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Slp-76 is a critical determinant of NK cell-mediated recognition of missing-self targets

Kristin Lampe[*], **Mehari Endale**[*], **Siobhan Cashman**[†], **Hao Fang**[‡], **Jochen Mattner**[§], **David Hildeman**[*], and **Kasper Hoebe**[*,¶]

[*]Division of Immunobiology, Cincinnati Children's Hospital Research Foundation, Cincinnati, Ohio, USA

[†]University of Oxford, Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences, Kennedy Institute of Rheumatology, Oxford, United Kingdom

[‡]Division of Infectious Diseases, Cincinnati Children's Hospital Research Foundation, Cincinnati, Ohio, USA

[§]Mikrobiologisches Institut - Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen and Friedrich-Alexander Universität (FAU) Erlangen-Nürnberg, D-91054 Erlangen, Germany

Abstract

Absence of MHC class I expression is an important mechanism by which NK cells recognize a variety of target cells, yet the pathways underlying "missing-self" recognition, including the involvement of activating receptors, remain poorly understood. Using ENU mutagenesis in mice, we identified a germline mutant, designated *Ace*, with a marked defect in NK cell-mediated recognition and elimination of "missing-self" targets. The causative mutation was linked to chromosome 11 and identified as a missense mutation [Thr428Ile] in the SH2 domain of Slp-76—a critical adapter molecule downstream of ITAM-containing surface receptors. The Slp-76 *Ace* mutation behaved as a hypomorphic allele—while no major defects were observed in conventional T cell development/function, a marked defect in NK cell-mediated elimination of β2-Microglobulin (β2M)-deficient target cells was observed. Further studies revealed Slp-76 to control NK cell receptor expression and maturation, however, activation of *Slp-76*$^{ace/ace}$ NK cells through ITAM-containing NK cell receptors or allogeneic/tumor target cells appeared largely unaffected. Imagestream analysis of the NK-β2M$^{-/-}$ target cell synapse, revealed a specific defect in actin recruitment to the conjugate synapse in *Slp-76*$^{ace/ace}$ NK cells. Overall these studies establish Slp-76 as a critical determinant of NK cell development and NK cell-mediated elimination of missing-self target cells.

[¶]Correspondence to: Kasper Hoebe; Cincinnati Children's Hospital Research Foundation; MLC7021, Room S5.421; 3333 Burnet Avenue; Cincinnati, OH USA; phone: +1 (513) 803-1056; fax: +1 (513) 636-5355; kasper.hoebe@cchmc.org.

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Introduction

NK cells are able to recognize and eliminate numerous target cells, including tumor cells, allogeneic cells, or pathogen-infected cells[1–4]. Activation of NK cells can occur through cytokines such as IL-12 and IL18, but also involves the integrated signals derived from inhibitory and activating surface receptors expressed on NK cells. Specifically, ligation of activating receptors expressed on NK cells such as FcγRIIIA, activating Ly49 receptors (i.e. Ly49D, Ly49H), natural-killer group 2, member C and D (NKG2C and NKG2D) and natural cytotoxicity receptor NKp46, drive signaling via adaptor molecules containing ITAMs. The Src-family adaptors CD3ζ and DAP12 are critical for NK cell activation downstream of activating receptors[5] and are highly conserved between various lymphocyte subsets including T cells.

Inhibitory signals involve NK cell-mediated recognition of constitutive expression of major histocompatibility complex (MHC) class I molecules through surface receptors either directly or indirectly[2, 6–9]. In mice, direct recognition of MHC class I molecules is mediated by members of the Ly49 family (i.e. Ly49I). Alternatively, indirect recognition occurs through CD94/NKG2A receptor binding of MHC-derived leader peptides expressed by Qa1—a non-classical MHC class I. More recently, interaction between the inhibitory receptor Ly49A with the non-classical MHC locus H2-M3 was found to assists in the "licensing" of $Ly49A^+$ NK cells in C57BL/6J mice[9]. Specifically, interaction between $Ly49A^+$ and H2-M3 resulted in fully mature NK cells, highly competent to recognize and eliminate infected or neoplastic cells without attacking self[9]. The inhibitory signals involve ITIM-mediated recruitment of the lipid phosphatase SHIP-1 and tyrosine phosphatases SHP-1 and SHP-2 that target tyrosine phosphorylation of ITAM motifs. When these inhibitory receptors are not engaged by MHC-I molecules— a condition referred to as "missing self"— the inhibitory signals are lost and activation of NK cells ensues. Biologically, missing-self is an important mechanism by which tumor cells, often exhibiting reduced MHC-I expression, are targeted[8, 10]. Importantly, absence of MHC-I expression alone is sufficient to activate NK cells. This process however requires "education" or "licensing" of NK cells i.e. prior interaction of inhibitory NK cell receptors with cognate MHC-I molecules resulting in competent "killer" cells. The importance of education/licensing is illustrated by the observation that MHC-deficient hosts (e.g. *β2M*-deficient) fail to recognize missing-self targets. Notably, the ability of healthy hosts to recognize and eliminate syngeneic *β2M*-deficient cells suggests the expression of ligands for activating NK receptors on these cells. The identification of these ligands and the molecular pathways underlying missing-self recognition remain incompletely understood.

In previous work we described two independent ethyl-N-nitrosourea (ENU) germline mutants, *Chip* —carrying a missense mutation in the ITSM motif of CD244— and a second mutant line designated *Ace* [11]. Both mutant lines failed to recognize and eliminate missing-self targets. Here we identify the causative mutation for the *Ace* phenotype as a missense mutation in Slp-76 resulting in impaired NK cell development and function. The studies provide new insight into the molecular pathways underlying missing-self recognition.

RESULTS

Identification of Ace—an ENU germline mutant with impaired "missing-self" target clearance

Using an ENU mutagenesis approach, we previously reported a germline mutant—designated *Ace* —that exhibited a reduced capacity to eliminate *β2M*-deficient target cells as determined by *in vivo* cytotoxicity assay[11]. The G3 mouse was selected for breeding with C57BL/6J mice to remove non-relevant ENU mutations and a homozygous colony was established that was used for further phenotypic characterization and genetic analysis. The *Ace* mutation exhibited a Mendelian distribution and behaved as a strictly recessive trait—heterozygote mutant mice were unaffected in their ability to eliminate *β2M*-deficient target cells (results not shown). Moreover, whereas the *Ace* mutation seemed to impair NK cell function, homozygote *Ace* mice showed a normal capacity to mount antigen-specific $CD8^+$ T cell responses following immunization[11], suggesting a selective defect in the NK but not $CD8^+$ T cell development/function.

