**NK CELLS**

**Gab3 is required for IL-2– and IL-15–induced NK cell expansion and limits trophoblast invasion during pregnancy**

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The scaffolding protein Grb2-associated binding protein 3 (Gab3) is a member of the Gab family, whose functions have remained elusive. Here, we identify Gab3 as a key determinant of peripheral NK cell expansion. Loss of Gab3 resulted in impaired IL-2 and IL-15–induced NK cell priming and expansion due to a selective impairment in MAPK signaling but not STAT5 signaling. In vivo, we found that Gab3 is required for recognition and elimination of “missing-self” and tumor targets. Unexpectedly, our studies also revealed that Gab3 plays an important role during pregnancy. Gab3-deficient mice exhibited impaired uterine NK cell expansion associated with abnormal spiral artery remodeling and increased trophoblast invasion in the decidua basalis. This coincided with stillbirth, retained placenta, maternal hemorrhage, and undelivered fetoplacental units at term. Thus, Gab3 is a key component required for cytokine-mediated NK cell priming and expansion that is essential for antitumor responses and limits trophoblast cell invasion during pregnancy.

**INTRODUCTION**

The Grb2-associated binding (Gab) family proteins support assembly of activated signaling complexes through scaffolding and docking functions. Gab family members, comprising Gab1–3, contain a highly conserved N-terminal pleckstrin homology (PH) domain as well as various phosphorylation sites specific for tyrosine kinases (1–3). The PH domain mediates plasma membrane recruitment by interacting with lipids such as phosphatidylinositol 3,4,5-triphosphate (PIP3), whereas the phosphorylated tyrosines are recruitment sites for binding to Src homology 2 domain–containing proteins (1,2,4). Gab3, unlike Gab1 and Gab2, is expressed exclusively in cells of the hematopoietic lineage, with the highest expression occurring in natural killer (NK) cells, mast cells, and memory CD8+ T cells (http://biogps.org, www.immgen.org) (5). Although the roles of Gab1 and Gab2 have been defined, the functions of Gab3 have remained elusive.

NK cells play an important role in the recognition and elimination of tumor cells, alloimmune cells, and pathogen-infected cells (6). Activation of NK cells can occur via different receptor pathways that integrate signals derived from inhibitory and activating surface receptors (7). Moreover, NK cells can be activated by cytokines such as interleukin-2 (IL-2) and IL-15, cytokines that share common receptor signaling components, including the common γ chain and the IL-2 receptor β chain (CD122) (8). Both IL-2 and IL-15 initiate NK cell priming, leading to a boost in NK cell effector function while also driving peripheral NK cell expansion (9). NK cells are found in circulation and lymphoid organs but can also reside in various tissues. Uterine NK (uNK) cells residing in the maternal tissues (decidua and mesometrial lymphoid aggregate of pregnancy) of the maternal-fetal interface are required for the early development and vascular remodeling of the decidua basalis (DB) during pregnancy (10–12). This unique subset of NK cells is highly proliferative and represents the main leukocyte subset within the early mouse and human DB, expanding to peak levels at gestational day (gd) 5.5 to 9.5 in mice, whereas in humans, their numbers increase in the first trimester and then decline as pregnancy progresses (11). uNK cells in the mouse can be defined by expression of a glycan recognized by Dolichos biflorus agglutinin (DBA) lectin (13,14) or expression of CD122 and NK1.1. Moreover, on the basis of their expression of CD49a and eomesodermin (EOMES), they can be differentiated into innate lymphoid cell 1 (ILC1), tissue-resident NK cells (trNK cells), and conventional NK cells (cNK cells) (15–17). Besides vascular remodeling, uNK cells play an essential role in regulating fetal trophoblast invasion during DB development and pregnancy (18). Although the importance of uNK cells for a successful pregnancy is well appreciated, the pathways that drive their expansion and activation remain poorly defined.

Our current studies identify a key role for Gab3 in NK cells. Specifically, we show that Gab3 is essential for mitogen-activated protein kinase (MAPK) signaling downstream of the IL-2/IL-15 receptor in NK cells. Gab3 deficiency was associated with a markedly impaired ability to clear tumor cells in vivo, whereas IL-2– and IL-15–induced priming and expansion of NK cells in vitro was abrogated. Moreover, we show a key role for Gab3 in the expansion of uNK cells that is associated with abnormal vascular remodeling and increased frequency of failed pregnancies characterized by stillbirth, retained placenta, maternal hemorrhage, and undelivered fetoplacental units at term.

