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Cutting Edge

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Cutting Edge: Priming of NK Cells by IL-18[1]

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Recent evidence suggests that NK cells require priming to display full effector activity. In this study, we demonstrate that IL-18 contributed to this phenomenon. IL-18 signaling-deficient NK cells were found to be unable to secrete IFN-γ in response to ex vivo stimulation with IL-12. This was not due to a costimulatory role of IL-18, because blocking IL-18 signaling during the ex vivo stimulation with IL-12 did not alter IFN-γ production by wild-type NK cells. Rather, we demonstrate that IL-18 primes NK cells in vivo to produce IFN-γ upon subsequent stimulation with IL-12. Importantly, IL-12-induced IFN-γ transcription by NK cells was comparable in IL-18 signaling-deficient and -sufficient NK cells. This suggests that priming by IL-18 leads to an improved translation of IFN-γ mRNA. These results reveal a novel type of cooperation between IL-12 and IL-18 that requires the sequential action of these cytokines. ***The Journal of Immunology,*** **2008, 181: 1627–1631.**

Natural killer cells are lymphocytes of the innate immune system that play a role in the protection against pathogens and tumors by means of cell cytotoxicity and the secretion of IFN-γ (1, 2). Recent evidence has shown that, similar to other lymphocytes, NK cells need to be primed to acquire their full effector functions. This priming involves an encounter with activated dendritic cells (DC)[3] in lymphoid organs (3). Mechanistically, NK cell priming has been found to be mediated in part by IL-15, *trans*-presented by IL-15Rα on DC. Accordingly, NK cells isolated from IL-15-deficient mice display low cytotoxicity and reduced IFN-γ secretion (4, 5). In this study, we aimed at identifying other molecules involved in NK cell priming using a candidate gene approach. We focused our attention on MyD88, a crucial integrator of innate immune responses required for signaling through most TLR and receptors of the IL-1 receptor family.

Materials and Methods

Mice

Inbred mice were purchased from Charles River. All mice were on a C57BL/6 background. Polyinosinic:polycytidylic acid (poly(I:C); Invivogen) was injected i.p. (200 μg/mouse). Bone marrow chimeras were generated as described (6). IL-18 knockout (KO) mice and IL-18R KO mice were purchased from The Jackson Laboratory.

NK cell culture

NK cells were enriched by negative selection as described (6). They were cultured with YAC1 cells (1:1 ratio), cytokines, or coated Abs (clone 29A1.4, anti-NKp46; clone 4E5, anti-Ly49D) and anti-CD107a/GolgiStop (BD Biosciences). IL-2 (3,000 U/ml; Peprotech), IL-12 (20 ng/ml), IL-18 (5 ng/ml; R&D Systems), anti-IL-18 (20 μg/ml; MBL International), and anti-IL-18R (12.5 μg/ml; R&D Systems) were used. Following stimulation, IFN-γ production by NK cells was measured as described (6). For lymphokine-activated killer (LAK) generation, splenocytes were cultured in medium with recombinant human IL-2 (2000 IU/ml; Peprotech). IFN-γ was also measured by ELISA (R&D Systems). In some experiments, NK cells were cultured for 4 h with IL-18 (5 ng/ml), washed three times, and then stimulated as described above.

Quantitative RT-PCR

RNA was extracted with the RNeasy micro kit (Qiagen). Reverse transcriptase (Invitrogen) was used to generate cDNA. PCR was conducted with a SYBR Green-based kit (Qiagen) using the following primers: mouse Hprt 5′, GCC CCAAAATGGTTAAGGTT; mouse Hprt 3′, TTGCGCTCATCTTAGG CTTT, mouse IFN-γ 5′, GAACTGGCAAAAGGATGGTGA; and mouse IFN-γ 3′, TGTGGGTTGTTGACCTCAAAC.

Statistics

A nonparametric permutation test was used for statistical analysis using StatXxact 8 software (Cytel Studio). Two-sided p values are shown as follows: *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

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[3] Abbreviations used in this paper: DC, dendritic cell; IRAK4, IL-1R-associated kinase 4; KO, knockout; LAK, lymphokine-activated killer; poly(I:C), polyinosinic:polycytidylic acid; TIR, TLR/IL-1R.

