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ARTICLE

Quantifying NK cell growth and survival changes in response to cytokines and regulatory checkpoint blockade helps identify optimal culture and expansion conditions

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Abstract

NK cells are innate lymphocytes critical for immune surveillance, particularly in eradication of metastatic cancer cells and acute antiviral responses. In contrast to T cells, NK cell-mediated immunity is rapid, with spontaneous cytotoxicity and cytokine/chemokine production upon pathogen detection. The renaissance in cancer immunology has cast NK cell biology back into the spotlight with an urgent need for deeper understanding of the regulatory networks that govern NK cell antitumor activity. To this end, we have adapted and refined a series of quantitative cellular calculus methods, previously applied to T and B lymphocytes, to dissect the biologic outcomes of NK cells following stimulation with cytokines (IL-15, IL-12, IL-18) or deletion of genes that regulate NK cell proliferation (*Cish*), survival (*Bcl2l11*), and activation-induced-cell-death (AICD; Fas). Our methodology is well suited to delineate effects on division rate, intrinsic apoptosis, and AICD, permitting variables such as population half-life, rate of cell division, and their combined influence on population numbers in response to stimuli to be accurately measured and modelled. Changes in these variables that result from gene deletion, concentration of stimuli, time, and cell density give insight into the dynamics of NK cell responses and serve as a platform to dissect the mechanism of action of putative checkpoints in NK cell activation and novel NK cell immunotherapy agents.

KEYWORDS

NK cell, survival, proliferation, quantification, cytokines, checkpoints, culture, stimulation, homeostasis, etc.

1 | INTRODUCTION

Targeting NK cells to enhance cancer immunotherapy has attracted significant attention in recent years. Despite their discovery in the early 1970s as "killer cells with rapid cytolytic, specific activity" toward murine leukemia cells,[1] exploiting NK cells in human disease has remained elusive due to limited understanding of the mechanisms regulating their function. Growing evidence for the role of NK cells in cancer immune surveillance and clearance has included human data demonstrating that patients with high intratumoral NK cell frequency have significantly improved survival outcomes.[2] Thus, it is not surprising that NK cells have become a high priority in immunotherapy development.[3–5]

Clinical investigation of NK cells for the treatment of malignancies has followed similar rationale to the work horses of immunotherapy: $CD8^+$ T cells; hence, clinical applications have been comparable. Cytokine therapy[4,6,7] and adoptive cell therapy including generation of chimeric antigen receptor effector cells[8–10] are amongst the methods currently under clinical investigation for both NK and T cells. However, $CD8^+$ T cells have garnered considerably greater traction in the clinic due, in part, to our limited understanding of NK cell regulation and control in vitro and in vivo. We anticipate that a more thorough understanding of NK cell homeostasis and functional biology is still needed to fully harness the potential of NK cells as a therapeutic tool.

Fundamental to NK cell homeostasis and activation is IL-15, a member of the gamma common (γc) family of cytokines. IL-15 was discovered to support the proliferation of the IL-2-dependent cell line CTLL[11,12] and has since been found to share biologic activities with IL-2, including recognition of the IL-2$\beta\gamma$ receptor,[13] stimulation of

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peripheral T cell proliferation, and induction of antibody secretion in B cells.[14,15] Unlike IL-2, however, NK cells critically depend on IL-15 for their survival and development.[16,17] Mice deficient in IL-15 lack NK cells[18] and fail to maintain adoptively transferred mature NK cells[19]; whereas mice deficient in IL-2 have normal NK cell numbers.[20] IL-15 transcripts are found at high levels in a variety of cell types, however extensive post-transcriptional regulation restricts IL-15 protein expression to a limited number of hematopoietic (dendritic cells, monocytes, macrophages) and nonhematopoietic (epithelial cells, stromal cells, fibroblasts, keratinocytes) cell types.[21–24] In addition, in vivo bioactive IL-15 levels are further limited by the required coexpression of IL-15Rα in IL-15-secreting cells, which binds monomeric IL-15 and presents it in *trans* to cells expressing the remaining 2 components of the receptor: the IL-15R$\beta\gamma$ heterodimer.[25] Activation of the IL-15R$\beta\gamma$ induces autophosphorylation and activation of JAK1/3 and three distinct signaling pathways: JAK-STAT, MAPK, and PI3K/AKT-mTOR. These molecular pathways of IL-15 signaling have been extensively reviewed.[26,27]

NK cells and CD8 T cells initiate an "immune-enhancing" signaling cascade upon IL-15R ligation; this includes extensive crosstalk between signaling pathways and results in transcription of genes that directly and indirectly control development and survival, activation and effector molecules, and a number of proto-oncogenes. Importantly, biologic responses to IL-15 also demonstrate variance depending on the strength of stimulation; mTOR activity (and subsequent signaling for NK cell growth and metabolism) is induced at higher IL-15 concentrations than required for survival signals through JAK/STAT activation.[28]

Survival signals induced through IL-15 stimulation result from changes in pro-apoptotic and anti-apoptotic protein levels, either directly or indirectly through target gene expression by the three signaling cascades. *Mcl-1* is a STAT5 target gene whose expression is continuously required for NK cell survival.[16,17] IL-15 deprivation in NK cells results in a rapid loss of MCL1, triggering apoptosis. However, NK cells isolated from *Bcl-2* transgenic mice show rescued survival when adoptively transferred into IL-15-depleted mice,[19] suggesting that BCL-2 or related anti-apoptotic proteins also contribute to NK cell survival, but are not critical. Notably, our group have previously shown that IL-15-mediated survival signals also occur through the down-regulation of pro-apoptotic protein BIM (encoded by *Bcl2l11*), and this can rescue BCL-2-deficient NK cells.[16,29] IL-15 inhibits BIM activation through PI3K-dependent inactivation of transcription factor FOXO3a and promotes its degradation through activation of MAPK kinases ERK1 and ERK2.[16]

In light of such critical dependence of NK cells on one cytokine, the IL-15 signaling pathway seems a logical target when attempting to improve NK cell expansion for adoptive cell therapy. Enhancing the IL-15 signaling cascade upstream of its divergence into three pathways might serve to boost expression of a multitude of factors, culminating in improvements in NK cell longevity, proliferation potential, and antitumor function. Our group addressed this hypothesis by targeting *Cish*: a STAT5-target gene in NK and T cells and founding member of the suppressor of cytokine signaling protein family, known to bind the IL-15Rβ chain.[30,31] We identified *Cish* as a potent checkpoint in NK cell activation, which is induced by IL-15 stimulation in NK cells and dampens IL-15 responses through inhibition of JAK1 kinase activity.[32] Deletion of *Cish* preferentially enhanced JAK/STAT signaling in NK cells, demonstrating clearly enhanced mitosis and antitumor function in response to IL-15. These findings suggest that targeting cytokine responsiveness is a promising strategy for NK cell immunotherapy.

There is currently no consensus on how to culture NK cells for adoptive cell therapy, in contrast to the extensive experimentation performed to determine optimal culture conditions for the expansion of T cells and development of the *rapid expansion protocol*.[33–37] These T cells studies have been made possible by a comprehensive understanding of B and T cell expansion mechanisms and kinetics, provided in part by our group.[38–43] Here, we apply our methods for quantifying lymphocyte kinetics to NK cells and document the dynamic range of NK cell responses over a broad range of IL-15 concentrations in vitro. In establishing the boundaries of biologic responses induced by IL-15 in NK cells, we gain insight into how key regulatory proteins and environmental inputs impact NK cell fate by tuning individual biologic parameters in response to IL-15. Our results provide further guidance for NK cell expansion approaches that will assist in improving NK cell adoptive cell therapies.