**RESULTS**

**Gab3 is a critical protein required for NK cell function**

As part of our N-ethyl-N-nitrosourea (ENU) mutagenesis approach to identify genes involved in NK cell–mediated missing-self recognition (19), we identified a G3 germline mutant (A961) that failed to clear β2-microglobulin–deficient target cells while exhibiting normal
antigen-specific CD8+ T cell responses after immunization (Fig. 1A). Linkage analysis (19) established the causative mutation to reside on chromosome X with the critical region between 0 and 93.6 Mb (Fig. 1B). Subsequent whole-exome sequencing (WES) revealed a nucleotide change [C to T at X:722788707 base pair (bp)] in Gab3 that was predicted to be damaging by sorting intolerant from tolerant (SIFT) and polymorphism phenotyping (PolyPhen) analysis. Specifically, the C→T missense mutation causes a single amino acid change (Arg→Cys or R27C) in the PH domain of Gab3 (Fig. 1C and fig. S1A). Alignment studies show that the Arg residue is highly conserved in PH domains; however, a rare human missense variant exists for the same Gab3 residue (Arg→His: http://exac.broadinstitute.org/). We hypothesized the Gab3R27C

Fig. 1. Identification of Gab3 as a critical determinant of NK cell function, antitumor responses, and pregnancy. (A) Identification of an ENU germline mutant (A961) exhibiting impaired NK cell function compared with a littermate control (A962). Control and ENU mice were immunized with SE1 TAKO cells. Seven days post immunization, mice were injected intravenously with control splenocytes [carboxyfluorescein diacetate succinimidyl ester (CFSE)–low], NK targets (β2M−/− splenocytes; CFSE-medium), and CD8+ targets (EBI192–200–loaded splenocytes; CFSE-high). After 2 days, the frequency of target populations in blood was determined by flow cytometry. (B) Coarse mapping on 34 mice (15 control and 19 mutant mice) using 150 genome-wide SNPs, identified the causal mutation to reside on chromosome X. (C) WES identified a C→T nucleotide change at position X:722788707 bp causing a single-residue change (Arg27→Cys27) in the PH domain of Gab3. (D and E) C57BL/6 ( ), Gab3R27C ( ), Gab3KO ( ), and NK cell–depleted ( ) mice were injected with 1 × 105 B16-F10 melanoma cells intravenously. After 3 weeks, mice were euthanized, and tumor burden was determined (bars represent mean ± SEM). N.S., not significant. (F) Frequency of dystocia observed in WT, Gab3R27C, and Gab3KO mice. (G and H) Representative images of stillborn pups, retained placentas (G), and maternal hemorrhaging (H) of a Gab3KO female with dystocia. Statistical analysis was performed using one-way ANOVA with Tukey posttest. *P < 0.05, **P < 0.01, and ***P < 0.0001.

mutation might be causal for the observed NK cell deficiency, and to confirm the role of Gab3 in NK cell function, we generated Gab3 knockout (KO) mice using CRISPR-Cas9. Specifically, guide RNAs were developed targeting exon 2, causing a single nucleotide insertion, resulting in a frameshift, alternative translation and a premature stop (fig. S1, B to D). Gab3KO mice were viable and confirmed the importance of Gab3 in recognition of KDM-deficient targets in vivo (fig. S1E). Characterization of other lymphocyte subsets from Gab3R27C and Gab3KO mice, including CD8^+^, CD4^+^, and B cells, suggests no differences in the frequency or development of these subsets and normal CD8^+^ T cell effector function as assessed by immunization with transporter associated with antigen processing (TAP)–deficient mouse embryonic fibroblasts expressing the human adenovirus type 5 early region 1 (5E1-TAKO) cells (fig. 1A). Further characterization of peripheral NK cell populations in homozygous Gab3R27C or Gab3KO mice revealed no change in the frequency of NK cells in the spleen (fig. S2A). In addition, NK cells from Gab3R27C and Gab3KO mice showed normal NK cell maturation (fig. S2B), expression of activating receptors Ly49D and Ly49H (fig. S2C), or expression of inhibitory Ly49 receptors, although the frequency of Ly49G2^+^ NK cells was reduced in Gab3R27C and Gab3KO mice (fig. S2D). Nonetheless, stimulation of the NK cells ex vivo using a variety of activating stimuli, (e.g., anti-NK1.1, YAC-1 tumor target cells, or combined IL-12/IL-18 activating cytokines) revealed similar interferon-γ (IFN-γ) production (fig. S2E). Last, we assessed development of NK cells in the bone marrow by flow cytometry as previously described (20). Consistent with the normal peripheral NK cell numbers, no changes were observed in the frequency of NK-committed progenitors or NK1.1^+^ NK cells in the bone marrow (fig. S3, A and B).

