IMMUNOLOGY

TIPE2 is a checkpoint of natural killer cell maturation and antitumor immunity

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The maturation process of NK cells determines their functionality during which IL-15 plays a critical role. However, very few checkpoints specifically targeting this process have been discovered. Here, we report that TIPE2 expression gradually increased during NK cell ontogenesis correlating to their maturation stages in both mice and humans. NK-specific TIPE2 deficiency increased mature NK cells in mice, and these TIPE2-deficient NK cells exhibited enhanced activation, cytotoxicity, and IFN-γ production upon stimulation and enhanced response to IL-15 for maturation. Moreover, TIPE2 suppressed IL-15–triggered mTOR activity in both human and murine NK cells. Consequently, blocking mTOR constrained the effect of TIPE2 deficiency on NK cell maturation in response to IL-15. Last, NK-specific TIPE2-deficient mice were resistant to tumor growth in vivo. Our results uncover a potent checkpoint in NK cell maturation and antitumor immunity in both mice and humans, suggesting a promising approach of targeting TIPE2 for NK cell–based immunotherapies.

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that emerge as potential therapeutic targets for tumor immunotherapy (*1*–*3*). They develop and start the process of maturation in the bone marrow and reach an optimally functional status when they egress to the peripheral and reach the mature status, represented by an optimal NK cell population size and optimal effector functions at the single–NK cell level (*4*), both of which are required to launch an efficient antitumor NK cell response. Impaired NK maturation compromises tumor surveillance (*5*–*7*), while enhanced NK cell maturation increases host resistance to tumor growth in vivo (*8*–*10*).

The maturation process of NK cells is enabled by signaling cascade triggered through extracellular cytokines (*4*). Among them, interleukin-15 (IL-15) is critical for NK cell maturation by supporting survival, resisting apoptosis, and promoting proliferation of NK cells (*11*). NK cells receive IL-15 signaling via IL-2Rβ and γc subunits, which recognize IL-15 transpresented by IL-15Rα$^+$ neighboring cells (*12*, *13*). The major components of IL-15 signaling include the Janus kinase 1/3 (JAK1/3)–signal transducer and activator of transcription 5 (STAT5) pathway (*14*, *15*) and mTOR pathway (*16*, *17*). Our understanding of molecular regulation of IL-15 signaling is limited, in which negative regulators of IL-15 signaling are seldom reported in NK cells. For example, CIS (cytokine-inducible SH2-containing protein) inhibits IL-15 signaling in NK cells via targeting the JAK-STAT5 pathway (*18*). Deficiency in CIS renders NK cells with a more mature phenotype (*19*), enhanced response to IL-15 (*18*), and increased effector functions (*18*), which facilitates antitumor immunity, synergizes with checkpoint blockade therapies (*18*, *20*), and benefits NK cell adoptive therapies (*21*, *22*). Despite these studies on the negative regulator in the IL-15–JAK-STAT pathway, negative regulators that are involved in the IL-15–mTOR pathway with potential roles in NK cell–mediated tumor immune responses are still elusive.

TIPE2 [tumor necrosis factor–α (TNF-α)–induced protein-8 like-2] is a negative regulator of innate and adaptive immunity that maintains immune homeostasis (*23*). Mice globally lacking TIPE2 are resistant to tumor growth (*24*), suggesting that TIPE2 might suppress antitumor immune response. In line with this, TIPE2 specifies the polarization of myeloid-derived suppressor cells toward a tumor-promoting phenotype (*24*). TIPE2 also promotes the differentiation of immunosuppressive M2 macrophages (*25*) and is required for the immunosuppressive function of CD4$^+$CD25$^+$ regulatory T cells (*26*). Despite these effects on the differentiation and/or functions of immunosuppressive leukocytes, the role of TIPE2 in the differentiation of NK cells, the essential population of antitumor effector lymphocytes, has not been revealed.

Here, in this study, we identified TIPE2 as a potential intrinsic checkpoint molecule in NK cell maturation and antitumor immunity. TIPE2 expression in NK cells correlates with mouse ontogenesis and NK cell maturation in both mice and humans. Loss of TIPE2 specifically in NK cells resulted in an increased generation of mature NK cells, elevated NK cell effector functions, and enhanced NK cell response to IL-15 via promoted IL-15–driven mTOR signaling. Mice specifically lacking TIPE2 in NK cells are more resistant to tumor growth in vivo. Therefore, TIPE2 represents a checkpoint in NK cell maturation and antitumor immunity, and we suggest that targeting TIPE2 might potentially serve as a new NK cell–based immunotherapeutic approach.

RESULTS

TIPE2 expression correlates with NK cell maturation

Similar to what was previously reported (*23*), TIPE2 was widely expressed in various immune cells including NK cells at both mRNA

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and protein levels in mice (fig. S1, A and B). To investigate the regulation mechanisms of NK cell maturation, we profiled TIPE2 protein expression in wild-type splenic NK cells during mouse ontogeny. We found that TIPE2 expression increased along mouse ontogeny as early as 2 weeks after birth and maintained at a high level 6 weeks after birth (Fig. 1A). Next, to characterize different NK cell subsets based on their maturation status via unbiased transcriptome, we performed single-cell RNA sequencing on mouse splenic NK cells and projected these cells into two dimensions by uniform manifold approximation and projection (UMAP) analysis. UMAP analysis revealed the clustering of mouse splenic NK cells into six different subsets, among which three major subsets displayed gene expression features representing resting conventional NK cells, hereafter referred to as mNK-Sp0, mNK-Sp1, and mNK-Sp2 (Fig. 1B). On the other hand, two minor subsets act. NK1 and act. NK2 displayed gene expression features of an activated status, while the rest of the minor subset corresponded to CD49a^{+} TRAIL^{+} tissue-resident NK cells (Fig. 1B). On the basis of the characterization of the three resting conventional NK cell subsets, as well as the gene expression profile during mouse NK cell maturation (*27*), we corresponded these three resting NK cell subsets with distinct stages in NK cell maturation: from mNK-Sp0, through mNK-Sp1, and then to mNK-Sp2 (Fig. 1C). These three stages displayed key features in gene expression changes along NK cell maturation, in that genes for effector molecules (e.g., *Gzmb* and *Prf1*), genes for maturation markers (e.g., *Itgam* encoding CD11b and *Klrg1*), and genes for essential transcription factors driving NK cell maturation (e.g., *Tbx21* encoding T-bet), were lowest in mNK-Sp0, medium in mNK-Sp1, and highest in mNK-Sp2 (Fig. 1C). Meanwhile, Cd27 and Mki67 expressions, which inversely correlate with NK cell maturation (*27*), were highest in mNK-Sp0, medium in mNK-Sp1, and lowest in mNK-Sp2 (Fig. 1C). On the basis of these three subsets corresponding