The NK phenotype in Ace mice is due to a Thr428→Ile missense mutation in Slp-76

The causative mutation in *Ace* mice was identified by coarse mapping and whole genome sequencing (WGS). Specifically, *Ace* C57BL/6J homozygotes males were outcrossed to C57BL/10J females and female F1 offspring were backcrossed to homozygote males. A total of 21 offspring (8 mutant- and 13 wildtype-phenotypes) were analyzed for both phenotype and genotype as described before[11]. Coarse mapping revealed a single peak with a LOD score of ~4.37 for adjacent SNPs rs6371316 (chr.11:38,330,231bp) and rs13481003 (chr.11:44,730,640bp) (Figure 1A). The entire critical region was defined by proximal marker rs13480859 (8,478,423bp) and distal marker rs6371316 (chr.11:38,330,231 bps) and consisted of ~29.8Mbp genomic DNA containing 230 annotated genes as defined by the NCBI build GRCm37-C57BL/6J database. Within the critical region, only one mutation was identified that affected coding sequence. This particular nucleotide change (C→T at 33,988,067bp) represented a non-synonymous mutation causing an amino-acid change (T428I) in the Src homology domain of the adapter molecule Slp-76 (aka Lcp2) (Figure 1B,C). Because the T428I mutation was predicted to be damaging (Polymorphism Phenotyping v2[12]) and the only mutation affecting coding sequence, and because Slp-76 is expressed in NK cells serving a role as adapter molecule downstream of ITAM receptors[13–15]—— we considered this mutation to be causal for the observed phenotype in *Ace (*from here on *Slp-76*$^{ace/ace}$) mice. To confirm allelic causality, we generated compound heterozygote *Slp-76*$^{null/ace}$ mice by crossing heterozygote *Slp-76* null mice with homozygote *Slp-76*$^{ace/ace}$ mice. The compound heterozygote F1 offspring were viable and exhibited a similar, albeit slightly more dramatic, reduced recognition and killing of $\beta2M^{-/-}$ target cell *in vivo* than regular *Slp-76*$^{ace/ace}$ mice (Figure 1E). These findings unambiguously confirm the *Ace* mutation in *Slp-76* to be causal in the impaired recognition of missing-self targets.

The T428I Ace mutation is a hypomorphic Slp-76 allele resulting in normal T cell development

Slp-76 expression is observed in a variety of hematopoietic cells including lymphocytes, myeloid cells and platelets (reviewed in [16]). Immunoblot analysis of total spleen, isolated NK cells or $CD8^+$ T cells revealed a markedly reduced Slp-76 protein expression in spleen, NK cells and $CD8^+$ T cells (Figure 1F). In T cells, Slp-76 is a critical adapter involved in TCR signaling; *Slp-76* null mice exhibit impaired thymic development and T cells fail to develop beyond stage III at the double-negative stage[13, 17]. Interestingly, we identified *Slp-76*$^{ace/ace}$ mice because of their aberrant missing-self recognition, while they appeared to exhibit normal antigen-specific $CD8^+$ T cell responses. We thus performed a careful examination of T cell development/function in *Slp-76*$^{ace/ace}$ mice. Flow cytometric analysis of the thymus revealed a normal T cell development with comparable numbers of DP-CD4/CD8, SP-CD4 and SP-CD8 cells between wildtype and *Slp-76*$^{ace/ace}$ mice (Figure S1A). In addition, no abnormalities were observed in the DN population, where similar percentages of stage I-IV cells were observed between wildtype and *Slp-76*$^{ace/ace}$ (Figure S1A). Moreover, peripheral $CD4^+$ and $CD8^+$ T cell populations in the spleen were unaffected (Figure S1B). Analysis of compound heterozygote *Slp-76*$^{null/ace}$ revealed similar results with regard to T cell development (results not shown). Further analysis revealed that *Slp-76*$^{ace/ace}$ T cells (and NK cells) express normal class I MHC expression (Figure S1C) and to functionally assess T cells, we enumerated $CD8^+$ T cell responses following immunization with irradiated 5E1-TAKO cells (1×10^6 cells/mouse s.q.). After 8 days, splenocytes were isolated and restimulation *ex vivo* with $E1B_{192–200}$ peptides revealed no differences in the number/percentage of antigen-specific $IFN\gamma^+$ $CD8^+$ T cells (Figure S1D). Moreover, no defects in secondary expansion of antigen-specific $CD8^+$ T cells were observed (Figure S1E). Together these data suggest that the missense mutation in the SH2 domain causes a hypomorphic *Slp-76* allele that has limited effect on conventional T cell development and function.

Slp-76$^{ace/ace}$ NK cells exhibit altered NK cell receptor expression

To provide detailed insight into the NK cell defects in *Slp-76*$^{ace/ace}$ mice, we next characterized NK cell maturation (defined by CD11b and CD27 surface expression) and surface expression of activating/inhibitory NK cell receptors. Whereas overall NK cell numbers in *Slp-76*$^{ace/ace}$ mice were unaffected (Figure 2A), a subtle but significant increase in the percentage of mature NK cells ($CD11b^+;CD27^-$), was observed in the spleen (Figure 2B). Consistent with an increased percentage of mature NK cells, surface expression of KLRG-1 was slightly increased for the overall NK cell populations, while the percentage of $KLRG1^+$ NK cells within $CD27^+/CD11b^-$, $CD27^+/CD11b^+$ or $CD27^-/CD11b^+$ NK cell subsets remained identical (Figure 2C). The increased maturation of NK cells was more pronounced in the BM, where ~50% of *Slp-76*$^{ace/ace}$ NK cells were $CD11b^+;CD27^-$ compared to ~25% in wildtype (Figure S2A). We next determined surface expression of NK cell receptors in various lymphoid tissues. Interestingly, slight but significant changes in Ly49 expression were observed in the BM from *Slp-76*$^{ace/ace}$ mice with a reduced percentage of $Ly49G2^+$ and $Ly49D^+$ NK cells in mutant mice (Figure S2B). In the spleen a significant reduction was observed in in the percentage of NK cells that express Ly49A,

Ly49G2, Ly49F, and Ly49D whereas the percentage of NKG2A$^+$ NK cells was increased (Figure 2D). Similar results were observed for NK cell surface expression in compound heterozygote *Slp-76$^{null/ace}$* mice (Supplemental figure 2D). These effects were more pronounced in NK cells from peripheral LNs where a marked decrease in the number of Ly49I, Ly49G2, Ly49D, Ly49H and NKG2A positive NK cells were observed (Figure 2E). We next compared the percentage of NK cells that express 0, 1, 2, 3 or 4 Ly49 inhibitory receptors (i.e. NK cells expressing Ly49I, Ly49G2, Ly49A, and/or Ly49F). Compared to WT mice, *Slp-76$^{ace/ace}$* mice had significantly more NK cells that lacked all 4 inhibitory receptors; whereas a reduced percentage of *Slp-76$^{ace/ace}$* NK cells expressed 1 or 2 Ly49 inhibitory receptors (Figure 2F). On the other hand, the percentage of NK cells that expressed 3 or 4 inhibitory Ly49 receptors was similar if not increased in *Slp-76$^{ace/ace}$* mice (Figure 2F). Overall, these data reveal that Slp-76 expression controls NK receptor expression in a tissue specific manner—observations that are largely in agreement with data obtained from Slp-76 null mice[18].