**In vivo functional Gab3 is essential for tumor cell clearance and successful pregnancy**

Missing-self recognition is an important mechanism by which NK cells recognize tumor cells, and we therefore investigated whether Gab3R27C and Gab3KO mice are susceptible to a melanoma tumor challenge in vivo. Specifically, we injected wild-type (WT), NK cell–depleted, Gab3R27C, and Gab3KO mice with 1 × 10^5^ B16-F10 melanoma tumor cells intravenously. After 3 weeks, we quantified pulmonary tumor burdens in homozygous Gab3R27C in vitro. Both Gab3R27C and Gab3KO mice showed markedly reduced proliferation as assessed by CellTrace Violet dilution and Ki67 expression (fig. 2D), whereas no apparent loss of NK cell viability was observed. Consistent with the survival, we observed a normal induction of Mcl-1, a critical survival factor induced by IL-2 or IL-15 (27), in Gab3R27C and Gab3KO NK cells compared with WT (fig. S3C). Last, we investigated cell cycle progression using EdU incorporation in conjunction with 7-aminomethoxycin D (7-AAD) staining during IL-2 and IL-15 expansion. We observed significantly fewer Gab3KO NK cells in S phase, whereas more NK cells resided in G1 compared with WT NK cells. Similar trends were observed in the Gab3R27C NK cells, although differences did not reach statistical significance (fig. 2E). These findings again suggest that the Gab3R27C mutation behaves as a hypomorphic allele. These data identify a critical role for Gab3 in IL-2– and IL-15–driven priming and expansion of NK cells.

**Gab3 is selectively required for MAPK signaling downstream of IL-2/IL-15 receptors**

IL-15Rα and IL-15Rβ chains display a marked reduction in peripheral NK cell numbers (28–31). Whereas our studies establish a role for Gab3 in IL-15–induced expansion of NK cells, peripheral NK cell numbers remain unaffected in Gab3-deficient mice. Thus, we hypothesized that Gab3 is required for a selective signaling pathway downstream of the IL-2/IL-15 receptor. Activation of the IL-2/IL-15 receptor triggers three main signaling pathways including the JAK (Janus kinase)/STAT5 (signal transducers and activators of transcription) 5 pathway, the phosphoinositide 3-kinase (PI3K) pathway, and MAPK pathway (32, 33). We investigated downstream signaling in WT, Gab3R27C, and Gab3KO NK cells with IL-2 or IL-15 by assessing phosphorylation of STAT5 (JAK/STAT5 pathway), Akt, p70 S6 kinase, mTORC1, ribosomal protein S6 (Rps6), and ERK (extracellular signal–regulated kinase). The activation of Gab3KO NK cells revealed no change in STAT5 (JAK/STAT5 pathway) at various time points. Both IL-2 and IL-15 induced robust and normal activation of STAT5, Akt, and p70 S6 kinase in WT, Gab3R27C, and Gab3KO NK cells (fig. 3, A to C). In contrast, both Gab3R27C and Gab3KO NK cells showed a profound defect in activation of the MAPK pathways (i.e., phospho-ERK, phospho-JNK, and phospho-p38) (Fig. 3, E to G). The Gab3KO displayed a partial reduction, whereas the Gab3R27C exhibited a near complete lack of MAPK activation, confirming that the Gab3R27C allele behaves as a hypomorphic allele. These studies also revealed a partial yet significant reduction in the phosphorylation of the Rps6 (fig. 3D) in Gab3R27C and Gab3KO NK cells. Rps6 is a key component of the 40S ribosomal subunit required for RNA translation and cell growth, and previous studies revealed that phosphorylation of Rps6 can be mediated by
both the PI3K and ERK pathway (34, 35). Thus, the partial reduction in RpS6 phosphorylation is likely derived from the selective loss of ERK, but not PI3K, signaling in Gab3-deficient NK cells. These findings establish a critical and selective role for Gab3 in the activation of the MAPK pathway downstream of the IL-2/IL-15R, whereas Gab3 is dispensable for the JAK/STAT and PI3K pathways (Fig. 3H).

