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Innate immunodeficiency following genetic ablation of *Mcl1* in Natural Killer cells

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Summary

The cytokine IL-15 is required for Natural Killer (NK) cell homeostasis, however the intrinsic mechanism governing this requirement remains unexplored. Here, we identify the absolute requirement for myeloid cell leukemia sequence-1 (*Mcl1*) in the sustained survival of NK cells *in vivo*. *Mcl1* is highly expressed in NK cells and regulated by IL-15 in a dose-dependent fashion via STAT5 phosphorylation and subsequent binding to the 3'UTR of *Mcl1*. Specific deletion of *Mcl1* in NK cells results in the absolute loss of NK cells from all tissues owing to a failure to antagonizing pro-apoptotic proteins in the outer mitochondrial membrane. This NK lymphopenia results in mice succumbing to multi-organ melanoma metastases, being permissive to allogeneic transplantation and being resistant to toxic shock following polymicrobial sepsis challenge. These results clearly demonstrate a non-redundant pathway linking IL-15 to *Mcl1* in the maintenance of NK cells and innate immune responses *in vivo*.

Introduction

Innate immune cells are responsible for pathogen detection, rapid inflammation and priming of sterilizing immunity in vertebrates. Innate lymphoid cells (ILCs) represent a diverse family of bone marrow-derived lymphocytes whose development depends on expression of the inhibitor of DNA-binding 2 (Id2) and cytokine signaling via the gamma common (γC) receptor[1]. ILCs are grouped into three subsets (ILC1-3) based on their functions and dependency on specific transcription factors[2]. ILCs do not express antigen receptors that are formed from rearrangement of gene segments and thus, rely primarily on cytokines, not antigenic stimulation, to dictate their development, activation and homeostasis. Natural killer (NK) cells are the most prevalent ILC member (ILC1) being capable of spontaneous cytokine, chemokine and lytic granule production upon activation[3]. NK cell development takes place in the bone marrow of adults and requires the pleiotropic cytokine, interleukin-15 (IL-15)[4, 5] with high expression of the IL-15Rβ chain (CD122), an essential component of IL-15 signaling[6] induced soon after commitment to the NK cell lineage[7]. IL-15 responsive progenitors need to express dimers of IL-2Rβ and the common gamma chain (γ_c/IL-2Rγ/CD132) and receptor proximal kinases (Jak1/3) and Signal Transducer and Activator of Transcription 5 (STAT5A/B) in order to survive, expand and differentiate[8].

Deletion of IL-15 or IL-15Rα *in vivo* largely blocks NK cell development in mice however when IL-15$^{-/-}$ or IL-15Rα$^{-/-}$ progenitors were transferred into wild type mice, NK cell development was partially restored indicating a role for IL-15 and IL-15Rα 'in trans' expressed by non-hematopoietic cells[9, 10, 11, 12, 13]. Both IL-2 and IL-15 utilize IL-2Rγ/β heterodimers to transmit proliferative and survival signals to NK cells and have been shown to induce, enhance or maintain various members of the Bcl-2 family of anti-apoptotic proteins including Bcl-2, Bcl-xL and Mcl-1[14, 15, 16, 17, 18, 19, 20]. On the flipside, we previously proposed that IL-15 regulates NK cell survival by inhibiting the activation of the BH3-only protein Bim[14]. In this instance, Bim protein levels dramatically increased in NK cells upon removal of IL-15 correlating with accelerated apoptosis. Stimulation of NK cells with IL-15 induced activation of the PI3K and MAPK pathways with phopho-Erk1/2 being responsible for Bim phosphorylation and degradation, whereas IL-15 mediated activation of the PI3K pathway was required to phosphorylate and inhibit Foxo3a (also called FKHR-L1), a member of the family of Forkhead box class O transcription factors known to induce *Bim (Bcl2l11)* transcription[21]. The dependency on Bim for NK cell apoptosis is evident by the resistance of *Bim*$^{-/-}$ NK cells to cell death in the absence of IL-15 and their ability to persist when transferred in *IL15*$^{-/-}$ mice[14]. IL-7, IL-15 and IL-2 are known to promote the survival of various T cell subsets with upregulation of Mcl-1 being observed following stimulation with these cytokines and deletion of *Mcl1* in differentiated T cells resulting in a significant reduction in their numbers *in vivo*[22, 23], indicating a key requirement for Mcl-1 in their steady state survival. In addition to the well characterized outer mitochondrial membrane role of Mcl-1 in antagonizing apoptotic proteins such as Bak, Bim and Noxa, it was recently proposed that the inner amino-terminally truncated isoform of Mcl-1 is imported into the mitochondrial matrix where it participates in mitochondrial fusion, ATP production and respiration[24].

We hypothesized that IL-15-mediated signaling is essential for NK cell homeostasis *in vivo* by primarily regulating the level of the highly labile anti-apoptotic protein Mcl-1 *in vivo*. Using a novel Mcl1-hCD4 mouse strain, whereby Cre-mediated deletion of the *Mcl1* coding sequence results in surface expression of human CD4 (hCD4) under control of the *Mcl1* regulatory elements[25, 26], we conclusively demonstrate that *Mcl1* expression is directly regulated by IL-15 via STAT5 binding to its 3'UTR. The *in vivo* consequence of failing to express *Mcl1* in differentiated NK cells was explored using *Ncr1* (NKp46) mediated deletion (*Ncr1-Cre*) of *Mcl1*. NK cells are completely absent from all tissues when both copies of *Mcl1* are deleted. This result in itself represents a milestone for the NK cell field, as this is the first genetic model specifically and constantly lacking NK cells. Our findings underline the non-redundant role for *Mcl1* in NK cell survival downstream of IL-2Rγ/β and permit the accurate investigation into the *in vivo* role of NK cells during pathogenesis.