2 | MATERIALS AND METHODS

2.1 | Mice

C57Bl/6 mice were bred and maintained in pathogen-free conditions in the Walter and Eliza Hall Institute of Medical Research (WEHI) animal facilities (Kew and Parkville, Victoria, Australia). Experimental protocols were conducted in accordance to WEHI animal ethics committee regulations. Mice deficient for expression of *Cish*[44] were generously provided by J. Ihle and E. Parganas (St. Jude Children's Research Hospital) and were maintained on a C57Bl/6 background. Mice from a C57Bl/6 background with an NK cell-specific deficiency in BIM (*Bcl2l11*$^{fl/fl}$ or "*Bim*$^{-/-}$") were generated by breeding mice with the *Bcl2l11* gene "flanked by LoxP sites" (termed floxed) with mice expressing the NK cell-specific *Ncr1*-Cre recombinase transgene. All control mice were phenotypically wild-type (WT), age-matched as well as gender-matched when possible. Both male and female mice were used, typically aged between 8 and 12 weeks.

2.2 | Purification and culture of NK cells

Murine NK cells were harvested from spleens and single-cell suspensions prepared by forcing of organs through 70 μm sieves. Partial enrichment by negative selection was first performed by staining cell suspensions with a cocktail of biotin-conjugated antibodies specific for Ly6G, F4/80, ter199, CD19, CD3, CD4, CD8 and MHC-II-A/E (Biolegend), and MagniSort™ SAV Negative Selection Beads (Thermo Fisher Scientific), for 10 and 5 min (respectively) at room temperature. Stained suspensions were placed on an EasyEights™ EasySep™ Magnet for 5 min and cells were harvested from supernatant.

Partially enriched NK cells were then sorted by positive selection by staining with a cocktail of fluorochrome-conjugated antibodies

specific for NK1.1, NKp46, CD49b, CD3, and CD45 (BD Bioservices), plus LiveDead-PI (Sigma-Aldrich) to exclude dead cells, for 30 min at 4°C. Sorting was performed on FACS Aria or Fusion (BD Bioservices).

NK cells (5000 per well) were seeded into 96-well round-bottom plates (Falcon; cat#353077) and cultured in 200 μl lymphocyte culture medium made of IMDM (Gibco) supplemented with 10% (vol/vol) FCS, HEPES, penicillin, streptomycin, L-glutamine, GlutaMAX, β-2-mercaptoethanol, and specific concentrations of recombinant human IL-15 (Miltenyi Biotech). Cells were incubated in a humidified environment at 37°C in 5% CO_2.

2.3 | CellTrace Violet labelling

Purified NK cells were resuspended in PBS 0.1% BSA at up to 2×10^7 cells/ml and incubated with 5 μM CellTrace violet (CTV) at 37°C for 20 min. CTV labelling was quenched with 5 mL ice cold IMDM 10% FCS.

2.4 | Flow cytometry

Flow cytometry was performed on a FACSVerse (BD Bioservices) using BD FACSuite software. Exported data were analyzed using FlowJo software version 10.4.2.

2.5 | Cell number determination

Invitrogen 123count eBeads™ Counting Beads (Thermo Fisher Scientific; cat#01-1234-42) (5000 per well) were added to cultures prior to flow cytometry analysis, along with 200 nM propidium iodide (Sigma-Aldrich) for dead cell exclusion. Total live cell counts were determined as a ratio of the counted live cells to beads.

Cell numbers in each generation beyond the original seeded precursors were determined by gating peaks of CTV histogram plots (Fig 1A).

2.6 | Total cohort number and mean division number determination

The principles of the precursor cohort method used to estimate and separate the division and survival rates of a stimulated population has been published previously.[38,39,42] In this method, the total "cohort" number relates to the number of founding cells initially present within a population at the start of an analysis, and is used to quantify cell dynamics thereafter. Typically, over time, cells simultaneously manipulate their rate of cell death and proliferation, leading to decreasing and increasing total cell numbers. To quantify changes in the rate of cell death, the confounding influence of proliferation on cell numbers is first removed. This is achieved using division tracking dyes and by dividing the number of cells within generation i by 2^i to provide the cohort number for generation i. Total cohort number at any harvest time is then determined by summing cohort numbers for all generations:

$$\text{Total cohort number} = \sum \frac{\text{cell number}_i}{2^i}$$

where i is generation number. Plotting total cohort number over time typically leads to a pattern of loss that is indicative of the survival of the population (Fig 1A).

Plotting the mean division number (MDN) of cell cohorts against time further enables estimates and comparisons of division rates. MDN is the average number of divisions the founding cohort has undergone. To determine this, each generation number i is multiplied by the fraction of the cohort that had undergone i divisions, and these values are summed for each generation:

$$\text{MDN} = \sum \left(i \times \frac{\text{cohort number}_i}{\text{total cohort number}} \right)$$

where i is generation number. Typically, MDN increases linearly over time until further culture limitations are reached (Fig 1A). Average interdivisional time can be estimated from the reciprocal of the slope of this line as the time required for the MDN of the population to increase by 1 (Fig 1A).

2.7 | Regression analysis

To enable comparisons of stimulation conditions on division and survival rates, regression fitting was used for selected data. *(1) Proliferation:* Plots of NK cell MDN against time were typically linear between 50 and 100 h of culture before plateauing due to culture growth media becoming limiting. For this reason, data in the linear range were selected in Prism 7.0 and fitted by linear regression. The average division rate was then derived from the gradient. *(2) Survival*: Plots of total cohort number against time were typically found to be approximately exponential between 24 and 100 h. Data points taken at zero hours were unreliable since rapid early death (presumably due to the cell preparation procedure rather than the physiologic response to stimuli[39]) within the first 24 h introduces errors when fitting lines to the data points (as discussed in results). Thus, data points from the selected time range were fitted to exponential growth equations (Prism 7.0), with Y0 constrained to the average of the value estimated from the survival inducing concentrations, 5 and 10 ng/ml IL-15. Half-lives were subsequently derived from the estimated exponential constant.

3 | RESULTS

3.1 | NK cell survival and proliferation quantified using division tracking data

We have previously performed extensive experimentation and modelling of T and B cell in vitro division and survival kinetics under different conditions to delineate the contributions of changing division rates, reversion to cell senescence and cell death to the overall responses. Methods developed include a graphical data interrogation protocol, the Precursor Cohort Method[39], and fitting of a fully probabilistic Cyton Model directly to cell division tracking data.[40] These analyses have proven effective in measuring cell survival and proliferation parameters and predicting cell number response patterns to a range of stimuli with surprising accuracy.[41,42] We therefore wished to evaluate the application of such methods to NK cells, to quantify their response kinetics to stimulation and allow direct comparisons to T and B cells. Figure 1 illustrates the general method applied to NK

