IL-17 constrains natural killer cell activity by restraining IL-15–driven cell maturation via SOCS3

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Increasing evidence demonstrates that IL-17A promotes tumorigenesis, metastasis, and viral infection. Natural killer (NK) cells are critical for defending against tumors and infections. However, the roles and mechanisms of IL-17A in regulating NK cell activity remain elusive. Herein, our study demonstrated that IL-17A constrained NK cell antitumor and antiviral activity by restraining NK cell maturation. It was observed that the development and metastasis of tumors were suppressed in IL-17A-deficient mice in the NK cell-dependent manner. In addition, the antiviral activity of NK cells was also improved in IL-17A-deficient mice. Mechanistically, ablation of IL-17A signaling promoted generation of terminally mature CD27[−]CD11b⁺ NK cells, whereas constitutive IL-17A signaling reduced terminally mature NK cells. Parabiosis or mixed bone marrow chimeras from *Il17a*^{−/−} and wild-type (WT) mice could inhibit excessive generation of terminally mature NK cells induced by IL-17A deficiency. Furthermore, IL-17A desensitized NK cell responses to IL-15 and suppressed IL-15-induced phosphorylation of signal transducer and activator of transcription 5 (STAT5) via up-regulation of SOCS3, leading to down-regulation of Blimp-1. Therefore, IL-17A acts as the checkpoint during NK cell terminal maturation, which highlights potential interventions to defend against tumors and viral infections.

IL-17A | NK cells | IL-15 | SOCS3 | terminal maturation

NK cells are derived from hematopoietic stem cells via a series of developmental stages, including NK cell precursors (lin[−]CD122⁺NK1.1[−]), immature (Imm) NK cells (NK1.1⁺DX5[−]CD27⁺CD11b[−]), mature 1 NK cells ([M1], NK1.1⁺DX5⁺CD27⁺CD11b⁺), and mature 2 NK cells ([M2], NK1.1⁺DX5⁺CD27[−]CD11b⁺) (1, 2). The developmental process of NK cells is regulated by multiple factors, among which the IL-15-JAK-STAT signaling pathway is the most important for promotion of NK cell maturation (3). STAT5 deficiency dramatically reduces NK cell numbers and abrogates NK cell maturation (4, 5). The IL-15-dependent transcription factor Blimp-1 is critical for NK cell maturation, which is characterized by a decrease in CD27 and increases in CD11b, KLRG1, and CD43 expression (6). In contrast, it has been reported that TGF- β signaling suppresses NK cell maturation to maintain NK cell homeostasis by constraining IL-15 signaling (7). Moreover, multiple intrinsic factors have also been found to regulate IL-15 signaling and NK cell maturation. For example, the balance between E-protein target genes and ID2 tunes the sensitivity of NK cells to IL-15 (8); Src homology-2-containing protein (CIS) suppresses IL-15–driven Janus kinase (JAK)–STAT signaling in NK cells (9); and FOXO1 inhibits the terminal maturation and effector functions of NK cells by repressing TBX21 expression (10). However, the underlying endogenous mechanisms for controlling the maturation and activity of NK cells remain elusive.

It is well established that NK cells make a critical contribution to immune defenses against tumors and infections and act in the first line by directly killing transformed cells and/or secreting cytokines (11, 12). The activity of NK cells is regulated by acti-

vating and inhibitory receptors during responsive or developmental process (13–15). Compromise of NK cell activity increases susceptibility to infection and malignancies, while excessive NK cell responses can cause severe tissue damage (16–18). Therefore, maintenance of NK cell homeostasis is important for a healthy immune status. Moreover, increased understanding of the mechanisms involved in the maintenance of NK cell homeostasis will be essential for the development of improved immunotherapy approaches to combat tumors and infections.

IL-17A has important functions in autoimmunity, infection, and cancer (19). Binding of IL-17A to the IL-17RA/IL-17RC receptor complex induces the activation of nuclear factor- κ B (NF- κ B), mitogen-activated protein kinase, and CCAAT/enhancer binding proteins (20). Recent studies demonstrate that IL-17A mediates the cancer development promoted by commensal microbiota (21, 22). Moreover, accumulating evidence illustrates that IL-17A displays protumor roles by recruiting neutrophils and myeloid-derived suppressor cells, promoting angiogenesis, or suppressing CD8⁺T cells (23). The crosstalk between IL-17A and NK cells in the cancer development has yet to be explored, albeit that the negative correlations between NK cell activity and IL-17A levels are observed in some types of cancer (24, 25). In addition, it is reported that increased IL-17A is accompanied by decreased NK cell numbers/activity in patients with atopic dermatitis who are susceptible to viral infection (26, 27). Moreover, IL-17 facilitates the

Significance

IL-17A promotes tumorigenesis, metastasis, and viral infection. However, the underlying mechanisms remain elusive. By using diverse gene-deficient mice, antibody depletion, and animal models, we show that IL-17A promotes tumorigenesis, metastasis, and viral infection by constraining NK cell antitumor and antiviral activity via inhibition of NK cell maturation. The ablation of IL-17A signaling increases terminally mature CD27[−]CD11b⁺ NK cells, whereas constitutive IL-17A signaling reduces terminally mature NK cells. IL-17A suppresses IL-15–induced phosphorylation of STAT5 via up-regulation of SOCS3 in NK cells, leading to inhibition of NK cell terminal maturation. Therefore, IL-17A acts as the checkpoint during NK cell terminal maturation, which suggests potential interventions to defend against tumors and infections.

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induction of severe skin lesions by the vaccinia virus through inhibiting NK cell activity (28), indicating that high levels of IL-17A may mediate viral immune escape through the induction of NK cell dysfunction. However, the role and mechanism of IL-17A in regulating NK cell activity during cancer development and viral infection remains unclear.

In the current study, we demonstrated that IL-17 constrains NK cell antitumor and antiviral activity through the inhibition of terminal maturation by desensitizing them to IL-15 stimulation via SOCS3. This information provides opportunities for the development of potential interventions to treat chronic viral infections and tumors exacerbated by targeting inflammation.

Results

IL-17A Deficiency Enhances NK Cell Antitumor and Antiviral Activity.