The Slp-76^{ace} mutation has limited impact on activating NK cell receptors in vitro

Slp-76 is thought to function as a critical adapter molecule downstream of NK cell receptors that utilize CD3ζ, or DAP12 (reviewed in [15]). Absence of Slp-76 may therefore affect signaling downstream of activating NK cell receptors and we thus assessed the ability of *Slp-76$^{ace/ace}$* NK cells to induce IFNγ and degranulate upon activation with NK cell stimuli. Specifically, NK cells from wildtype and *Slp-76$^{ace/ace}$* mice were stimulated with plate-bound anti-NK1.1(PK136), Ly49D, NKG2D, PMA/Ion, or exposed to YAC-1, RMA-RAE1, B16 melanoma cells or IL-12/IL18 in the presence of IL-2 *ex vivo*. IFNγ production and CD107 surface expression were similar between C57BL/6J wildtype and *Slp-76$^{ace/ace}$* NK cells following activation with various stimuli (Figure 3A–D). Also in the absence of IL-2, a dose response of plate-bound NK1.1 stimulation of WT or *Slp-76$^{ace/ace}$* NK cells resulted in similar intracellular accumulation of IFNγ and/or CD107 surface expression (Figure 3E,F). To assess changes or defects in proximal signaling pathways we performed immunoblot analysis on phosphorylated and total VAV1, PLCγ2, AKT and ERK on PMA/Ion-stimulated LAK cells from C57BL/6J or *Slp-76$^{ace/ace}$* mice (Figure S3A). With the exception of Slp-76 expression itself, no major differences in the overall expression and activation of downstream signaling components were found (Figures S3 A,B), confirming a normal intrinsic activation cascade in *Slp-76$^{ace/ace}$* NK cells following activation via this receptor. When NK cells were exposed to plate bound NK1.1 or β2M-deficient target cells, no significant differences in ERK phosphorylation were observed for NK1.1, while *Slp-76$^{ace/ace}$* NK cells exhibited a reduced ERK phosphorylation to β2M-deficient target cells (Figure 3G,H). We next assessed whether the reduced recognition of missing-self targets was due to a selective impairment of “licensed” (Ly49I$^+$) NK cells. Incubation of WT or *Slp-76$^{ace/ace}$* NK cells with β2M-deficient blast cells resulted in overall reduced IFNγ production and CD107 surface expression, independent of the expression of Ly49I expression (Figure 3I,J). Finally, NK cells from *Slp-76$^{ace/ace}$* mice were equally effective in killing YAC-1, RMA-RAE1 and B16 target cells (Fig. 3K–M), suggesting a normal cytolytic potential of *Slp-76$^{ace/ace}$* NK cells. Overall, these data suggest that the *Slp-76$^{ace/ace}$* allele caused a selective impairment of NK cell function with normal activation of NK cells observed following exposure to tumor cells, IL12/IL18 or NK1.1, Ly49D or NKG2D

activating antibodies. These findings also suggest that NK cells in *Slp-76*$^{ace/ace}$ mice are fully "educated" and possess an efficient capacity to kill tumor cells.

Aberrant missing-self but normal allogeneic target cell recognition by Slp-76$^{ace/ace}$ NK cells in vivo

To further define NK cell defect in *Slp-76*$^{ace/ace}$ mice, we assessed their ability to eliminate *β2M*-deficient and allogeneic (BALBc/J) target cells. Using an *in vivo* cytotoxicity assay we injected C57BL/6J, *β2M*$^{-/-}$ and BALB/cJ splenocytes in wildtype, *Slp-76*$^{ace/ace}$ mice and NK-depleted controls (anti-asialo GM-1 treated). While a significant reduction in the elimination of *β2M*$^{-/-}$ deficient cells in *Slp-76*$^{ace/ace}$ mice was observed, allogeneic BALB/cJ splenocytes were effectively removed compared to C57BL/6 wildtype mice (Figure 4A,B), again suggesting a target cell-specific NK cell defect in *Slp-76*$^{ace/ace}$ mice. Thus, both allogeneic and NK1.1-driven activation are separable from "missing-self" recognition on the basis of their differential dependence upon Slp-76.

Improved recognition of missing-self targets in Slp-76$^{ace/ace}$ mice containing a congenic SV129.NKC (Ly49) cluster

Previous work suggest the 129 congenic NKC cluster to mediate a more potent recognition of missing-self targets when exposed to MHC-I deficient target cells[19]. To further provide insight into the role of Slp-76 on NK cell receptors involved in missing-self recognition, we generated a congenic mouse strain containing a 129 NK cell receptor complex on chromosome 6 (*C6.NKC*$^{129/129}$) defined by SNPs rs6200835 (~126.4 Mb) to rs3658783 (145.2 Mb) (Figure 5A). The region contains significant cluster variation both in duplication of Ly49 receptors as well as polymorphisms within the Ly49 receptors between both strains, with 129/SvJ mice carrying an increased number of (inhibitory) receptors compared to C57BL/6J[20, 21]. We thus crossed the *C6.NKC*$^{129/129}$ congenic region onto a homozygote *Slp-76*$^{ac/acee}$ background, allowing us to assess the role of Slp-76 on Ly49 haplotype variance following target cell exposure. Interestingly, whereas no differences were observed for the recognition of allogeneic cells, the recognition and elimination of *β2M*$^{-/-}$ targets was markedly increased in congenic *C6.NKC*$^{129/129}$; *Slp-76*$^{+/+}$ mice (Figure 5B). When bred to a *Slp-76*$^{ace/ace}$ background, a single NKC^{129} allele improved elimination of *β2M*$^{-/-}$ target cells to wildtype C57BL/6J levels, whereas homozygote *C6.NKC*$^{129/129}$;*Slp-76*$^{ace/ace}$ mice showed increased elimination of *β2M*$^{-/-}$ target cells compared to C57BL/6J mice albeit with a lower efficiency then *C6.NKC*$^{129/129}$;*Slp-76*$^{+/+}$ mice. These data are consistent with previous observations[19], but again suggest that NK cell education/licensing *per se* does not depend on functional Slp-76.

Slp-76 affects actin recruitment to the interface in a target cell-specific manner

Previous work showed that Slp-76 interacts with the adapter molecule Nck and the Rho-family guanine nucleotide exchange factor (GEF) VAV1, mediating WASp activation and F-actin rearrangement towards the immune synapse in DC-T cell conjugates[22]. We therefore examined the ability of *Slp-76*$^{ace/ace}$ NK cells to form conjugates with either missing-self or allogeneic target cells and assessed their ability to recruit actin to the conjugate interface (CI). MACS-isolated NK cells were cocultured *in vitro* for 0′, 1′, 2′, 5′,

and 10′ with either Cell-trace violet-labeled β2M$^{-/-}$ or BALBc/J blasts. The actin-specific recruitment was determined following intracellular phalloidin staining and the mean pixel density (MP) was determined in NK cells masked either on the interface of NK cells versus MP in the non-interface membrane for each NK-target cell conjugate (Figure 6A). This approach allows for a quantitative, population-based, analysis of actin-rearrangement at the CI between NK and target cells. Interestingly, whereas actin recruitment to the interface was observed in WT NK cells after 1 minute exposure to *β2M*$^{-/-}$-target cells, NK cells from *Slp-76*$^{ace/ace}$ mice failed to recruit actin to the interface (Figure 6B, D). In contrast, exposure of C57BL/6J or *Slp-76*$^{ace/ace}$ NK cells to BALB/cJ target cells resulted in an early actin recruitment followed by a displacement of actin at the interface that was followed by a robust late (10′) actin recruitment (Figure 6C,E). Interestingly, *Slp-76*$^{ace/ace}$ NK cells exhibited a similar yet slightly delayed profile of actin recruitment (Figure 6C,E).