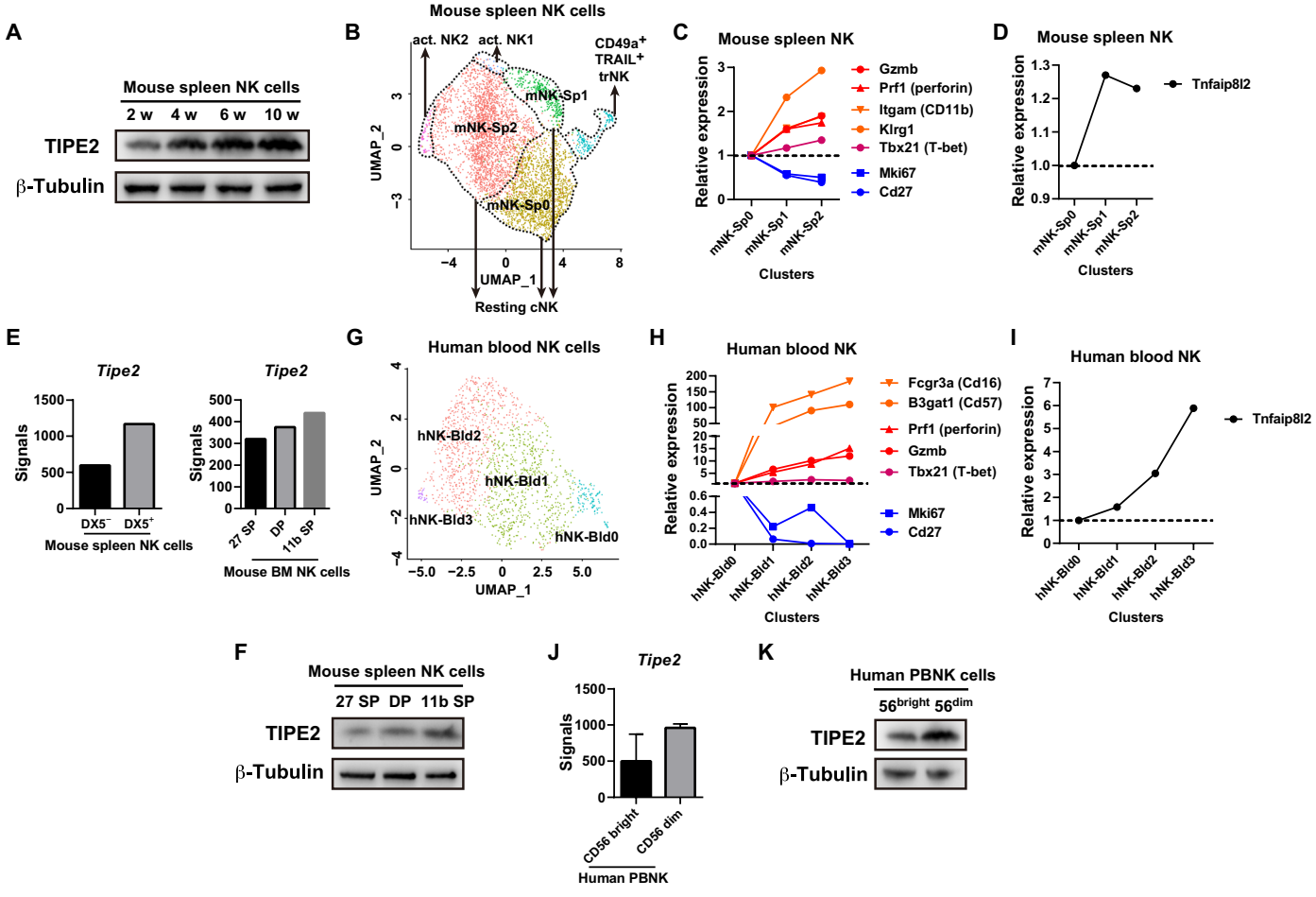

Fig. 1. TIPE2 expression correlates with NK cell maturation in both mice and humans. (**A**) TIPE2 expression of purified splenic NK cells from 2-, 4-, 6-, or 10-week-old wild-type mice was examined by Western blot. (**B**) UMAP of splenic NK cells from *Tipe2*$^{WT/WT}$ mice. Cells are color coded according to the defined subset. (**C**) Relative expression of the indicated genes in the defined subsets from (B). (**D**) *Tipe2* relative expression levels were shown for clusters from (B). (**E**) *Tipe2* transcript levels of purified DX5^{-} or DX5^{+}murine liver NK cells or CD27 SP, DP, or CD11b SP murine bone marrow NK cells obtained from the dataset GSE43339 or the Immunological Genome Project database (www.immgen.org), respectively. (**F**) TIPE2 expression of purified CD27 SP, DP, or CD11b SP splenic NK cells from wild-type mice was examined by Western blot. (**G**) tSNE of human peripheral blood NK cells. Cells are color coded according to the defined subset. (**H**) Relative expression of the indicated genes in the defined subsets from (G). (**I**) *Tipe2* relative expression levels were shown for clusters from (G). (**J**) *Tipe2* transcript levels of purified CD56^{bright} or CD56^{dim}peripheral blood NK cells from healthy donors were obtained from the dataset GSE79939 (*61*). (**K**) TIPE2 expression of purified CD56^{bright} or CD56^{dim} peripheral blood NK cells from healthy donors was examined by Western blot. (A, F, and K) Data are representative of at least two independent experiments.

with NK cell maturation stages, we found that TIPE2-encoding *Tnfaip8l2* gene expression showed an increasing trend from less matured mNK-Sp0 to more matured mNK-Sp1 and mNK-Sp2 (Fig. 1D), suggesting that TIPE2 expression increases along NK cell maturation. Consistently, more matured DX5$^+$NK cells expressed higher level of *Tnfaip8l2* gene compared with less matured DX5$^-$NK cells (Fig. 1E), and according to the CD11b- and CD27-based maturation program of mouse NK cells (*27*), the *Tnfaip8l2* gene expression increased from the CD11b$^-$CD27$^+$ subset, through the CD11b$^+$CD27$^+$ subset, then to the CD11b$^+$CD27$^-$ subset in mouse bone marrow NK cells (Fig. 1E). This increase of TIPE2 expression along NK cell maturation was confirmed at the protein level on mouse splenic NK cells (Fig. 1F). These data indicated that TIPE2 expression in mouse NK cells increases along mouse ontogeny and during NK cell maturation.

We wondered whether human NK cells also displayed this pattern of TIPE2 expression. It was noted that human NK cells expressed much higher TIPE2 mRNA level than T cells and NKT cells (fig. S1C). Therefore, we performed t-distributed stochastic neighbor embedding (tSNE) analysis on the single-cell transcriptomic data of human peripheral blood NK cells, which revealed the clustering of human peripheral blood NK cells into four different subsets, hereafter referred to as hNK-Bld0, hNK-Bld1, hNK-Bld2, and hNK-Bld3 (Fig. 1G). Similar with the above analysis on mouse splenic NK cells, we corresponded these four subsets with four stages of human NK cell maturation: hNK-Bld0 ➔ hNK-Bld1 ➔ hNK-Bld2 ➔ hNK-Bld3 (Fig. 1H), in that genes for effector molecules (e.g., *Gzmb* and *Prf1*), genes for maturation markers (e.g., *Fcgr3a* encoding Cd16 and *B3gat1* encoding Cd57), and genes for essential transcription factors driving NK cell maturation (e.g., *Tbx21* encoding T-bet), increased along hNK-Bld0 ➔ hNK-Bld1 ➔ hNK-Bld2 ➔ hNK-Bld3 (Fig. 1H). On the other hand, Cd27 decreased along hNK-Bld0 ➔ hNK-Bld1 ➔ hNK-Bld2 ➔ hNK-Bld3 (Fig. 1H). In addition, the proliferation capability of NK cells decreased along maturation process (*27*), as shown by an overall decline of proliferation marker *Mki67* expression along hNK-Bld0 ➔ hNK-Bld1 ➔ hNK-Bld2 ➔ hNK-Bld3 (Fig. 1H). We found that *Tnfaip8l2* expression increased along human NK cell maturation (Fig. 1I). Consistent with this, mature CD56^{dim} human NK cells expressed much higher amount of TIPE2 than immature CD56^{bright} human NK cells at both the mRNA level and protein level (Fig. 1, J and K). These data indicated that TIPE2 expression in NK cells increases along NK cell maturation not only in mice but also in humans, suggesting that TIPE2 plays an important role in NK cell maturation.

NK-specific TIPE2 deficiency promotes NK cell maturation

To investigate the role of TIPE2 in NK cell maturation, we analyzed the level and phenotypes of NK cells in NK-specific TIPE2-deficient *Tipe2*$^{\Delta NK/\Delta NK}$ mice and control *Tipe2*$^{WT/WT}$ mice. We found that *Tipe2*$^{\Delta NK/\Delta NK}$ mice contained significantly more NK cells in the spleen, both in the percentage of NK cells among total CD45$^+$ cells and in the absolute number of NK cells (Fig. 2A). Higher NK cell numbers were also observed in the liver and only in the compartment of CD49a$^-$ conventional NK cells but not in CD49a$^+$ liver-resident NK cells (Fig. 2A). The increased numbers of NK cells was not a result of enhanced proliferation or decreased apoptosis at resting state since we detected comparable levels of both proliferation marker Ki-67 and apoptosis marker annexin V in the splenic NK cells of *Tipe2*$^{\Delta NK/\Delta NK}$ mice and *Tipe2*$^{WT/WT}$ mice (Fig. 2B). On the other hand, although there were more NK cells in the bone marrow of *Tipe2*$^{\Delta NK/\Delta NK}$ mice than *Tipe2*$^{WT/WT}$ mice, the numbers of NK precursors ("NKp") but not NK cells were similar between two groups (Fig. 2C). These data demonstrated that NK-specific TIPE2 deficiency results in the increased numbers of NK cells.

We next assessed NK cell maturation markers in these two mouse strains and found that *Tipe2*$^{\Delta NK/\Delta NK}$ mice contained significantly more CD11b$^+$CD27$^-$ terminally matured NK cells in the percentage among total NK cells and in the absolute number, from both spleen and bone marrow (Fig. 2D). In addition, TIPE2-deficient NK cells expressed increased level of maturation marker KLRG1 (Killer cell lectin-like receptor subfamily G member 1) than control NK cells (Fig. 2E), while TIPE2-deficient and control NK cells expressed similar levels of CD43, another marker associated with NK cell maturation (Fig. 2E) (*28*). To evaluate the intrinsic effects of TIPE2 on NK maturation, we purified splenic CD11b$^-$CD27$^+$ immature NK cells or CD11b$^+$CD27$^-$ mature NK cells from *Tipe2*$^{\Delta NK/\Delta NK}$ mice and *Tipe2*$^{WT/WT}$ mice and transferred into immune-deficient nonobese diabetic (NOD)–*Scid*-$\gamma c^{-/-}$ mice (Fig. 2F and fig. S2). We found that only transfer of immature, but not mature, NK cells resulted in the increased numbers of total NK cells, as well as CD11b$^+$CD27$^-$ mature NK cells in the bone marrow 6 days later (Fig. 2F and fig. S2). Together, these data suggested that TIPE2 intrinsically suppresses NK cell maturation.