R27C mutation disrupts Gab3 PH domain phosphoinositide lipid binding

On the basis of three-dimensional modeling of the PH domain in complex with PIP3, we predicted that the R27C mutation would affect the ability of the PH domain to recognize and bind lipids (Fig. 4A). We thus generated and purified PH domains from WT Gab3 or R27C Gab3 and assessed their ability to bind to various phosphatidylinositols and membrane lipids using PIP strips incubated with the WT or R27C PH domain of Gab3 (Fig. 4B). WT bound the monophosphorylated (PIP), as well as the bisphosphorylated (PIP2) and triphosphorylated (PIP3) phosphatidylinositols (Fig. 4B). In contrast, lipid binding was significantly disrupted in the presence of the R27C mutation; only minimal binding was observed for lipids that strongly bind WT Gab3 (Fig. 4B). To confirm PIP specificity and quantitatively assess differences between the lipid-binding capacities of WT or R27C Gab3 domains, we repeated the protein-lipid overlay assay using serial dilutions of specific phosphatidylinositols. The strongest binding of the Gab3 PH domain was observed for PIP2 species, with the highest binding observed for PtdIns(3,4)P2 and PtdIns(3,5)P2, whereas limited binding for these lipids was observed with the Gab3R27C PH domain (fig.S4). These studies suggest that the R27C missense mutation may hamper recruitment of Gab3 to the membrane and/or IL-2/IL-15R complex upon IL-2/IL-15 stimulation. To test this hypothesis, we examined the localization of WT or R27C Gab3 protein in resting and IL-2/IL-15–activated NK cells. Specifically, we generated lentiviral vectors expressing green fluorescent protein (GFP)–coupled Gab3WT or Gab3 R27C mutant proteins and subsequently transfected primary Gab3 KO NK cells to assess protein localization. After cytokine stimulation, we assessed Gab3 colocalization with the IL2Rβ (CD122) component in time using ImageStream. During resting conditions, WT Gab3-GFP or Gab3 R27C-GFP fusion proteins showed similar levels of expression, whereas no significant differences were observed in cellular distribution and limited colocalization with the IL2Rβ component (Fig. 4, C and D). After stimulation with IL-2, the cellular distribution of the Gab3R27C protein is significantly different from WT Gab3. Whereas WT Gab3 is rapidly recruited to CD122 (IL-2Rβ) locations (Fig. 4, C and D), this recruitment was largely abrogated in the case of Gab3 R27C–GFP. Thus, these findings suggest that the Gab3 R27C variant in the PH domain impairs phosphoinositide lipid binding, thereby abrogating the recruitment of the scaffolding protein Gab3 to the IL-2/IL-15 receptor complex, ultimately resulting in a failure to activate MAPK signaling.
Loss of Gab3 impairs uNK cell function and expansion

Given the increased frequencies of dystocia and pregnancy-associated abnormalities seen in Gab3R27C and Gab3KO females (Fig. 1, F to H), we sought to identify the underlying mechanisms for these observations. The uNK population includes unique subsets that can be defined by a variety of surface markers, including NK1.1, CD122, DBA lectin binding, CD49a, and EOMES (13, 14, 16, 17). Given the observed pregnancy complications, we posited that uNK cell expansion/function may be impaired during early gestation in Gab3R27C and Gab3KO mice. Flow cytometric analysis of WT and Gab3KO gd8.5 implantation sites revealed a markedly reduced frequency of CD45+, CD122+ CD3-, DBA- NK1.1+ NK cells (down to ~30% of WT levels) in Gab3KO mice, whereas the CD45+, CD122+ CD3-, DBA- NK1.1+ population remained relatively unperturbed (Fig. 5A), suggesting that the NK1.1+/CD122+ uNK subset specifically requires Gab3 for its expansion early during pregnancy. Further, characterization of the innate lymphoid populations to differentiate ILC1, cNK cells from trNK cells based on CD49a and EOMES expression (15, 16),
revealed a selective reduction in the number of trNK cells in Gab3KO mice compared with WT uNK cells. The frequency of cNK and ILC1 cells at gd8.5 remained relatively unperturbed compared with WT implantation sites (Fig. 5, B and C). Previous work suggested only trNK cells to show evidence of proliferation (17). Ex vivo activation of isolated uNK cells with IL-15 further confirmed a selective defect in the NK1.1+/CD122+ NK cells observed in IL-15–stimulated NK cells (Fig. 5, F and G, and fig. S5F). These granzymes are thought to be involved in tissue remodeling and angiogenesis (18). Thus, their reduced expression is consistent with the overall reduction in trNK cells observed in Gab3KO uNK cells. In contrast, uNK cells from Gab3KO mice exhibited a ~10-fold increase in the expression of gonadotropin-releasing hormone 1 (GnRH1) and macrophage migration inhibitory factor (Mif), and macrophage migration inhibitory factor (Mif) was observed for uNK cells in the DB of Gab3KO females (fig. S5G). No differences were observed in Ifng expression levels and angiogenic factors (i.e., Vegfa and Vegfc) between WT and Gab3KO uNK cells. In contrast, uNK cells from Gab3KO mice exhibited a ~10-fold increase in the expression of gonadotropin-releasing hormone 1 (fig. S5G). The latter has previously been shown to be involved in implantation and is suggested to promote the attachment and invasion of trophoblasts into the endometrium (36).