Results and Discussion

MyD88 is required for IFN-γ production by NK cells in response to IL-12

We first compared the function of NK cells isolated from unchallenged wild-type and MyD88 KO mice. For this purpose, we used an ex vivo assay in which freshly purified NK cells were stimulated for 4 h with YAC-1 target cells or cytokines or with mAb specific to the activating NK cell receptors Ly49D and NKp46. IFN-γ secretion and cytotoxic granule release (which is proportional to surface CD107a exposure; Ref. 7) were measured. MyD88 KO NK cells produced four times less IFN-γ than wild-type NK cells in response to stimulation with IL-12 or IL-2 plus IL-12 (Fig. 1, *A–C*). This was true over a wide range of IL-12 concentrations (data not shown). Similar results were also obtained using ELISA and for longer periods of stimulation (Fig. 1*D*). By contrast, both cell types produced similar levels of IFN-γ in response to all other stimuli tested, i.e., IL-2, YAC1 cells, or cross-linking of Ly49D or NKp46 (Fig. 1, *A* and *B* and data not shown). Wild-type and MyD88 KO NK cells were equally potent in their degranulation response to YAC1 cells or to Ly49D or NKp46 cross-linking (Fig. 1, *B* and *C*). Moreover, NK cell development and maturation were found to be normal in MyD88 KO mice (data not shown). Unexpectedly, MyD88 KO NK cells produced similar levels of IFN-γ transcripts as wild-type NK cells in response to stimulation with IL-12 or IL-2 plus IL-12 (Fig. 1*E*). Thus, MyD88 regulates NK cell IFN-γ production induced by IL-12 at the posttranscriptional level.

We then tested whether MyD88 functioned in a way intrinsic to NK cells. We reconstituted irradiated recipient mice with a 1:1 mixture of wild-type CD45.1$^+$ and MyD88-deficient CD45.1$^-$ bone marrow cells. The NK cell response was assessed 8 wk after transplantation. MyD88 KO NK cells displayed reduced IFN-γ production in response to IL-12 or IL-2 plus IL-12 stimulation in comparison with CD45.1$^+$ wild-type NK cells (Fig. 2*A*). Their response was otherwise similar to that of wild-type NK cells (Fig. 2*A*). These results indicate that MyD88 has a cell autonomous function in IL-12-induced production of IFN-γ by NK cells. Next, we analyzed the response of NK cells in other mutants of the IL-1/TLR/MyD88 pathway. MyD88 associates with receptors of the TLR and IL-1R families through a homotypic interaction of their respective TLR/IL-1R (TIR) domains (8). The *Pococurante* point mutation in the mouse MyD88 TIR domain abrogates this interaction (9). Signaling downstream of MyD88 is also dependent on IL-1R-associated kinase 4 (IRAK4) (10). We found that both *Pococurante* and IRAK4 KO mice phenocopied MyD88 KO mice for the NK cell response (Fig. 2*B*). These results show that the NK cell response to IL-12 requires the association of MyD88 with a TIR domain-containing receptor signaling through the canonical MyD88/IRAK4 signaling pathway.

IL-18 signaling is required for optimal IL-12-induced IFN-γ production by NK cells

We sought to determine which MyD88-coupled receptor(s) were involved in the NK cell response to IL-12. NK cells isolated from TLR1, TLR2, TLR3, TLR4, TLR 5, TLR7, or TLR9 knockout mice responded normally to stimulation with IL-12 (data not shown). NK cells from *3d* mutant mice, bearing a mutation in Unc93b impairing TLR3, TLR7, and TLR9 (11) also responded normally to stimulation with IL-12 (data not