Results

NK cells express high *Mcl1* levels throughout development

To investigate cytokine-mediated survival in ILCs *in vivo*, we focused on NK cells since their development is dependent on a single growth factor (IL-15), their differentiation is well characterized and they are abundant in lymphoid organs of mice[3]. Peripheral NK cell maturation is defined by differential expression of Mac-1, CD27 and KLRG1 with immature (Imm.) NK cells being Mac-1$^-$CD27$^+$KLRG1$^-$, Mature 1 (M1) NK cells being Mac-1$^+$CD27$^+$KLRG1$^-$ and Mature 2 (M2) being Mac-1$^+$CD27$^-$KLRG1$^+$ (Figure 1A)[27, 28, 29]. Measurement of mRNA levels of *Bcl-2* family members revealed that *Mcl1* is highly expressed in NK cells, and that this expression increases with maturation (Figure 1B and Supplementary Fig. 1). To visualize *Mcl1* expression *in vivo*, we utilized a Mcl1-floxed-hCD4 mouse strain (allele is termed *Mcl1*^{fl} from herein). In this system, Cre-mediated deletion of the *Mcl1* coding sequence results in surface expression of human CD4 (hCD4) under control of the *Mcl1* regulatory elements[25, 26]. This strain was bred to both the Rosa26 Cre-ERT2 (*CreERT2*) mice to facilitate *Mcl1* deletion *in vivo*. Treatment of *Mcl1*$^{fl/+}$*CreERT2* mice with tamoxifen via oral gavage resulted in stable expression of hCD4 (reporting on *Mcl1* transcription) on all hematopoietic cell types with the highest expression observed on bone marrow stem/progenitors (LSK; green) consistent with previous data[30], then CLPs (orange), while pre-pro NK cells (Lin$^-$c-kit$^-$Sca-1$^+$flt3$^-$IL-7Rα^+; red) and conventional NK cells (blue) expressed slightly lower levels (Figure 1C). In the periphery, *Mcl1* expression was found to increase as NK cells matured with M2 NK cells (red) expressing clearly higher levels of *Mcl1* compared to M1 (blue) and Imm. (green) NK cells in the spleen (Figure 1D) and liver (Figure 1E) consistent with our mRNA data (Figure 1B). We also verified *Mcl1* expression in the TCR-β$^-$NK1.1$^+$CD49b$^-$ hepatic resident NK cells (orange; Figure 1E) since their developmental origins and transcription factor requirements are distinct from conventional NK cells[31]. TCR-β$^-$NK1.1$^+$CD49b$^-$ hepatic resident NK cells expressed an *Mcl1* level equivalent to that of hepatic resident M1 NK cells (Figure 1E).

Mcl1 is induced by γ_C cytokines in a dose-dependent manner

We next wanted to generate a model where *Mcl1* was deleted specifically in NK cells via Cre-mediated deletion. The *Ncr1* gene (encoding NKp46) is highly expressed in NK cells in all organs and importantly, Ncr1 is not expressed to any significant level on other major immune cells. We investigated *Mcl1* expression specifically in NK cells by crossing the *Mcl1*$^{fl\text{-}hCD4/+}$ mice with the Ncr1-iCre (*Ncr1-Cre*) mice[32]. The strict requirement for IL-15 on NK cell development *in vivo* prompted us to investigate the relationship between IL-15 and *Mcl1* regulation in these cells. Culturing NK cells (TCR-β$^-$NK1.1$^+$NKp46$^+$) from *Mcl1*$^{fl\text{-}hCD4/+}$ *Ncr1-Cre* mice in graded concentrations of IL-15 resulted in a robust dose-dependent increase in *Mcl1* (hCD4) expression (Figure 2A). Western blot analysis of NK cell lysates confirmed that Mcl-1 and to a lesser extent Bcl-xL, but not Bcl-2, were elevated following IL-15 stimulation *in vitro* (Figure 2B) and that these same proteins were degraded when IL-15 was withdrawn from NK cells that had been cultivated in a high concentration of IL-15 for one week (Figure 2C). Since Bcl-xL was also increased in NK cells following IL-15 stimulation (Figure 2B), we next determined the contribution of Bcl-xL to NK cell development *in vivo* by conditional deletion of *Bcl-x (BCL2L1)* in NK cells using the Bcl-x loxP strain[33]. Bcl-$x^{fl/fl}$*Ncr1-Cre* mice presented a normal proportion and number of NK cells in all organs examined indicating that Bcl-xL is not required for NK cell development *in vivo* (Figure 2D).

Mcl1 is essential for the generation of NK cells in mice

Given the abundant expression of Mcl-1 driven by IL-15 in NK cells, we examined the importance of this pathway *in vivo* by deleting both *Mcl1* alleles specifically in NCR^+ cells using the *Ncr1-Cre* mice. The result was striking; *Mcl-1*$^{fl/fl}$*Ncr1-Cre*$^+$ mice contained essentially no NK cells (TCR-β^-NKp46$^+$NK1.1$^+$ or TCR-β^-CD49b$^+$NK1.1$^+$) in all organs examined (Figure 3A and B). The few NK cells that remained in *Mcl1*$^{fl/fl}$*Ncr1-Cre* mice were immature (Imm.; Figures 3C). The Imm. stage of NK cell differentiation is the stage at which Ncr1-mediated Cre expression begins[32] and hence *Mcl1* gene deletion is initiated (Supplementary Fig. 2), thus the appearance of only a very minor population of Imm. NK cells in *Mcl-1*$^{fl/fl}$*Ncr1-Cre*$^+$ mice confirms the absence of Mcl-1 is incompatible with NK cell viability.