Last, uNK cells from Gab3KO implantation sites showed an increased cytokine/IFN signature (fig. S5, H and I) that correlated with increased expression of Ifne measured in total RNA of implantation sites. Gab3KO uNK cells (249 genes were up and 364 genes were down in Gab3KO uNK cells) (fig. S5B). Gene enrichment analyses (TopFun) identified gene sets with reduced expression in Gab3KO uNK cells that were involved in (i) eukaryotic translation, (ii) citric acid and respiratory electron transport, and (iii) NK cell–mediated cytotoxicity (fig. S5, C to F). The reduced expression of gene sets in translation/elongation and metabolism are consistent with the signaling defects and reduced Rps6 phosphorylation observed in IL-15–stimulated NK cells (Fig. 3D) and suggest a reduced uNK cell growth/expansion in the DB. In addition, Gab3KO uNK cells exhibited significant changes in the RNA expression of surface NK cell receptors and non-cytotoxic granzymes such as Gzmd, Gzme, Gzmf, Gzmg, and Gzmn (fig. S5F). These granzymes are thought to be involved in tissue remodeling and highly expressed in trNK cells (15).
site (fig. S5J); however, *Ifne* was not produced by uNK cells directly, given that the RNA-seq revealed no significant IFN-ε expression in either WT or *Gab3*KO uNK cells. A number of these IFN signature genes are part of a highly enriched pathway in ILC1s involving antigen processing and presentation of peptide via major histocompatibility complex (MHC) class II (i.e., H2-Aa, H2-DMb1, H2-Ab1, and CD74) (15). Thus, the IFN signature observed may in part reflect the relative enrichment of ILC1s in our RNA-seq analysis.

Together, these data suggest that loss of Gab3 results in a reduced expansion of trNK cells in the DB. Moreover, the reduction of trNK cells affects genes implicated with important effector functions in the development of the placenta during pregnancy.

**Loss of Gab3 is associated with abnormally invasive trophoblast**

The role of maternally derived uNK cells during pregnancy is evident at an early stage when they expand rapidly in the DB and initiate spiral artery remodeling as well as controlling the depth and pattern
of interstitial and endovascular trophoblast invasion (26, 37–40). Nonetheless, their regulatory function in both successful and unsuccessful pregnancies remains poorly understood (41–44). Given the marked impact on uNK cell expansion in Gab3-deficient mice, we investigated whether this correlated with changes in placental development, spiral artery remodeling, and trophoblast giant cell (TGC) infiltration in gd12.5 placentas of Gab3-deficient mice. Histological assessment of the placentas revealed no major differences in the size of the labyrinth and junctional zone, whereas a trend for reduced decidual depth was observed, particularly in the Gab3KO compared with WT (fig. S6, A to C). The spiral artery walls appeared to be heavily invaded by TGCs, as determined by staining with cytokeratin-7, a pan-trophoblast maker (Fig. 6A) (45), resulting in significantly smaller lumens and thicker artery walls. We therefore assessed spiral artery remodeling by measuring the vessel-to-lumen ratio (i.e., the area measurement of the whole vessel divided by the vessel lumen area) and assessed the frequency of TGCs in the spiral arteries by cytokeratin-7 staining. Gab3R27C and Gab3KO females exhibited a significantly higher vessel-to-lumen ratio (Fig. 6B) while also showing a significant increase in the number of TGCs within spiral arteries, indicating that the increased vessel-to-lumen ratio is due to the invading cytokeratin-7+ TGCs (Fig. 6C).

These observations extended to gd18.5, at which point we observed invasion of TGCs into the uterine wall (fig. S7A). In addition, we observed a significant expansion of trophoblasts in the labyrinth and junctional zone of both Gab3R27C and Gab3KO placentas leading to an overall increase in the depth of labyrinth, junctional zone, and overall placenta (fig. S7, B to D). In the DB, we observed discontinuous trophoblast invasion, with select areas showing deep invasion reaching the uterine wall in Gab3R27C and Gab3KO placentas. By analyzing serial sections stained for proliferin, we next determined the smallest distance between areas of trophoblast invasion and the uterine wall, whereas in WT placentas, the average minimal distance was ~490 μm, more than triple the distance observed in both Gab3R27C and Gab3KO placentas (fig. S7, A and E). Together, these data suggest that loss of Gab3 results in a failure to control trophoblast invasion throughout pregnancy, ultimately leading to continuous trophoblast invasion toward the uterine wall up to term.