To confirm that these observations indeed correlated with impaired killing, we performed an overnight *in vitro* killing assay incubating *β2M*$^{-/-}$- or BALBc/J- target cells with different ratios of C57BL/6J or *Slp-76*$^{ace/ace}$ NK cells. Consistent with the *in vivo* data, *Slp-76*$^{ace/ace}$ NK cells exhibited a reduced killing of β2M-deficient cells (Figure 6E), while a similar killing of BALBc/J cells was observed (Figure 6F), suggesting the slightly delayed actin recruitment *in vitro* had limited impact on allogeneic target cell killing.

Together, these data suggest that the elimination of missing self-targets by NK cells, involves Slp-76-mediated actin recruitment. In contrast, interaction with allogeneic cells resulted in slightly delayed actin recruitment to the CI in *Slp-76*$^{ace/ace}$ NK cells that had little effect on the cytolytic potential.

Discussion

Here we describe a novel ENU germline mutant carrying a hypomorph mutation in the SH2 domain of Slp-76 that affects NK cell function but displayed limited effect on conventional T cell development/function. Specifically, NK cells showed alterations in NK cell receptor surface expression and exhibited a decreased recognition of missing-self targets, while signaling of ITAM-dependent NK cell receptors appeared unaffected.

Previous work established a critical role for Slp-76 in T cell development[13]. The selective defect on NK cells, but not T cells, in *Slp-76*$^{ace/ace}$ mice may be due to a number of factors. Firstly, immunoblot analysis suggests that the *Ace* mutation caused a marked loss of Slp-76 expression in NK cells, while significant expression was observed in T cells. The latter correlated with an overall increased baseline Slp-76 expression in T cells compared to NK cells. Moreover, studies using Slp-76 SH2-deletion mutants showed improved survival of T cells in the thymus and periphery compared to complete KO mice[23]; suggesting a limited requirement for the Slp-76 SH2 domain in T cell development. Functionally, the Slp-76 SH2 domain serves as a docking site for proteins such as the Adhesion- and Degranulation-promoting Adapter Protein (ADAP)[24, 25] and/or Hematopoietic progenitor kinase 1 (HPK1)[26]. ADAP is responsible for TCR-induced integrin activation, whereas HPK1 serves as a negative regulator of TCR signaling[27, 28]. Importantly, ADAP-deficient mice have relative normal NK cell development and cytolytic function, while they exhibit a

reduced NK cell-specific cytokine production [29, 30]. In addition, to our knowledge no role for HPK1 in NK cell development/function has thus far been reported. The more profound effect of the *Ace* mutation on Slp-76 function in NK cells is the overall loss of Slp-76 protein expression, thus affecting all pathways linked to Slp-76 function. These include the N-terminal tyrosine residues that are critical for activation of Vav1, Itk—a tyrosine kinase important for PLCγ activation, and recruitment of Nck1—an adapter critical for actin-recruitment. The marked loss of Slp-76 expression in *Slp-76*$^{ace/ace}$ NK cells is expected to impair all receptors that require Slp-76 for their activation.

The role of Slp-76 in NK cell function has been studied before with ambiguous results. Early studies suggested Slp-76 to be dispensable for NK cell function[31], while more recent work suggested NK cells to be critical for activation of Ly49D[18]. Slp-76 is thought to play a role in activating NK cell receptors that utilize CD3ζ or DAP12 for signaling. Our studies show that the hypomorphic *Ace* mutation in Slp-76 does not affect activation of NK cells through the NKG2D, Ly49D or NK1.1 pathway, while killing of allogeneic blasts and tumor cells is relatively normal. Nonetheless, *Slp-76*$^{ace/ace}$ NK cells showed marked changes in surface expression of activating/inhibitory receptors. Interestingly, Ly49I expression—an inhibitory receptor associated with NK cell education—appeared unaffected in the BM and spleen albeit slightly reduced in NK cells from LNs. Moreover, the *Ace* mutation had limited effect on the killing of missing-self targets in congenic *C6.NKC*$^{129/129}$ mice— exhibiting an increased number of self-recognizing inhibitory receptors. These observations suggest that Slp-76 is not required for “education” or “licensing” of NK cells.

Our finding that Slp-76 is important for NK cell-mediated elimination of missing-self targets is intriguing. Missing-self is thought to result in loss of inhibitory signals via ITIM-motifs within CD94/NKG2A, Ly49I and Ly49A[9, 19, 32]. The inhibitory Ly49 receptors can bind to MHC class I expressed on the same surface of NK cells (*in cis*) or can bind to class I MHC expressed on other cells (*in trans*)[33–35]. Previous studies suggested that *cis* interactions may play a role in NK cell education/licensing and limit inhibitory receptor recruitment to the immune synapse thereby reducing the inhibitory signals mediated by Ly49 inhibitory receptors[36, 37]. However, NK cells of *Slp-76*$^{ace/ace}$ mice exhibit normal class I MHC expression (Suppl. Figure 1C) and importantly, the NK cell response towards β2M-deficient target cells results in an overall reduced effector function (i.e. IFNγ production and CD107 surface expression) independent of Ly49I expression, again suggesting a defect independent of education/licensing. Nonetheless, the percentage of NK cells expressing inhibitory receptors relevant to missing-self-recognition (Ly49A, Ly49I, and/or NKG2A) is reduced in *Slp-76*$^{ace/ace}$ mice. The difference in Ly49A and Ly49I expression however is subtle and appears to be tissue specific— i.e. more or less pronounced in lymph nodes (Figure 2D). The slight changes observed may contribute but are not likely a main cause for impaired recognition of missing-self targets. More importantly, we identified a critical role for Slp-76 in actin recruitment to the conjugate interface between NK cells and *β2M*$^{-/-}$ target cells (Figure 5). Previous work in T cells has shown that Slp-76 is a critical regulator of actin-rearrangement following TCR signaling[38, 39], mediating recruitment of the NcK and activation of the Wiskott-Aldrich syndrome protein (WASp)/WAVE complex. Our previous studies indicate that actin-rearrangement is

essential for missing-self recognition in that mice deficient in the WAVE complex exhibit impaired elimination of *β2M*$^{-/-}$ cells[40]. Although our studies implicate a role for Slp-76 in the recognition/elimination of missing-self targets; the receptors involved in the activation of Slp-76 following interaction with β2M$^{-/-}$ cells, still remain elusive. Slp-76 activation can occur downstream of a number of receptor signaling pathways. Besides its involvement in signaling via the CD3/DAP12 pathways, Slp-76 has been implicated in integrin inside-out signaling, mediating a conformational change in LFA-1 and high affinity binding to ICAM-1. Studies in our lab, however, revealed comparable changes in LFA-1 affinity between C57BL/6J and *Slp-76*$^{ace/ace}$ cells following activation of NK cell receptors or PMA/ionomycin (results not shown). Moreover, previous work revealed a mild impact on NK cell-mediated killing of MHC-I-deficient targets in LFA-1-deficient (Itgb2) mutant mice[41], suggesting limited involvement of this pathway in missing-self recognition.