IL-17A has been identified to promote cancer development and metastasis. Consistently, it was observed in our study that the growth (size and weight) of colon cancer and melanoma was significantly inhibited in *Il17a*^{-/-} mice after MC38 and B16F10 were inoculated into *Il17a*^{-/-} and WT mice (Fig. 1 A and C). Furthermore, there were fewer metastatic colon cancer nodules in the liver and melanoma metastases in the lungs of *Il17a*^{-/-} and *Il17a*^{-/-}*Il17f*^{-/-} (DKO) mice (Fig. 1 B and D), suggesting IL-17A is detrimental in the host response to cancer metastasis. The depletion of CD8⁺ cells promoted the growth of colon cancer both in *Il17a*^{-/-} and in WT mice. But the tumor sizes in CD8⁺ cell-depleted *Il17a*^{-/-} mice were still smaller than those in CD8⁺ cell-depleted WT mice (SI Appendix, Fig. S14), suggesting that the inhibition of cancer growth due to IL-17A deficiency did not completely depend on CD8⁺ cells. But the depletion of NK cells significantly abolished the protection against the growth of colon cancer and melanoma observed in *Il17a*^{-/-} mice (Fig. 14). Moreover, the depletion of NK cells also profoundly abolished the protection against cancer metastasis observed in *Il17a*^{-/-} and DKO mice (Fig. 1B), suggesting that the enhanced in-

hibition of cancer metastasis due to IL-17A deficiency depends on NK cells. Therefore, these data reveal that IL-17A deficiency enhances host antitumor capacity partially in the NK cell-dependent manner, suggesting IL-17A constrains NK cell antitumor activity.

Our group has previously confirmed that IFN- γ -producing NK cells mediate virus-mimicking poly I:C-induced liver injury (18). To assess the role of IL-17A in NK cell-mediated liver injury, we injected mice with poly I:C/d-galactosamine (D-GalN) in which D-GalN can make hepatocytes more sensitive to IFN- γ -induced cell death. *Il17a* deficiency led to higher serum alanine aminotransferase (ALT) levels, more serious liver damage, higher levels of hepatic IFN- γ ⁺ NK cells, and elevated serum IFN- γ during *Il17a* deficiency after poly I:C/D-GalN injection (Fig. 2 A–D), suggesting IL-17A attenuates poly I:C/D-GalN-induced NK cell-mediated fulminant hepatitis. NK cells are also known to be the key defendant in the murine cytomegalovirus (MCMV) early-stage infection. To assess the influence of IL-17A deficiency on NK cell antiviral activity, *Il17a*^{-/-} and WT mice were infected intraperitoneally with MCMV. Viral titers were lower in the livers of *Il17a*^{-/-} than WT mice and were accompanied by a higher frequency of IFN- γ ⁺ NK cells in *Il17a*^{-/-} mice (Fig. 2 E and F), indicating that IL-17A deficiency enhanced the early control of MCMV infection by NK cells.

To confirm the inhibitory role of IL-17 in NK cell activity, we decipher the activity of NK cells from *Il17a*^{-/-} and WT mice. We observed that the frequency of CD69⁺ NK cells in the spleens of *Il17a*^{-/-} mice was higher than that in WT mice (SI Appendix, Fig. S1B). Splenocytes isolated separately from *Il17a*^{-/-} and stimulated with phorbol-12-myristate-13-acetate (PMA) (preactivated with poly I:C in vivo), or IL-2/IL-12 showed higher percentages of IFN- γ ⁺ NK cells than those from WT mice (Fig. 3 A and B), similar with the findings in hepatic NK cells (SI Appendix, Fig. S1C), suggesting IL-17A restricts the cytokine production by NK cells. Moreover, the specific lysis of YAC-1 cells by NK cells from *Il17a*^{-/-} mice was markedly higher ex vivo at the indicated

Fig. 1. IL-17 deficiency enhances antitumor activity of NK cells. (A) Tumor size in *Il17a*^{-/-} and WT mice treated with αASGM1 or PBS 4 wk after MC38 cell inoculation. (B) Metastatic nodules on the livers of WT, *Il17a*^{-/-}, and double knockout (DKO) mice treated with αASGM1 or PBS 2 wk after MC38 cell inoculation. (C) Tumor size in WT and *Il17a*^{-/-} mice treated with αASGM1 or PBS 3 wk after B16F10 cell inoculation. (D) Metastatic nodules on the lungs of WT, *Il17a*^{-/-}, and DKO mice 2 wk after B16F10 cell inoculation. Data are representative of, at least, 3 independent experiments and are shown as means ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005 (*n* = 5–7).

Fig. 2. IL-17 deficiency enhances antiviral activity of NK cells. (A–D) Serum ALT levels (A), liver damage areas (hematoxylin and eosin stained; original magnification, 100×) (B), frequency of IFN-γ⁺ hepatic NK cells (C), and serum IFN-γ levels (D) in *Il17a*^{-/-} and WT mice. *Il17a*^{-/-} and WT mice were treated with poly I:C/b-GalN. Tissue samples were analyzed 18 h after the poly I:C/b-GalN challenge. (E) Viral titers in the livers of *Il17a*^{-/-} and WT infected mice. (F) Frequencies of IFN-γ⁺ hepatic NK cells in *Il17a*^{-/-} and WT infected mice. *Il17a*^{-/-} and WT mice were infected with MCMV. Tissue samples were analyzed 36 h post MCMV infection. Data are representative of, at least, 3 independent experiments and are shown as means ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005 (*n* = 3–5).

E:T ratios (Fig. 3C). Additionally, in vivo killing experiments also demonstrated that the killing activity of NK cells from *Il17a*^{-/-} mice was higher as there were fewer remaining YAC-1 cells in *Il17a*^{-/-} mice (Fig. 3D and E), suggesting that IL-17A restricts the cytotoxicity of NK cells. Altogether, the data show that IL-17A constrains NK cell antitumor and antiviral activity.