Outer mitochondrial membrane Mcl-1 inhibits NK cell death

Mcl-1 has been recently proposed to contribute to cellular biogenesis in the inner mitochondrial membrane in addition to its known anti-apoptotic role in the outer membrane[24]. We next examined the ability of Mcl-1 (able to access the outer mitochondria membrane and the mitochondria matrix) and an Mcl-1 variant, Mcl-1^{OM} (expression restricted to the outer mitochondrial membrane)[24] in rescuing NK cell survival in the absence of wild type Mcl-1. To do this, *Mcl-1*$^{fl/fl}$*Ncr1-Cre* LSKs were transduced *in vitro* with retrovirus encoding vector alone, Mcl-1 or Mcl-1^{OM} and all co-expressing green fluorescent protein; GFP) (Figure 4A). Total LSKs were then cultured for 21 days in 50ng/mL IL-15 and analyzed for NK cell generation. Despite similar transduction efficiency to Mcl-1, *Mcl-1$^{fl/fl}$Ncr1-Cre* LSKs transduced with vector alone (GFP) failed to generate NK cells, underlining the requirement of Mcl-1 for NK genesis, even in high concentrations of IL-15. In contrast, ectopic expression of either Mcl-1 or Mcl-1^{OM} was equally efficient in rescuing NK cell development from *Mcl-1$^{fl/fl}$Ncr1-Cre* LSKs *in vitro* (Figure 4A). NK cell development in these conditions was robust and independent of Mcl-1 expression in the mitochondria matrix suggesting the primary defect following loss of *Mcl-1* in NK cells is the inability to antagonize the pro-apoptotic BH3-only and BAX/BAK proteins and not in mitochondrial fusion or cellular biogenesis. Collectively, these results clearly indicate Mcl-1 is the principle antagonist of apoptosis in NK cells and are consistent with our *in vitro* observation that Mcl-1 levels are proportional to NK cell viability following IL-15 withdrawal (Figure 2C)[14].

IL-15 directly regulates *Mcl1* via STAT5 binding the 3'UTR

To understand how the dynamic regulation of *Mcl1* in response to IL-15 is achieved we next investigated the molecular pathways activated by IL-15 in NK cells. IL-15 stimulation of NK cells resulted in the rapid induction of Jak1 and STAT5a/b phosphorylation that peaked between 30 - 60 minutes (Figure 4B). STAT5 is a transcription factor that dimerizes upon phosphorylation and translocates to the nucleus where it can drive the expression of a large number of genes via binding an evolutionarily conserved TTC(T/C)N(G/A)GAA motif[34 35]. We identified a conserved STAT motif in the promoter and a conserved STAT5 motif in 3'UTR of Mcl1 (Figure 4C). To determine whether STAT5 directly bound to *Mcl1* in NK cells, we cultivated NK cells in a high concentration of IL-15 to induce STAT5 phosphorylation and performed chromatin immuno-precipitation (ChIP) using STAT5 antiserum. NK cells not stimulated with IL-15 were used as a negative control as these cells displayed negligible STAT5 phosphorylation by western blot. STAT5 ChIP confirmed a robust enrichment of the 3'UTR sequence of Mcl1 but not the promoter in NK cells stimulated with IL-15 compared to untreated NK cells indicating that STAT5 directly binds the 3'UTR of *Mcl1* (Figure 4C). Enrichment of the well characterized STAT5 target gene *Cish* (cytokine-inducible SH2-containing protein) was used as a positive control as this gene has multiple TTC(T/C)N(G/A)GAA motifs in its promoter[36] (Figure 4C). Given that IL-15 directly maintains NK cell viability by driving Mcl-1 expression via STAT5, we next determined whether the requirement for IL-15 could be overcome by transgenic expression of *Mcl1* in NK cells. NK cells from Vav-Mcl-1 transgenic (Mcl-1 Tg) mice[37] displayed greatly enhanced Mcl-1 protein levels compared to littermate controls (Figure 4D) and when cultured in limiting concentrations of IL-15 Mcl-1 Tg NK cell survival was 10-50 fold greater than control NK cells (Figure 4E). Taken together these data identify *Mcl1* as a STAT5 target gene in NK cells and highlight the direct link between IL-15 signaling and NK cell survival.

Absence of innate cytotoxicity in NK lymphopenic mice

NK cells were the first innate lymphoid cells described owing to their spontaneous ability to kill target cells that had altered expression of MHC-I (non-self MHC-I or reduced/absent MHC-I) and this role has since been extended to the killing of cells expressing stress-induced activating ligands[38, 39]. NK cells are also potent producers of pro-inflammatory cytokines, namely IFN-γ upon pathogen encounter. Studying the *in vivo* role of NK cells has largely relied on NK cells being depleted with anti-NK1.1 antibody since a genetic mouse strain specifically lacking NK cells was, until now, unavailable. We next examined some functional consequences of the complete loss of NK cells in *Mcl1$^{fl/fl}$Ncr1-Cre* mice. Following injection with B16F10 murine melanoma cells (lacking MHC-I but expressing DNAM-1 ligand, CD155), *Mcl1$^{fl/fl}$Ncr1-Cre* mice needed to be sacrificed after 12 days due to acute onset of respiratory distress. Post-mortem analysis revealed that the lungs of these animals were overwhelmed with melanoma metastases whereas metastases were rarely observed in similarly challenged control mice (*Mcl1$^{+/+}$Ncr1-Cre*; Figure 5A and B). B16F10 is not thought to be a particularly metastatic variant of B16 and has been classically used to measure experimental lung metastasis however we discovered that at the low dose used, the tumor cells metastasized extensively and were located in additional sites in *Mcl1$^{fl/fl}$Ncr1-Cre* mice. This included the liver, bone marrow, kidney and lymph nodes, whereas metastases at these sites were never observed in control mice (Figure 5C and D).