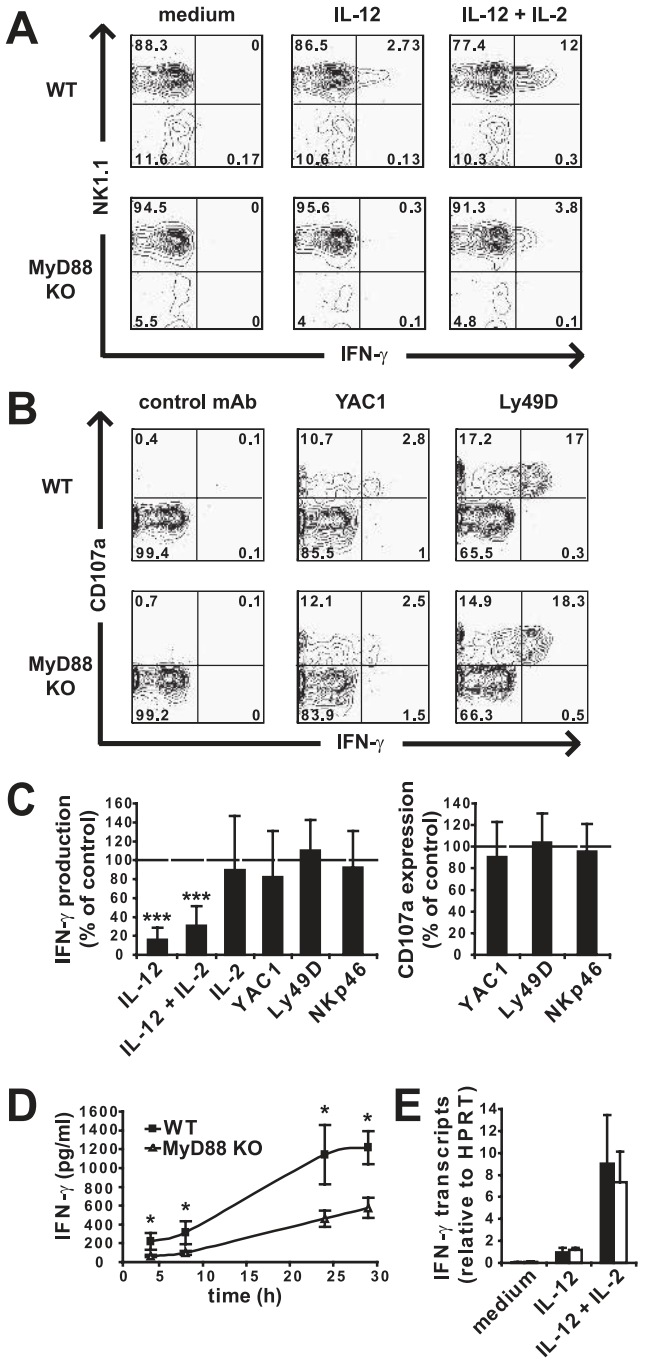

FIGURE 1. MyD88 is required for IFN-γ production by NK cells in response to IL-12. NK cells from wild-type (WT) and MyD88 KO mice were stimulated under the indicated conditions. *A–C*, The expression of NK1.1, CD3, CD107a, and IFN-γ were measured. *A*, Representative FACS analysis of NK1.1 and IFN-γ expression by gated CD3$^-$ cells. Cytokine stimulation did not induce CD107a surface exposure (data not shown). *B*, Representative FACS analysis of IFN-γ expression and CD107a surface exposure by gated NK1.1$^+$ CD3$^-$ cells. *C*, Mean production of IFN-γ (*left*) and CD107a surface exposure (*right*) by gated MyD88 NK cells, expressed as the percentage of the wild-type value in each condition. Data are mean ± SD of $n = 8$ mice. *D*, IFN-γ level was measured by ELISA in the culture supernatant over time. Data are mean ± SD for $n = 4$ MyD88 KO mice and $n = 3$ WT mice. *E*, Detection of IFN-γ transcripts as assessed by quantitative RT-PCR upon 4 h of stimulation. The relative quantity of IFN-γ transcripts was determined by normalization to *Hprt1* mRNA. Data are mean ± SD of $n = 3$.