IL-17A Deficiency Enhances the Terminal Maturation of NK Cells. NK cell activity is commonly regulated during the activating process or maturation process. It was observed that IL-17RA was partially expressed on NK cells (SI Appendix, Fig. S2A–C). Unexpectedly, the addition of IL-17A with IL-12/IL-18 failed to directly suppress IFN-γ production by NK cells from WT mice (SI Appendix, Fig. S2D), suggesting the inhibition of NK cell activity by IL-17 does not occur in the activating process of NK cells. To determine the role of IL-17A in NK cell maturation, we assessed NK cells at different developmental stages in *Il17a*^{-/-} and WT mice. The overall numbers of mononuclear cells and the frequencies of total (CD3⁺NK1.1⁺) NK cells and mature (CD3⁺DX5⁺NK1.1⁺) NK cells in the spleen, liver, bone marrow, and peripheral blood were comparable between *Il17a*^{-/-} and WT mice (SI Appendix, Fig. S2E and F); however, the frequency of the M2 NK cell subset was significantly higher in these tissues and organs from *Il17a*^{-/-} mice relative to those in WT mice (Fig. 4A and SI Appendix, Fig. S2G). Correspondingly, the frequencies

of Imm and M1 NK cell subsets but not CD27⁺CD11b⁺ NK cells were markedly lower in *Il17a*^{-/-} mice (Fig. 4A), suggesting that IL-17A can inhibit terminal maturation of NK cells by blocking the transition from CD27⁺ to CD11b⁺ NK cells. Additionally, the frequencies of M2 NK cell subsets in *Il17a*^{-/-} mice bearing colon cancer were also higher than those in WT mice bearing colon cancer (SI Appendix, Fig. S2H). To further confirm the hypothesis, the CD27⁺CD11b⁺ or CD27⁺CD11b⁺ NK cell subset from WT mice was adoptively transferred into WT and *Il17a*^{-/-} mice. The increased transition of the M1 NK cell subset into the M2 NK cell subset was observed in *Il17a*^{-/-} mice, whereas the M2 NK cell subset maintained the stable maturational stage (SI Appendix, Fig. S2I), suggesting IL-17A hampers the M1 to M2 transition but fails to induce the M2 to M1 transition. Terminally mature stages of NK cells are also characterized by CD43 or KLRG1 expression (1). Accordingly, the frequencies of CD27⁺CD43⁺, CD11b⁺CD43⁺, and CD11b⁺KLRG1⁺ NK cells were substantially higher in *Il17a*^{-/-} mice (Fig. 4B), indicating that IL-17A deficiency significantly promotes the terminal maturation of NK cells. IL-17A/IL-17F functions by binding the IL-17RA/IL-17RC receptor complex. To assess the role of IL-17R signaling in terminal NK cell maturation, we also assessed the levels of terminally mature NK cells in *Il17f*^{-/-}, DKO, and *Il17ra*^{-/-} mice. As expected, the frequencies of the M2 NK cell subset were higher in these deficient mice and much higher in DKO and *Il17ra*^{-/-} mice, relative to *Il17f*^{-/-} mice (Fig. 4C–E), suggesting that IL-17R signaling is essential for the inhibition of NK cell terminal maturation. To determine whether the effect of IL-17A on NK cells is ontogenetic, NK cells from neonatal (day 7) and infant (day 21) mice were analyzed. The frequencies of M2 NK cell subset in neonatal and infant *Il17a*^{-/-} mice were higher than those in their respective WT counterparts (Fig. 4F). Together, these data demonstrate that IL-17 signaling constrains NK cell terminal maturation in mice.

IL-17A Has a Physiological Role in Constraining Terminal Maturation of NK Cells. To investigate whether the physiological level of IL-17A could constrain NK cell terminal maturation, we constructed parabiosis between CD45.1 WT and CD45.2 *Il17a*^{-/-} mice. The excess of the M2 NK cell subset in CD45.2 *Il17a*^{-/-} mice returned to normal levels, similar to those of WT mice, 4 wk postsurgery (Fig. 5A). These findings were confirmed by the results of experiments using mixed bone marrow chimeras in which the frequency of terminally mature NK cells derived from *Il17a*^{-/-} mice was comparable to that from WT mice in the same recipient (Fig. 5B). In addition, the hematopoietic reconstitution showed that the frequency of the M2 NK cell subset derived from the bone marrow of WT mice was lower than that from *Il17a*^{-/-} mice in recipient mice (Fig. 5C), suggesting that physiological levels of IL-17A are sufficient to constrain terminal maturation of NK cells and that IL-17A is derived from hematopoietic cells. Moreover, the frequency of the M2 NK cell subset derived from *Il17ra*^{-/-} mice, which cannot respond directly to IL-17A, was higher than that from WT mice in the same recipient (Fig. 5D). The hematopoietic reconstitution in *Il17ra*^{-/-} mice also showed the frequency of the M2 NK cell subset derived from *Il17ra*^{-/-} mice was higher (Fig. 5E), suggesting that IL-17A signaling is required for IL-17A-mediated suppression of NK cell maturation. Taken together, these data demonstrate that IL-17A is a physiological suppressor of NK cell terminal maturation.

Constitutive IL-17A Signaling Constrains the Terminal Maturation of NK Cells. To confirm the IL-17A-mediated suppression on the maturation of NK cells, IL-17A was expressed in vivo to mimic the constitutive IL-17A signaling during disease progression since the pLIVE-IL-17A administered to recipient mice (WT, *Il17a*^{-/-}, and *Il17ra*^{-/-}) by hydrodynamic injection can lead to lasting expression of IL-17A in vivo, at least, for 3 wk. At 1 wk after injection, the frequencies of the M2 NK cell subset were

Fig. 3. IL-17A deficiency enhances NK cell activity. (A and B) Frequencies of IFN- γ ⁺ NK cells from *Il17a*^{-/-} and WT mice stimulated with poly I:C in vivo (A) or in vitro stimulated with IL-2/IL-12 (B). (C) Specific lysis of YAC-1 cells by NK cells from *Il17a*^{-/-} and WT mice at the indicated E:T ratios. (D and E) Frequencies (D) and number (E) of remaining carboxyfluorescein succinimidyl ester (CFSE)-labeled YAC-1 cells in the peritoneal cavities of *Il17a*^{-/-} and WT mice. CFSE-labeled YAC-1 cells were intraperitoneally injected into *Il17a*^{-/-} and WT mice. CFSE-labeled YAC-1 cells were evaluated 24 h postinjection. Data are representative of 3 independent experiments and are shown as means $\pm$ SEM. * P < 0.05 and ** P < 0.01 (n = 3–5).