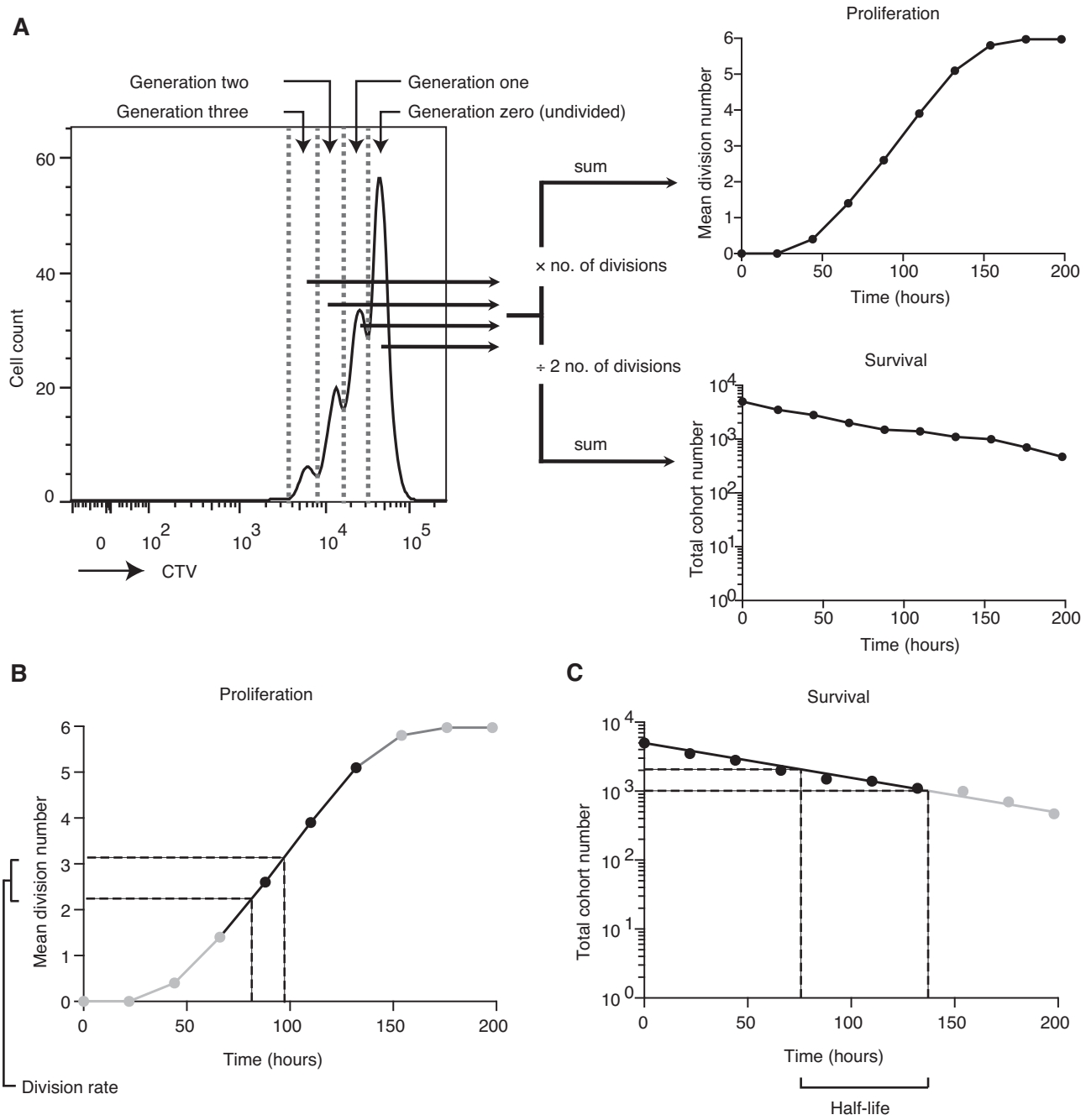

FIGURE 1 **Quantification of survival and proliferation using the cohort method.** (**A**) Average division numbers and total cohort numbers are calculated as shown. (**B**) Proliferation rate is quantified by the slope of the linear mean division number plot (usually between 50 and 150 h). A linear regression is fitted to the remaining data points to determine division rate. (**C**) Survival is quantified from an exponential decay curve fitted to the total cohort number plot (here shown as fitting a line to log values). Half-life is defined as the number of hours required for the total number of cells within the population to halve

cells. Cells labelled with CTV were followed over time, and the total cell number and number of cells in each successive generation was determined. For the latter information, CTV histograms were gated based on the position of peaks. For the graphical Precursor Cohort Method,[39,42] the number of founding cell descendants extant in each generation (i.e., the precursor cohorts) was determined by taking cells found in each respective gate and dividing by 2^i, where i represents the number of divisions that cells within that gate have undergone. These values were summed to show the change in population size due to effects other than cell division (i.e., cell death), presented as *total cohort number*. The number of cells within each gate was then multiplied by its respective i value and summed to show the MDN of that population (Fig 1A).

To simplify the presentation of survival and proliferation time course data, linear regressions were fitted to selected MDN data (Fig 1B) to determine cell division rates. To estimate and compare survival rates, exponential decay curves were found suitable for

fitting to *total cohort number* (Fig 1C) plots as described in *Materials and Methods*. Quantitative measures of survival and proliferation were then obtained from regression equations, namely the *division rate* and the *half-life* (i.e., time required for the cell number to halve).

3.2 | Regulation of NK cell survival is more sensitive to IL-15 than proliferation

In order to complete a conceptual picture of NK cell responsiveness to IL-15, we characterized the influence of a large dynamic range of IL-15 concentrations on total NK cell numbers over a 10-day period. NK cell population size was markedly altered by the intensity of IL-15 signaling, with concentrations below 5 ng/ml resulting in a net loss of cells, whereas concentrations above 5 ng/ml increasing NK cell numbers up to 50-fold above the seeding number (Fig 2A).

Using the cohort analysis method, we assessed the relative contribution of proliferation and cell death to these numbers over time (Figs. 2B and C). IL-15 levels above 10 ng/ml induced MDNs to exceed 1 during the 10-day period, indicating a majority of NK cells underwent division in these conditions (Fig 2B). Furthermore, increasing concentrations resulted in steeper slopes indicating faster average division rates. IL-15 concentrations below 5 ng/ml induced little proliferation, however as little as 0.5 ng/ml was sufficient to promote NK cell survival. Remarkably, 5 ng/ml provided long-term survival throughout the time course without invoking cell division (Fig 2C). Thus, careful manipulation of IL-15 concentration can differentially modify proliferation and survival.

Regression analysis was performed to quantify the delineated NK cell responses to IL-15 (Supplemental Figs. 1A and B). Half-life and division rate IL-15 dose–response curves were overlaid to gauge the contributions of both parameters to overall cell numbers across the IL-15 stimulation spectrum (Fig 2D). These data revealed that in IL-15 conditions equal or below 2 ng/ml, loss of cell numbers can be attributed to a lack of proliferation and a population half-life of less than 50 h. Maintenance of a steady population in 5 ng/ml is almost entirely due to an extended half-life of approximately 300 h, with little contribution of division; while 10 ng/ml demonstrated optimal cell survival and an induction of proliferation. IL-15 concentrations above 10 ng/ml increased NK cell division rate in a dose-dependent manner, however this was accompanied by a considerable dose-dependent reduction in half-life, which correlated closely with the rise in division rate (Fig 2D).

Given that virtually all bioactive IL-15 in vivo is complexed to IL-15Rα and is rarely in soluble monomeric form, the use of monomeric IL-15 to investigate NK cell responsiveness in vitro is unlikely to accurately recapitulate in vivo events. We hence sought to determine the relative potencies of monomeric and complex IL-15 on total NK cell number responses. To this end, we cultured NK cells in 3 concentrations of complex IL-15 and our standard 50 ng/ml monomeric IL-15 and compared total NK cell numbers over a 10-day period. While 0.5 and 1 ng/ml complex IL-15 induced little change in cell numbers, 5 ng/ml stimulated substantial NK cell growth that was comparable to 50 ng/ml monomeric IL-15 (Fig 2E), suggesting that complex IL-15 is close to 10 times more potent than monomeric IL-15.