To investigate the causal role of uNK cells in abnormal arterial remodeling and invasion of TGCs, we depleted uNK cells at gd0.5, gd3.5, and gd8.5 in pregnant WT females using depleting anti-NK1.1 antibodies (fig. S8). Subsequently, we examined both arterial remodeling and the presence of TGCs in the spiral arteries at gd12.5 using cytokeratin-7 immunohistochemistry. We observed a significant increase in the frequency TGCs in spiral arteries, whereas the vessel-to-lumen ratio was also similar to Gab3KO placentas (Fig. 6, A to C).

We next tested whether the adoptive transfer of WT NK cells into pregnant Gab3KO females could correct the increased trophoblast invasion in the maternal spiral arteries. Specifically, we isolated splenic NK cells from WT mice that were expanded in vitro with IL-15 for 4 days. Subsequently, 4 million NK cells were injected intravenously into pregnant Gab3KO females at gd9.5. The recipient females were euthanized 3 days after injection (gd12.5), and arterial remodeling and trophoblast invasion in the spiral arteries were assessed after hematoxylin and eosin and cytokeratin-7 staining. Intriguingly, both the vessel-to-lumen ratio and the average depth of cytokeratin-7+ cell layers in the spiral artery walls were reduced to WT levels (Fig. 6, A to C).

Together, these studies confirm the critical role of uNK cells in limiting trophoblast invasion and promoting spiral artery remodeling in the DB and reveal that Gab3 loss of function abrogates uNK cell expansion in the DB resulting in abnormal spiral artery remodeling and trophoblast infiltration that may ultimately impede a successful pregnancy outcome.

DISCUSSION

Although the functions of Gab family members Gab1 and Gab2 have been defined, the biological function of Gab3 has remained entirely elusive. Here, we identify Gab3 as a critical determinant of IL-2– and IL-15–induced activation of NK cells. Strikingly, consistent with a reduced ability to eradicate missing-self targets, we observed that Gab3 loss of function in vivo leads to a markedly impaired recognition and elimination of metastatic tumor cells. Moreover, our studies identify Gab3 to be important during pregnancy, because pregnant Gab3-deficient females exhibited reduced expansion of uNK cells, abnormal arterial remodeling, and increased trophoblast invasion into the DB. At the cellular/molecular level, we identified a clear defect in IL-2/IL-15–induced NK cell priming and expansion. Both cytokines share receptor signaling components including the IL2R β chain (CD122) and the common γ chain (CD132). Our studies reveal that Gab3 is selectively required for the activation of MAPK signaling pathways downstream of the IL-2/IL-15 receptor, whereas the STAT5 and PI3K signaling pathways were unaffected. As such, Gab3-deficient mice deviate from complete IL-15-deficient mice, retaining a relative normal peripheral development and survival of NK cells (28, 46, 47).

A previous study performed an immune analysis of independently generated Gab3 KO mice, and the authors reported no obvious immune phenotype (5). However, the study limited immune analysis to macrophages and T cells, whereas no assessment of NK cell function was reported. The latter may be challenging when working with a mixed C57BL/6J/129SvJ background causing variable background-specific NK cell receptor expression. Our current studies involve two independent mouse models on the C57BL/6J background, a complete loss-of-function knockout and an ENU germ line carrying a hypomorphic R27C missense mutation, both corroborating the critical role of Gab3 in NK cells, antitumor responses, and successful pregnancy.

