Precursor frequency of the DN1 population

To determine the precursor frequency for $NK1.1^+$ cells, we performed a limiting dilution analysis (LDA) by seeding sorted *Rag1*$^{-/-}$ DN1 $CD122^-NK1.1^-$ thymocytes in wells at different densities. After 19 days, all growth-positive wells were individually collected and analyzed; approximately 80–90% of the cells were $NK1.1^+$. Although the level of NK1.1 expression was somewhat variable, the NK1.1 mean fluorescent intensity (MFI) was independent of seeding dose (Supplemental Figure 1A). In addition, when growth-positive wells from one seeding dose were compared to each other, NK1.1 expression levels varied from well to well (Supplemental Figure 1B). These data suggest that the frequency of wells with $NK1.1^+$ cells, regardless of the level of NK1.1 expression, is a reliable outcome in LDA. Assuming the cells being titrated were randomly and independently distributed among all wells, then the number of cells in each well that differentiated into $NK1.1^+$ cells should have followed the Poisson distribution. We thus demonstrated a frequency of 0.24% with a 95% confidence interval of 0.3% to 0.17% (Supplemental Figure 2). These results indicate that one out of every 416 sorted DN1 $CD122^-NK1.1^-$ thymocytes from *Rag1*$^{-/-}$ mice is an NK cell precursor that can differentiate into an $NK1.1^+$ cell *in vitro*. While this may seem like a low frequency, it should be noted that this putative precursor population is negatively defined by lack of marker expression (CD4, CD8, CD122, NK1.1), rather than positively by markers it expresses, a topic beyond the scope of the current paper (see Discussion for more comments), and LDA has not been performed previously for NK cell precursors, to our knowledge. Nonetheless, these data suggest that it is possible to differentiate DN1 $CD122^-NK1.1^-$ cells into $CD122^+NK1.1^+$ *in vitro* from single precursors.