Residual NK cell activity following whole body γ-irradiation is associated with bone marrow allograft rejection[40, 41]. Consistent with the previous finding, C57BL/6 (H-2b) recipient mice lacking NK cells

($Mcl1^{fl/fl}$*Ncr1-Cre*) possessed significantly more splenic monocytes and spleen colony forming units (CFU-S) at day 8 post-transplantation with allogeneic BALB/c (H-2d) bone marrow cells compared to NK cell proficient control recipients ($Mcl1^{+/+}$*Ncr1-Cre*; Figure 5E-F). CFU-S counts are a reflection of bone marrow engraftment and the fact that recipients lacking NK cells possessed similar CFU-S counts after allogeneic bone marrow transplantation as C57BL/6 mice receiving syngeneic bone marrow (H-2b) demonstrates that Mcl-1 dependent survival of NK cells is critical for the early clearance of MHC mismatched bone marrow and a contributing factor in bone marrow transplant outcomes.

NK lymphopenia protects mice from lethal sepsis

Sepsis is a systemic inflammatory response to bacteria infection resulting in the death of over a million humans annually. Polymicrobial sepsis induced by cecal ligation and puncture (CLP) is the most common murine model of bacterial sepsis[42]. This model induces a hyper-inflammatory response by innate immune cells, characterized by high levels of IL-6, TNF and MCP-1 typically resulting in death within 48 h[43]. To investigate the contribution of NK cells in CLP-mediated sepsis, we compared mice with varying degrees of NK cell deficiency from including $Mcl1^{fl/fl}$*Ncr1-Cre* (~0% NK cells), anti-αasialoGM1 treated C57BL/6 (30-40% NK cells), anti-NK1.1 treated C57BL/6 (~10% reduction in NK cells) and control C57BL/6 $Mcl1^{+/+}$*Ncr1-Cre* (100% NK cells; Figure 6A). Strikingly, $Mcl1^{fl/fl}$*Ncr1-Cre* mice were resistant to toxic shock induced by CLP compared to control mice, whereas mice depleted of NK cells by antibody treatment (anti-NK1.1 and anti-αasialoGM1; Figure 6A) were also largely protected (Figure 6B). The presence or absence of NK cells had no bearing on the bacterial load with no differences in bacteria colony forming units observed in the blood at this early time point (Figure 6C). The protection offered by the absence of NK cells was however characterized by a significant reduction in IFN-γ and IL-6 protein level in the serum 12 h following CLP (Figure 6D. E). This finding indicates an NK cell dependent pro-inflammatory cytokine response contributes to the lethality following CLP in mice and that even a 50% reduction in NK cells is effective in reducing the incidence of death due to septic shock.

Discussion

NK cells evolved prior to adaptive lymphocytes and lack the somatically rearranged antigen receptors that control the development and survival of B and T cells[44, 45]. Here we demonstrate conclusively that NK cells instead rely on a non-redundant pathway for IL-15 in directly regulating the expression of the anti-apoptotic protein Mcl-1 via STAT5 binding to the 3'UTR for their viability *in vivo*. Mcl-1 expression in NK cells requires IL-15 and is tightly regulated suggesting that changes in the levels of these cytokines will greatly impact on NK cell homeostasis *in vivo*. IL-15 is derived from both parenchymal and hematopoietic cells and plays an integral role in the homeostasis of various T cell subsets such as γ/δ T cells, NKT cells, CD8αα T cells as well as innate lymphocytes[46, 47]. A similar mechanism of IL-15 dependent Mcl-1 regulation in these cell types is also likely. Other members of the Bcl-2 family have been shown to be upregulated in lymphocytes following stimulation with IL-15 and proposed to antagonize the pro-apoptotic BH3-only and BAX/BAK proteins to prevent cell death[48 49]. Our observation and that of others indicate that Bcl-2 family members in addition to Mcl-1 are upregulated in NK cells by cytokines that stimulate γ_C chain containing receptors, however these apoptosis inhibitors must function in synergy with Mcl-1 to promote NK cell survival[14, 22]. For example, while we found Bcl-xL to be completely redundant in maintaining steady state NK cell survival, this is not to say that induction of Bcl-xL in NK cells is irrelevant for NK cell survival during different circumstances such as inflammation.

It is interesting that the requirement for Mcl-1 in NK cell survival appears greater than that of STAT5, a major transcriptional activator downstream of IL-15 signaling that we found to bind the 3'UTR of *Mcl1*. *In vivo* deletion of STAT5 specifically in NK cells via the Ncr1-Cre transgene (using a bacterial artificial chromosome approach) only resulted in 3-6 fold reduction in peripheral NK cells numbers *in vivo* [50], which is consistent with earlier germ-line deletion studies [51] and an order of magnitude less than the reduction in NK cells observed in *Mcl1*$^{fl/fl}$*Ncr1-Cre* mice (up to 100 fold reduction in some organs). This suggests that STAT3, which is phosphorylated following IL-15 stimulation in NK cells may also contribute to Mcl-1 expression in the absence of STAT5, as a similar pathway is proposed to exist in various tumor cells[52 53]. STAT3 phosphorylation is also induced following stimulation of IL-21R/γ_C heterodimers[54] thus it will be interesting to investigate if the synergy between IL-15 and IL-21 converge at a level of STAT3 activation leading to *Mcl1* expression.