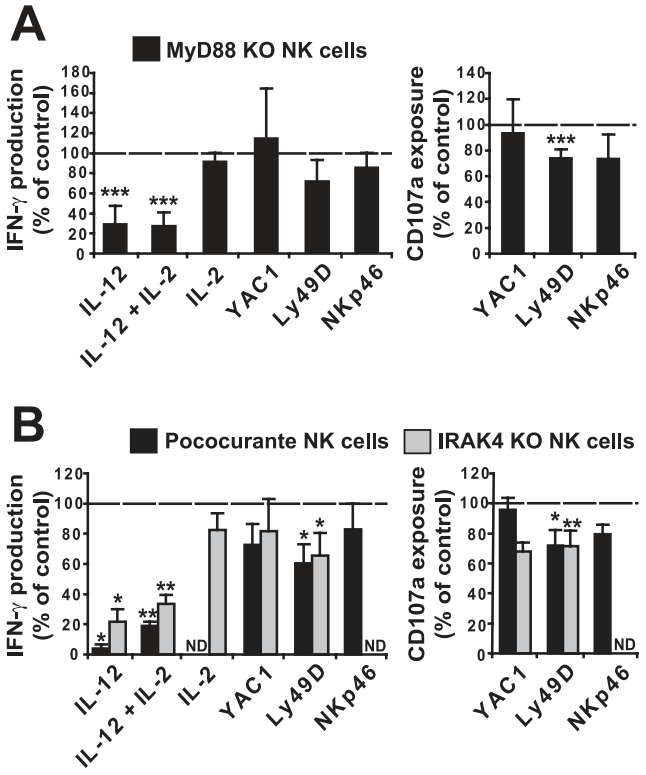

FIGURE 2. MyD88 and IRAK4 have an intrinsic role in NK cell response to IL-12. NK cells from mixed bone marrow chimera mice (wild-type CD45.1 with MyD88 KO or *Pococurante* or IRAK4 KO) were stimulated as indicated. The expression of NK1.1, CD45.1, CD3, CD107a, and IFN-γ were measured. *A*, Mean production of IFN-γ (*left*) and CD107a surface exposure (*right*) by gated MyD88 KO NK cells, expressed as the percentage of the wild-type value in each condition. Data are mean ± SD of $n = 8$ mice. *B*, Mean production of IFN-γ (*left*) and CD107a surface exposure (*right*) by gated *Pococurante* or IRAK4 KO NK cells, expressed as the percentage of the wild-type value in each condition. Data are mean ± SD of $n = 4$ mice.

shown). By contrast and similarly to MyD88 KO NK cells, IL-18R KO NK cells responded poorly to IL-12 and IL-2 plus IL-12 but normally to Ly49D and NKp46 cross-linking (Fig. 3*A*). Thus, the IL-18R/MyD88/IRAK4 pathway is required for

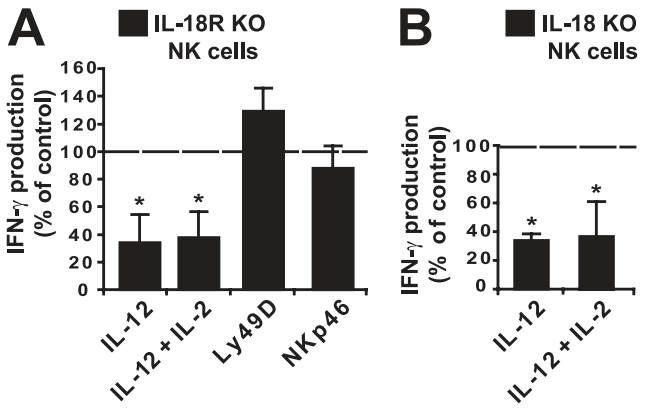

FIGURE 3. IL-18 signaling is required for IL-12-induced IFN-γ production by NK cells. NK cells from wild-type, IL-18R KO, or IL-18 KO mice were stimulated as indicated. The expression of NK1.1, CD3, and IFN-γ were measured. Results show the mean production of IFN-γ by gated NK cells from IL-18R KO mice (*A*) or IL-18 KO mice (*B*). Results are expressed as the percentage of the wild-type value in each condition. Data are mean ± SD of $n = 10$ mice for each genotype.

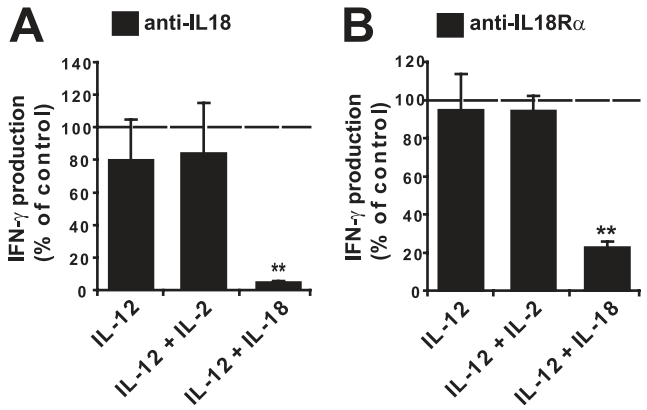

FIGURE 4. Endogenous IL-18 does not costimulate NK cells stimulated with IL-12. NK cells isolated from wild-type mice were stimulated 4 h in vitro as indicated in the presence or absence of anti-IL-18 Ab (*A*) or anti-IL-18Rα mAbs (*B*). Results show the mean production of IFN-γ by gated NK cells, expressed as the percentage of the control (no antibody) value. Data are mean ± SD of $n = 7$ (*A*) or $n = 8$ (*B*).