In conclusion, our data reveal a critical role for Slp-76 in actin recruitment following exposure to target cells. The non-redundant role of Slp-76 was predominantly observed for missing-self targets while NK cell-mediated killing of allogeneic and various tumor targets remained largely unaffected. Moreover, TCR as well as ITAM-dependent NK cell receptor signaling appeared unaffected; suggesting a non-canonical Slp-76 pathway to be involved in missing-self-recognition in NK cells.

Methods

Mice and reagents

All experiments were performed according to the US National Institutes of Health guidelines and were approved by the IACUC of The Cincinnati Children's Hospital. C57BL/6J and C57BL/10J mice were obtained from Jackson Laboratory. ENU (ENU-Isopac; Sigma-Aldrich) mutagenesis was performed as previously described[42]. *Slp-76*$^{ace/ace}$ mice were on a complete C57BL/6J background and housed as homozygote breeders. Wildtype or *Slp-76*$^{ace/ace}$ congenic *C6.NKR*$^{129/129}$ mice were housed as homozygote stocks and all strains were confirmed for C57BL/6J background purity performing whole genome SNP analysis.

In vivo cytotoxicity assay to assess NK and CD8^{+} T cell responses

G3 ENU germline mice were assessed *in vivo* for their ability to recognize and eliminate target cells representing "missing-self" (*β2M*-deficient; NK cell targets) and/or antigen-specific target cells (CD8^{+} T cell targets) as described before[43]. Where indicated, allogeneic BALB/cJ splenocytes were labeled with CFSE and served as NK cell targets.

SNP genotyping and whole genome mapping

To establish critical regions for the *Ace* mutation, we performed low resolution mapping using a custom designed genome-wide SNP map with 150 markers informative for the C57BL/6J and C57BL/10J genetic backgrounds[11]. Genotyping was performed using the Illumina GoldenGate Assay as described before[44].

Whole genome sequencing

Genomic DNA was sequenced on an Illumina Genome Analyzer IIx using the paired-end protocol and collecting 40 bases from each read. Read alignment against the mouse genome was performed using the CASAVA software from Illumina. The software program SeqMate was used for variant identification and post-alignment for read sequence visualization as described before[45]. Identified mutations in the *Ace* critical region were confirmed by PCR and Sanger sequencing using an Applied Biosystems 3730xl DNA Analyzer.

Flow cytometry

Isolated lymphocytes from spleen, peripheral lymph nodes (LN) or bone marrow (BM) were incubated with mixtures of fluorochrome-labeled antibodies directed against mouse NK cell receptors/surface markers including NKp46 (29A1.4), NK1.1 (PK136); Ly49A (A1); Ly49H (3D10); Ly49D (4E5); Ly49F (HBF-719, Southern Biotec); Ly49G2 (4D11); Ly49I (YLI90); NKG2D (CX3); NKG2A (16A11); CD11b (MI/70); CD27 (LG.3A10); CD16/32 (93) and $H2D^b$/$H2K^b$ (28-8-6). All antibodies were obtained from eBioscience and/or Biolegend unless otherwise indicated. Cells were analyzed on the FACSCanto (BD Biosciences) using FlowJo Software for analysis.

NK cell assays in vitro

MACS-isolated NK cells (1×10^5/mL) were cultured in complete RPMI (5%FBS, 1%penicillin/streptomycin and 50μM L-glutamine) plus 500U/ml recombinant human IL-2 (Miltenyi Biotec) and stimulated with either plate-bound NK1.1 (5–10μg/mL; 96-well overnight incubation at 4 C°, 50μL/well), PMA(50ng/ml)/ionomycin(1μg/mL), cytokines (IL12p70 [0.1μg/mL] and IL18 [1μg/mL]) or various target cells (RMA-RAE-1, YAC1, or B16) in the presence of GolgiPlug (BD Biosciences). To assess degranulation, CD107 (1D4B) antibodies were added to the medium and after 5 hours of incubation, cells were isolated, stained with anti-NKp46 (29A1.4) PBS+10%FCS (4°C) and fixed with fixation/permeabilization buffer (eBioscience) followed by intracellular staining for IFNγ. IFNγ was quantified by Flow cytometry.

The cytolytic potential of NK cells was assessed after coculturing MACS isolated NK cells with various tumor cells or concanavalinA(ConA; 5μg/mL)-stimulated *β2M*$^{-/-}$ or BALB/cJ) splenocytes in RPMI complete at indicated ratios. Target cell killing was determined after overnight incubation using a LDH assay (Pierce).

Immunoblotting

Immunoblot analysis was performed using lysates from total spleen or MACS-isolated NK, or $CD8^+$ T cells (Miltenyi) or LAK cells (MACS-isolated NK stimulated with 500U/ml rec.hIL-2 for 5 days; NK cell purity was generally ≥80% based on NKp46 expression). Immunoblotting were performed as described previously[46]. Antibodies against Slp-76, total ERK and pERK, pPLCγ2, tPLCγ2, VaV1, pAkt, tAkt and ADAP were obtained from Cell-signaling.

Conjugate analysis of NK-target cell interaction using Imagestream

To examine conjugate formation between NK cells and target cells, we performed Imagestream (Amnis) analysis. Briefly, conA (5 μg/mL)-stimulated *β2M*$^{-/-}$ or BALB/cJ splenocytes were cultured in IMDM complete medium for 3 days. Hereafter, blasts were collected and labeled with CellTrace™ violet (LifeTechnologies). Subsequently, NK cells were isolated using a negative NK cell isolation kit (Miltenyi Biotec) and 1×10^6 NK cells were cocultured with either 1×10^6 *β2M*$^{-/-}$ or 1×10^6 BALB/cJ blasts (1:1 ratio) *in vitro* for the indicated time points at 37°C in RPMI (40μL volume; 5% FCS). Subsequently, cells were transferred to ice, stained with anti-NKp46, washed at 4°C and fixed in 1% formalin. Cells were permeabilized using permeabilization buffer (eBioscience) and labeled with AF647-labeled Phalloidin to stain F-actin. Conjugate analysis was performed on doublet cells containing one NK and one target cell. The actin-specific mean pixel density (MP) was determined in NK cells masked either on the interface of NK cells versus MP in the non-interface membrane.