Previous genome-wide association studies linked single-nucleotide polymorphisms (SNPs) in the promoter region of Gab3 with risk for human type 1 diabetes (48, 49), whereas recent studies link Gab3 overexpression with tumor cell growth (50, 51). The ExAC database (a database containing >80,000 WES from human patients/controls) reveals an absence of human complete loss-of-function mutations, suggesting that the latter may be incompatible with successful human survival or reproduction. Nonetheless, missense variants in human Gab3 have been identified, including a rare missense mutation affecting the same ArgR27 (Arg→His: http://exac.broadinstitute.org/) that is affected in our ENU mouse model. Alignment studies show that the Arg residue is highly conserved in PH domains, and our studies suggest this residue to be critical for the interaction of the PH domain with phosphoinositides. Thus, our ENU mouse model not only presents a unique model to molecularly assess the role of the PH domain in PIP binding, it may also be predictive of a potential NK cell deficiency in humans carrying this R27H missense mutation.
Fig. 6. Functional Gab3 is required for controlling TGC invasion. (A) Representative hematoxylin and eosin (H&E)– or cytokeratin-7–stained images of gd12.5 placentas showing DB with spiral arteries (SpA), junctional zone (JZ), and labyrinth from WT, Gab3R27C, Gab3KO, NK cell–depleted (PK136 treatment) WT, or Gab3KO injected with WT NK cells at gd9.5 females. (B) Spiral artery remodeling was assessed by measuring the vessel-to-lumen ratio of spiral arteries on midsagittal sections of gd12.5 placentas stained with H&E (n = 4 mice; each symbol represents an individual placenta, lines represent mean ± SEM). ns, not significant. (C) Loss of Gab3 is associated with increased TGC invasion within the maternal spiral arteries. The average depth of cytokeratin-7+ cell layer in SpA walls were quantified on midsagittal sections of gd12.5 placentas stained with cytokeratin-7 antibody and a hematoxylin counterstain (n = 4 mice; each symbol represents an individual placenta, lines represent mean ± SEM). Statistical analysis was done using a one-way ANOVA with Tukey posttest. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
The pregnancy complications in Gab3-deficient mice associated with invasive fetal trophoblasts in the uterine wall resemble the human condition placenta accreta. The latter presents a clinical condition when placental trophoblasts invade deeper into the uterine wall and fail to separate from the uterine wall. The incidence of placenta accreta has increased significantly over the past decades and is currently estimated to be 1 in ~530 births. Although this increase may be linked to the increase in cesarean delivery rate, immunological and/or genetic factors are likely to play a role as well. For instance, recent studies link the development of placenta accreta with significantly reduced numbers of uNK, whereas no significant association with the number of uterine scars was observed (52, 53). Whether the rare R27H missense mutation presents a hypomorph allele in humans that results in impaired uNK cell expansion like in the mouse model and/or is associated with the development of placenta accreta in humans remains to be addressed.

It is evident that uNK cells play a critical role in the DB development and determine the successful outcome of a pregnancy (11, 26, 37, 39, 40, 54). NK cell deficiencies have previously been linked with impaired regulation of trophoblast invasion in both mice and rats. Abnormal uNK cell numbers have been associated with human gestational complications, including recurrent spontaneous abortion and placenta accreta (55–59). Moreover, IL-15–deficient murine models lack peripheral and uNK cells and display a robust invasive trophoblast phenotype and spiral artery remodeling similar to what is observed in Gab3-deficient mice, although significant differences exist between spiral artery remodeling in IL-15–deficient mice and rats (24, 26, 60). Nonetheless, these studies corroborate the importance of IL-15 in placental development and pregnancy. Although Gab3-deficient mice exhibit reduced uNK cell numbers, they have relatively normal peripheral NK cell numbers in the blood circulation that have the potential to interact with fetal trophoblasts aligned within the spiral arteries. Given the impaired recognition and clearance of MHC-I–deficient target cells or tumor cells by Gab3-deficient NK cells, the possibility exists that this mechanism may contribute to the invasive trophoblast phenotype. Fetal trophoblasts express a unique pattern of predominantly non-MHC antigens and lack the expression of a number of classical MHC antigens (61–63), thus representing a “missing-self” target. Whether peripheral NK cells within the maternal arteries play a role in containing trophoblast invasion is currently unclear and remains to be investigated.

Gab3 is expressed in a variety of cell types including CD8+ T cells and mast cells. Although we have not observed abnormal CD8+ T cell responses or changes in their development, we cannot exclude a role for these cell types in the abnormal pregnancy phenotype observed. Nonetheless, our studies establish an important role for Gab3 in uNK cell expansion and the control of decidual trophoblast invasion. Our “add-back” experiments, in which we transplant IL-15–stimulated WT NK cells in Gab3KO recipients at gd9.5, can largely overcome decidual trophoblast invasion as analyzed by gd12.5. These experiments point to an effective therapeutic approach to limit trophoblast invasion in the maternal spiral arteries that deserves further investigation.

Gab3 expression is predominantly observed in hematopoietic cells and is particularly high in NK cells. Our current study reveals a key role for Gab3 in IL-2/IL-15–induced NK cell expansion, specifically in mediating MAPK activation downstream of the IL-2/IL-15R. This pathway provides a new opportunity to selectively induce or repress NK cell function in settings of autoimmunity, antitumor immunity, transplantation, and other therapeutically important settings. Moreover, we posit that Gab3 loss of function is associated with increased trophoblast invasion and development of placenta accreta, a significant pregnancy complication in humans.