ex vivo IFN-γ production by NK cells in response to IL-12. In support of this conclusion, NK cells isolated from IL-18 KO mice produced four times less IFN-γ than wild-type NK cells in response to stimulation with IL-12 or IL-2 plus IL-12 (Fig. 3*B*).

IL-12 and IL-18 are well-known to synergize in inducing IFN-γ production by NK cells when coadministered in vitro (12, 13). It has also been previously reported that during a 24-h culture of splenocytes with exogenous IL-12, endogenous production of IL-18 can occur and subsequently costimulate IL-12-induced IFN-γ production (14). This phenomenon could potentially explain why, in our experiments, NK cells deficient for the IL-18 pathway were impaired for IFN-γ production in response to stimulation by exogenous IL-12. However, the addition of blocking mAbs to IL-18 and IL-18R or NF-κB inhibitors did not block the production of IFN-γ by wild-type NK cells induced by IL-12 or IL-2 plus IL-12 stimulation (Fig. 4 and data not shown). As a control, these reagents efficiently blocked the production of IFN-γ by wild-type NK cells induced by exogenously added IL-18 (Fig. 4 and data not shown). Thus, IL-18 signaling is not required for NK cells to respond to IL-12 during a 4-h in vitro assay but it is mandatory in vivo to prime NK cells to respond to a subsequent stimulation with IL-12.

In vitro or in vivo NK cell preactivation restores IL-12-induced IFN-γ production by MyD88 KO NK cells

Next, we asked whether the defective NK cell response to IL-12 observed in IL-18 signaling-deficient mice could be overcome by prior in vivo or in vitro NK cell activation. We first cultured WT and IL-18 KO NK cells with IL-18 before the stimulation with either IL-12 or IL-2 plus IL-12. In these conditions, IL-18 KO NK cells produced IFN-γ as efficiently as WT NK cells in response to both stimuli (Fig. 5*A*). Second, MyD88 KO and WT mice were injected with poly(I:C) to activate NK cells in vivo. IFN-γ production by in vivo activated MyD88 KO NK cells in response to IL-12 or IL-2/IL-12 was lower than that of WT NK cells (Fig. 5*B*), but the difference was less than that between resting WT and MyD88 KO NK cells (Fig. 1*A*). Third, NK cells from MyD88 KO and WT mice were cultured for 6 days in vitro with IL-2 to generate LAK. The response of

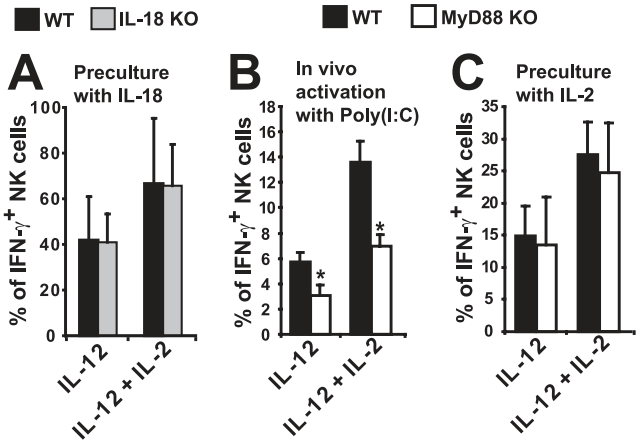

FIGURE 5. In vitro or in vivo NK cell preactivation restores IL-12-induced IFN-γ production by MyD88 KO NK cells. *A*, NK cells from wild-type (WT) and IL-18 KO mice were cultured for 4 h in vitro with IL-18, extensively washed, and then stimulated for 4 h as indicated. The percentage of IFN-γ^+ NK cells was measured; $n = 4–5$. *B*, NK cells from poly(I:C)-injected wild-type and MyD88 KO mice were stimulated for 4 h in vitro as indicated. The percentage of IFN-γ^+ NK cells was measured; $n = 4$. *C*, LAK cells from wild-type and MyD88 KO NK cells were stimulated for 4 h in vitro as indicated. The percentage of IFN-γ^+ LAK cells was measured; $n = 4$.