We are only now beginning to understand the intrinsic pathways required for NK cell homeostasis. Following upregulation of Id2 in pre-pro NK cells[7], expression of the IL-2/15Rβ (CD122) is acquired and NK cells become dependent on IL-15. The near total loss of NK cells following *Mcl1* deletion may suggest their dependency on Mcl-1 for survival is more stringent than that of B and T cells since specific deletion of *Mcl1* using CD19-Cre (B cell specific) and Lck-Cre (T cell specific) resulted in a less dramatic loss of these cell types *in vivo* [22] than what we observed for NK cells. It remains possible that this may also reflect the efficiency of Cre expression from the respective transgenes. The aforementioned conclusion would, however, explain why transgenic Bcl-2 overexpression failed to rescue NK cell development in $\gamma_C^{-/-}$ mice but could (partially) rescue T cell development in these animals[55]. Furthermore, specific deletion of STAT5 in lymphoid progenitors via Rag1-Cre expression results in an absence of Pro-B cells that can also be rescued by Bcl-2 overexpression[56]. In these "rescued" STAT5-null Pro-B cells, Mcl-1 protein was absent compared to STAT5 sufficient Pro-B cells suggesting a role for STAT5 in directly activating Mcl1 in the B lineage in response to IL-7[56].

The fidelity and efficiency of Cre-expression in the Ncr1-iCre strain is paramount to our success in generating an NK cell null mouse model. This clear and sustained lack of innate effector functions in *Mcl1*$^{fl/fl}$*Ncr1-Cre* mice offers the most relevant model to date in addressing the contribution of NK cells in mammalian immune defense. This model does not require *in vivo* administration of antibodies or diphtheria toxin to deplete NK cells or effect additional cell types, caveats of all current approaches[4, 5, 6, 57, 58, 59]. Functionally, our results demonstrate that NK cells (ILC1) are a rapid source of pro-inflammatory cytokines following bacteria detection *in vivo* contributing to septic shock and are central to the clearance of cells lacking self MHC-I expression *in vivo*. Collectively, these findings unequivocally demonstrate for the first time that induction of anti-apoptotic Mcl-1 expression by IL-15 is required for the survival of NK cells *in vivo*.

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CONFLICT OF INTEREST

E.V. is a co-founder and shareholder in InnatePharma. Remaining authors have no conflicting financial interest.

AUTHORSHIP

PS, RBD, MC, TBK, LCR, CS, LAM, CJV and FSFG designed and performed experiments. IV, SEN, SG and EV provided key reagents. MJS, AS, SC, SLN, GTB and NDH supervised experimental design, and provided input into interpretation of results and writing of the paper.

Materials and Methods

Mice. *Mcl1-loxp-hCD4* [25,26], *Rosa26-CreERT2* (TaconicArtemis), Bcl-x-loxp [33] *Rosa26-(loxP-stop-loxP)yfp* [60] *Vav-Mcl1 Tg*[37] and *Ncr1-iCre* mice [32] were bred and maintained at the Walter and Eliza Hall of Medical Research. The *Mcl-1-loxp-hCD4*, *Rosa26-CreERT2, Vav-Mcl1 Tg* and *Rosa26-EYFP* mice were generated on a C57BL/6 background using C57BL/6-derived ES cells. The *Ncr1-iCre* mice were generated on a mixed C57BL/6x129SV background using 129SV derived ES cells and then backcrossed with C57BL/6 mice for at least eight generations. The relevant Animal Ethics and Experimentation Committees approved animal experiments according to the guidelines of the National Health and Medical Research Council Australia. Both female and male mice aged between 8-15 weeks of age were used in this study. Age and sex matching was performed for each independent experiment.

Flow cytometry and cell sorting. Antibodies specific for NK1.1 (PK136; 1:400), Sca-1 (E13-161.7; 1:200), c-kit (2B8; 1:500), hCD4 (RPAT4; 1:100), Gr-1 (RB6-8C5; 1:500), CD244.2 (244F4; 1:200), CD19 (1D3; 1:500), CD3 (KT31.1; 1:500), CD122 (TM-β1; 1:200), NKp46 (29A1.4; 1:100), Flt3 (A2F10.1; 1:100), CD127 (A7R34; 1:200), CD11b (M1/70; 1:800), TCR-β (H57-5921; 1:500), CD45.2 (104; 1:500, KLRG1 (2F1; 1:100), CD27 (LG.7F9; 1:200), H-2b (AF6-88.5.3.3; eBioscience; 1:100) H-2d (34-2-12; 1:100) and CD49b (DX5; 1:200 and HMa2; 1:200) were from BD PharMingen unless stated otherwise. Single-cell suspensions were prepared by forcing of organs through 70 μM sieves. Lymphocytes from liver were isolated by suspension in isotonic percoll (Amersham Pharmacia Biotech) and centrifugation at 1800 g. For flow cytometry, single-cell suspensions were stained with the appropriate monoclonal antibody in PBS containing 2% (vol/vol) FCS. FACS Verse, Fortessa and AriaII (BD Biosciences) were used for cell sorting and analysis, with dead cells excluded by propidium iodide staining. Depletion of differentiated cells was performed by incubating bone marrow suspensions with a cocktail of rat mAb against CD3 (KT3), CD8 (53-6.7), CD19 (1D3), Ter119 (TER119), CD11b (M1/70) and Ly6G (1A8). Supernatants from these hybridoma cultures were generated in house, titrated on bone marrow suspensions and visualized with anti-rat FITC or anti-rat Al700 to control for any variability in labeling efficiency over time. Antibodies were added to the cocktail at the dilution found to give optimal labeling by FACS, constituting 2 - 10 % of the final cocktail volume. The bone marrow suspension was then incubated with polyclonal sheep anti-rat IgG magnetic beads at a ratio of 8 beads per cell, and bead-bound cells magnetically depleted. Remaining differentiated cells were visualised by staining with anti-rat Al700. All single cell suspensions were diluted in PBS prior to analysis and enumeration using the Advia hematology analyzer (Siemens).