3.3 | Deletion of *Cish* alters NK cell responsiveness to IL-15

Having shown that we can separate and analyze the effects of IL-15 on cell proliferation and survival, we were interested in examining how known modifications to the IL-15 signaling pathway enhance or diminish these parameters. We have previously identified *Cish* as a critical checkpoint in IL-15 signaling in NK cells, with *Cish*-null NK cells displaying a hyper-proliferative and hyper-functional phenotype.[32,45] To further delineate the contribution of *Cish* in NK cell survival and proliferation, $Cish^{-/-}$ and WT NK cells were cultured in IL-15 concentrations ranging from 0.5 to 100 ng/ml and were routinely analyzed for total cell numbers and CTV fluorescence, to determine half-lives and division rates. In IL-15 concentrations below 5 ng/ml, $Cish^{-/-}$ NK cells displayed enhanced survival compared with WT cells in a dose-dependent manner, with 5 ng/ml demonstrating the greatest survival difference (Figs. 3A and B). IL-15 concentrations above 5 ng/ml demonstrated the reverse effect, with survival consistently reduced in $Cish^{-/-}$ NK cells compared with WT. Deletion of *Cish* induced consistently greater proliferation of NK cells compared with WT in IL-15 conditions above 5 ng/ml, while 5 ng/ml induced proliferation of $Cish^{-/-}$ NK cells but not WT NK cells (Fig 3C). Notably, enhanced $Cish^{-/-}$ NK cell death balanced their enhanced proliferation in high IL-15, largely preventing cell accumulation (Fig 3A). Overall, the net differences in NK cell responsiveness following deletion of *Cish* can be attributed to hypersensitivity to IL-15 for both survival and proliferation responses.

3.4 | Deletion of *Bcl2l11* promotes survival in low IL-15

We next wanted to examine survival alone to understand how its manipulation influenced total cell numbers. We utilized $Bcl2l11^{fl/fl}Ncr1^{Cre}$ (herein called "$Bim^{-/-}$") mice where NK cells specifically lack the key inducer of intrinsic apoptosis BIM. $Bim^{-/-}$ NK cells were compared with WT NK cells for their total cell numbers, half-lives, and division rates across a 5-point IL-15 titration. As predicted, $Bim^{-/-}$ NK cells demonstrated enhanced survival to WT NK cells in limited IL-15 conditions (Figs. 4A and B). Similarly to $Cish^{-/-}$ NK cells, $Bim^{-/-}$ NK cells demonstrated optimum survival at 5 ng/ml IL-15, with a half-life 4 times that of WT NK cells. Stimulation at 50 ng/ml abolished this survival benefit in $Bim^{-/-}$ NK cells. To our surprise, loss of BIM also reduced the proliferation rate of NK cells in 10 and 50 ng/ml IL-15 compared with WT (Fig 4C).

3.5 | Treatment of NK cells with stimulatory cytokines creates distinct survival and proliferation profiles

Having investigated the manipulation of intrinsic regulators of NK cell survival and proliferation, we next wanted to examine further extrinsic factors, in addition to IL-15, that control NK cell homeostasis. Additional cytokines known to stimulate NK cells include IL-12 and IL-18 which, along with low-doses of IL-15, induce persistence, sustained

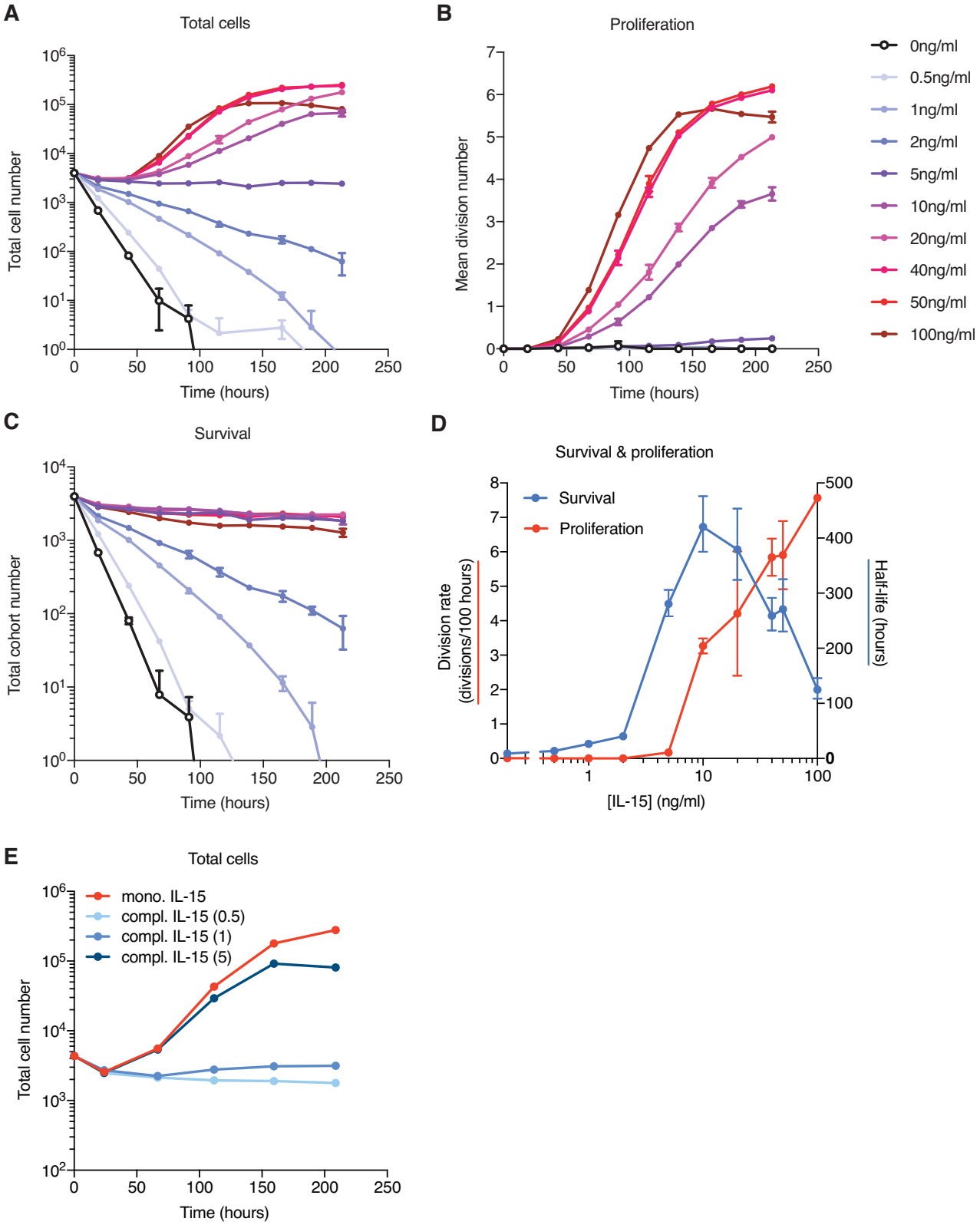

FIGURE 2 **NK cell survival and proliferation kinetics vary with IL-15 stimulation strength.** NK cells were cultured in IL-15 and analyzed over a 10-day period for total cell numbers (**A**), and CTV fluorescence to calculate mean division numbers (**B**) and total cohort numbers (**C**) over time. (**D**) Quantification of division rates and half-lives from mean division numbers and total cohort numbers, respectively, to allow survival and proliferation to be directly compared across IL-15 concentrations. (**E**) NK cells were cultured in monomeric IL-15 (mono. IL-15; 50 ng/ml), or IL-15 bound to the IL-15Rα chain (compl. IL-15; 0.5, 1, 5 ng/ml), and analyzed over a 10-day period for cell numbers. Data in (**A–D**) representative of 2 independent experiments. Data in (**E**) representative of 1 experiment. Error bars in (**A–C**) and (**E**) represent mean ± SD. Error bars in (**D**) represent 95% confidence intervals obtained in Prism 7.0 based on the likelihood that regressions were perfectly fitted