To further confirm the role of TIPE2 in NK cell maturation, we performed single-cell RNA sequencing on splenic NK cells from both *Tipe2*$^{\Delta NK/\Delta NK}$ mice and *Tipe2*$^{WT/WT}$ mice. UMAP analysis revealed the clustering of splenic NK cells from both genotypes into six subsets, among which three major subsets—mNK-Sp0, mNK-Sp1, and mNK-Sp2—displayed gene expression features representing resting conventional NK cells (Fig. 2, G to I). On the basis of the key gene expression of NK cell effector molecules, transcriptional factors, maturation markers, and proliferation marker, we identified NK cell maturation program as mNK-Sp0 ➔ mNK-Sp1 ➔ mNK-Sp2 by which immature NK cells should go through to gain optimal effector functions at the cost of proliferation ability (Fig. 2, J and K). According to this model, *Tipe2*$^{\Delta NK/\Delta NK}$ mice contained more NK cells belonging to the terminally mature mNK-Sp2 than *Tipe2*$^{WT/WT}$ mice (up to 10% more) (Fig. 2L), which is consistent with what we observed by flow cytometry (Fig. 2D). Therefore, these results collectively showed that NK-specific TIPE2 deficiency promotes NK cell maturation.

NK-specific TIPE2 deficiency promotes NK cell effector functions

NK cells acquire optimal effector functions along maturation process. The effects of TIPE2 deficiency in promoting NK cell maturation suggest that TIPE2 deficiency might also promote NK cell effector functions. An in vivo cytolysis assay against NK-sensitive MHC-I^{low} RMAS cells showed that *Tipe2*$^{\Delta NK/\Delta NK}$ mice eliminated significantly more RMAS cells in vivo (Fig. 3A). Consistently, TIPE2-deficient NK cells also displayed more potent cytolytic activity against NK-sensitive YAC-1 cells in vitro (Fig. 3B), as well as against NK-insensitive MC38 cells after stimulated with IL-12, IL-15, and IL-18 (Fig. 3C). Besides, TIPE2-overexpressed human NK cell line YTS showed reduced cytolysis against 721.221 cells (Fig. 3B), while TIPE2-overexpressed human NK cell line NK92 also showed decreased cytolysis against HCT116 cells (Fig. 3C). These data indicated that TIPE2 suppresses NK cell cytolytic functions. Although resting TIPE2-deficient NK cells and control NK cells expressed comparable

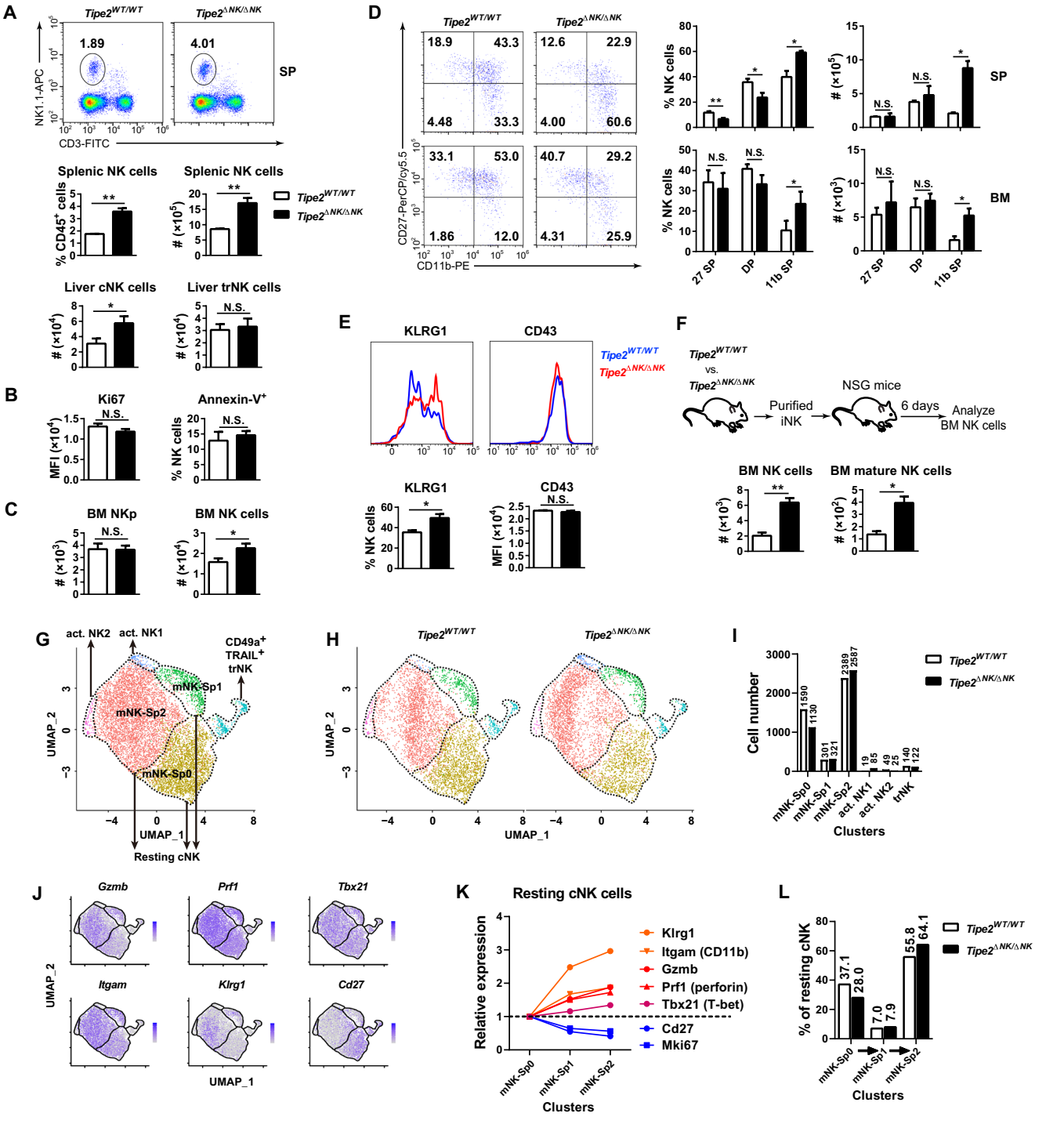

Fig. 2. NK-specific TIPE2 deficiency promotes NK cell maturation. (**A** to **E**) *Tipe2*$^{WT/WT}$ mice and *Tipe2*$^{\Delta NK/\Delta NK}$ mice were compared for NK cell or precursor absolute numbers, percentages, and phenotypes. (A) Percentages among CD45$^+$ cells or absolute numbers of total NK cells, cNK (conventional CD49a$^-$ NK cells), and lrNK (liver-resident CD49a$^+$ NK cells) in the spleen or in the liver. (B) Ki-67 and annexin V levels in splenic NK cells. MFI, mean fluorescence intensity. (C) Absolute numbers of Lin$^-$c-Kit$^-$CD122$^+$NKG2D$^+$NKP or CD3$^-$NK1.1$^+$NK cells in the bone marrow. (D) Percentages or absolute numbers of the CD11b$^-$CD27$^+$ (27 SP), CD11b$^+$CD27$^+$ (DP), and CD11b$^+$CD27$^-$ (11b SP) subsets in the spleen or bone marrow. (E) Left: Percentages of KLRG1$^+$ cells among NK cells in the spleen. Right: CD43 levels in splenic NK cells. (**F**) Purified CD11b$^-$CD27$^+$ immature NK cells from the spleen were transferred into NOD-*Scid*-γc$^{-/-}$mice. Six days later, total NK cell numbers and CD11b$^+$CD27$^-$mature NK cell numbers in the bone marrow of recipient mice were analyzed.(**G** and **H**) UMAP of pooled splenic NK cells from both *Tipe2*$^{WT/WT}$ mice and *Tipe2*$^{\Delta NK/\Delta NK}$ mice. Cells are color coded according to the defined subset. Cells are shown as a pool in (G) or separately by the indicated genotypes in (H). (**I**) Cell numbers of each population in (H) were shown. (**J**) Feature plot showing the relative expression levels of the indicated genes in each cluster from (G). (**K**) Relative expression of the indicated genes in the defined subsets from (G). (**L**) Percentages of each cluster among total resting conventional NK cells of the indicated genotypes. (A to F) Data are representative of at least three independent experiments and are presented as means ± SEM ($n = 3$). N.S., not significant. *$P < 0.05$ and **$P < 0.01$ (unpaired Student's t test).