unchanged; however, they decreased at 2 wk after injection (*SI Appendix, Fig. S3A and B*). Moreover, at 3 wk after injection, the frequency of the M2 NK cell subset was significantly lower in IL-17A-expressing WT mice with higher frequencies of Imm and the M1 NK cell subsets (Fig. 6A and *SI Appendix, Fig. S3C*), suggesting the inhibitory effect of IL-17A on NK cell maturation is time dependent mainly due to the natural turnover of NK cells in the host. Additionally, constitutive IL-17A signaling could reverse the increase in the M2 NK cell subsets induced by IL-17A deficiency as demonstrated by the reduced frequency of the M2 NK cell subset in IL-17A-expressing *Il17a*^{-/-} mice (Fig. 6B and *SI Appendix, Fig. S3D and E*). However, the frequency of the M2 NK cell subset in IL-17A-expressing *Il17a*^{-/-} mice was comparable to that in controls (Fig. 6C), suggesting that IL-17A constrains terminal maturation of NK cells in an IL-17A-dependent manner. Additionally, constitutive IL-17A signal-

ing decreased the numbers of total NK cells in WT and *Il17a*^{-/-} but not in *Il17a*^{-/-} recipient mice (*SI Appendix, Fig. S3F*). Taken together, these data demonstrate that constitutive IL-17A signaling constrains the terminal maturation of NK cells.

IL-17A Desensitizes NK Cells to IL-15 Signaling during Their Maturation. IL-15 is the key factor that determines NK cell survival, proliferation, and maturation (29). To determine whether IL-17A counteracts the effects of IL-15, splenocytes were isolated and stimulated with vehicle + IL-15 or IL-17A + IL-15 in vitro for 1 wk. The frequency of the total NK cells and DX5⁺ NK cells increased with increasing IL-15 concentration (Fig. 7A), while IL-17A efficiently inhibited this increase in NK cells (Fig. 7A). Moreover, the frequency of the M2 NK cell subset in the IL-17A + IL-15 group was significantly lower than that in the vehicle + IL-15 group (Fig. 7B), suggesting that IL-17A antagonizes

Fig. 4. IL-17 deficiency accelerates the terminal maturation of NK cells. (A) Frequencies (Left) and statistical analysis (Right) of NK cells at different stages of maturity, labeled with CD27 and CD11b in spleens from *Il17a*^{-/-} and WT mice. (B) Frequencies of the M2 NK cell subset in spleens from *Il17a*^{-/-} and WT mice, labeled with CD27 and CD43, CD11b and CD43, or CD11b and KLRG1. (C–E) Frequencies of the M2 NK cell subset in spleens from *Il17f*^{-/-}, *Il17a*^{-/-}, *Il17f*^{-/-} (DKO), *Il17a*^{-/-}, and WT mice. (F) Frequencies of the M2 NK cell subset in neonatal (day 7) and infant (day 21) mice of the indicated genotypes. Data are representative of, at least, 3 independent experiments and are presented as means $\pm$ SEM. * P < 0.05, ** P < 0.01, and *** P < 0.005 (n = 3 to 4).

Fig. 5. Physiological level of IL-17A constrains NK cell terminal maturation. (A) Comparison of the M2 NK cell subset in spleens and blood from parabiont *Il17a*^{-/-}, *Il17a*^{-/-}, and WT mice. Representative dot plot of spleen and blood samples gated on live CD3⁺NK1.1⁺DX5⁺ cells followed by CD45.1 and CD45.2 gates for each parabiont as indicated. *Il17a*^{-/-} (CD45.2) mice were parabiosed to congenic WT-CD45.1 mice; 4 wk after surgery, spleens and blood were harvested, and flow cytometry performed. (B) Frequencies of the M2 NK cell subset derived from *Il17a*^{-/-} or WT mouse donor bone marrow cells in mixed bone marrow chimeras. WT (CD45.1) recipient mice were transplanted with donor bone marrow cells containing mixtures (1:1) of WT (CD45.1) and *Il17a*^{-/-} (CD45.2) mice bone marrow donor cells. (C) Frequencies of M2 NK cell subset from *Il17a*^{-/-} or WT donor mouse bone marrow cells. WT or *Il17a*^{-/-} recipient mice were injected with donor bone marrow cells from WT or *Il17a*^{-/-} mice, respectively. (D) Frequencies of the M2 NK cell subset from *Il17a*^{-/-} or WT donor mouse bone marrow cells. *Il17a*^{-/-} recipient mice were injected with donor bone marrow cells from WT or *Il17a*^{-/-} mice, respectively. (E) Frequencies of the M2 NK cell subset derived from *Il17a*^{-/-} or WT mouse donor bone marrow cells in mixed bone marrow chimeras. WT (CD45.1) recipient mice were transplanted with donor bone marrow cells containing mixtures (1:1) of WT (CD45.1) and *Il17a*^{-/-} (CD45.2) bone marrow donor cells. Data are representative of, at least, 3 independent experiments and are presented as means $\pm$ SEM. **P* < 0.05 and ***P* < 0.01 (*n* = 3–5).

IL-15-mediated proliferation of NK cells. NK cells, purified from the spleens of WT mice, were also stimulated with vehicle + IL-15 or IL-17A + IL-15. The proportion of the M2 NK cell subset in the IL-17A + IL-15 group was significantly lower than that in the control group 7 d after stimulation (Fig. 7C). In addition, the apoptosis ratio of NK cells was higher in the IL-17A + IL-15 group (*SI Appendix*, Fig. S3G), indicating that IL-17A inhibits IL-15-mediated survival of NK cells. Therefore, IL-17A can directly antagonize the effects of IL-15 on NK cells.