***In vivo* analysis of NK cells.** Melanoma metastasis: 4 x 10^4 B16F10 melanoma cells (perforin-sensitive, FasL- and TRAIL-insensitive, H-2^b; ATCC) were injected IV into mice and monitored for respiratory difficulty and weight loss. At 12 days mice were sacrificed. Lymphoid organs, kidney, liver and lungs were harvested, fixed in Bouin's solution and B16F10 metastases counted[61]. Allogeneic bone marrow transplants: 1.5×10^7 allogeneic (H-2d) and syngeneic (H-2b) total bone marrow cells were injected i.v. into lethally-irradiated mice (2×5.5Gy). Eight days later, mice were sacrificed, lymphoid organs harvested and analyzed for H-2d hematopoietic engraftment. Colony assays (CFU-S) were performed with 0.6% agar and 2×DME + 40%FCS. 1 × 10^5 splenocytes/plate were added into a tube containing equal concentrations of 2×DME/FCS and Difco Bacto Agar. 100µl of GM-CSF made up at 100ng/ml was added to the plate. Plates were incubated at 10% CO2 for 7 days and colonies were counted using a light microscope. Colonies were classified as greater than 50 cells[62, 63]. Cecal ligation and puncture (CLP) was performed at QIMR Berghofer Medical Research Institute. Briefly, mice were anesthetized with isoflurane, the abdomen was shaved, disinfected using betadine antiseptic spray and a midline incision made. Cecum was externalized with a cotton bud and 75% was ligated and punctured once using a 25-gauge needle to extrude a small amount of cecal content. The cecum was returned to the abdomen, the peritoneal was closed via continuous suture, and the skin was sealed using an auto clip wound clip applier (Becton, Dickinson). Buprenorphin (Reckitt Benckiser Pharmaceutical) was applied at 0.05 mg per kg body weight at the incision site for postoperative analgesia.[64]. NK cell depletion was performed by treating mice with 100µg of purified antibody with anti-NK1.1 (PK136) or anti-αasialoGM1 antibody (Wako Pure Chemical Industries) at day -3 and day 0 prior to CLP. Tamoxifen oral gavage was performed as in [25, 26].

***In vitro* NK cell assays**

NK cell cultivation was performed in Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) FCS plus gentamycin (50ng/mL; Sigma) and 40ng/mL recombinant hIL-15 (Peprotech). NK cells co-cultured (1x10^5 of each) *in vitro* with various doses of hIL-15 (Peprotech) for 5 days. Mcl-1 induction studies were performed by culturing purified $Mcl1^{fl\text{-}hCD4/+}$*Ncr1-Cre* NK cells in hIL-15 (Peprotech) in 96-well, flat-bottomed plates. Live and dead cells were discriminated by staining with propidium iodide followed by analysis on a FACSVerse (Becton-Dickinson). Bone marrow LSK transduction was performed using retrovirus packaged in 293T cells. Briefly, 10 cm tissue culture plates containing 80% confluent 293T cells were treated with chloroquine (25mM) for 30 min at 37°C before transfection with plasmids encoding eco-MLV (0.2µg/mL), gag-pol (0.3ug/mL) and either GFP, Mcl-1 or Mcl-1^{OM} [14] (1ug/mL) using $CaCl_2$ (20mM) and HBSS (2x) [24]. LSKs were transduced with a combination of RetroNectin (Takara) and spin inoculation. Twelve well non–tissue culture plates were coated for 12 h with RetroNectin (4mg/cm^2 in PBS) at 4°C before blocking with PBS containing 2% (wt/vol) BSA at 25°C. Viral supernatant (2ml) was added and plates were then centrifuged for 2 hours at 37°C and 1,200g. LSKs were cultured for 48 h in IL-15 (50ng/ ml) at 2 x 10^6 cells per ml.

Polybrene (2.5μg/mL) was added to these cultures, and the cultures added to each virus coated well from above after the viral supernatant was removed. Cells were then cultured for 12 h at 37°C. Fresh wells were then coated with 2 ml viral supernatant as above, and the LSK cultures transferred to these wells for 12 h at 37°C. Cultures were transferred to tissue culture treated plates after this second round of transduction, and infection efficiency was determined by flow cytometry of GFP expression

Quantitative PCR and STAT5 ChIP. Total RNA from 3 x 10^5 Imm. M1 and M2 NK cells was purified using RNeasy mini columns (Qiagen). SuperScript II reverse transcriptase (Roche) was used for first-strand cDNA synthesis according to the manufacturer's instructions. Quantitative PCR was performed using Sensimix SYBR HI-ROX (Bioline) and the Bio Rad CFX384 detection system and software (Bio Rad). Primers used [*Mcl1*, *Bcl2*, *Bcl-x* (*Bcl2l1*), *Bcl-w* (*Bcl2l2*) and *A1* (*Bcla1*), Puma (Bbc3), Noxa (Pmaip1), Bim (Bcl211)] have been described [65]. The relative expression of each gene was normalized to *Hprt*. For STAT5 ChIP, 1.5 x 10^7 *in vitro* expanded splenic NK cells were cultured in IL-15 (40ng/mL) or media alone for 5 h. NK cells were then crosslinked for 10 min in presence of 1% paraformaldehyde (Sigma) in PBS then lysed (1%SDS + 1mM EDTA + proteases inhibitors). Cross-linked DNA was sonicated with the Branson sonifier 250 (Branson). Lysates were incubated overnight with 10 μg of Pan human/mouse Stat5a/b antibody (R&D). 100 μl of Protein G Dynabeads (Invitrogen) were added, and incubated for 2 hours at 4°C under gentle rotation. Unbound chromatin was removed using a series of five washes (low salt, high salt, liCL and 2xTE). Following elution, bound chromatin was reverse cross-linked and subjected to phenol/chloroform immunoprecipitation. Recovered DNA was resuspended in TE buffer and enrichment for specific region of the genome was measured by real time PCR using the primers described (Supplementary Table 1). Raw CT values are shown in Supplementary Table 2.