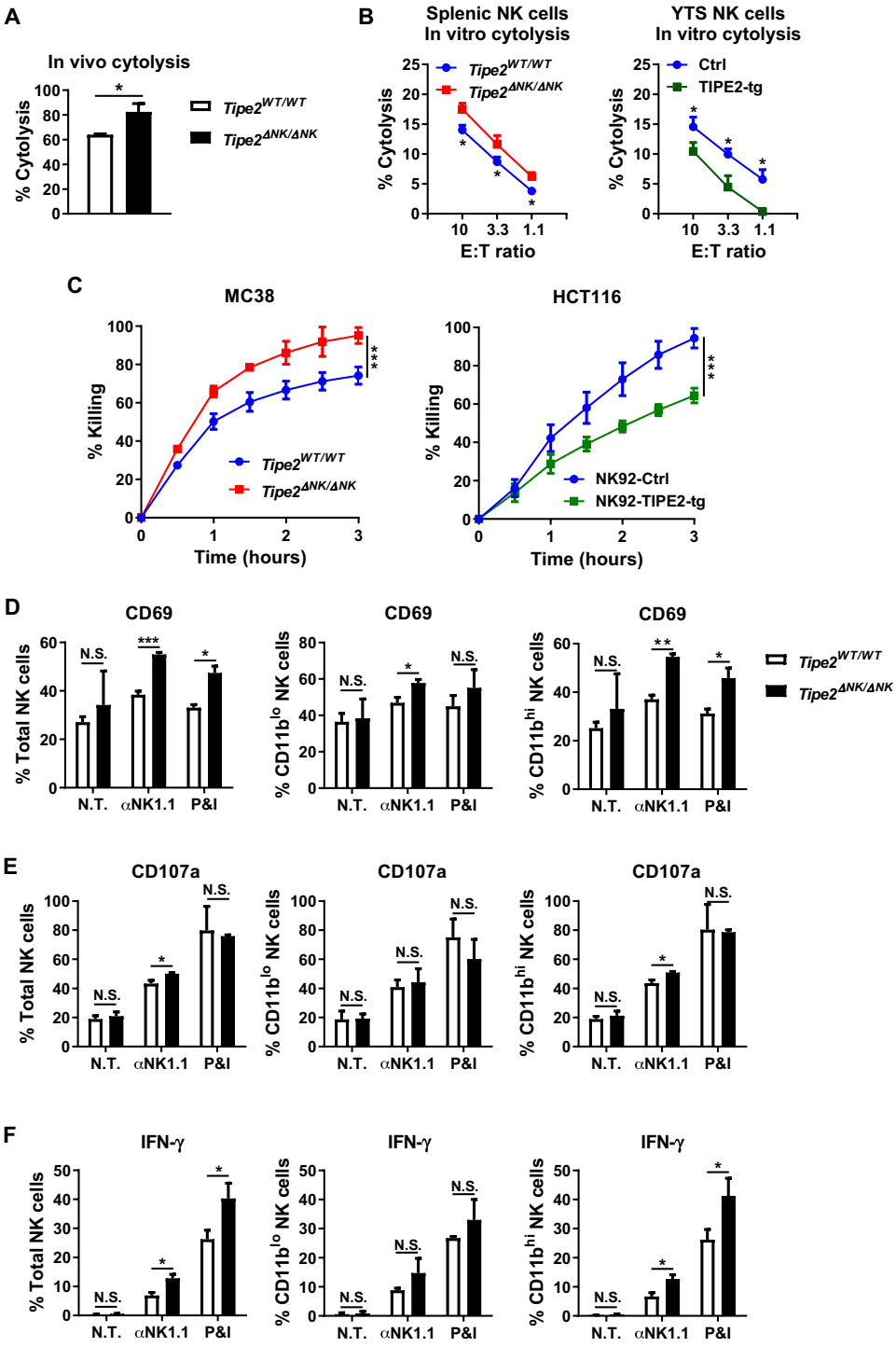

Fig. 3. NK-specific TIPE2 deficiency promotes NK cell effector functions. (**A**) CellTrace Violet (CTV) or carboxyfluorescein diacetate succinimidyl ester–labeled MHC-I^{low} RMAS and parental MHC-I–sufficient RMA cells were mixed and injected into *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice. Fourteen hours later, peritoneal cells were harvested for analysis of remaining RMAS cells relative to RMA cells to evaluate in vivo cytolysis levels. (**B**) Left: Purified splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were tested for in vitro cytolytic activity against YAC-1 cells. Right: TIPE2-overexpressed or control YTS NK cells were evaluated for in vitro cytolytic activity against 721.221 cells. (**C**) Left: Splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ were stimulated with IL-12, IL-15, and IL-18 for 2 days before a cytolytic assay against MC38 target cells at an effector:target (E:T) ratio of 3:1. Right: Cytotoxicity of TIPE2–overexpressed or control NK92 cells against HCT116 target cells at an E:T ratio of 3:1 was evaluated. (**D** to **F**) Total, CD11b^{lo} immature, or CD11b^{hi} mature splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were assessed for CD69 (D), CD107a (E), or IFN-γ (F) expression either left untreated or stimulated with plate-bound anti-NK1.1 or with PMA and ionomycin (P&I). (A to F) Data are representative of at least two independent experiments and are presented as means ± SEM. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$ [unpaired Student's *t* test (A, E, and F), one-way analysis of variance (ANOVA) (B), or two-way ANOVA (C)]. N.T., not treated.

levels of CD69, CD107a, and interferon-γ (IFN-γ; Fig. 3, D to F), anti-NK1.1–triggered or phorbol 12-myristate 13-acetate (PMA)/ionomycin–stimulated TIPE2-deficient NK cells expressed significantly higher levels of activation marker CD69 and effector cytokine IFN-γ than control NK cells (Fig. 3, D and F). In addition, degranulation of TIPE2-deficient NK cells upon anti-NK1.1 triggering was also significantly higher (Fig. 3E). Further analysis of $CD11b^{lo}$ less matured and $CD11b^{hi}$ more matured NK cell subsets showed that mature TIPE2-deficient NK cells displayed similar patterns of elevated expression of CD69, CD107a, and IFN-γ after stimulation when compared with total TIPE2-deficient NK cells; however, the changes in immature TIPE2-deficient NK cells were not statistically significant (Fig. 3, D to F), indicating that increased effector functions of TIPE2-deficient NK cells is observable not only on total NK cell population but also on a per cell basis. Collectively, the above data showed that NK-specific TIPE2 deficiency promotes NK cell effector functions.

NK-specific TIPE2 deficiency promotes NK cell response to IL-15

To investigate the underlying mechanisms of TIPE2-mediated suppression of NK cell maturation and effector functions, we set out to study whether TIPE2 regulates the response of NK cells to IL-15, the cytokine that plays a critical role in NK cell maturation and effector functions. Upon the IL-15/IL-15 receptor complex (referred to as "IL-15c" below) stimulation in vitro, mouse splenic NK cells maintained TIPE2 expression for at least 24 hours (Fig. 4A). NK-specific TIPE2 deficiency resulted in significantly more NK cells than the control group 3 days after IL-15c stimulation (Fig. 4B), among which more terminally matured $CD11b^{+}CD27^{-}$ ("CD11b SP") NK cells were detected in the $Tipe2^{\Delta NK/\Delta NK}$ group (Fig. 4C). Meanwhile, although NK cell proliferation at the steady state was not affected by TIPE2 deficiency (Fig. 2B), enhanced level of proliferation, as assessed by Ki-67 expression, was detected in TIPE2-deficient NK cells after IL-15c stimulation, as compared with control NK cells (Fig. 4D). In addition, control NK cells and TIPE2-deficient NK cells showed comparable levels of annexin V staining, indicating similar apoptosis in both groups under the effect of IL-15 (Fig. 4D). These data demonstrated that NK-specific TIPE2 deficiency promotes NK cell proliferation in response to IL-15.

We also examined the effect of TIPE2 overexpression on human NK92 cell response to IL-15 and found that IL-15 stimulation resulted in significantly fewer TIPE2-overexpressed NK92 cells than control NK92 cells (Fig. 4E), supporting a role of TIPE2 in suppressing NK cell response to IL-15. Moreover, bone marrow–derived dendritic cells (BMDCs) are essential for IL-15–mediated regulation of NK cells. Here, TIPE2 deficiency also led to increased accumulation of NK cells after coculture with BMDCs (Fig. 4F). These effects were abrogated in the presence of anti–IL-15/IL-15 receptor antibody (Fig. 4F), indicating that TIPE2 deficiency promotes NK cell survival by interacting with BMDCs in an IL-15–dependent manner. We observed that the cellular size and granularity of NK cells, as measured by FSC (forward scatter) and SSC (side scatter) intensity of NK cells, were comparable between TIPE2-deficient and control NK cells at steady state. However, TIPE2-deficient NK cells displayed significantly higher signals of both FSC and SSC than control NK cells in response to IL-15 stimulation in vitro (Fig. 4G), indicating enhanced activity in TIPE2-deficient NK cells in response to IL-15. Furthermore, TIPE2-deficient NK cells displayed significantly enhanced up-regulation of activation marker CD69 and nutritional receptor CD71 in response to IL-15, while the enhanced up-regulation of CD98, another nutritional receptor, was relatively modest (Fig. 4H). Together, these results indicated that NK-specific TIPE2 deficiency promotes NK cell response to IL-15, suggesting that TIPE2 might suppress IL-15 signaling in NK cells.

TIPE2 suppresses IL-15–mediated mTOR activation in NK cells

mTOR kinase activation is essential for IL-15 signaling during the development of NK cells (*16*). The above observation that TIPE2 suppressed IL-15 response by NK cells prompted us to assess the phosphorylation status of S6 and Akt Ser^{473}, the downstream effectors of the IL-15–phosphatidylinositol 3-kinase (PI3K)–mTOR pathway (*29*, *30*). Upon IL-15 stimulation, TIPE2-overexpressed NK92 cells suppressed phosphorylation of both S6 and Akt Ser^{473}, as shown either by Western blot or flow cytometry (Fig. 5, A and B), while TIPE2 deficiency in mouse NK cells increased phosphorylation of S6 (Fig. 5, C and D) but not Akt Ser^{473} (Fig. 5, C and D). These effects on p-S6 by the overexpression or absence of TIPE2 were IL-15 dose dependent (fig. S3). On the other hand, overexpression or absence of TIPE2 showed no effect on IL-15–dependent phosphorylation of STAT5, the transcription factor that we believed is not involved in the mechanisms of action of TIPE2 (Fig. 5, E and F, and fig. S3). These data indicated that TIPE2 suppresses IL-15–mediated mTOR activity, possibly through indirect suppressive effects on mTOR in the IL-15–mTOR pathway in NK cells since we did not find the interaction of TIPE2 with S6, AKT, or mTOR.