To confirm the antagonistic effect of IL-17A on IL-15, the vectors pLIVE-IL-17A and pLIVE-IL-15 were simultaneously administered to recipient mice by hydrodynamic injection (17–15 group); null vector and pLIVE-15 vector were administered as a control (null-15 group). Analysis of NK cells 2 wk after injection revealed that the frequency of total NK cells was lower in the 17–15 than in the null-15 group (Fig. 7D). Moreover, the frequency of the M2 NK cell subsets in the 17–15 group was also markedly lower than that in the null-15 group (Fig. 7E). Correspondingly, the frequencies of the Imm and M1 NK cell subsets but not CD27⁺CD11b⁺ NK cells increased markedly in the 17–15 group, suggesting that IL-17A antagonized the effect of IL-15 on NK cell maturation, consistent with the observed effects of

the absence or lasting expression of IL-17A. Additionally, the frequency of Ki67⁺ NK cells decreased significantly in mice in the 17–15 group relative to the null-15 group (Fig. 7F), suggesting IL-17A counteracts IL-15-induced NK cell proliferation. Together, these results reveal that IL-17A suppresses the IL-15-mediated effect on NK cell during NK cell maturation.

IL-17A Constrains IL-15-Supported NK Terminal Maturation via SOCS3. We noted that the deficiency or lasting expression of IL-17A had no influence on serum levels of IL-15 or levels of IL-15R α , IL-15R β (CD122), and IL-15R $\beta\gamma$ (CD132) on NK cells (*SI Appendix*, Fig. S4A–E). There was no difference in the expression of IL-15R α on CD11b⁺ myeloid cells in the bone marrow of IL-17A-deficient mice (*SI Appendix*, Fig. S4F), indicating that IL-17A does not directly target IL-15/IL-15R. Therefore, we tested levels of pSTAT5, a critical molecule downstream of IL-15 (5) in NK cells from *Il17a*^{-/-} and WT mice. Consistently, pSTAT5 levels were increased in NK cells from *Il17a*^{-/-} mice (Fig. 8A and *SI Appendix*, Fig. S5A). The addition of IL-17A significantly reduced IL-15-supported pSTAT5 levels in NK cells (Fig. 8B and *SI Appendix*, Fig. S5B). Next, we evaluated molecules downstream of IL-15-STAT5 in *Il17a*^{-/-} and WT mice. Levels of Helios,

Fig. 7. IL-17 desensitizes NK cells to IL-15 signaling during their maturation. (A) Frequencies of total NK cells (Left) and DX5⁺ NK cells (Right) among splenocytes treated with IL-17A (50 ng/mL) and the indicated dose of IL-15 for 7 d. (B) Frequencies of the M2 NK cell subset in splenocytes treated with IL-17A (50 ng/mL) and IL-15 (5 ng/mL) for 7 d. (C) Frequencies of the M2 NK cell subset among purified NK cells treated with IL-17A (50 ng/mL) and IL-15 (5 ng/mL) for 7 d. (D–F) Total NK cells (D), the M2 NK cell subset (E), and Ki67⁺ NK cells (F) among splenic NK cells from mice treated with IL-17A vector + IL-15 vector or null vector + IL-15 vector. IL-17A vector or null vector (20 μ g/mouse) were hydrodynamically injected into recipient WT mice simultaneously with the IL-15 vector (5 μ g/mouse). NK cells were analyzed 2 wk after injection. Data are representative of, at least, 3 independent experiments and are shown as means $\pm$ SEM. * P < 0.05 and ** P < 0.01 (n = 3 to 4).

that negatively regulate the process of NK cell maturation other than TGF- β signaling inhibition of NK cell development, which can promote viral infection and cancer progression (7, 36). In this study, we found that IL-17A suppressed the terminal maturation of NK cells as evidenced by the increased levels of the M2 NK cell subset, pSTAT5, and Blimp-1 in *Il17a*^{-/-} compared with WT mice. Ontogenetic analysis of NK cells and the results of long-term lasting-expression experiments confirmed that IL-17A suppressed NK cell terminal maturation. IL-15 is important for NK cell development. In *Il17a*^{-/-} mice, only the M2 NK cell subset was profoundly altered. But in IL-17A-overexpressed mice, whole NK cells were observed to be influenced, indicating the effect of IL-17A on the NK cell and IL-15 signaling is dose dependent. Furthermore, we hypothesize that the suppression of IL-15 signaling by IL-17A occurs extensively in immune cells rather than exclusively in NK cells since IL-15 is also an important regulator of NKT cells and memory-phenotype CD8⁺ T cells (37, 38). Zhao et al. demonstrated that IL-17A negatively regulates NKT cell function in Con A-induced fulminant hepatitis (39); therefore, the desensitization of IL-15 signaling by IL-17A appears to be a general occurrence under physiological and pathological conditions.

The inhibitory effects of IL-17A are extensive and achieved via multiple mechanisms. IL-17A can down-regulate TNF- α -induced CCL5 (CC chemokine ligand 5) expression through inhibition of IRF-1 DNA-binding activity (40) and can inhibit activation of C/EBP β via sequential phosphorylation (41). As there was no dif-

ference in the level of IL-15 in the serum or of CD122 and CD132 on NK cells, we hypothesized that IL-17A suppressed IL-15 signaling in NK cells via intrinsic molecular pathways. IL-17A treatment significantly reduced the level of pSTAT5, suggesting that IL-17A signaling suppressed the phosphorylation of STAT5. CIS is a critical negative regulator of IL-15 signaling in NK cells (9); however, we found that IL-17A induced the expression of SOCS3 but not CIS in NK cells, in accordance with the finding by Delconte's group that the deletion of SOCS3 restores IL-15 signaling after ID2 deficiency (8). Knockdown of SOCS3 enhanced phosphorylation of STAT5 after stimulation with IL-17A and IL-15. An inhibitor of SOCS3 could rescue the IL-17A-mediated decrease in terminal maturation of NK cells; hence, IL-17A-induced SOCS3 suppresses IL-15-induced STAT5 phosphorylation in NK cells. Nevertheless, there are other negative regulators of JAK-STAT signaling and the identification of the precise intracellular mechanisms via which IL-17A desensitizes IL-15 signaling requires further investigation. In contrast, the findings by Bär et al. show that IL-17 receptor signaling promotes the development of functional NK cells and IL-17 enhances NK cell-derived GM-CSF against fungal infection (42). The contradictory findings might attribute to the used mice, the disease models, or the experimental system and require further investigation to decipher the reasons.