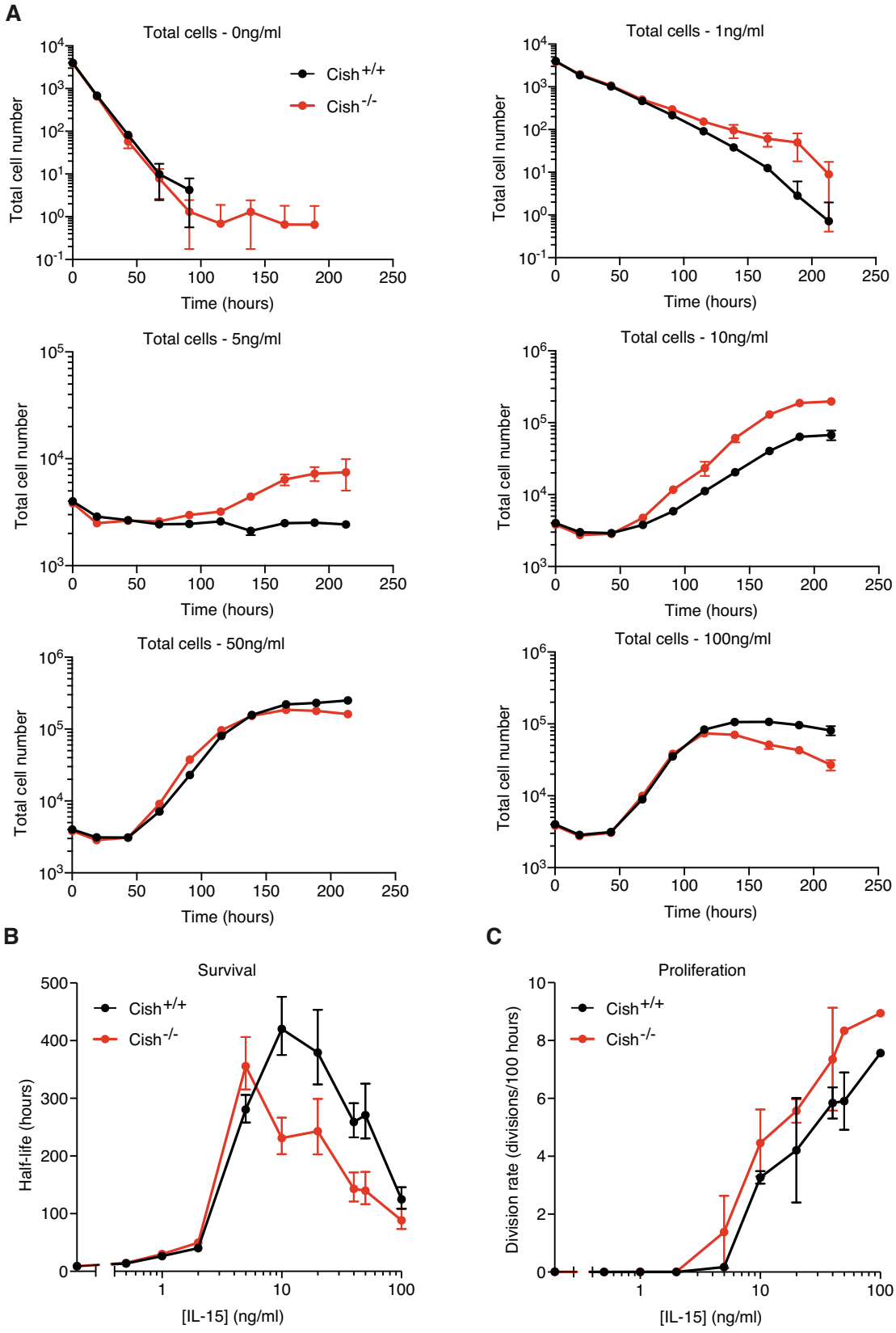

FIGURE 3 **Deletion of *Cish* alters NK cell survival and proliferation in IL-15.** *Cish*$^{-/-}$ and wild-type NK cells were cultured in IL-15 and analyzed over a 10-day period. (**A**) Total cell numbers were determined during analysis and are shown for 0, 1, 5, 10, 50, and 100 ng/ml IL-15. Half-lives (**B**) and division rates (**C**) were obtained using regression analysis and are shown for all IL-15 concentrations. Data representative of 2 independent experiments. Error bars in (**A**) represent mean ± SD. Error bars in (**C**) and (**D**) represent 95% confidence intervals obtained in Prism 7.0 based on the likelihood that regressions were perfectly fitted

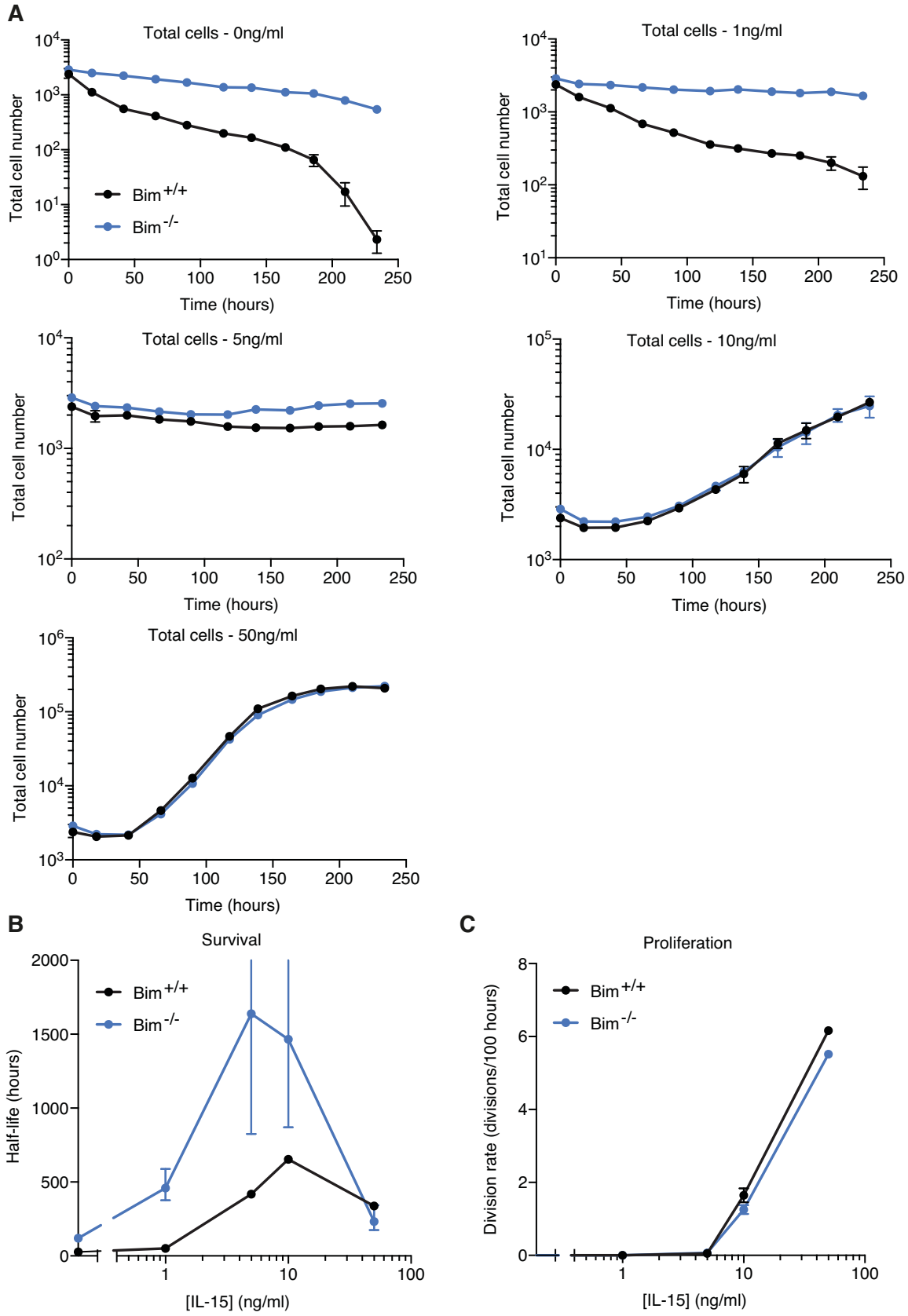

FIGURE 4 **Deletion of *Bcl2l11* enhances NK cell survival in low IL-15.** *Bim*$^{-/-}$ and wild-type NK cells were cultured in IL-15 and analyzed over a 10-day period for total cell numbers (**A**), half-lives (**B**), and division rates (**C**). Data representative of 3 independent experiments. Error bars in (**A**) represent mean ± SD. Error bars in (**C**) and (**D**) represent 95% confidence intervals obtained in Prism 7.0 based on the likelihood that regressions were perfectly fitted