To determine whether TIPE2 suppresses IL-15–triggered NK cell response via mTOR, we used an mTOR inhibitor, rapamycin, in the IL-15–triggered NK cell expansion assay (Fig. 5, G and H). The results showed that the presence of rapamycin abrogated both the inhibitory effect of TIPE2 overexpression (Fig. 5G) and the promoting effect of TIPE2 deficiency (Fig. 5H) on IL-15–triggered expansion of NK92 cells (Fig. 5G) or mouse NK cells (Fig. 5H). Therefore, TIPE2 suppresses IL-15–triggered NK cell response by influencing mTOR activity in vitro.

Next, we asked whether TIPE2 suppresses NK cell maturation via mTOR in vivo. We injected both $Tipe2^{\Delta NK/\Delta NK}$ mice and control $Tipe2^{WT/WT}$ mice with dimethyl sulfoxide (DMSO) or rapamycin for five consecutive days and assessed NK cell levels in the bone marrow, where they begin the maturation process. We found that although $Tipe2^{\Delta NK/\Delta NK}$ mice contained significantly higher levels of both total NK cells and $CD11b^{+}CD27^{-}$ terminally matured NK cells in bone marrow than control mice, the injection of rapamycin eliminated these effects (Fig. 5I). These data demonstrated that the higher levels of total or mature NK cells in $Tipe2^{\Delta NK/\Delta NK}$ mice, when compared with control $Tipe2^{WT/WT}$ mice, were dependent on mTOR activity, further suggesting that TIPE2 suppresses NK cell maturation by influencing mTOR activity in vivo.

NK-specific TIPE2 deficiency enhances antitumor immunity

The above data showed that NK-specific TIPE2 deficiency resulted in the increased level of mature NK cells and enhanced overall effector functions of NK cells; therefore, we wondered whether NK-specific TIPE2 deficiency also affects tumor surveillance in vivo. We found that $Tipe2^{\Delta NK/\Delta NK}$ mice were more resistant to tumor growth in vivo than $Tipe2^{WT/WT}$ mice, as evidenced by significantly reduced growth of MC38, RMAS, and LLC (Lewis lung carcinoma cell) tumors, as well

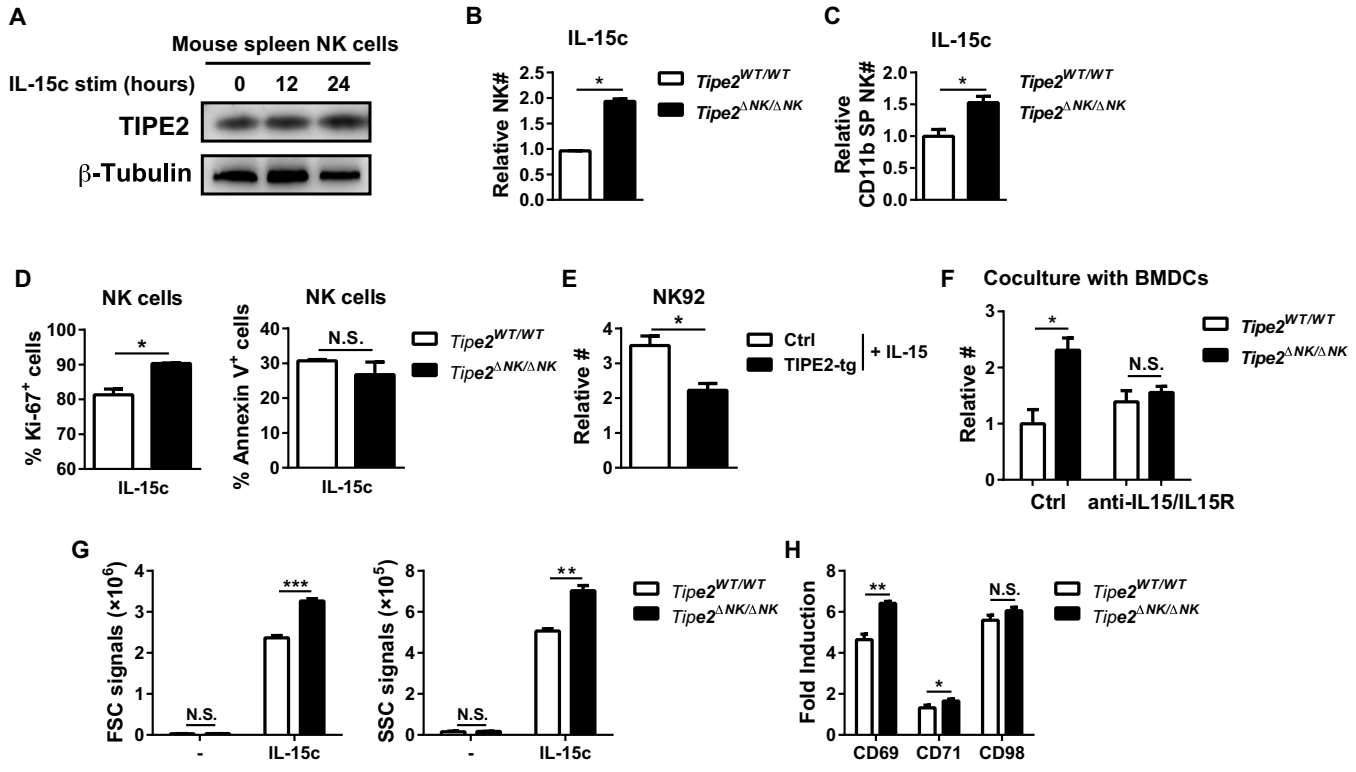

Fig. 4. NK-specific TIPE2 deficiency promotes NK cell response to IL-15. (**A**) Purified mouse splenic NK cells from wild-type mice were stimulated with IL-15/IL-15 receptor complex (10 ng/ml) for the indicated time for determining TIPE2 expression by Western blot. (**B** and **C**) Purified splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were stimulated with IL-15/IL-15 receptor complex (10 ng/ml) for 3 days before determining cell numbers of total (B) or CD11b SP NK cells (C) relative to the numbers of total (B) or CD11b SP (C) NK cells on day 0. (**D**) Ki-67 or annexin V levels in NK cells 1 day after stimulation as in (B). (**E**) TIPE2–overexpressed or control NK92 cells were stimulated with IL-15 (1 ng/ml) for 3 days before determining the cell numbers relative to NK92 cells of the same genotypes without IL-15 stimulation on day 3. (**F**) Splenic NK cells were cocultured with BMDCs for 3 days before determining the NK cell numbers relative to the control group with BMDCs alone on day 3. (**G**) Splenocytes from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were stimulated with IL-15/IL-15 receptor complex (10 ng/ml) for 3 days. FSC and SSC signals of NK cells were detected by flow cytometry and quantified as mean fluorescence intensity. (**H**) Splenocytes from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were stimulated with IL-15/IL-15 receptor complex (80 ng/ml) for 1 day. Expression of CD69, CD71, and CD98 on NK cells was detected by flow cytometry and was quantified as mean fluorescence intensity. The results are presented relative to unstimulated cells of respective genotypes set as 1. (A to H) Data are representative of at least two independent experiments. (B to H) Data are presented as the means ± SEM. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.001$. [unpaired Student's *t* test (B to E, G, and H) or one-way ANOVA (F)].

as decreased end point tumor mass (Fig. 6, A to C). These indicated that NK-specific TIPE2 deficiency suppresses tumor growth in vivo. Moreover, we detected elevated number of tumor-infiltrating NK cells (Fig. 6D) and higher percentage of IFN-γ–producing or perforin-producing cells among tumor-infiltrating NK cells (Fig. 6E), indicating enhanced functions of tumor-infiltrating NK cells. Therefore, NK-specific TIPE2 deficiency could promote NK cell antitumor functions.

DISCUSSION

NK cells have to go through the maturation process to reach optimal functional status for host immune surveillance, and the maturation process has to be strictly regulated. However, negative mechanisms that constrain NK cell maturation remain poorly understood. In this study, we showed that TIPE2 protein displays an expression pattern correlated with NK cell maturation and acted as a negative regulator to inhibit NK cell maturation. Specific deletion of *Tnfaip8l2* in NK cells resulted in the increased level of mature NK cells with enhanced effector functions and response to IL-15 stimulation via elevated IL-15–triggered mTOR activity. As a consequence, NK-specific TIPE2-deficient mice were more resistant to tumor challenges.