MyD88 KO LAK in response to IL-12 or IL-2/IL-12 was comparable to the response of WT LAK (Fig. 5*C*). Thus, the responsiveness of MyD88 KO NK cells to IL-12 can be restored by in vitro activation with IL-2 and partially recovered by in vivo activation with poly(I:C). Altogether, these results show that the defective production of IFN-γ by NK cells in IL-18 signaling-deficient mice is not due to a developmental deficiency but rather to a defect of priming.

Our results reveal a novel type of cooperation between IL-12 and IL-18 that requires a sequential activity of these cytokines. What is the molecular basis of this phenomenon? IL-12 and IL-18 have been previously reported to act synergistically to induce IFN-γ secretion by T cells and NK cells (12, 13). Synergy between IL-12 and IL-18 signaling arises from the distinct transcription factors induced and by the cooperation between these factors. For instance, IL-12-induced STAT4 up-regulates the binding activity of IL-18-induced AP-1 on the IFN-γ promoter (15). However, we found that the IFN-γ gene was transcribed at a similar level in WT and MyD88 KO NK cells in response to IL-12 stimulation. Accordingly, we found the following: 1) IL-12R chains were expressed normally in MyD88 KO NK cells at both the mRNA and protein levels; and 2) STAT4 was phosphorylated at a normal level in MyD88 KO NK cells upon stimulation with IL-12 (data not shown). These results suggest an unexpected role of the IL-18 pathway in the posttranscriptional regulation of IFN-γ induced by IL-12. Posttranscriptional regulation of IFN-γ has been shown to occur at different stages, from the localization of IFN-γ mRNA (16) to the stability (17) and even the translation of this mRNA (18, 19). Our results do not favor a role for IL-18 in IFN-γ mRNA stabilization, as the level of IFN-γ mRNA is similar in wild-type and MyD88 KO NK cells stimulated with IL-12. They are, however, compatible with the two other possibilities. Interestingly, Hodge et al. found that IL-12 induces IFN-γ mRNA accumulation in the nucleus of cells of the NK92 line. They also found that stimulation with IL-2 improves the translocation of IFN-γ mRNA to the cytoplasm, which could be linked to an improved translation of this mRNA (16). Thus, it is possible that the stimulation of NK cells with IL-12 induces a suboptimal production of IFN-γ by NK cells, because translation is limiting. In this model, a prior stimulation by IL-18 could result in a better nuclear export and translation of IFN-γ mRNA. Of note, other NK cell effector functions such as cytotoxicity have been found to be primed at the level of translation. In particular, perforin and granzyme B mRNA are translated upon IL-15-mediated priming (20). Thus, the control of mRNA translation could be central in NK cell priming. Interestingly, although we found no effect of IL-18 signaling on the capacity of NK cells to degranulate, others previously reported that NK cells isolated from IL-18 KO mice had defective cytotoxicity against YAC1 cells (21). One possible explanation for this discrepancy could be that the level of granule proteins are also limiting in IL-18 KO NK cells.

Another open question is the source of IL-18 in vivo. IL-18 has been shown to be produced at the basal level (14) by different cell types including macrophages (22) and DC (23). Interactions between macrophages or DC and NK cells have been documented in recent years. In particular, DC have been shown to be essential for IL-15-mediated NK cell priming (3). Therefore, they could also potentially prime NK cells through IL-18 production. This could occur during NK cell development, as bone marrow NK cells were found to produce IFN-γ in response to stimulation with IL-12 (24). The absence of one cytokine could be partly compensated by the others during inflammation. Indeed, we found that in vitro stimulation with IL-2 or in vivo stimulation with poly(I:C) restored at least partially the ability of MyD88 KO NK cells to produce IFN-γ in response to stimulation with IL-12. Similarly, IL-18 deficiency has been shown to be compensated by other pathways in the production of IFN-γ in the liver upon infection with mouse CMV (25). Redundancy in different cytokine pathways might thus contribute to the robustness of NK cell responses.

Disclosures

The authors have no financial conflict of interest.

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