Immunoblotting. Protein extracts were prepared in RIPA buffer (300 mM NaCl, 2% IGEPAL CA-630, 1% deoxycholic acid, 0.2% SDS, 100 mM Tris-Hcl pH 8.0) and 30 μg of protein was loaded into NuPAGE 10% Bis-Tris gels. Western blotting was performed according to standard procedures. Blots were probed with the following antibodies: Mcl-1 (1:1000 clone 19C4-15, WEHI mAb lab); Bcl-2 (1:500) clone 7, BD Biosciences); Bcl-xL (1:1000) polyclonal, BD Biosciences); pY-STAT5 (Tyr694; 1:1000 Millipore), pY-Jak1 (1:1000 1022/1023; Invitrogen), Erk1/2 (1:1000 Cell Signaling), Bim (1:2000 polyclonal, Enzo Life Sciences); and β−actin (1:2000 clone AC-74, Sigma). Un-cropped Immunoblots are displayed in Supplementary Fig. 3.

Statistical analysis. A standard Student's t-test with two-tailed distributions for two-samples with equal variance was used for statistical analysis. *P* values are provided. In Fig. 6B significance of survival differences were determined using Mantel-Cox test whereas for Fig. 6C, E and F, t-tests using the Sidak-Bonferroni method were performed.

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Figure 1. ***Mcl1*** **is expressed throughout NK cell development.**

(A) Peripheral NK cell subsets were FACS sorted from spleen based on TCR-β, NK1.1, CD122, NKp46, CD27, Mac-1 and KLRG1 expression (Imm. – green; M1 - blue; M2 - red) and (B) expression of *Mcl1*, *Bcl2*, *Bcl-x* (*Bcl2l1*), *Bcl-w* (*Bcl2l2*) and *A1* (*Bcl2a1*) mRNA was determined by qPCR and normalized to HPRT. Data represent the mean ± SD of 2 independent experiments. (C) 12 week old $Mcl1^{fl(loxp\text{-}hCD4)/+}$*CreERT2* mice were treated with tamoxifen by oral gavage and Mcl-1 expression (reflected by surface hCD4) analyzed at 15 weeks by FACS in LSKs (green), CLPs (orange), pre-pro NK cells (red) and conventional NK cells (blue) in the bone marrow. (D) Imm., M1 and M2 splenic NK cell subsets from $Mcl1^{fl(loxp\text{-}hCD4)/+}$*CreERT2* mice were analyzed for *Mcl1* (hCD4) expression by FACS. Black histograms in (C, D) represent mononuclear cells from tamoxifen treated $Mcl1^{+/+}$*CreERT2* mice. $Mcl\text{-}1^{fl(loxp\text{-}hCD4)/+}$*CreERT2* mice were treated with tamoxifen by oral gavage. (D) Imm., M1 and M2 splenic NK cell subsets from $Mcl1^{fl(loxp\text{-}hCD4)/+}$*CreERT2* mice were analyzed for *Mcl1* (hCD4) expression by FACS. $Mcl\text{-}1^{fl(loxp\text{-}hCD4)/+}$*CreERT2* mice were treated with tamoxifen by oral gavage and (E) Imm. (green), M1 (blue) and M2 (red) and $CD49b^-$ (orange) hepatic NK cell subsets were analyzed for Mcl-1 (hCD4) expression by FACS. Black histograms in (C-F) represent mononuclear cells from tamoxifen treated $Mcl1^{+/+}$*CreERT2* mice. Data are representative of 2 independent experiments.

Figure 2. ***Mcl1*** **is induced by IL-15 in a dose-dependent manner.**

(A) Freshly isolated splenic NK cells ($NKp46^+NK1.1^+TCR\text{-}\beta^-$) from $Mcl1^{fl\text{-}hCD4/+}$*Ncr1-Cre* mice were cultured for 18 h in the indicated concentrations of IL-15 and hCD4 (*Mcl1*) expression analyzed by FACS. (B) Splenic NK cells were sorted and stimulated for 0, 3 or 6 h with 40 ng/mL IL-15. NK cell lysates were analyzed for Bcl-xL, Mcl-1, Bcl-2 and β-actin (loading control) by Western blotting. (C) Splenic NK cells were sorted and grown in 40 ng/mL IL-15 for 7 days and then starved in media alone for 0, 3 or 6 h. NK cell lysates were analyzed for Bcl-xL, Mcl-1, Bcl-2 and β-actin (loading control) by Western blotting. (D) $NK1.1^+NKp46^+$ cells in $Bcl\text{-}x^{+/+}$*Ncr1-Cre* and $Bcl\text{-}x^{fl/fl}$*Ncr1-Cre* mice were analyzed amongst $TCR\text{-}\beta^-$ mononuclear cells from bone marrow, spleen and liver. Data are representative of 4 mice.

Figure 3. Mcl-1 is essential for the generation of NK cells *in vivo*.

