Supplementary Materials and Methods

Generation of IL15 and IL15Rα plasmids

The IL15 cDNA sequence containing the IL-2 signal peptide was kindly provided by M. Caligiuri (1). Primers were used to add a BsaI site to the 5' end (5'GGGGTCTCGC **ATG** TAC AGC ATG CAG CTC G 3') and an XhoI site to the 3' end (5' GGC TCG AGC TAT TTG TCA TCG TCG TCC TTG 3') by PCR. The PCR product was then cloned upstream of an IRES ECFP sequence in the MFG vector to make the pMFG-IL15HS-IRES-ECFP plasmid. IL15Rα was amplified from pcDNA3.1-IL15Rα (generated from cDNA); the following primers were used to add a BsaI and a NotI restriction site: 5'-GGG GTC TCT CAT GGC CTC GCC GCA G-3' and 5'- GTG CGG CCG CTT AGG CTC CTG TGT C-3'. The PCR product was then cloned into the pMFG-GFP vector, which was cut with NcoI and NotI to obtain the plasmid pMFG-IL15Rα.

Transduction of cells lines

To transduce 8215, 9604, AG104A, and MC57 cell lines, we used supernatants of phoenix-ampho cells transfected with pMFG-IL15HS-IRES-ECFP, pMFG-IL15Rα (for plasmids, see Supplementary Materials and Methods) or the empty vector using the CalPhos Mammalian Transfection Kit (Clontech, Mountain View, CA). To enrich for IL-15 producers, ECFP positive cells were sorted. IL15Rα was stained with anti-IL15Rα and sorted.

In vivo antibody treatments

For IL15 neutralization experiments, one week prior to tumor challenge mice were injected with 45μg anti-IL15 antibody (clone M96) intraperitoneally and 95μg weekly thereafter. Each experiment varied slightly depending on tumor sizes. For NK depletions, mice were treated intraperitoneally every 3-4 days with 100μg anti-NK1.1 (clone PK136, BioXCell, West Lebanon, NH). NK cell depletion was confirmed by flow cytometry of peripheral blood samples with anti-NK1.1 and anti-CD122.

Bone marrow transfers

Bone marrow was harvested from the tibias and femurs of donor *Rag1*$^{-/-}$ mice and single cell suspensions were generated and passed through a 70μm nylon mesh. Red blood cells were lysed with TrisNH_4Cl and cells were washed and resuspended in PBS. Approximately 3x10^7 cells were injected into each recipient mouse. Cells were injected intravenously into the retro-ortibal plexus in 100μL into tumor-bearing *Rag2*$^{-/-}\gamma c^{-/-}$ mice. Animals that died before a conclusive result was established were excluded from the experiment.

Flow cytometry

Single cell suspensions from mouse tissue or trypsinized MC57 cells were Fc-blocked with anti-CD16/anti-CD32 antibody at 1μg/mL in FACS buffer (PBS, 2% BSA and azide) for 10-15min at 4°C. Conjugated antibodies were then used to stain the cells at 1μg/mL in FACS buffer for 20-30min at 4°C. Cells were then

washed with FACS buffer. Peripheral blood cells were lysed with TrisNH_4Cl, washed and resuspended in FACS buffer. FACS data were collected on a FACSCalibur or LSRII, and sorting was performed on a FACS Aria (all BD Biosciences) and analyzed using FlowJo software (Treestar Inc., Ashland, OR). Anti-NK1.1 APC and PE (PK136), anti-CD122 FITC (TM-b1), anti-CD49b PE (DX5), anti-NKp46 FITC (29A1.4), anti-CD137 PE (4-1BB 2A), anti-NKG2D PE (CX5), anti-CD11b APC (M1/70), and anti-IL10 (JES5-16E3) antibodies were purchased from eBioscience (San Diego, CA) and anti-IL12(p70) (C15.6) from BD Pharmingen (San Jose, CA). Anti-TRAIL PE (N2B2) and anti-CD27 PE (LG.3A10) were purchased from BioLegend (San Diego, CA). Anti-CD16/CD32 and the biotinylated 2B4 antibodies were purchased from BD Pharmingen. The biotinylated polyclonal anti-IL15Rα antibody was purchased from R&D Systems (Minneapolis, MN).

Histology and Immunohistochemistry

Tissue was frozen in OCT using isopentane in dry ice and stored at -80°C. For immunohistochemistry, 4 μm sections were freshly cut, dried for one hour at room temperature, and mounted and fixed in acetone for 8 minutes. Prior to staining, endogenous peroxidase was blocked using Dako dual endogenous enzyme block (Dako, Carpinteria, CA) for 10 minutes followed by blocking of unspecific Fc binding with anti-CD16 and anti-CD32 antibodies for ten minutes. The FITC conjugated primary antibodies, anti-NK1.1 and anti-granzyme B were used at 1 μg/mL and 2.5 μg/mL, respectively, for 32 minutes. Isotype controls

were run in parallel. The secondary antibody, anti-FITC (Pierce) was used at 1:200 for 30 minutes, as recommended by the manufacturers. Immunostaining was performed on the Ventana BenchMark instrument (Ventana Medical Systems, Inc, Tucson, AZ). Horseradish peroxidase was developed using DAB (Dako) for 10 minutes. Slides were counterstained with hematoxylin, dehydrated in alcohols and mounted in mounting medium (Sakura Finetek, USA, Torrance, CA).