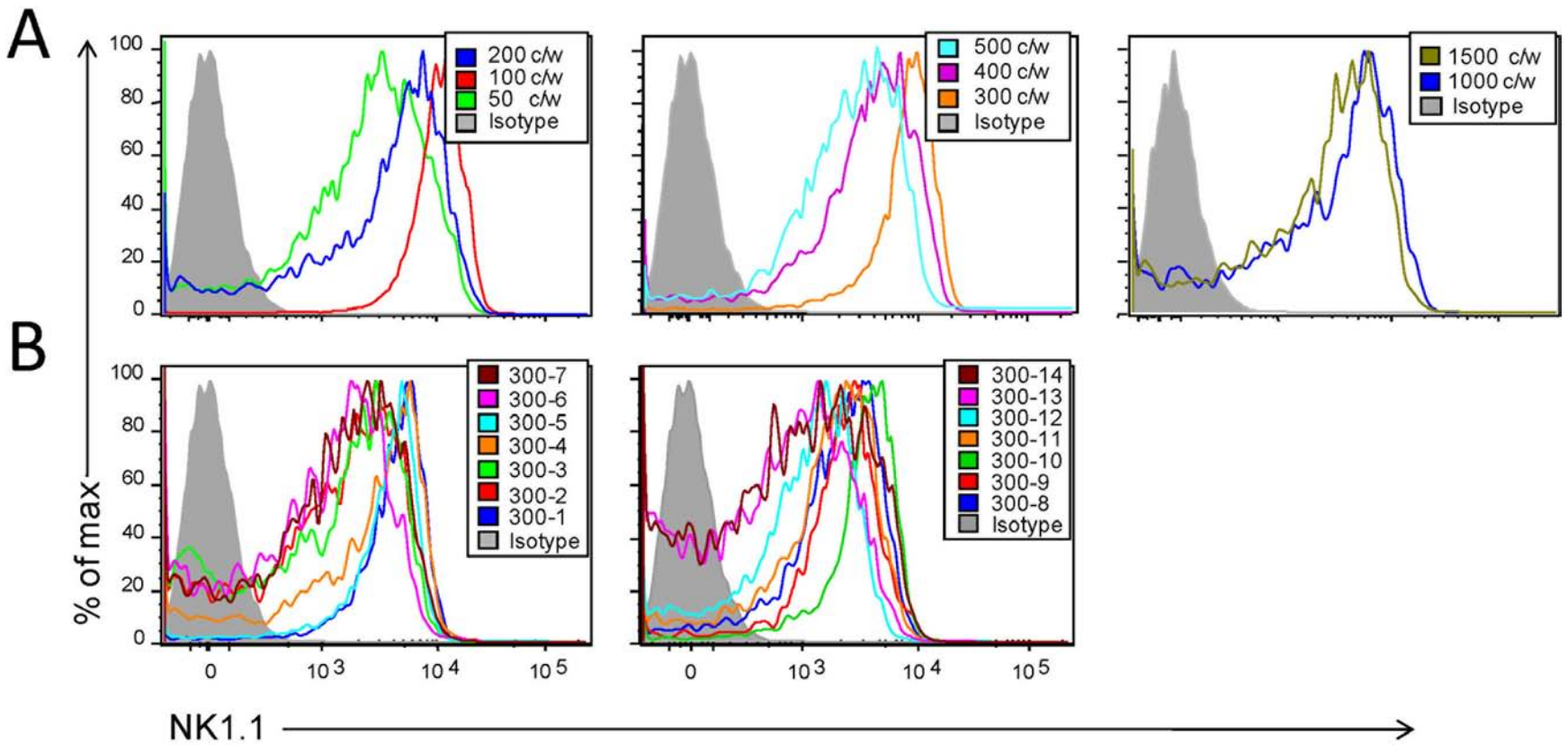

Figure S1. All visually growth positive wells were NK1.1^{+}
Sorted DN1 CD122^{-} NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines at limiting cell densities for 19 days. Growth-positive wells were analyzed for NK1.1 expression by flow cytometry. Cells were gated on the lymphocyte population. (A) Histograms from one representative well for each seeding dose (cell/well) are shown. (B) Histograms show all growth-positive wells for progenitors seeded at 300 cells/well. Data represent three experiments.

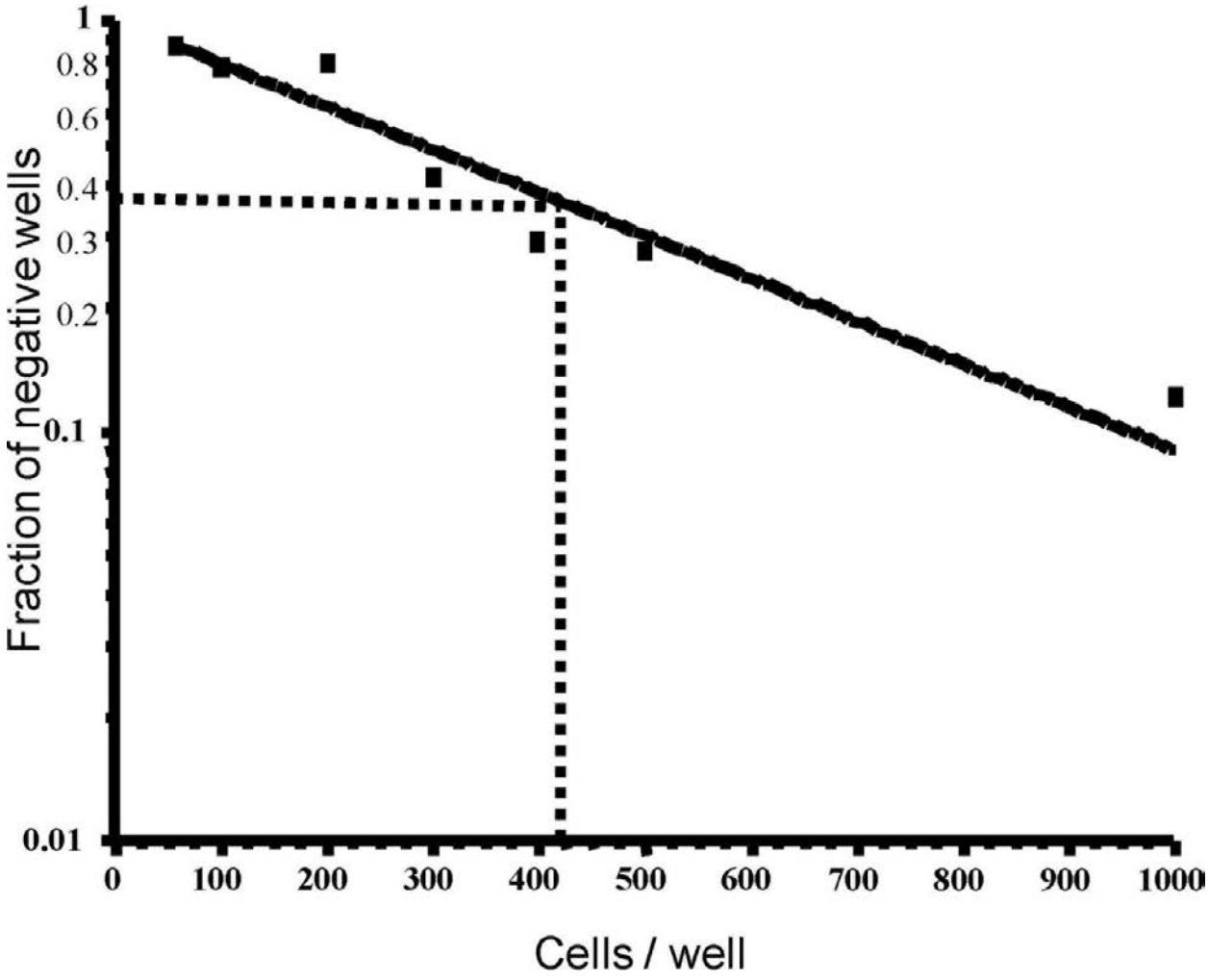

Figure S2. Limiting dilution analysis (LDA) of DN1 CD122^{-} thymocytes to determine progenitor frequency

