Proinflammatory cytokine signaling required for the generation of natural killer cell memory

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Although natural killer (NK) cells are classified as innate immune cells, recent studies demonstrate that NK cells can become long-lived memory cells and contribute to secondary immune responses. The precise signals that promote generation of long-lived memory NK cells are unknown. Using cytokine receptor–deficient mice, we show that interleukin-12 (IL–12) is indispensible for mouse cytomegalovirus (MCMV)–specific NK cell expansion and generation of memory NK cells. In contrast to wild-type NK cells that proliferated robustly and resided in lymphoid and nonlymphoid tissues for months after MCMV infection, IL–12 receptor–deficient NK cells failed to expand and were unable to mediate protection after MCMV challenge. We further demonstrate that a STAT4–dependent IFN–γ–independent mechanism contributes toward the generation of memory NK cells during MCMV infection. Understanding the full contribution of inflammatory cytokine signaling to the NK cell response against viral infection will be of interest for the development of vaccines and therapeutics.

The Journal of Experimental Medicine

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947

www.jem.org/cgi/doi/10.1084/jem.20111760

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 209 No. 5 947-954
www.jem.org/cgi/doi/10.1084/jem.20111760
dendritic cells and can act on many cell types including B cells, T cells, NK cells, NK T cells, and even other dendritic cells and hematopoietic progenitor cells (Trinchieri, 2003). Studies comparing the response of NK cells that can or cannot sense IL-12, in a setting where pleiotropic effects are reduced or eliminated, have not been done. Furthermore, long-lived memory NK cell generation in the absence of IL-12 signaling was not investigated, have not been done. Additionally, long-lived memory NK cells preferentially expanded over 7 d and became the predominant NK cell subset in the spleen (Fig. 2 a). A roughly fivefold increase in total numbers of WT compared with Il12rb2−/− mice was determined by CD27, CD11b, KLRG1, and CD69 expression (Fig. 1 a). In the absence of infection, WT and Il12rb2−/− mice cleared B2m−/− mice equally well (Fig. 1 b), suggesting that Il12rb2−/− NK cells exhibited normal in vivo cytotoxic function. When activating NK cell receptors were triggered with plate-bound antibodies, Il12rb2−/− NK cells were able to degranulate similar to WT NK cells (Fig. 1 c). Lastly, Il12rb2−/− NK cells were able to kill m157-bearing target cells as well as WT NK cells ex vivo (Fig. 1 d), demonstrating that Ly49H-mediated cytotoxicity is not dependent on IL-12 signaling at steady state.

Defective expansion of Il12rb2−/− NK cells during MCMV infection

Previous studies examined the role of IL-12 on NK cells by one of three methods: injecting the IL-12 cytokine directly, blocking IL-12 with neutralizing antibodies, or infecting cytokine-deficient mice (Magram et al., 1996; Orange and Biron, 1996; Wu et al., 1997, 2000; Cousens et al., 1999; Pien and Biron, 2000). In each of these experimental systems, uncontrolled global effects are expected as a result of the expression of the IL-12R by many immune cell populations. Infection of IL-12−/− mice results in higher viral titers (compared with WT mice) in many instances (Cousens et al., 1999). To circumvent this problem, we generated mixed bone marrow chimeric mice, where approximately half of the hematopoietic compartment expressed the IL-12Rβ2 and the other half was deficient. Reconstitution of all immune cell populations was found to be equally distributed between WT and Il12rb2−/−, including the NK cell compartment (Fig. 2 a and not depicted).

