Seaman WE, Ryan JC. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. J Exp Med 1997;**185**:673–684.
53. Maeda A, Kurosaki M, Ono M, Takai T, Kurosaki T. Requirement of SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 for paired immunoglobulin-like receptor B (PIR-B)-mediated inhibitory signal. J Exp Med 1998;**187**:1355–1360.
54. Olcese L, et al. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTPlD protein tyrosine phosphatases. J Immunol 1996;**156**:4531–4534.
55. Bruhns P, Marchetti P, Fridman WH, Vivier E, Daëron M. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. J Immunol 1999;**162**:3168–3175.

56. Burshtyn DN, Shin J, Stebbins C, Long EO. Adhesion to target cells is disrupted by the killer cell inhibitory receptor. Curr Biol 2000;**10**:777–780.
57. Brumbaugh KM, et al. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. J Exp Med 1997;**186**:1965–1974.
58. Binstadt BA, et al. SLP-76 is a direct substrate of SHP-1 recruited to killer cell inhibitory receptors. J Biol Chem 1998;**273**:27518–27523.
59. Palmieri G, et al. CD94/NKG2-A inhibitory complex blocks CD16-triggered Syk and extracellular regulated kinase activation, leading to cytotoxic function of human NK cells. J Immunol 1999;**162**:7181–7188.
60. Zhang W, et al. Essential role of LAT in T cell development. Immunity 1999;**10**:323–332.
61. Peterson EJ, Clements JL, Ballas ZK, Koretzky GA. NK cytokine secretion and cytotoxicity occur independently of the SLP-76 adaptor protein. Eur J Immunol 1999;**29**:2223–2232.
62. Colucci F, et al. Redundant role of the Syk protein tyrosine kinase in mouse NK cell differentiation. J Immunol 1999;**163**:1769–1774.
63. Timms JF, et al. Identification of major binding proteins and substrates for the SH2-containing protein tyrosine phosphatase SHP-1 in macrophages. Mol Cell Biol 1998;**18**:3838–3850.
64. Mizuno K, et al. Src homology region 2 (SH2) domain-containing phosphatase-1 dephosphorylates B cell linker protein/SH2 domain leukocyte protein of 65 kDa and selectively regulates c-Jun NH2-terminal kinase activation in B cells. J Immunol 2000;**165**:1344–1351.
65. Xie ZH, Zhang J, Siraganian RP. Positive regulation of c-Jun N-terminal kinase and TNF-α production but not histamine release by SHP-1 in RBL-2H3 mast cells. J Immunol 2000;**164**:1521–1528.
66. Yang J, Cheng ZL, Niu TQ, Liang XS, Zhao ZZJ, Zhou GW. Structural basis for substrate specificity of protein-tyrosine phosphatase SHP-1. J Biol Chem 2000;**275**:4066–4071.
67. Lanier LL. On guard-activating NK cell receptors. Nat Immunol 2001;**2**:23–27.
68. Sivori S, et al. 2B4 functions as a co-receptor in human NK cell activation. Eur J Immunol 2000;**30**:787–793.
69. Tangye SG, Lazetic S, Woollatt E, Sutherland GR, Lanier LL, Phillips JH. Human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. J Immunol 1999;**162**:6981–6985.
70. Bottino C, et al. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. Eur J Immunol 2000;**30**:3718–3722.
71. Nakajima H, et al. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. Eur J Immunol 2000;**30**:3309–3318.
72. Parolini S, et al. X-linked lymphoproliferative disease: 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein–Barr virus-infected cells. J Exp Med 2000;**192**:337–346.
73. Tangye SG, Phillips JH, Lanier LL, Nichols KE. Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. J Immunol 2000;**165**:2932–2936.
74. Benoit L, Wang X, Pabst HF, Dutz J, Tan R. Defective NK cell activation in X-linked lymphoproliferative disease. J Immunol 2000;**165**:3549–3553.
75. Sayos J, et al. Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4. Int Immunol 2000;**12**:1749–1757.
76. Dustin ML, Chan AC. Signaling takes shape in the immune system. Cell 2000;**103**:283–294.
77. Sedwick CE, Morgan MM, Jusino L, Cannon JL, Miller J, Burkhardt JK. TCR, LFA-1, and CD28 play unique and complementary roles in signaling T cell cytoskeletal reorganization. J Immunol 1999;**162**:1367–1375.