Interestingly, IL-15 induces binding of STAT5 to the *Il17* locus and down-regulates IL-17A production (43). Moreover, our group

Fig. 8. IL-17A constrains IL-15-supported NK terminal maturation via SOCS3. (A) pSTAT5 levels in splenic NK cells from *Il17a*^{-/-} and WT mice. (B) pSTAT5 in splenic NK cells from mice treated with IL-17A vector + IL-15 vector or null vector + IL-15 vector. (C) Levels of Blimp-1 in NK cells from *Il17a*^{-/-} and WT mice. (D) Levels of Blimp-1 in NK cells from mice treated with IL-17A vector + IL-15 vector or null vector + IL-15 vector. (E) mRNA levels of members of the SOCS family in NK cells from mice treated with IL-17A vector or null vector. (F) SOCS3 protein levels in NK cells from mice treated with IL-17A vector or null vector. (G) pSTAT5 levels in NK cells transfected with *Socs3* siRNA or control siRNA. Purified splenic NK cells were electronically transfected with *Socs3* siRNA or negative control siRNA, and then stimulated with IL-17A+IL-15. (H and I) mRNA (H) and protein (I) levels of SOCS3 in NK cells from mice treated with null vector + PBS, IL-17A vector + PBS, or IL-17A vector + ZA. (J) Frequency of the M2 NK cell subset in spleens from mice treated with null vector + PBS, IL-17A vector + PBS, or IL-17A vector + ZA. Data are representative of, at least, 3 independent experiments and are shown as means $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$.

found that NK cells can inhibit Th17 cells via IFN- γ (44). Here, we demonstrate the role of IL-17A in the terminal maturation of NK cells, suggesting bilateral rather than unilateral crosstalk between IL-17A-producing cells and NK cells and between IL-17A and IL-15. The IL-17A–IL-15 interaction is a potential target for disease treatment. STAT5 suppresses the transcription of VEGFA in NK cells (45), hence it is possible that IL-17A promotes tumor development through deactivation of STAT5 in NK cells and enhancement of VEGFA. Therefore, a therapeutic regimen that blocks IL-17A may be a means of strengthening immune defenses against tumors. In contrast, inhibition of IL-15 signaling by IL-17A could be useful for treatment of inflammatory autoimmune diseases and large granular lymphocyte leukemia.

Under physiological conditions, IL-17A is primarily produced by CD4⁺ or $\gamma\delta$ T cells. IL-17A levels were undetectable in serum samples from both *Il17a*^{-/-} and WT mice at steady state. Using bone marrow transplantation and chimera experiments, we determined that the IL-17A acting on NK cells is generated by hematopoietic cells. Although CD4⁺ T cells were possible IL-17A producers under steady state conditions, CD4-specific deficiency of IL-17A might help confirm the resource of IL-17A under steady state conditions. *Staphylococcus epidermidis* on the skin and segmented filamentous bacteria in the gut can induce

IL-17A production under steady state conditions (46, 47). Therefore, we hypothesize that trace levels of IL-17A acting on NK cells may be derived from peripheral sites. In chronic inflammation, multiple cell populations can be sources of IL-17A (48). IL-17A reporter mice may help to precisely identify and trace IL-17A-producing cells under steady state or pathological conditions in vivo.

To summarize, our study provides evidence that IL-17A-activated SOCS3 counteracts IL-15-induced STAT5 activation during NK cell terminal maturation thereby constraining NK cell maturation and effector function; however, the mechanisms underlying suppression of IL-15 signaling by IL-17A require further investigation. This study not only provides insights into the role of IL-17A in regulating NK cell homeostasis, but also suggests approaches for interventions in chronic inflammatory conditions, such as viral infections and tumors where NK cells become dysfunctional because of elevated IL-17 levels.

Materials and Methods

Mice. Male C57BL/6J WT mice (6–8 wk old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy Sciences. Male C57BL/6N mice (6–8 wk old) were purchased from Beijing Vital River Company. Congenic CD45.1 mice (C57BL/6J background) were purchased from Jackson Laboratories. Mice used included CD4^{-/-}, *Il17a*^{-/-}, and *Il17f*^{-/-} strains (kindly provided by Professor Zhexiong Lian), *Il17a*^{-/-}*Il17f*^{-/-} DKO mice were generated

in our laboratory by cross breeding *Il17a*^{-/-} and *Il17f*^{-/-} strains. *Il17a*^{-/-} mice were kindly provided by Amgen, Inc., Seattle, WA. All mice were housed in microisolator cages under humidity- and temperature-controlled specific pathogen-free conditions in the animal facility of the School of Life Sciences, University of Science and Technology of China. Mice were maintained on an irradiated sterile diet and provided with autoclaved water. Animal experimental ethical approvals were obtained from the ethics committee of the University of Science and Technology of China.

Cell Isolation. Single cell suspensions were prepared from murine spleen, bone marrow, peripheral blood, and liver for flow cytometry analysis. Briefly, spleen and bone marrow (from the tibia and femur) were harvested, put through a 200-gauge stainless steel mesh, and lysed to deplete erythrocytes. Peripheral blood was diluted with PBS, then gently transferred to 70% Percoll (Gibco BRL), and centrifuged for 30 min at 1260 × *g* (room temperature). Livers were harvested, pressed through a 200-gauge stainless steel mesh, and suspended in PBS. Suspensions were centrifuged at 50 × *g* for 1 min, supernatants transferred into fresh tubes, and centrifuged again at 800 × *g* for 10 min. Pellets were resuspended in 40% Percoll and gently transferred to 70% Percoll, followed by centrifugation at 1260 × *g* for 30 min at room temperature.