TIPE2 belongs to a protein family that consists of four proteins—TIPE, TIPE1, TIPE2, and TIPE3 (*31*)—whose aberrant expression has been reported in many human diseases (*32*–*37*). Among these four proteins, TIPE2 is expressed mainly in leukocytes (*23*) and has been reported to promote the differentiation and/or the regulatory functions of various immunosuppressive cells (*24*–*26*). TIPE2 is an inducible negative regulator upon certain stimulations such as TNF-α (*23*), reactive oxygen species, IL-6, and L-arginine (*24*). In addition, TIPE2 is down-regulated in CD8$^+$ T cells of patients with hepatitis B virus, correlating with the increased expression of perforin, granzyme B, and IFN-γ by these cells (*38*), suggesting that TIPE2 might play a role in suppressing the effector functions of cytotoxic lymphocytes. TIPE2 is a negative regulator of innate immunity (*23*), and its global deficiency leads to reduced tumor growth (*24*), suggesting that TIPE2 might regulate the innate surveillance of tumor by the host. NK cells are the first line in tumor surveillance. Our data showed that TIPE2 plays an essential role in NK cell biology by displaying an expression pattern correlating with NK cell

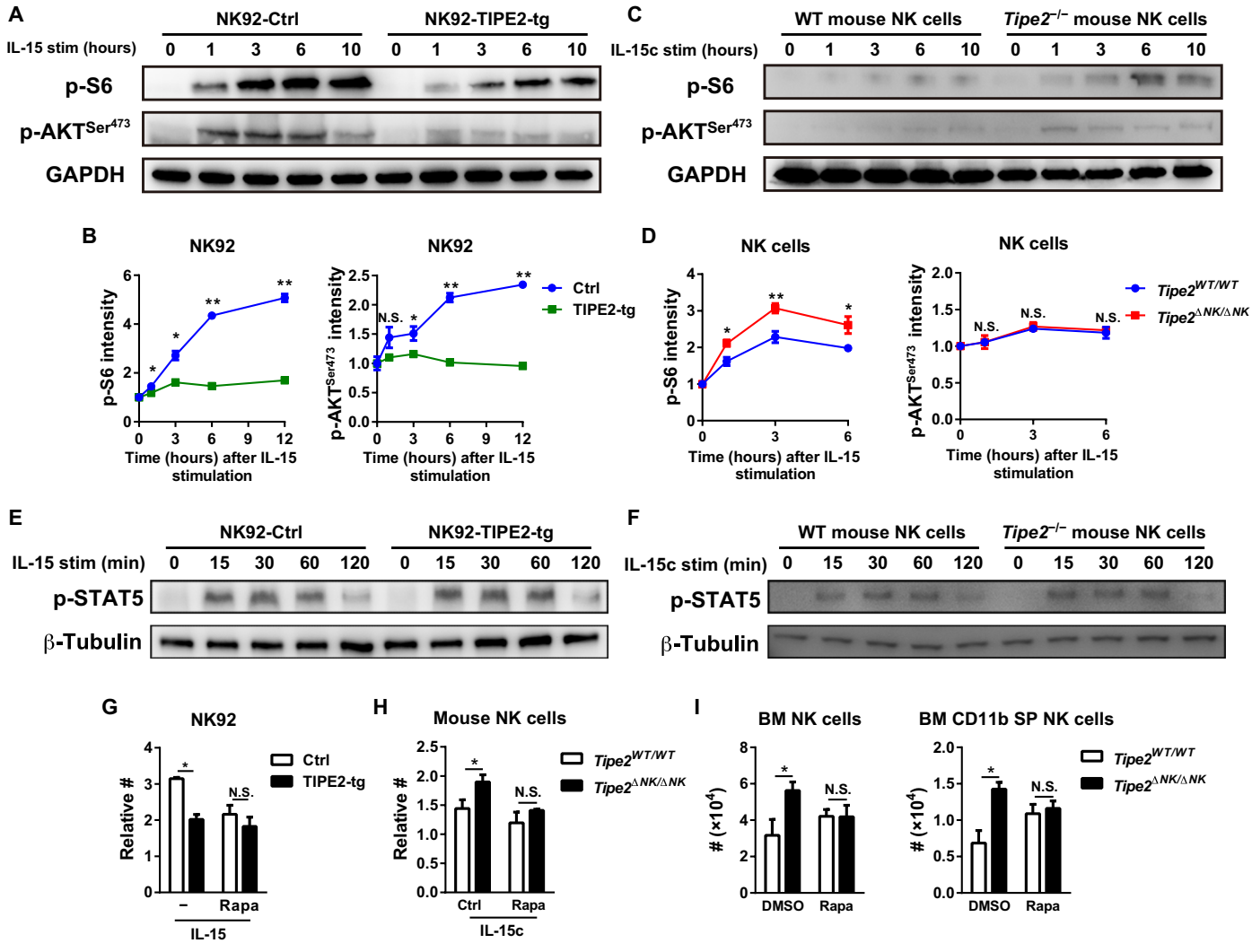

Fig. 5. TIPE2 suppresses IL-15–mediated mTOR activation in NK cells. (**A**, **B**, and **E**) TIPE2-overexpressed or control NK92 cells were stimulated with IL-15 (10 ng/ml) for the indicated time for determining the phosphorylation levels of S6 and Akt Ser^{473} (A and B) or STAT5 (E) by Western blot (A and E) or flow cytometry (B). (**C**, **D**, and **F**) Purified splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were stimulated with IL-15/IL-15 receptor complex (10 ng/ml) for the indicated time for determining the phosphorylation levels of S6 and Akt Ser^{473} (C and D) or STAT5 (F) by Western blot (C and F) or flow cytometry (D). (**G**) TIPE2-overexpressed or control NK92 cells were left untreated or stimulated with IL-15 (10 ng/ml) in the presence of rapamycin or equal volume of DMSO as vehicle. Cell numbers relative to the group without IL-15 stimulation were assessed 3 days after stimulation. (**H**) Splenic NK cells from *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were stimulated with IL-15/IL-15 receptor complex (10 ng/ml) in the presence of rapamycin or equal volume of DMSO as vehicle. NK cell numbers relative to those of each genotypes on day 0 were assessed 3 days after stimulation. (**I**) *Tipe2*$^{WT/WT}$ mice or *Tipe2*$^{\Delta NK/\Delta NK}$ mice were injected with 25 μg of rapamycin or equal volume (5 μl) of DMSO in 400 μl of phosphate-buffered saline for four consecutive days. Absolute numbers of total NK cells or CD11b SP NK cells in the bone marrow were assessed 2 days after final injection. (A to I) Data are representative of at least three independent experiments. (B and D to I) Data are presented as means ± SEM. *$P < 0.05$ and **$P < 0.005$ (one-way ANOVA).

maturation process and by negatively regulating NK cell maturation and antitumor immunity. Although our Western blot results in Fig. 4A showed that total protein levels of TIPE2 in NK cells were not further induced by IL-15 receptor signaling upon IL-15 stimulation, TIPE2 has been reported to interact and inhibit the activation of molecular "on-and-off" switches such as Rac (*39*) or signaling effectors such as RalGDS (Ral guanine nucleotide dissociation stimulator) (*40*) upon the triggering of signaling cascade by stimulations. In addition, TIPE2 is subcellularly localized in the leading edge after exposure of the cells to chemoattractants that activate the PI3K-AKT pathway (*41*). Therefore, upon IL-15 stimulation, TIPE2 might be "inducibly" recruited to the intracytoplasmic membrane to interact with other molecular switches to play a role in the regulation of signaling pathways downstream the IL-15 receptor.

The NK cell maturation process relies on IL-15 signaling cascades to support the survival, to stimulate the proliferation, or to antagonize apoptosis of NK cells (*4*). This process also depends on the presence of transcription factors T-bet, Eomes (Eomesodermin homolog), STAT5, TOX, PRDM1 (PR domain zinc finger protein 1), Zeb2 (Zinc finger E-box-binding homeobox 2), etc. to direct the differentiation and expression of effector molecules (*4*). Although the factors required for NK cell maturation have been extensively investigated,