Statistical analysis

Data were analyzed using the GraphPad Prism4® software (GraphPad Software, San Diego, CA). Statistical significance of the differences between groups was determined from the mean and standard deviation by Student's two-tailed test or by ANOVA followed by Dunnett's test for three or more groups. Data was considered significant when $P<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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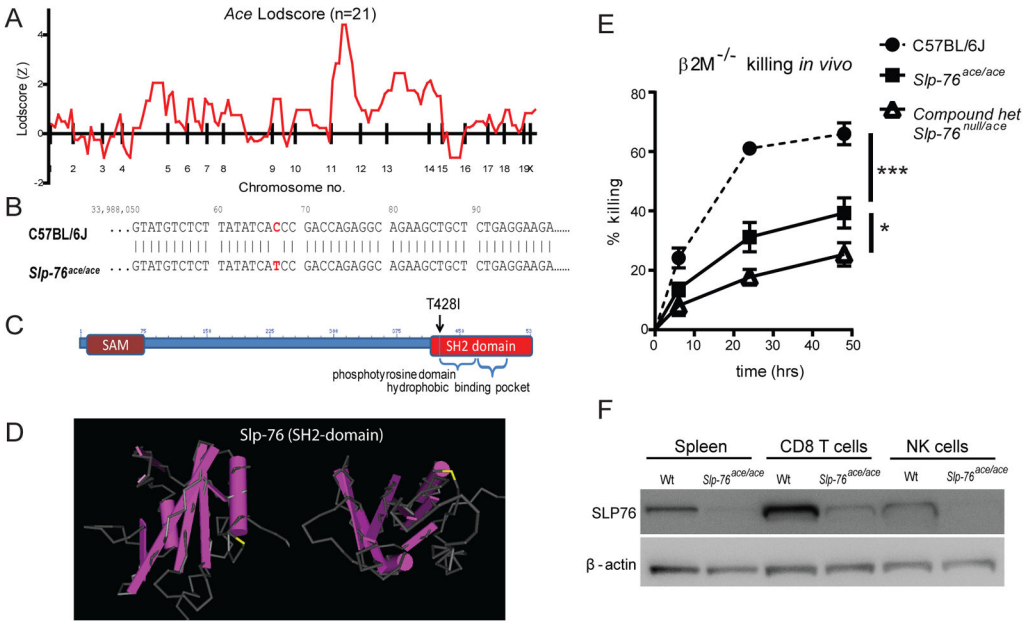

Figure 1. Identification of a missense mutation in the SH2 domain of Slp-76 as the causative variant responsible for impaired missing self-recognition in *Ace* mutants

(**A**) Coarse mapping of the *Ace* mutation based on twenty-one mice (mutants, wildtype phenotype) and a panel of 150 SNPs covering the entire genome, revealed linkage of the *Ace* phenotype to chromosome 11. (**B–D**) the C→T missense mutation (**B**) causes a single amino-acid change in the SH2 domain of Slp-76 (**C–D**). (**E**) Both *Slp-76*$^{ace/ace}$ and compound heterozygote *Slp-76*$^{null/ace}$ exhibit reduced elimination of β2M-deficient target cells in vivo, compared to control C57BL/6J mice. The kinetic removal of target cells was performed using an *in vivo* CFSE cytotoxicity assay. (**F**) Slp-76 protein expression in total spleen, MACS isolated CD8^{+} and NK cells from wildtype and *Ace* mice as determined by immunoblot analysis.

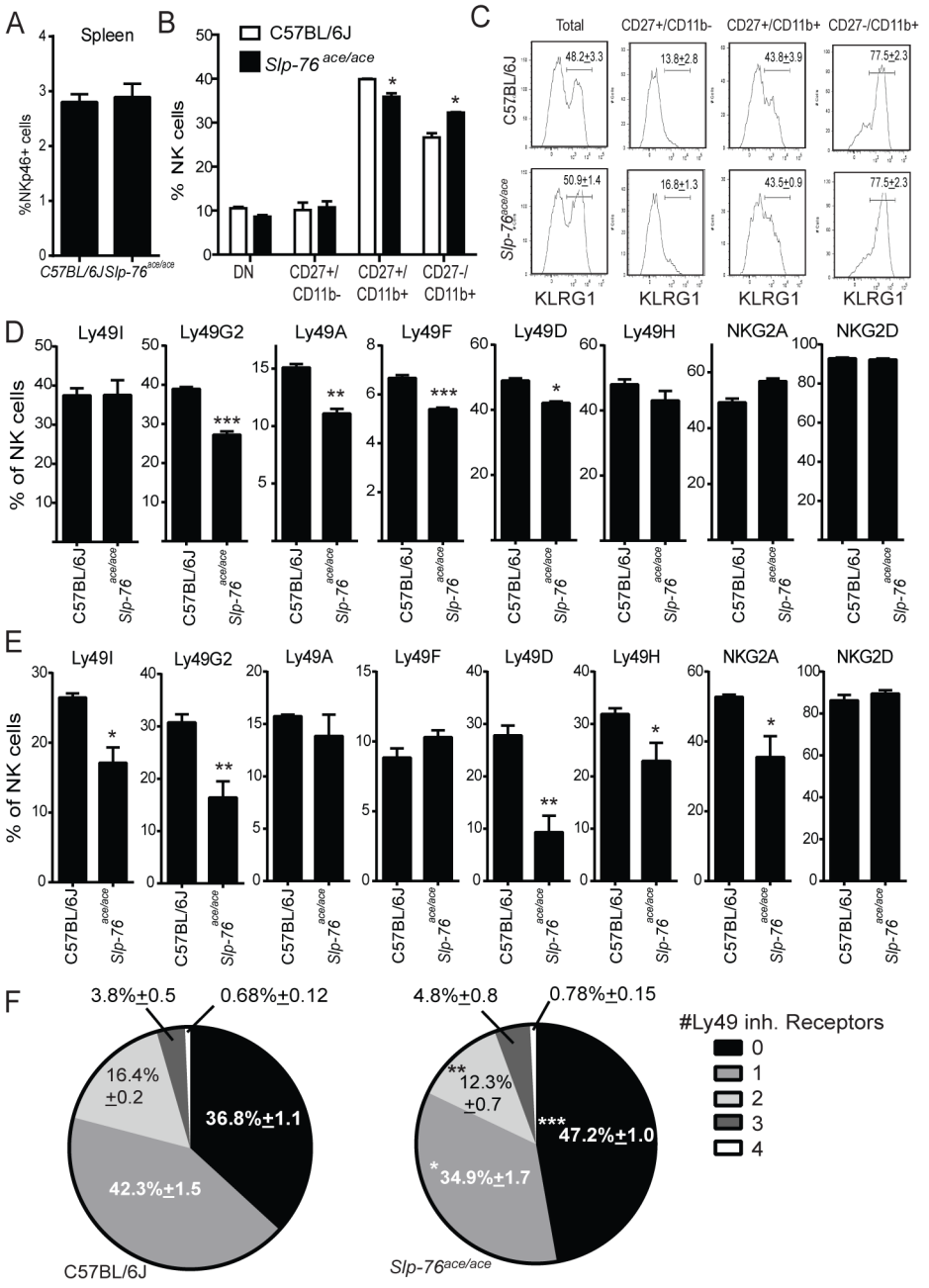

Figure 2. the *Slp-76*$^{ace/ace}$ mutation causes alterations in NK cell maturation and NK cell receptor expression

(**A**) Percentage of NK cells in spleen from wildtype and *Slp-76*$^{ace/ace}$ mice as determined by NKp46^{+}/NK1.1^{+} staining. (**B**) Maturation of NK cells in C57BL/6J and *Slp-76*$^{ace/ace}$ as measured by CD27 and CD11b surface expression (NKp46-gated) (n > 4). (**C**) KLRG1 expression on total NK cells, and immature (CD27^{+}/CD11b^{-}), transitional stage (CD27^{+}/CD11b^{+}) and mature NK cells (CD27^{-}/CD11b^{+}). (**D,E**) Surface expression of NK cell receptors on NKp46^{+}/NK1.1^{+} cells isolated from spleen (**D**) and peripheral lymphnodes (**E**) from 6-week-old C57BL/6J and *Slp-76*$^{ace/ace}$ mutant mice in. (**F**) The percentage of NK cells in the spleen from control C57BL/6J or *Slp-76*$^{ace/ace}$ mice that express, 0, 1, 2, 3 or 4

inhibitory Ly49 receptors (Ly49I, Ly49G2, Ly49A, Ly49F). Data represents mean values + SD of at least 4 mice. * $P<0.05$; ** $P< 0.01$; *** $P<0.001$.