Sorted DN1 CD122^{-} NK1.1^{-} thymocytes from *Rag1*$^{-/-}$ mice were co-cultured with OP9 cells and cytokines at limiting cell densities for 19 days. Growth-positive wells were analyzed for NK1.1 expression by flow cytometry. The frequency of growing cells was determined using Poisson distribution : Y=(e − m)(mr)/r!, where *Y* is the expected fraction of wells with *r* precursors when there is a mean of *m* precursors per well. Growth-negative wells were plotted as a function of the number of sorted thymocytes added per well using the Graphpad Prism software. The frequency is 0.24%. Standard error is 0.027% and 95% confidence interval is 0.3% to 0.17%. Data represent three experiments.

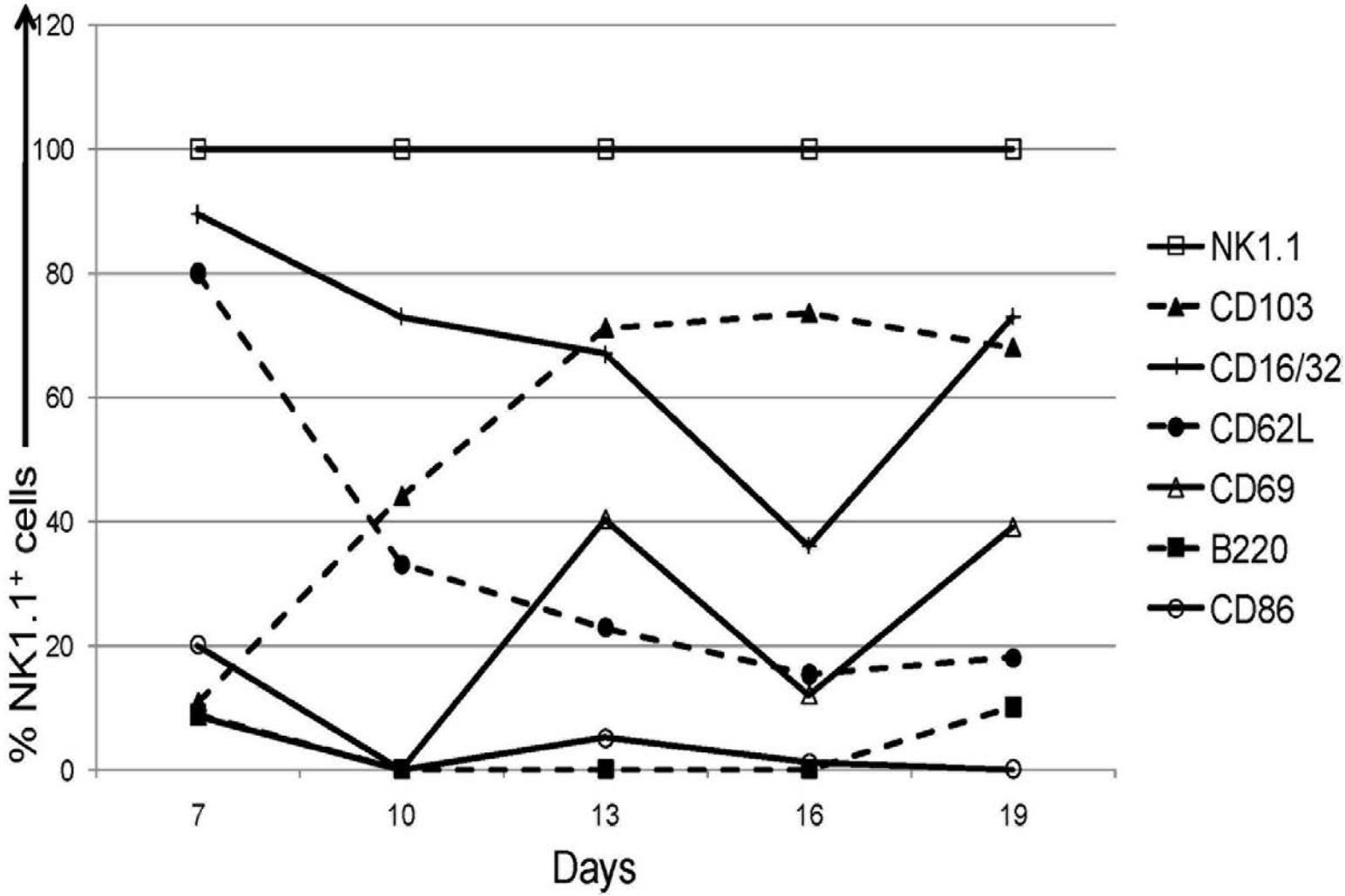

Figure S3. **Developmental kinetics of sorted thymocytes**
In vitro differentiated NK1.1^{+} cells were assessed for marker expression on days 7, 10, 13, 16, and 19 via six-color flow cytometry. Data are representative of 3–5 experiments.