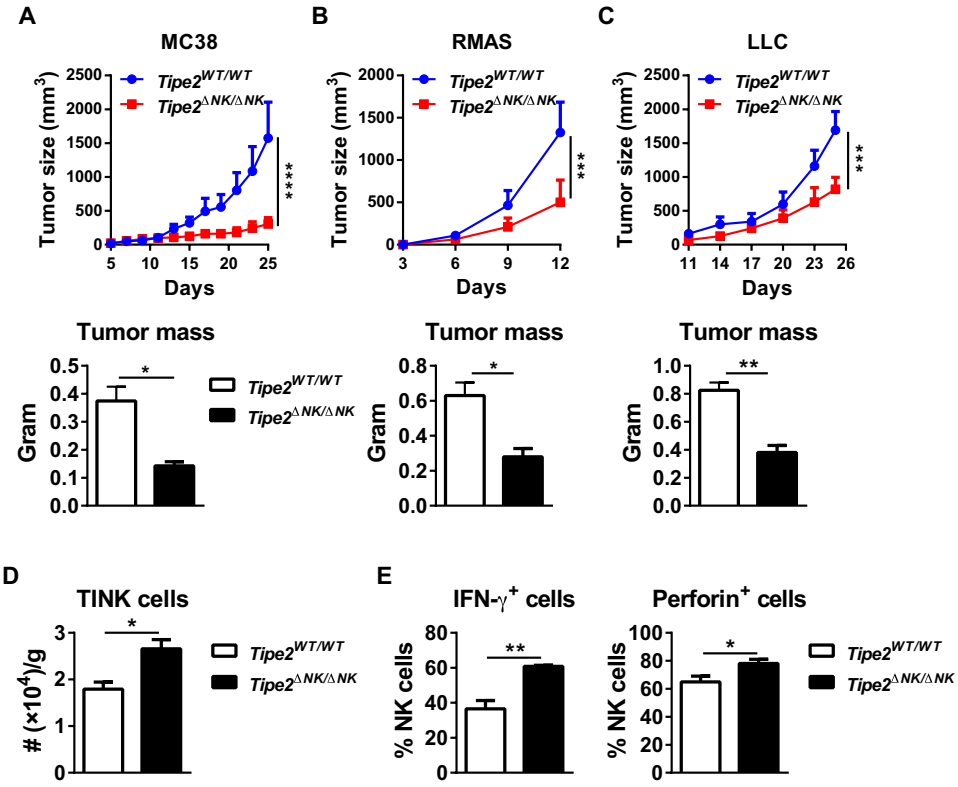

Fig. 6. NK-specific TIPE2 deficiency promotes NK cell antitumor functions. (**A** to **C**) *Tipe2*$^{WT/WT}$ mice and *Tipe2*$^{\Delta NK/\Delta NK}$ mice were subcutaneously inoculated with MC38 (A), RMAS (B), or LLC (C) tumor cells. Tumor growth and end point tumor mass were shown. (**D** and **E**) Absolute numbers (D) and percentages of IFN-γ or perforin-producing cells (E) of tumor-infiltrating NK (TINK) cells were shown in the MC38 model from (A). (A to E) Groups of six mice per experiment were used. Data are representative of at least three independent experiments and are presented as means ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, and ****$P < 0.0001$ [two-way ANOVA (A to C) or unpaired Student's *t* test (A to E)].

very few negative regulators specifically targeting this process are reported (*8*, *9*). Here, in this study, we found that the expression of TIPE2, a previously reported negative regulator of innate and adaptive immunity, correlated with the NK cell maturation process. We uncovered TIPE2 as a previously unidentified negative regulator in NK cell maturation in mice by suppressing the IL-15–mTOR signaling cascade. As for the role of TIPE2 in human NK cells, we showed that TIPE2 expression not only correlated with human NK cell maturation stages analyzed by single-cell transcriptomic analysis but was also higher in more matured CD56^{dim} human NK cells than in less matured CD56^{bright} human NK cells. In addition, TIPE2 suppressed the cytolytic activity and IL-15 response of human NK cell lines in vitro. These data suggest that human TIPE2 might have a similar role as mouse TIPE2 in suppressing NK cell maturation.

Our results showed that *Tipe2*$^{\Delta NK/\Delta NK}$ mice contained more mature NK cells than control mice, while Ki-67 and apoptosis parameters at the steady state in vivo remained unaltered. These data suggest that changes in cell numbers could not just be attributed to changes in the proliferation/apoptosis parameters at steady state, especially at the whole population level. This sometimes happens during lymphocyte differentiation, as already reported. Previous studies have shown that changes in NK cell transition from immature to mature NK cells result in changes in total and mature NK cell levels; however, the cause of which is not demonstrated by parameters of in vivo proliferation, survival, or death of the whole NK cell population at steady state (*5*, *42*, *43*). In addition, rather than constantly responding to soluble IL-15, NK cell precursor (NKP) and NK cell development intermediates reside in specialized bone marrow niches that are characterized by IL-15Rα–expressing cells that transpresent IL-15, which is fundamental for NK cell homeostasis and development (*44*–*46*). Since these intercellular interactions in the specific "niche" are spatial and dynamic, some changes on this process might not be fully reflected by proliferation markers at the whole NK population level. In an in vitro system, to mimic such process, our data from Fig. 4F showed that TIPE2-deficient NK cells cocultured with BMDCs that express IL-15Rα transpresenting IL-15 resulted in significantly more NK cell numbers than wild-type NK cells in an IL-15Rα/IL-15–dependent manner, suggesting that in the NK cell "niches" of physiological conditions, TIPE2 deficiency might promote IL-15 signaling in NK cells to stimulate higher level of NK cell proliferation, which, however, might not be able to show changes in proliferation marker by the whole NK cell population. On the other hand, the increased fraction of NK cells in *Tipe2*$^{\Delta NK/\Delta NK}$ mice was predominantly CD11b^{+}CD27^{-} mature NK cells (Fig. 2D). Transferring immature, but not mature, TIPE2-deficient NK cells into NSG mice resulted in increased mature NK cells (Fig. 2F and fig. S2). These data suggest that TIPE2 deficiency might promote the differentiation process from immature NK cells to mature NK cells, resulting in increased accumulation of mature NK cells in these mice. Meanwhile, the niche vacated by previous immature NK cells might

dynamically allow increased production of new immature NK cells from NK precursors, possibly leading to overall stable levels of immature NK cells between *Tipe2*$^{\Delta NK/\Delta NK}$ mice and control mice. These dynamic changes in differentiation altogether result in an increased number of total NK cells in normal hematopoietic condition; however, these changes might not be reflected by the proliferation markers at the whole NK cell population level.

As an essential factor downstream of IL-15 signaling in NK cells, mTOR plays an important role in NK cell development, maturation, and activation (*16*, *17*, *47*). IL-15 activates mTOR and boosts bioenergetic metabolism for the growth and nutrient uptake of NK cells, which is essential for the proliferation and acquisition of cytolytic potential during NK cell activation (*16*). Defective mTOR activity resulted in reduced expression of transcriptional factors that govern NK cell development and maturation, leading to reduced cell numbers, impaired maturation, and diminished effector functions of NK cells (*16*, *47*–*49*). In this study, we showed that TIPE2 suppressed IL-15–triggered mTOR activity. In addition, we also detected decreased level of phosphatidylinositol 3,4,5-trisphosphate, the product of mTOR-upstream PI3K, in TIPE2-overexpressed YTS human NK cells (data not shown), suggesting that TIPE2 might also suppress the activity of PI3K resulting in the observed suppression of mTOR. Considering that TIPE2 could serve as the binding protein for lipid second messenger to promote PI3K activity at the leading edge of migrating cells during leukocyte chemotaxis (*41*), our results suggest that TIPE2 might display opposite functions depending on the type of stimulation and the subcellular localization. Alternatively, TIPE2 might suppress mTOR activity independently of PI3K. TIPE2 can directly bind and inhibit Rac (*39*), which was confirmed in TIPE2-overexpressed NK92 cells (data not shown). Rac1 was previously shown to directly bind mTOR and mediate localization of mTORC1 and mTORC2 to specific membranes for their activation (*50*), and inhibition or deletion of Rac suppresses mTOR activity independently of PI3K (*50*–*53*). Therefore, TIPE2 might suppress mTOR activation by inhibiting Rac1.

Our data demonstrated that TIPE2 suppressed the mTOR activity downstream of IL-15 receptor, suggesting that TIPE2 might also suppress mTOR activity triggered by other mTOR–stimulating cytokines, such as IL-2, IL-12, IL-21, and IL-18 (*54*–*58*). However, compared with other mTOR–stimulating cytokines that majorly promote NK cell effector functions, IL-15 plays an important role in NK cell development and maturation (*4*, *58*). On the other hand, when NK cells are stimulated with IL-15 together with other mTOR–stimulating cytokines, these other cytokines might affect TIPE2 regulation of mTOR; the outcome of which, however, should be determined by the complex interactions of the signaling network downstream of various cytokines, requiring further investigations in the future.

NK cells play an important role in tumor surveillance; however, the molecular regulation of NK cell antitumor immunity is poorly understood. Here, we found that TIPE2 deficiency in NK cells resulted in the enhanced tumor control of tumor-bearing mice, which represents an overall increased antitumor effector functions at the whole NK cell population level. Besides, our analysis of parameters in CD11b^{hi} mature NK cell effector functions indicated that TIPE2 also suppressed NK cell functions on a per cell basis. In line with this, changes in NK cell maturation are commonly accompanied by changes in effector functions on a per cell basis (*5*, *47*). On the basis of our analysis on TIPE2-deficient NK cell total numbers, maturation level, and effector functions, the better antitumor immunity that we observed could be attributed to a collective contribution of increased NK cell maturation, numbers, and enhanced NK cell activity on a per cell basis. On the other hand, although IL-15 signaling is not directly involved in the in vitro functional assays in Fig. 3 (D to F), IL-15 signaling could have already affected the functional potential of NK cells used in these assays before they were isolated from the mice. IL-15 signaling in NK cells was constantly stimulated by physiological IL-15 in vivo, which, according to our data in Fig. 4, should be regulated by TIPE2. This possibly leads to the increased effector functions on a per cell basis that we observed in Fig. 3 (D to F).