Antibody Staining and Flow Cytometry. Cells (1 × 10⁶) were stained with PE-anti-CD69, Percp-Cy5.5-anti-CD3, and APC-anti-NK1.1 to assess preactivated NK cells. Cells (1 × 10⁶) were stained with FITC-anti-CD27, FITC-CD122, PE-anti-NKp46, PE-anti-IL-17RA, PE-anti-CD43, Percp-Cy5.5-anti-CD11b, APC-anti-KLRG1, Alexa660-anti-NKp46, APC-CY7-anti-CD3, PE-CY7-NK1.1, APC-CY7-anti-CD45.2, PE-CY7-anti-CD45.1, BV421-anti-DX5, BV510-anti-NK1.1, and BV786-anti-CD3 to detect NK cells at different stages of maturity. Cells (1 × 10⁶) were stained with FITC-anti-CD3 and Percp-Cy5.5-anti-NK1.1, then intracellularly stained with PE-anti-Blimp1, PE-anti-T-bet, APC-anti-GATA3, PE-anti-Helios, and APC-anti-Eomes to analyze transcription factors, and intracellularly stained with Alexa 660-anti-Ki67 to assess NK cell proliferation after fixation and permeabilization using fixation/permeabilization diluent (eBioscience Company). Monoclonal antibodies and isotype controls were purchased from BD Pharmingen (San Jose, CA), eBioscience Company, or BioLegend Company, other than PE-anti-Blimp1 and its isotype control, which were purchased from Santa Cruz Biotechnology (California). Stained cells were analyzed using a FACSCalibur, BD LSR II, BD LSRFortessa (BD Biosciences, San Jose, CA). Acquired data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Western Blotting. NK cells were purified from the spleens of *Il17a*^{-/-} and WT mice and from WT mice treated with null vector + IL-15 vector or IL-17A vector + IL-15 vector (*SI Appendix, Supplemental Experimental Procedures*) using a MACS kit (NK Cell Isolation Kit II, Miltenyi Biotec) and a FACS sorting (BD FACSARIA III, San Jose, CA). Purified NK cells (purity > 90%) were lysed in radioimmunoprecipitation assay buffer (P0013, Beyotime, China) supplemented with protease inhibitors (Pierce Biotechnology). Cell debris was removed by centrifugation at 12,000 × *g* for 5 min. Protein concentrations in supernatants were determined by bicinchoninic acid assay (Pierce Biotechnology). Equal amounts of protein were separated by SDS/PAGE and then transferred to PVDF membranes. Proteins of interest were probed with primary antibodies (SOCS3, STAT5, phospho-STAT5, phospho-p38, and phospho-NF-κB p65 from CST Company, and GAPDH, β-actin from Boster Company, all at 1:1,000) overnight at 4 °C, then incubated with HRP-conjugated secondary antibodies (1:5,000) for 1 h at room temperature, and detected by chemiluminescence autoradiography.

Quantitative PCR Analysis. Total RNA was isolated from NK cells using total RNA purification solution (Invitrogen) and 2 μg aliquots reverse transcribed at 25 °C for 15 min, 42 °C for 50 min, and 70 °C for 10 min using a reverse transcription kit (Sangon Biotech, Shanghai, China). cDNA fragments were amplified using the following gene-specific primers: *Cis* (sense 5'-ACCTTCGGGAATCTGGGTG-3'; antisense 5'-GGGAAGGCCAGGATTCGA-3'); *Socs1* (sense 5'-CCGCTCCCACTCCGATTA-3'; antisense 5'-GCACCAAGAAGGTGCCCA-3'); *Socs2* (sense 5'-CCCTTAGGTAGTTTATGCTGAATG-3'; antisense 5'-TTTAAAGGGCCATTGATCTT-3'); *Socs3* (sense 5'-TTTCGCTTCGGGACTAGCTC-3'; antisense 5'-TTGCTGTGGGTGACCATGG-3'); *Id2* (sense 5'-GGTGGACGACC-GATGAGT-3'; antisense 5'-TGCCTGCAAGGACAGGATG-3'); *Blimp-1* (sense 5'-GACGGGGTACTTCTGTTC-3'; antisense 5'-GGCATTCTTGGGAACGTGTG-3'); *Hprt* (sense 5'-GCGATGATGAACAGGTTATGA-3'; antisense 5'-ACAATGTGATGGCTCCCAT-3'). Quantitative RT-PCR was performed to measure mRNA expression of *Cis*, *Socs1*, *Socs2*, *Socs3*, *Id2*, and *Blimp-1* using SYBR Premix ExTaq (TaKaRa Biotechnology, Dalian, China) and specific primers in a reaction with an optimal number of cycles at 95 °C for 10 s, then 60 °C for 30 s in a Corbett Rotor-Gene 3000 real-time PCR system (Corbett Research). Gene expression levels were calculated relative to those of *Hprt*.

Ex Vivo Stimulation of NK Cells. To access the apoptosis of NK cell ex vivo, purified NK cells were treated with IL-17A (50 ng/mL) and IL-15 (5 ng/mL) for 4 d, and then the frequency of Annexin-V⁺ NK cells was measured by FACS. To access the role of NF-κB in SOCS3 expression, purified NK cells were pretreated with NF-κB inhibitor BAY11-7082 (Beyotime, China) and then stimulated with IL-17A. The levels of SOCS3 in NK cells were tested by qPCR. To access the role of SOCS3 in NK cell activity, premade siRNAs targeting mouse *Socs3* or negative control siRNA were designed and synthesized by GenePharma (Shanghai) and electronically transfected into purified NK cells. NK cells were plated in RPMI-1640 complete medium and stimulated with IL-17A and IL-15. pSTAT5 and total STAT5 were analyzed by Western blotting. Sequences of siRNAs were as follows: *Socs3* siRNA 5'-GGAACCCUGUCCGAAGUUTT-3'; control siRNA 5'-UUCUCCGAACGUGUCACGUTT-3'.

Overexpression of Cytokines In Vivo. The expression vectors pLIVE-IL-17A (IL-17A vector) and pLIVE-IL-15 (IL-15 vector) were constructed, and the indicated doses administered by hydrodynamic injection separately or jointly. The pLIVE null (null vector) was used as a control. Concentrations of mouse IL-17A and IL-15 were measured using ELISA kits (Dakewe Biotech Company, Shenzhen, China and R&D Systems, Inc., Minneapolis, respectively). Mice were treated with ZA (100 μg/kg, i.p.) or sodium orthovanadate (20 mg/kg, i.p.) every 2 d for 14 d from day 2 after the expression vector pLIVE-IL-17A injection.

Adoptive Transfer of NK Cells. The purified CD45.1 NK cells were sorted with a magnetic-activated cell sorter (Miltenyi Biotec) and then labeled with FITC anti-CD27 mAb and Percp-Cy5.5-anti-CD11b mAb. CD27⁺CD11b⁺ or CD27⁻CD11b⁺ NK cells were sorted out with BD FACSARIA and then adoptively transferred into *Il17a*^{-/-} and WT mice. The CD45.1 NK cell subsets were assessed 2 wk after adoptive transfer.