MATERIALS AND METHODS

Study design

The goal of these studies was to characterize the role of Gab3 in NK cell function. The studies were performed using groups of age- and gender-matched mice, 5 weeks or older. Timed pregnancy studies and dystocia assessment were performed using homozygous WT, Gab3R27C, and Gab3KO breeding colonies. Power analysis indicated that analyses of three to four mice and three placentas per mouse would give 80% power (P < 0.05, two sided) to detect a 50% increase/decrease in the depth of trophoblast invasion. Histological analyses were performed double-blinded where appropriate.

Generation of Gab3KO mice through CRISPR-Cas9 genome editing

Gab3KO mice were generated by CRISPR-Cas9 genome editing. Gab3-specific short hairpin RNA (shRNA) guides were designed using Benchling software and full sequence of Gab3. The following guide sequences were used: Gab3 shRNA guide_1, 5′ACACTATCT- GGAGTCACTTA3′; Gab3 shRNA guide_2, 5′CTACCTAGTAGC- CGAAGACTG3′; and Gab3 shRNA guide_3, 5′AGTCATCTGGAAGAT- GGTGTC3′. The guides were designed to target a 201-bp region of exon 2 (Ensembl Genome Browser, ENSMUSG00000032750, genome assembly version GRCh38.p6). Microinjections into C57BL/6 embryonic stem cells were done by the Transgenic Animal and Genome Editing Core at Cincinnati Children’s Hospital Medical Center (CCHMC). Founder animals were screened for mutations in Gab3 by polymerase chain reaction and sequencing. A founder animal was selected that carried a frameshift causing a premature stop at residue 104, resulting in expression of a truncated protein presenting the proximal PH domain. The genome editing was functionally confirmed at the RNA transcript (complementary DNA) level.

Statistics

Two-way analysis of variance (ANOVA) with a Tukey or Sidak’s posttest for multiple comparisons and one-way ANOVA were used where noted; statistical tests were run using GraphPad software (v 7.02), SEM was reported for all experiments, and P < 0.05 was taken as significant for all tests. Further information on the materials and methods used can be found in the Supplementary Materials.
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Table S1. Raw data in Excel spreadsheet.
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Acknowledgments: We thank M. Kofron for excellent support in conducting confocal imaging and microscopy studies. We also thank the staff of the CCHMC Core facilities including the research flow cytometry core, the Transgenic Animal and Genome Editing Core, and the pathology research core for providing exceptional technical support. We thank the Veterinary Services Core at CCHMC for their excellent animal care, and last, we thank the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati for support in the RNA-seq studies. Funding: The research was funded by NIH grant P30 DK078392 (Integrative Morphology Core of the Cincinnati Digestive Disease Research Core Center), NIH grant R21 AI135380, and the Maren Foundation. Author contributions: A.S. performed and designed experiments, analyzed the data, and contributed in writing. K.C.S.L., K.L., and A.G. performed experiments. D.R.P., E.M.J., H.J., and A.B.H. helped with the experimental design and data analyses, and A.H. was involved in experimental design, data analyses, and writing of the manuscript. Competing interests: A.B.H. serves as a Scientific Advisory Board member for Hoth Therapeutics Inc. and has equity interests in Hoth Therapeutics Inc. and Chelexa BioSciences LLC. The other authors declare that they have no competing interests. Data and materials availability: The RNA-seq data is available from the Gene Expression Omnibus under accession number GSE133313. The Gab3 mutant mouse strains and the Gab3-GFP fusion proteins are available through a material transfer agreement with the Cincinnati Children's Hospital Medical Center. Requests for mice should be directed to H.J., and requests for the fusion proteins should be directed to A.B.H. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

Submitted 12 September 2018
Accepted 3 July 2019
Published 2 August 2019
10.1126/sciimmunol.aav3866

Gab3 is required for IL-2– and IL-15-induced NK cell expansion and limits trophoblast invasion during pregnancy


DOI: 10.1126/sciimmunol.aav3866

Gab3 adaptor prevents NK cell dysfunction

Natural killer (NK) cells are innate effector cells that help defend mammals from viral infections and cancer while acting locally in the uterus to support successful pregnancy outcomes. Sliz et al. used a chemical mutagenesis screen in mice to identify Gab3 as a scaffolding protein required for NK cell priming and peripheral expansion in response to cytokines. Gab3 mutant mice exhibited defects in their ability to control tumors and successfully complete pregnancies because of impaired NK cell function. This study identifies an adaptor protein required to achieve full NK cell function and enhances our understanding of how subtle uterine NK cell perturbations can contribute to the demise of pregnancies. See related Focus by Colucci.

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