effector function, and enhanced antitumor activity in vivo.[46–48] For these reasons, these cytokines are attracting considerable attention for clinical therapy. To probe the effects of stimulatory cytokines IL-12 and IL-18 on IL-15-mediated survival and proliferation, combinations of these 3 cytokines were added to NK cell cultures and total cell numbers and CTV fluorescence were routinely analyzed. Given that IL-15-deprived NK cells have a half-life of approximately 8.5 h, measuring proliferation in IL-12 or IL-18 alone becomes challenging after a few days due to lack of cells. IL-12, IL-15, and IL-18 combinations were hence also tested on *Bim*$^{-/-}$ NK cells, whereby cytokine deprivation-induced apoptosis is blocked.

NK cells treated with IL-18 alone (yellow) underwent mild proliferation but failed to surpass a MDN of 1, indicating IL-18 was unable to stimulate a majority of NK cells to divide (Figs. 5A and B). IL-18 also enhanced cell death during the first 24 h compared with unstimulated cells (Fig 5C; black). This effect was absent in *Bim*$^{-/-}$ NK cells, suggesting IL-18-induced death is BIM-dependent (Fig 5F). In contrast, NK cells treated with IL-12 alone (blue) showed little difference in survival or proliferation compared with unstimulated cells, beyond a slight improvement in late survival (Figs. 5B and C). However, cell numbers at these late stages are low and hence interpretations of these data should be approached with caution.

NK cells treated with the combination of IL-15/18 (orange) underwent significantly faster proliferation compared with treatment with IL-15 alone (red), suggesting synergy between IL-15 and IL-18 for this parameter (Fig 5B). This synergy was surprisingly countered by a reduction in cell survival compared with IL-15 alone (Fig 5C). In contrast, adding IL-12 to IL-15 (purple) reduced late proliferation compared with IL-15 alone (Fig 5B), suggesting an inhibitory property of IL-12. A mild reduction in survival was also observed with addition of IL-12 to IL-15 (Fig 5C). The addition of IL-12 to the combination of IL-15/18 (green) also mildly dampened the dual effect of IL-15 and IL-18 (Fig 5B). The combined effects of these cytokines also reduced IL-15-mediated cell survival to a level equivalent to NK cells deprived of cytokine (Fig 5C). *Bim*$^{-/-}$ NK cells showed these same survival trends in IL-15/18 and IL-12/15/18, suggesting IL-12/18-enhanced cell death is occurring independently of BIM and the intrinsic apoptosis pathway (Figs. 5D and F).

3.6 | IL-12/18-enhanced NK cell death occurs by homotypic NK cell interactions via Fas–FasL

To determine the cause of NK cell death following stimulation by IL-12 and IL-18, we turned to potential mechanisms of apoptosis alternate to intrinsic BIM-dependent apoptosis. Ligation of the TNF family of death receptors (TNFR) is known to activate downstream caspases and induce apoptosis via the extrinsic apoptosis pathway. Fas, a member of the TNFR family, induces this apoptotic pathway in activated T cells and was hence our candidate. Fas and its ligand, Fas ligand (FasL), are both expressed on NK cells and hence have the potential of inducing apoptotic signaling. To test whether IL-12/18-induced NK cell death was mediated by Fas–FasL interaction, we utilized C57Bl/6By*Jgld* (B6/*gld*) mice, which lack functional FasL. B6/*gld* and WT NK cells were isolated and cultured in a titration of IL-15, plus combinations of IL-12, IL-15, and IL-18, and were analyzed for total cohort numbers. While WT NK cells demonstrated enhanced cell death in IL-15/18 and IL-12/15/18 compared with IL-15 alone (Fig 6A), this effect was completely abolished in IL-15/18-treated B6/*gld* NK cells and partially removed in those treated with IL-12/15/18 (Fig 6B). To rule out the possibility that this survival enhancement was the result of a compensatory FasL-mediated reduction in proliferation rate, MDN of these cultures were analyzed. B6/*gld* and WT NK cells demonstrated similar rates of proliferation for all conditions (Figs. 6C and D), hence the survival enhancement is unlikely to be attributed to a proliferation defect.

4 | DISCUSSION

B and T lymphocyte immune responses are initiated following antigen detection by a small subset of antigen-specific cell clones, inducing rapid exponential cell expansion that is potentiated by cytokine signals. The strength of antigen and cytokine stimulation determines the number of divisions the parental cell pool undergo before returning to a nondividing state—resulting in a cell number plateau and subsequent decline.[40–43] These insights, particularly when applied to CD8$^+$ T cell responses, paved the way for our analysis of NK cells, their closest innate cell relative; however, there are essential differences between CD8$^+$ T cells and NK cells that must be understood to potentiate NK cell-based therapies.

Unlike T and B cells, NK cells do not undergo clonal expansion in response to antigen recognition. NK cells will undergo continuous proliferation provided sufficient IL-15 is present and can be grown in vitro in IL-15 for several weeks, and this is exploited in generating adoptive cell therapy products. Overall cell health or *fitness* must be considered as well as killing capacity when generating ideal NK cells for adoptive cell therapy in order to maximize in vivo persistence and tumor killing efficiency following culture. The absolute dependence of NK cells on IL-15R signaling and the ease with which they are grown in vitro have made these cells ideal for investigating pathways involved in cytokine-induced fitness, namely their capacity to survive and divide for extended periods. The Precursor Cohort Method[39] has proven useful for measuring these 2 parameters with high precision during the stimulation process. Applying this method, we were able to determine that NK cell numbers can be modulated by tempered changes in average division rates and in survival times, and both kinetic features can be altered by stimulation strength, intrinsic elements, and combining extrinsic signals.

To enumerate and quantify differences, we utilized regression fitting to generate half-life and division rate plots. We chose exponential growth equations to fit total cohort number plots as these provided the best estimates of data points, and constrained Y0 to the average for 5 and 10 ng/ml IL-15 to maximize fitting accuracy. Since NK cells experience little death in 5 and 10 ng/ml IL-15, their Y0 values represent the approximate starting cohort number once inevitable early cell death resulting from the cell preparation (see Ref.39) was