The *Mcl1* gene was specifically deleted in Ncr1$^+$ (NKp46$^+$) cells by intercrossing the *Mcl1*^{fl} and *Ncr1-Cre* strains. (A) NK1.1$^+$NKp46$^+$ and NK1.1$^+$CD49b$^+$ cells in *Mcl1*$^{+/+}$*Ncr1-Cre* and *Mcl1*$^{fl/fl}$*Ncr1-Cre* mice were analyzed amongst TCR-β$^-$ mononuclear cells from bone marrow, spleen, liver, lymph node and blood. Data in are representative of 6 mice. (B) TCR-β$^-$NK1.1$^+$CD49b$^+$ NK cells from the indicated organs were enumerated using the Advia blood analyser. Data are mean ± SEM of 6 mice of each genotype. Student t-test; P > 0.001. (C) Splenic TCR-β$^-$NK1.1$^+$CD49b$^+$ NK cells from (A) were further analyzed for Mac-1 and KLRG1 expression by FACS and proportions of NK cell subsets (Imm. – green; M1 - blue; M2 - red) amongst these cells shown. Data in (C) are mean ± SEM of 6 mice of each genotype.

Figure 4. Outer mitochondria membrane Mcl-1 protects from apoptosis

(A) LSK (linage$^-$Sca-1$^-$c-kit$^-$) cells were FACS sorted from the bone marrow of *Mcl1*$^{fl/fl}$*Ncr1-Cre* mice and transduced using retrovirus encoding Mcl-1, Mcl-1^{OM} (outer mitochondria membrane only) or empty vector (GFP). Total LSKs were analyzed for transduction efficiency (GFP) by FACS and then cultured for 21 days in 50ng/mL IL-15. Cultures where then analyzed for GFP and hCD4 expression amongst resulting NK cells. Data are representative of 2 experiments. (B) Splenic NK cells were sorted and grown in 40ng/mL IL-15 for 7 days and then starved in media alone for 5 h. NK cell were then stimulated with 40 ng/mL IL-15 for the indicated time. NK cells were then lysed and analyzed for tyrosine phosphorylated Jak1, STAT5 and total ERK1/2 (loading control) by Western blotting. Blots are representative of 5 independent experiments. (C) STAT5 ChIP. A diagram of STAT binding sites in the *Mcl1* locus is shown. Splenic NK cells were sorted and grown in 40 ng/mL IL-15 for 7 days and then starved in media alone or maintained in 40 ng/mL IL-15 for 5 h. STAT5 chromatin immuno-precipitation (ChIP) was preformed on formaldehyde fixed NK cell lysates. Data represents fold enrichment of *Mcl1* promoter or 3'UTR DNA sequences in IL-15 treated versus starved NK cell lysates. Enrichment of *Cish* is used as a positive control. Histograms are representative of two independent experiments. (D) Splenic NK cells from Vav-Mcl-1 transgenic and wild type (WT) mice were sorted and grown in 40 ng/mL IL-15 for 7 days. NK cell lysates were analyzed for Mcl-1 and β-actin (loading control) by Western blotting. (E)

Vav-Mcl-1 transgenic mice (Mcl-1 Tg) and WT NK cells were culture for 48 h in the indicated doses of IL-15. Data represents fold-difference in NK cell viability (PI^-) comparing Vav-Mcl-1 transgenic mice to wild type.

Figure 5. Absence of innate cytotoxicity in NK lymphopenic mice

$Mcl1^{+/+}$Ncr1-Cre (negative control) and *$Mcl1^{fl/fl}$Ncr1-Cre* mice were injected intravenously with 40,000 B16F10 melanoma cells. At day 12, mice were sacrificed and peripheral organs analyzed for B16F10 metastases. (A) The frequency of lung metastases were enumerated. Data are mean ± SEM of 5 mice of each genotype. Student t-test; ***$P < 0.0001$. (B) Representative whole mounts of lungs are shown. (C) Representative whole mounts of diseased bone marrow, liver, kidney and lymph nodes from *$Mcl1^{fl/fl}$Ncr1-Cre* mice are shown. (D) The frequency of mice with B16F10 metastases in additional organs was enumerated. (E) 1.5×10^7 allogeneic (H-2d) bone marrow cells were transplanted into lethally irradiated *$Mcl1^{fl/+}$Ncr1-Cre* and *$Mcl1^{fl/fl}$Ncr1-Cre* (H-2b) recipients and (E) splenic monocyte ($Gr\text{-}1^+$) reconstitution analyzed by FACS. (F) CFU-S were enumerated after 8 days. 1.5×10^7 autologous (H-2b) bone marrow cells (H-2b) were transplanted into lethally irradiated *$Mcl1^{fl/+}$Ncr1-Cre* mice as a control. Data in (E) are representative and in (F) are the mean ± SEM of 3 mice of each genotype. Student t-test; *$P < 0.03$.

Figure 6. NK lymphopenia protects mice from lethal sepsis

Cecal ligation and puncture (CLP) was performed on mice lacking NK cells (*$Mcl1^{fl/fl}$Ncr1-Cre)* or with reduced numbers of NK cells (anti-NK1.1 or anti-αasialoGM1 antibody treated). (A) NK cell frequencies were enumerated in the blood of the indicated mice on the day of CLP. Student t-tests using the Sidak-Bonferroni method; **$P < 0.01$; *$P < 0.03$. (B) Kaplan-Meier survival curve following CLP-induced septic shock. Mantel-Cox test; *$P < 0.04$. (C) Bacteria CFUs from peripheral blood were enumerated on agar plates 12 h after CLP induction. (D) Serum IFN-γ and (E) IL-6 levels were measured at 12 h after CLP. t-tests using the Sidak-Bonferroni method ; **$P < 0.009$; *$P < 0.05$. Data are mean ± SEM of 5-6 mice of each genotype/treatment group.