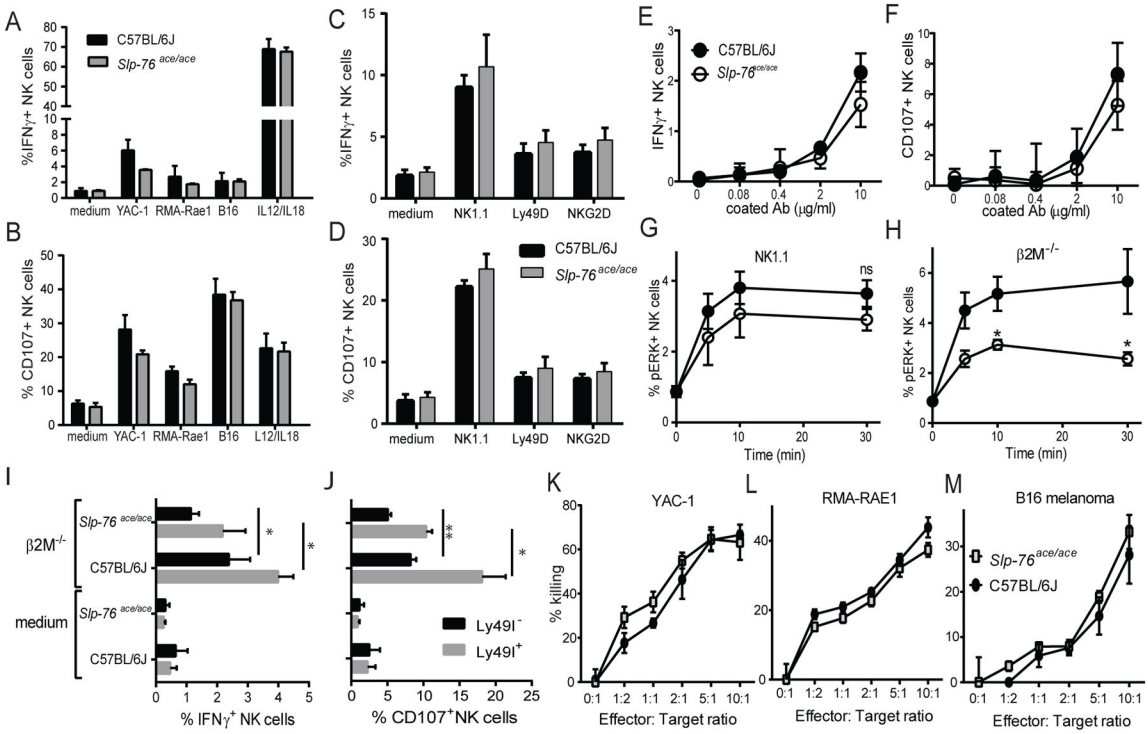

Figure 3. Normal NK cell activation and elimination of tumor targets by *Slp-76*$^{ace/ace}$ NK cells

(**A,B**) Activation of NK cells isolated from wildtype C57BL/6J or *Slp-76*$^{ace/ace}$ mice as measured by IFNγ production (**A**) or CD107 surface expression (**B**) left unstimulated or following stimulation with indicated tumor cells, IL12/IL18 or NK1.1 in the presence of IL-2. (**C–D**). Activation of NK cell in vitro using αNK1.1, Ly49D, or NKG2D-activating antibodies in the presence of IL2. NK cell activation was quantified by intracellular IFNγ accumulation measured by flow cytometry or CD107 surface expression during 5 hours incubation. (**E,F**) Dose-response activation of isolated control C57BL/6J or *Slp-76*$^{ace/ace}$ NK cells, stimulated with various concentrations of plate-bound αNK1.1 in the absence of IL-2. Activation was measured by intracellular IFNγ accumulation (**E**) or CD107 surface expression (**F**). (**G–H**) Phosphorylation of ERK in NK cells from control C57BL/6J or *Slp-76*$^{ace/ace}$ NK cells 0, 5, 10 or 30 minutes after stimulation with plate-bound αNK1.1 (**G**) or β2M-deficient target cells (**H**) in the absence of IL-2. (**I–J**) Activation of Ly49I^{+} or Ly49I^{-} NK cells from WT or *Slp-76*$^{ace/ace}$ mice with β2M-deficient target cells as measured by IFNγ production (**I**) or CD107 surface expression (**J**). (**K–M**) *In vitro* killing assays using different effector: target ratios wt or *Slp-76*$^{ace/ace}$ NK cells and YAC1 (**K**), RMA-Rae1 (**L**), or B16 (**M**) melanoma cells during a 24 hour incubation time. For these experiments, NK cells from 2 spleens per genotype were pooled and 3 mice per group/ experiment were included. Data represent mean values ± SD. Experiments were repeated 3 times. * P<0.05; ** P< 0.01

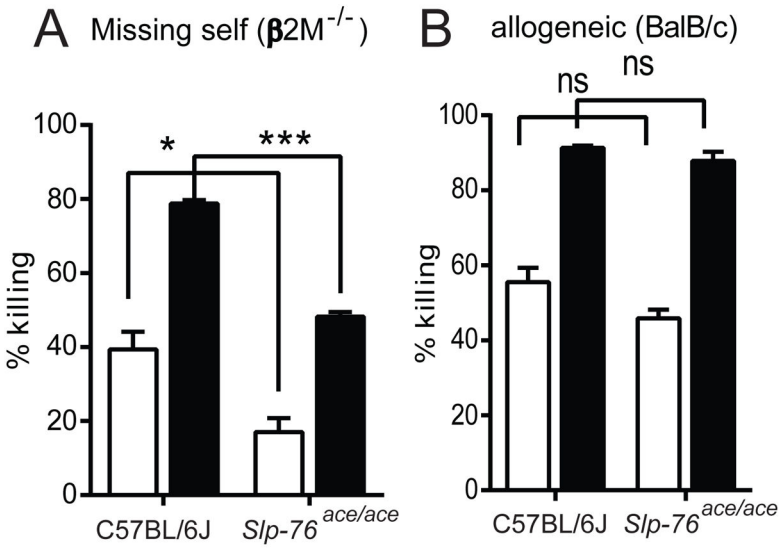

Figure 4. Impaired killing of missing-self but normal allogeneic recognition in *Slp-76*$^{ace/ace}$ mice

(**a**) Reduced clearance *in vivo* of CFSE labeled *β2M*-deficient but not allogeneic splenocytes in *Slp-76*$^{ace/ace}$ mutants compared to C57BL/6J control mice. Mice were injected with different target cells and six (white bars) or twenty-four hours (black bars) after transfer, blood samples were collected and analyzed for the relative presence/absence of target cells compared to reference wildtype splenocytes. The percentage killing is calculated from the ratio between target cell and C57BL/6J control cells administered to NK cell depleted and control C57BL/6J recipients. Numbers in graph represent the mean % killing ± SD (n=4).