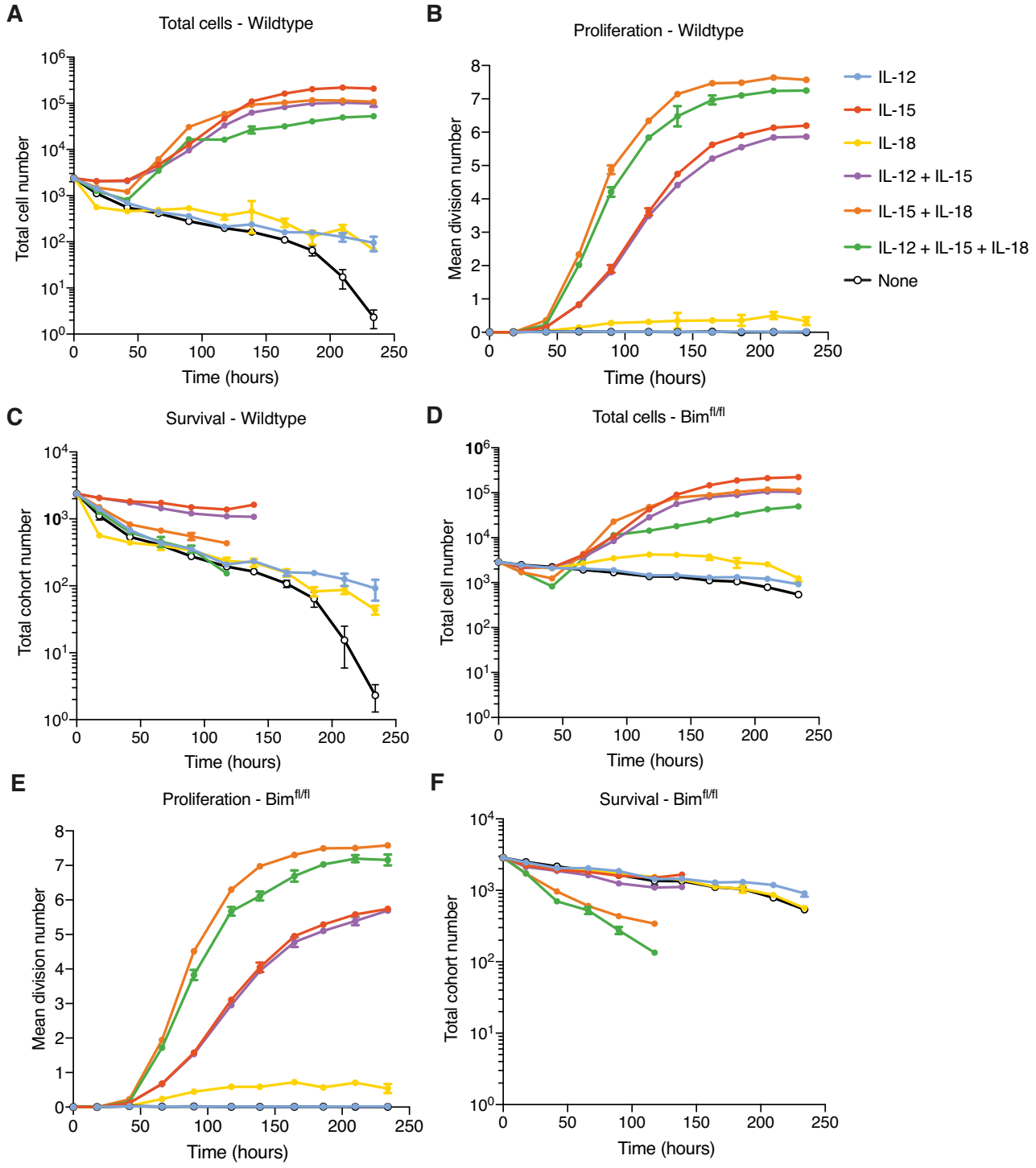

FIGURE 5 **Stimulation of NK cells with IL-12 and IL-18 alters IL-15-mediated survival and proliferation.** Wild-type (**A–C**) and *Bim*$^{-/-}$ (**D–F**) NK cells were cultured in combinations of IL-12 (100 pg/ml), IL-15 (50 ng/ml), and IL-18 (50 ng/ml) and analyzed over a 10-day period. Panels (**A**) and (**C**) show total cell numbers, (**B**) and (**E**) show mean division numbers, and (**C**) and (**F**) show total cohort numbers. Data points in (**C**) and (**F**) were excluded once CTV diluted to autofluorescence levels or culture nutrients were depleted, as observed by plateaus in mean division numbers in (**B**) and (**E**), respectively. Data representative of 3 independent experiments. Error bars represent mean ± SD

accounted for. The average of these 2 Y0 values provided the best approximation of data points, with the exception of *Bim*$^{-/-}$ NK cells (discussed below). Generation of half-life and division rate plots visually demonstrated the effect of a range of nanomolar concentrations of IL-15 on proliferation and survival in NK cells, in both homeostatic and genetically modified settings.

While our assays here suggest that NK cell turnover requires at least nanomolar IL-15 concentrations, estimations suggest IL-15 levels

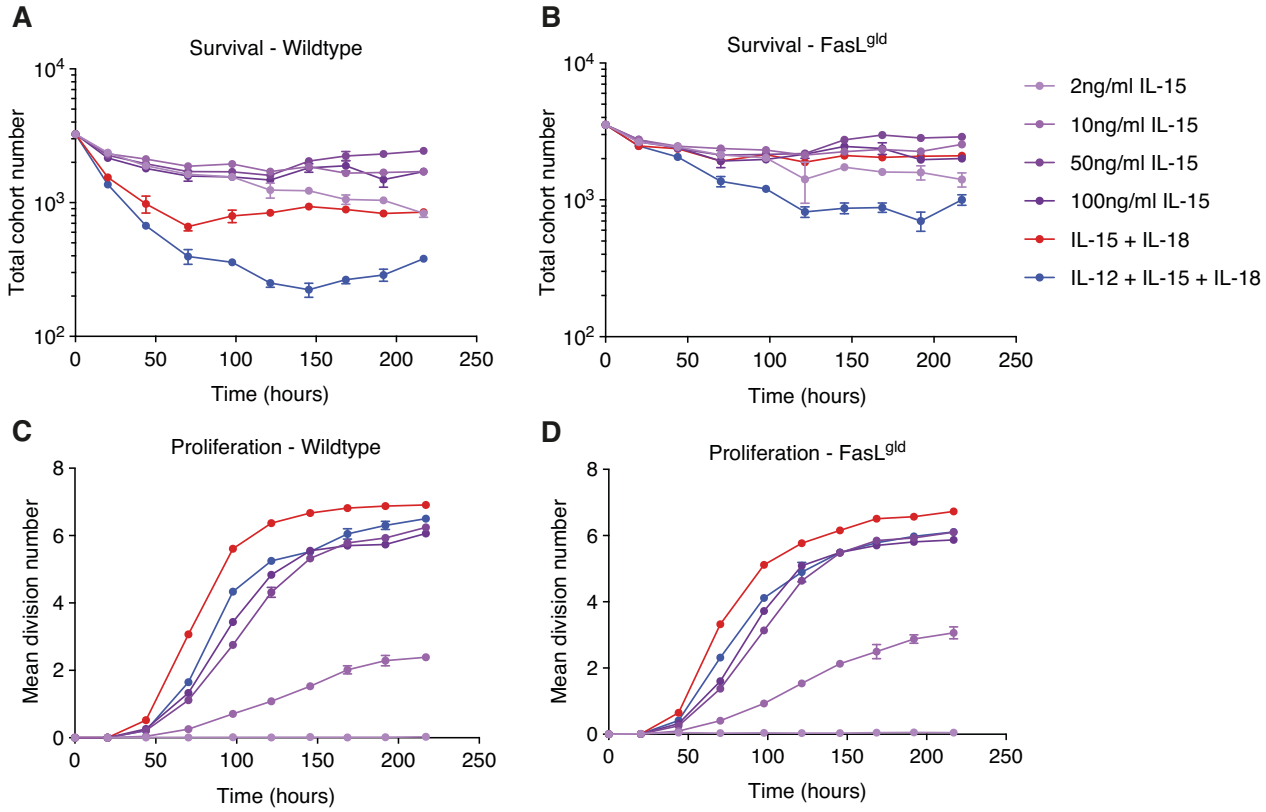

FIGURE 6 **Loss of functional FasL enhances NK cell survival under excessive stimulation.** Wild-type (**A** and **C**) and B6/*gld* (**B** and **D**) NK cells were cultured in IL-15 or combinations of IL-12 (100 pg/ml), IL-15 (50 ng/ml), and IL-18 (50 ng/ml) and analyzed over a 10-day period. Panels (**A**) and (**B**) show total cohort numbers, (**C**) and (**D**) show mean division numbers. Data representative of 2 independent experiments. Error bars represent mean ± SD