Ex vivo production of cytokines by cancer and stromal cells

For the analysis of cancer and stromal cells *ex vivo*, tumors were surgically excised and placed in PBS, minced into 1mm^3 pieces, and incubated for 30min in 2mg/mL collagenase D and 100U/mL DNase I (both Roche Diagnostics, Mannheim, Germany) in an atmosphere of 5% CO_2 at 37ºC, shaking/stirring the samples every 10min. Tumor fragments were pipetted up and down for 2min after having added pre-warmed trypsin (0.025% final concentration, Gibco). Suspensions were spun down, washed, and filtered through a 70µm nylon mesh, resulting in a single-cell suspension, which was used for flow cytometry analysis or sorts; tumor cells were separated into stromal cells ($CD45^+CD11b^+$) and cancer cells ($CD45^-ECFP^+$). Sorted tumor cells were intracellularly stained for IL-12(p70) and IL10 immediately after the sort using the Cytofix/Cytoperm kit (BD Biosciences). $1x10^5$ sorted cancer and $2x10^5$ stromal cells were cultured in 96-well plates in serum-free advanced MEM (Gibco, Life Technologies, Grand Island, NY). 24h supernatants were tested for IL10,

IL12(p70) (both OptEIA, BD Pharmingen, San Jose, CA), IL18 (Platinum ELISA, eBioscience) and TGF-β1 (Emax ImmunoSystem Assay, Promega).

Splenocyte cultures

Splenocytes were seeded at $1x10^6$ cells/mL in complete RPMI medium (10% FCS, Pen/Strep) and treated with human recombinant IL15 at 10ng/mL (eBioscience, San Diego, CA).

Supplementary References

1. Fehniger TA, Suzuki K, Ponnappan A, VanDeusen JB, Cooper MA, Florea SM, et al. Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8+ T cells. J Exp Med. 2001;193:219-31.

Supplementary Figure Legends

Figure S1. IL15 expression and secretion by MC57 cancer cells

A) IL15 expression was detected by ECFP fluorescence in M-IL15 cells; MC57 cells were analyzed as negative control. Rather than displaying cell counts, histograms have been normalized to the maximum cell count (Normalized maximum). **B)** Amount of IL15 in 48-hour supernatants from 2.0 x 10^5 cells was analyzed by ELISA.

Figure S2. Induction of densely granulated NK cells requires IL15 and the tumor microenvironment.

A) PAS-stained cytospin of a 72 h culture *in vitro* of wild type and IL15-transgenic splenocytes activated with IL15. Lymphocytes are activated as seen by frequent mitosis in the cultures but they do not show PAS-positive granules. Scale bars are 50 μm. Insets show flow cytometry analyses of the cultures stained with anti-NK1.1 and anti-CD3. Numbers indicate the percentage of cells within the respective quadrants of all living leukocytes in the cultures. B) PAS-staining (with diastase) of M-IL15 tumor and spleen from a *Rag1*$^{-/-}$ mouse treated with anti-IL15 antibody to allow the tumor to form. Antibody-treatment was ended 13 days prior to excision of tumor and spleen. Scale bars are 20 μm. C) PAS-staining (with diastase) of M-control tumor grown in a *Rag1*$^{-/-}$*Prf1*$^{-/-}$ mouse for 22 days. Scale bars are 50 μm and 20 μm for inset showing densely granulated NK cells in the center of the lower magnification picture.

Figure S3. Granular PAS$^+$ NK cells require maturation in the M-IL15 tumor.

Serial sacrifices of *Rag1$^{-/-}$Prf1$^{-/-}$* mice injected with M-IL15 tumors show that the appearance of the granular PAS$^+$ cells does not occur immediately. Images were taken 3, 12 and 16 days after injection of cancer cells. Size of scale bars (in μm) is indicated in panels.

Figure S4. Stromal cells in M-IL15 tumors secrete IL10 and IL12.

CD45$^-$ECFP$^+$ cancer cells and CD45$^+$CD11b$^+$ stromal cells were obtained from a 28-day-old M-IL15 tumor grown in a *Rag1$^{-/-}$Prf1$^{-/-}$* mouse by FACS. **A)** Both sorted populations were directly analyzed *ex vivo* for IL12 and IL10 production by intracellular cytokine staining. Percentage of cytokine-positive cells in entire sorted population is shown (iso, isotype-control). **B)** Supernatants of cancer and stromal cells cultured for 24 h post sort were analyzed by ELISA for IL12, IL18, IL10 and TGF-β. Bars represent the mean of cytokine content in two wells of cultured cells with standard deviation.

Figure S5. IL15-activated NK cells have bystander killing activity

A) *Rag1$^{-/-}$* mice were injected with mixed inocula of 1 x 10^7 total cells composed of M-IL15 cells and 5-20% M-GFP cells, to identify non-IL15-secreting cells (white symbols), or control inocula containing the equivalent number of M-GFP cells only (black symbols). **B)** Tumors that grew from a 10% mixed inoculum were readapted to culture and analyzed for EGFP expression (thick black line) by

flow cytometry. For comparison cultured M-IL15 and M-GFP cells are shown in red and gray, respectively. The bars indicate the IL15-negative, GFP-positive cells.

Figure S6. IL15 and IL15Rα expression by cancer cells

IL15 expression was detected by ECFP fluorescence in AG104A-Rα-IL15, 9604-Rα-IL15 and 8215-IL15 cells (left panels, red lines); AG104A, 9604 and 8215 cells were analyzed as negative controls (black lines). Expression of IL15Rα is shown in the right panels (red lines). The same transfected cell lines were stained with only streptavidin-FITC as negative controls (2° only, shaded gray). Rather than displaying cell counts, histograms have been normalized to the maximum cell count (Normalized maximum %).