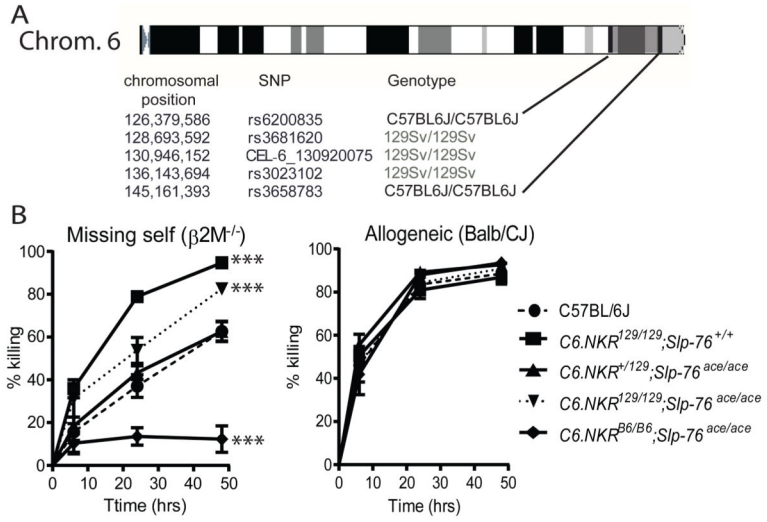

Figure 5. Missing-self recognition by NK cells is defined by the chromosome 6 NCR gene cluster expression; the C6.NKC$^{129/129}$ region can largely overcome the Slp-76-defect

(**A**) Generation and analysis of C57BL/6J mice containing a 129 congenic region of the NK cell receptor cluster on chromosome 6. The region was defined by chromosome 6 proximal SNP marker rs6200835 (126.4 Mbps) and distal SNP rs3658783 (145,16Mbps). The congenic region was confirmed after crossing the *C6.NKC$^{129/129}$* to the *Slp-76$^{ace/ace}$* background. (**B**) The effect of the *C6.NKC$^{129/129}$* cluster improves missing-self recognition both in C57BL/6J mice and *Slp-76$^{ace/ace}$* mice in a gene dose-dependent manner. Data represents mean values ± SD of at least 4 mice. *** P<0.001 compared to control C57BL/6J mice.

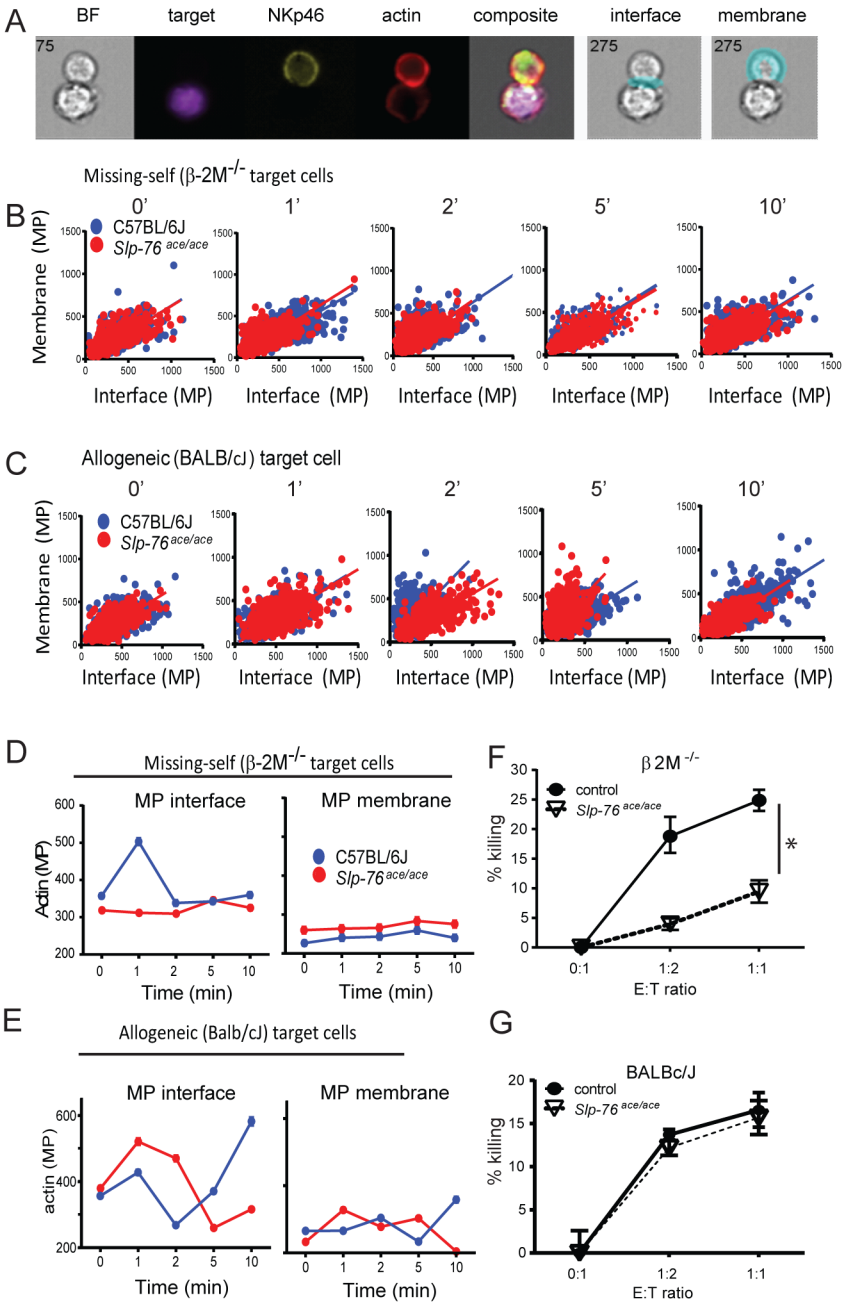

Figure 6. *Slp-76*$^{ace/ace}$ NK cells exhibit impaired F-actin recruitment towards the immune synapse following conjugate formation between NK and target cells

(**A**) Image stream analysis of NK-target cell conjugates with defined interface and non-specific membrane masks as used in the analysis. (**B, C**) F-actin mean particle (MP) density as measured by intracellular phalloidin staining in the interface or membrane within NKp46^{+} cells during various incubation times. Each dot represents a single NK-target cell conjugate with **B** presenting F-actin recruitment in NK-*β2M*$^{-/-}$ conjugates and **C** F-actin recruitment in NK-allogeneic BALB/cJ conjugates. (**D–E**) Kinetic changes in F-actin density towards interface or membrane calculated as an average of all NK-target cell

conjugates for both *β2M*$^{-/-}$ targets (**D**) and allogeneic targets (**E**). (**F–G**) *In vitro* killing potential of NK cells isolated from control C57BL/6J or *Slp-76*$^{ace/ace}$ mice incubated with conA-stimulated *β2M*$^{-/-}$ (**F**) or allogeneic BALB/cJ (**G**) splenocytes. NK cells from 2 spleens per genotype were pooled and 4 mice per group/experiment were included. Data depicted is representative for all experiments which were repeated 3 times. *P<0.05.