in vivo are in the order of picomolars.[25,49] This disparity between in vitro and in vivo settings may be explained by the *trans*presented nature of IL-15 with the heterotrimeric IL-15R, which includes IL-15Rα to significantly increase IL-15 affinity for its receptor. Lack of IL-15Rα expression on NK cells and hence inability to facilitate high affinity IL-15 *trans*presentation likely limits the IL-15 sensitivity of NK cell-rich cultures in vitro.[49,50] This is evidenced by complex IL-15 being approximately 10 times more potent than monomeric IL-15. In addition, it is plausible that NK cells may only be exposed to very low IL-15 levels in vivo as their nature is not to undergo the clonal expansion response seen in T cells. While significant proliferation was not observed in IL-15 concentrations below 10 ng/ml, it is possible that these cells are still undergoing division, simply at a rate undetectable by the Precursor Cohort Method within a 240-h period. Low IL-15 levels may be sufficient to maintain slow but sustained, healthy NK cell turnover and prevent superfluous NK cell outgrowth in vivo. Or perhaps only during heightened pathogenic detection by antigen-presenting cells may IL-15 levels peak and locally boost NK cell numbers as required to combat infection.

We were surprised to find that deletion of BIM in NK cells slowed their proliferation in high IL-15, given a direct role for BIM in mitosis is not evident. This result, however, is further suggestive of a regulatory mechanism linking apoptosis and mitosis. While enhanced proliferation appears to be accompanied by an increase in cell death, the converse may also be true: under a state of enhanced survival, proliferation may slow in order to regulate cell numbers. This idea is consistent with similar *Bim*$^{-/-}$ and WT NK cell numbers observed in 10 and 50 ng/ml IL-15 throughout the time course. Such a phenomenon would suggest a mechanistic link between pathways involved in survival and proliferation, or more specifically, that apoptosis- and mitosis-inducing proteins in NK cells have regulatory actions towards each other. Given the documented role of BIM in cytokine deprivation-induced apoptosis,[16] it was of little surprise that loss of BIM prevented largely all apoptosis in low IL-15 within the 10-day period. Nevertheless, this served as a valuable proof-of-principle, demonstrating that changes in survival and proliferation induced by genetic manipulation can be detected with ease using the Precursor Cohort Method. The significant alteration to survival patterns induced by deletion of BIM, particularly in low and absent IL-15, led to improper regression fitting when Y0 was constrained (Supplemental Fig. 2C). Hence, these constraints were removed in *Bim*$^{-/-}$ NK cell analysis to accurately fit these data (Supplemental Fig. 2E). In the clinic, in vivo NK cell persistence is a major hurdle for adoptive cell therapy and research is continuously exploring new ways to increase the longevity of NK cells after transfer. In light of the survival enhancement provided by removal of BIM, we could postulate that deletion of *Bcl2l11* in NK cells during in vitro cultivation could enhance in vivo persistence and overall clinical efficacy.

IL-18 has been previously documented as a synergistic cytokine, enhancing function of NK cell stimulatory cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21 through activation and metabolism pathways.[51–54] Indeed, our results confirmed that combining IL-15 and IL-18 induced extremely rapid NK cell proliferation, far exceeding the rate of IL-15 alone. The little proliferation or survival induced by IL-18 alone further suggests a more prominent role of IL-18 as a synergistic cytokine. Of note, however, IL-18 alone induced greater cell death within the first 24 h compared with cultures absent of cytokine. This is concordant with an increase in cell metabolism and early induction of death due to lack of survival signals to maintain high metabolism. Similarly, IL-12 alone had little effect on proliferation or survival. Despite substantial proliferation of IL-12/15/18-treated NK cells, NK cells displayed better proliferation *and* survival in IL-15/18 than did when stimulated by all three cytokines. In light of this, it may be worth considering the use of IL-15 and IL-18—without IL-12—as a more efficient method of NK cell cultivation, given the greater proliferation and reduced cell death. Nevertheless, it is important to recognize that IL-12 has an essential role of boosting NK cell functionality, particularly in terms of IFN-γ secretion.[55,56] Despite the loss of cell fitness and overall cell numbers induced by combined IL-12/15/18, IL-12 may be essential for the generation of fully functional NK cells for immunotherapy. Finding the balance between NK cell fitness and functionality is likely to be an ongoing issue, and it is highly likely that the most effective NK cell culture conditions will require sequential changes in cytokine compositions and concentrations during the cultivation process.

Given their highly cytotoxic and inflammatory capabilities, tight regulatory mechanisms in NK cells are essential to ensure homeostasis is maintained. Remarkably, among the plethora of genetic mouse models affecting genes associated with NK cell homeostasis, very few result in an accumulation of NK cells.[16,29,32,57,58] Possible explanations could be that expansion of other cell types limits IL-15 availability, that in vivo NK cell production slows to compensate for longer lifespans or faster proliferation, or that cell death increases to balance enhanced division. The inverse correlation between NK cell half-life and division rate observed in high IL-15 supports the latter. Upon antigenic stimulation, T cells undergo successive rounds of clonal expansion to rapidly increase their numbers, followed by a decline in numbers by programmed cell death. This secondary contraction phase is known as peripheral deletion and occurs due to the engagement of apoptosis pathways during signaling for clonal expansion. Such a phenomenon has been termed "activation-induced cell death" or AICD, and is mediated by the interaction of Fas on activated T cells with its ligand, FasL. When stimulated by antigen, T cells up-regulate FasL and increase their sensitivity to Fas-mediated apoptosis. T cell numbers decline as Fas–FasL interactions between T cells trigger their cell death. However, it has been demonstrated in T cells and other cell types that FasL expression can be induced without antigen–receptor ligation; DNA-damaging agents and other stressors also up-regulate FasL and can induce Fas–FasL-mediated apoptosis.[59,60] Under excessive stimulation, rapid mitosis and metabolic processing can impose stress on the cell's internal machinery, increasing the likelihood of error-prone DNA replication and promoting damage control pathways and stress-induced ligand expression.[61–63] We postulate that excessive stimulation by IL-12, IL-15, and IL-18 induces—either directly or through DNA-damage pathways—the expression of Fas and/or FasL in NK cells and promotes their AICD via NK cell fratricide, independently of BIM. Enhanced death observed in hyper-proliferative *Cish*$^{-/-}$ NK cells supports the idea that IL-15 alone can also induce AICD.

The insights on NK cell stimulation presented here offer valuable ideas for the development of a consensus on NK cell culture conditions, both for research purposes and in the clinic. In the revolutionary era of immunotherapy, understanding the most efficient methods to cultivate NK cells, in terms of both fitness and functionality, is paramount for the development of effective NK cell adoptive therapies or other NK cell-enhancing immunotherapies. It is essential to have a thorough understanding of basic NK cell biology in order to translate this knowledge to therapeutic avenues, and this could include exploitation of this knowledge to screen novel targets for drug discovery. Looking forward, improvements to current NK cell immunotherapies will require a deeper understanding of how homeostatic parameters, such as survival and proliferation, impact on NK cell functionality. NK cells clearly demonstrate highly regulatory features for their homeostasis and this likely also translates to their functional roles, in terms of both viral responses and tumor immunity. Developing a comprehensive model of how these various parameters controlling NK cell responses are regulated in vivo will revolutionize our approaches to NK cell-based immunotherapy.

DISCLOSURES

The authors declare no conflicts of interest.

AUTHORSHIP

N.D.H. and J.R are cofounders and shareholder in oNKo-Innate. N.D.H. has funded research agreements with Servier, Anaxis, and Paranta Bioscience. Therefore, all authors declare that this was written in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. P.D.H. and N.D.H. contributed equally to this work.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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