Ex Vivo Stimulation of NK Cells. Splenocytes were isolated from WT mice. Some 1 × 10⁶ splenocytes were stimulation with IL-17A (50 ng/mL) and the indicated dose (0, 5 ng/mL, 10, and 20 ng/mL) of IL-15 for 7 d. The frequencies of total NK cells and mature NK cells were measured by FACS lastly. The frequency of the M2 NK cell subset in splenocytes treated with IL-17A (50 ng/mL) and IL-15 (5 ng/mL) for 7 d was also measured by FACS at the end. The purified NK cells with a magnetic-activated cell sorter (Miltenyi Biotec) were treated with IL-17A (50 ng/mL) and IL-15 (5 ng/mL) for 7 d, and then the frequency of the M2 NK cell subset was measured by FACS.

Assessment of NK Cell Function. IFN-γ production by splenic NK cells was determined by intracellular cytokine staining after stimulation with poly(I:C (5 μg/mouse) for 18 h in vivo and with PMA/Ion for 4 h ex vivo, by stimulation with IL-12 (20 ng/mL) and IL-2 (1000 IU/mL), or through incubation with YAC-1 cells for 4 h ex vivo. To evaluate NK cell cytotoxic activity, splenic NK cells were purified 18 h post poly(I:C injection and cocultured with 2 × 10⁴ CFSE-labeled YAC-1 cells at the indicated effector/target ratios (1:1; 1:5; 10:1) for 4 h. The viability of YAC-1 cells was assessed by 7-AAD staining combined with flow cytometry, and killing efficiency was calculated as the percentage of 7-AAD positive YAC-1 cells. To determine NK cell cytotoxic activity, 2 × 10⁶ CFSE-labeled YAC-1 cells were intraperitoneally injected into WT mice and *Il17a*^{-/-} mice, respectively. Cells in the peritoneal cavity were harvested, and CFSE-labeled YAC-1 cells measured 24 h postinjection.

Parabiosis. To construct parabiosis in mice, surgery was performed as previously described (49). Briefly, longitudinal skin incisions were cut in the flanks of WT (CD45.1) and *Il17a*^{-/-} (CD45.2) male mice. Their elbows and knees were joined, and the incisions closed with sutures. Buprenex compound was administered for pain management. Nutritional gel packs were provided in each cage and antibiotics (Sulfatrim) added to the drinking water for the duration of the experiment. NK cells were analyzed 4 wk postsurgery.

Bone Marrow Transplantation and Chimeras. To construct the transplantation model, WT recipient mice were lethally irradiated (10 Gy) and i.v. injected with donor bone marrow cells (1 × 10⁶) from WT or *Il17a*^{-/-} mice. *Il17a*^{-/-} recipient mice were lethally irradiated (10 Gy) and i.v. transplanted with donor bone marrow cells (1 × 10⁶) from WT or *Il17a*^{-/-} mice. *Il17a*^{-/-} recipient mice were lethally irradiated (10 Gy) and i.v. transplanted with donor bone marrow cells (1 × 10⁶) from WT or *Il17a*^{-/-} mice. To generate mixed bone marrow chimeras, WT recipient mice (CD45.1) were lethally irradiated (10 Gy) and i.v. transplanted with a mixture (1:1) of WT (CD45.1) and *Il17a*^{-/-} (CD45.2) donor bone marrow cells (1 × 10⁶). NK cells were analyzed 8 wk posttransplantation.

NK Cell-Mediated Liver Injury. *Il17a*^{-/-}, WT, and chimeras mice (WT: *Il17a*^{-/-} or *Il17a*^{-/-}: *Il17a*^{-/-}) were injected with poly I:C (1 µg/mouse, i.v.) and D-GalN (10 mg/mouse, i.p.). Serum samples were collected to evaluate the degree of liver injury by measurement of ALT levels 18 h after drug treatment. ALT levels were measured using a diagnostic kit (Rongsheng, Shanghai, China). Mouse IFN-γ concentrations were measured using ELISA kits (Dakewe Biotech Company, Shenzhen, China). Liver specimens were fixed using 4% paraformaldehyde, dehydrated with graded alcohol, embedded in paraffin, cut into tissue sections, and stained with hematoxylin and eosin. Hepatic mononuclear cells (1 × 10⁶) were stained with Percp-Cy5.5-anti-CD3 and APC-anti-NK1.1, then intracellularly stained with PE-anti-IFN-γ or PE-Rat IgG1 and κ as isotype controls to detect IFN-γ⁺ NK cells.

Viral Infection and Quantification. Experimental mice were infected with the MCMV strain Smith (kindly provided by Professor Mingli Wang, Anhui Medical University) by i.v. injection of 5 × 10⁴ plaque-forming units in 0.5 mL or PBS as control. Mice were euthanized 1.5 d after infection, and viral titers assessed by plaque assay as previously described (50). IFN-γ in NK cells after viral infection was analyzed by flow cytometry 4 h after treatment with Monensin and IL-2 (500 U/mL).

Mouse Tumor Models. Two mouse tumor models were induced in *Il17a*^{-/-} mice and WT mice by s.c. inoculation with 2 × 10⁵ colon cancer cells (MC38) or

melanoma cells (B16F10) into the right flanks of mice. NK cells were depleted using αASGM1 3 d before inoculation and then twice per week following inoculation. Tumor volumes were monitored with a caliper and calculated using the formula: V (in mm³) = 0.5 (ab²), where a is the longest diameter and b is the shortest diameter. For metastasis studies, 2 × 10⁵ of B16F10 cells (intravenously) or MC38 cells (intrasplenically) were administered into *Il17a*^{-/-}, DKO, and WT mice. The number of B16F10 melanoma surface nodules in the lungs or MC38 colon tumors in the livers of each mouse were counted. Samples were collected at the indicated time points for further analysis.

Statistical Analysis. Data are presented as means ± SEM and were analyzed using the Student's *t* test or ANOVA. Differences were considered significant when *P* < 0.05 (**P* < 0.05; ***P* < 0.01; ****P* < 0.005). All analyses were performed using Prism 6 software (GraphPad Software, San Diego, CA).

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