In summary, TIPE2 suppresses IL-15–triggered mTOR activity in NK cells to inhibit NK cell maturation and effector functions. Deletion of TIPE2 renders NK cells with a more matured phenotype and antitumor functions. Our study coincides with previous reports in other immune cells regarding the idea that TIPE2 protein functions as a negative regulator of antitumor immune responses in the immune system (*24*–*26*), suggesting that TIPE2 is a checkpoint in NK cell maturation and antitumor immunity and targeting TIPE2 might benefit NK-based tumor immunotherapy.

MATERIALS AND METHODS

Mice

Tnfaip8l2$^{fl/fl}$ mice were generated by the Cam-Su Genomic Resource Center (Suzhou, China). *Ncr1-iCre* mice and NOD-*Scid*-$\gamma c^{-/-}$mice were purchased from Biocytogen (Beijing, China). *Tnfaip8l2*$^{fl/fl}$ mice were crossed with *Ncr1-iCre* mice to generate NK-specific TIPE2-deficient mice (referred to as "*Tipe2*$^{\Delta NK/\Delta NK}$ mice"); age and sex matched *Tnfaip8l2*$^{fl/fl}$ mice were used as control (referred to as "*Tipe2*$^{WT/WT}$ mice"). All mice used were 5 to 8 weeks old, had a B6 background, and were housed in the specific pathogen–free facility at the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Western blot

The following primary antibodies were used for Western blot: antibodies to glyceraldehyde-3-phosphate dehydrogenase (1A6, Bioworld, Nanjing, China), β-tubulin (Proteintech, Rosemont, USA), β-actin (AC-15, Sigma-Aldrich, Santa Clara, USA), TIPE2 (Proteintech, Rosemont, USA), and phospho-STAT5 (94-10C-9-10C-2, Millipore, Burlington, USA), and phospho-S6 (D57.2.2E) and phospho-AKT (Ser^{473}) (D9E) were purchased from Cell Signaling Technology (Danvers, USA). The use of peripheral blood from healthy donors was approved by the ethical committee of Shenzhen Institutes of Advanced Technology.

Flow cytometry

Single-cell suspensions were stained with the appropriate monoclonal antibody in phosphate-buffered saline containing 5% rat serum. When necessary, intracellular staining was performed using True-Nuclear Transcription Factor Buffer Set (BioLegend) according to the manufacturer's instructions. CytoFLEX (Beckman Coulter, Brea, USA) and FACSAria III (BD Biosciences, San Diego, USA) were used for analysis and cell sorting, with dead cells excluded by the LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, USA). Antibodies specific for CD45 (30-F11), NK1.1 (PK136), mouse CD3 (17A2), CD56 (HCD56), human CD3 (UCHT1), CD11b (M1/70),

CD27 (LG.3A10), KLRG1 (2F1/KLRG1), CD43 (S11), Ki-67 (16A8), c-Kit (2B8), CD122 (TM-β1), NKG2D (CX5), Lineage Cocktail (anti-CD3ε, 145-2C11; anti–Ly-6G/Ly-6C, RB6-8C5; anti-CD45R/B220, RA3-6B2; anti–TER-119, Ter-119; and anti-CD11b, M1/70), CD107a (1D4B), CD69 (H1.2F3), IFN-γ (XMG1.2), and perforin (S16009A) were purchased from BioLegend (San Diego, USA). Antibodies specific for phospho-S6 (N7-548) and phospho-AKT (Ser^{473}) (M89-61) were purchased from BD Biosciences (San Diego, USA). Annexin V Apoptosis Detection Kit (BioLegend, San Diego, USA) was used to assess the levels of annexin V^+ apoptotic cells according to the manufacturer's instructions.

Single-cell RNA sequencing and data processing

Splenic NK cells pooled from three mice of each indicated genotypes were enriched by MACS (magnetic-activated cell sorting) for library preparation and subsequent RNA sequencing by Berry Genomics (Beijing, China). The 10x Genomics Chromium single-cell 3′ v4 kit and protocol was used to prepare the library. RNA sequencing was performed on a NovaSeq 6000 machine with sequencing depth of about 80,000 reads per cell. Raw FASTQ files were processed with Cell Ranger software (v1.3.1) for alignment, filtering, barcode counting, and UMI (Unique Molecular Identifier) counting. Further analysis was performed with Seurat (*59*, *60*) by selecting highly variable genes using FindVariableGenes functions with default parameters, reducing the dimensionality by principal components analysis, and clustering the cells using FindNeighbors and FindClusters. We then applied RunUMAP for visualization. Specific parameters used were described below.

For analysis of *Tnfaip8l2* expression in wild-type mouse splenic NK cells, 4488 NK cells were identified by the positive expression of *Ncr1* for cluster analysis. We clustered the cells using FindNeighbors (dims = 1:10) and FindClusters (resolution = 0.2) and applied RunUMAP (dims = 1:10) for visualization. For analysis of *Tnfaip8l2* expression in human primary NK cells from the peripheral blood, dataset GSM3738543,which includes 1530 cells, was analyzed similarly as above except for the following parameters: FindNeighbors (dims = 1:10), FindClusters (resolution = 0.3), and RunUMAP (dims = 1:10).

For comparison of splenic NK cells from $Tipe2^{\Delta NK/\Delta NK}$ mice or control $Tipe2^{WT/WT}$ mice, the data including 4270 NK cells from $Tipe2^{\Delta NK/\Delta NK}$ mice and 4488 NK cells from control $Tipe2^{WT/WT}$ mice were merged for analysis. FindNeighbors (dims = 1:10) and FindClusters (resolution = 0.2) were applied for clustering, and RunUMAP (dims = 1:10) was used for visualization.

Adoptive transfer

$CD11b^-CD27^+$ immature or $CD11b^+CD27^-$ mature splenic NK cells were sorted from $Tipe2^{\Delta NK/\Delta NK}$ mice or control $Tipe2^{WT/WT}$ mice and transferred into NOD-*Scid*-$\gamma c^{-/-}$ mice (3000 cells per mice for immature NK cells or 20,000 cells per mice for mature NK cells). Six days later, absolute numbers of total NK cells or $CD11b^+CD27^-$ mature NK cells per femur of the recipient mice were analyzed.

Cytolytic assays

For cytolytic assays against YAC-1 cells or 721.221 cells, CellTrace Violet (CTV; Invitrogen, Carlsbad, USA)–labeled target cells were cocultured with effector cells (mouse splenic NK cells or YTS cells) at the indicated effector:target (E:T) ratios for 4 hours. After that, cell mixtures were stained with 7-AAD (7-aminoactinomycin D) to determine percentages of 7-AAD^+ dead cells among CTV^+ target cells.

For cytolytic assays against MC38 cells or HCT116 cells, levels of live target cells were measured using a real-time electric cell impedance sensing system (CP96, Applied Biophysics, Troy, USA). Target cells were first seeded into the wells of CP96 plates in 100 μl of media. After target cells formed a monolayer (within 24 hours), effector cells [mouse splenic NK cells stimulated with IL-12 (20 ng/ml), IL-15 (50 ng/ml), and IL-18 (10 ng/ml) for 48 hours or NK92 cells] were added to wells containing target cells. After addition of effector cells, the system continued to take measurements every 30 min for 3 hours.

For in vivo cytolytic assay, 10^6 CTV–labeled MHC-I–sufficient RMA cells and 10^6 CellTrace Far Red (CTFR; Invitrogen, Carlsbad, USA)–labeled MHC^{low} RMAS cells were mixed before intraperitoneal injection. Fourteen hours later, peritoneal cells were harvested to calculate the ratio of remaining $CTFR^+$ RMAS cells to remaining CTV^+ RMA cells as percentage of surviving RMAS cells ("surviving RMAS %"). The in vivo cytolysis percentage was calculated as 100% − surviving RMAS %.

Cell lines

YTS and NK92 human NK cell lines, 721.221 B lymphoblastoid cell line, MC38 colon cancer cells, *Tap2*-deficient RMAS lymphoma cells, and parental RMA lymphoma cells were preserved in-house. LLC lung cancer cells and HCT116 colon cancer cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). YTS, RMA, and RMAS cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and penicillin and streptomycin (100 IU/ml). MC38, LLC, and HCT116 cells were cultured in Dulbecco's modified Eagle medium with 10% FBS and penicillin and streptomycin (100 IU/ml). NK92 cells were cultured in α-MEM with 12.5% FBS, 12.5% horse serum, penicillin and streptomycin (100 IU/ml), and IL-2 (10 ng/ml).

Tumor models

Groups of six mice per experiment were used. The group size ensured enough power to determine biological differences. No mice were excluded in this study, and no active randomization was applied to groups. The investigators were not blinded to group allocation during the experiment and/or when assessing the outcome. Single-cell suspension of MC38 colon cancer, RMAS lymphoma, or LLC lung cancer cells were injected intravenously into the tail vein of the indicated strains of mice (2×10^5 cells per mouse). Mice were euthanized on days 16 to 21 following tumor injection for analysis of tumor infiltrating lymphocytes, as previously described (*3*).

Statistical analysis

Statistically significant differences between two groups were determined by the Student's *t* tests or one-way analysis of variance (ANOVA) when appropriate, except that two-way ANOVA was used in the cytolytic assays against MC38 and HCT116, as well as in the comparison of tumor growth. $P < 0.05$ was considered significant in all analyses.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abi6515

View/request a protocol for this paper from *Bio-protocol*.

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