After MCMV infection of mixed chimeric mice, WT NK cells preferentially expanded over 7 d and became the predominant NK cell subset in the spleen (Fig. 2 a). A roughly fivefold increase in total numbers of WT compared with Il12rb2−/− NK cells was observed at day 7 post infection (P1; Fig. 2 b).
A similar outcome was observed in nonlymphoid organs such as the liver (unpublished data). The expansion of the WT NK cell compartment was a result of the rapid proliferation of Ly49H-bearing cells (Fig. 2 c). Although the uninfected chimeric mice contained similar numbers of WT and $I_l 12b2^{-/-}$ NK cells, by 7 d PI the WT Ly49H+ NK cells were nearly 10-fold higher in absolute number compared with $I_l 12b2^{-/-}$ Ly49H+ NK cells (Fig. 2 d). Consistent with a role for IL-12 in IFN-γ induction, fewer $I_l 12b2^{-/-}$ NK cells produced IFN-γ compared with WT NK cells at day 1.5 PI, and the $I_l 12b2^{-/-}$ NK cells made less IFN-γ per cell (as measured by mean fluorescence intensity). Interestingly, WT and $I_l 12b2^{-/-}$ NK cells similarly up-regulated the activation marker CD69 (Fig. 2 e). Induction of CD69 expression correlates with type I IFN-mediated activation, and our current data supports a mechanism whereby IFN-γ secretion and CD69 expression result from two segregated signaling pathways even though both are determinants of activation status. Phosphorylation of the signaling component STAT4 has been shown to be a consequence of IL-12R signaling leading to IFN-γ induction (Trinchieri, 2003); thus, we examined splenic NK cells for phosphorylation of STAT4 early after infection. Whereas robust phosphorylation of STAT4 was observed in nearly all WT NK cells on day 1.5 PI, $I_l 12b2^{-/-}$ NK cells showed minimal levels of phosphorylated STAT4 (Fig. 2 e). Nearly half of the $I_l 12b2^{-/-}$ NK cells remained unphosphorylated for STAT4, at levels comparable to uninfected WT and $I_l 12b2^{-/-}$ mice (Fig. 2 e). Splenic T cells from the same infected mice showed minimal amounts of STAT4 phosphorylation in both WT and $I_l 12b2^{-/-}$ mice (unpublished data), consistent with previous findings that NK cells are the early and major producers of IFN-γ during infection (Yokoyama et al., 2004). At day 7 PI, WT Ly49H+ NK cells strongly up-regulated KLRG1, Ly6C, CD90 (Thy-1), and Ki67 (a nuclear marker of cellular proliferation) and down-regulated CD27, in contrast to $I_l 12b2^{-/-}$ Ly49H+ NK cells (Fig. 2 f), suggesting that IL-12 signals during MCMV infection are crucial toward achieving a full maturation program in virus-specific NK cells.

We determined whether the $I_l 12b2^{-/-}$ NK cells were expanding as extensively as WT NK cells, or were dying faster once activated. Adoptive transfer of CFSE-labeled WT or $I_l 12b2^{-/-}$ NK cells into Ly49H-deficient mice confirmed that both WT and $I_l 12b2^{-/-}$ NK cells proliferated after infection, with the WT NK cells dividing more extensively (Fig. 2 g). By day 6 PI, the WT NK cells had fully diluted their CFSE, whereas the $I_l 12b2^{-/-}$ NK cells remained intermediate for CFSE staining (Fig. 2 g). These results corroborate the higher expression of Ki67 detected on WT compared with $I_l 12b2^{-/-}$ NK cells at day 7 PI (Fig. 2 f). At day 4 PI, WT and $I_l 12b2^{-/-}$ NK cells showed comparable staining for Annexin V (Fig. 2 h), suggesting that a lack of IL-12 signal does not result in greater apoptosis.

**IL-12 signals required for memory NK cell generation and protection during MCMV infection**

A recent study demonstrated that although defective IL-12 signaling in CD8+ T cells also resulted in fewer effector cells, surprisingly higher numbers of memory cells were generated (Pearce and Shen, 2007). Therefore, we investigated the long-term consequences of IL-12 signaling during the NK cell response against MCMV infection. Using a previously described adoptive transfer system (Sun et al., 2009), we purified NK cells from WT and $I_l 12b2^{-/-}$ mice and transferred an equal number...
of Ly49H+ NK cells from each group into separate DAP12-deficient hosts, which are deficient in Ly49H-expressing NK cells. After MCMV infection, we found that WT NK cells proliferated robustly in the new hosts, in contrast to Il12rb2−/− NK cells (Fig. 3 a). The percentage and absolute number of WT Ly49H+ NK cells at day 7 PI were 15- to 20-fold higher than the Il12rb2−/− NK cells (Fig. 3 a and b). Strikingly, although WT NK cells were easily recovered from recipient mice several weeks later, adoptively transferred Il12rb2−/− NK cells were not detectable after week 2 PI (Fig. 3 b). Co-adoptive transfer of equal numbers of WT and Il12rb2−/− NK cells into recipient mice yielded the same outcome after infection, with WT NK cells preferentially expanding at day 7 PI to become the only memory NK cells detected months later in both spleen and liver (Fig. 3 c). To determine whether fewer Il12rb2−/− NK cells were recovered at later time point simply because they could not expand as well, equal numbers of WT and Il12rb2−/− effector Ly49H+ NK cells isolated at day 7 PI (1:1 mix) were adoptively transferred and memory cell percentages determined at later time points (Fig. 3 d). Although the day 7 effector NK cell numbers were experimentally normalized, only WT NK cells were detected 1 mo later in recipient mice (Fig. 3 d). Thus, IL-12 signals are crucial not only for the optimal expansion of virus-specific NK cells during infection but also for the generation of a long-lived NK cell population in lymphoid and nonlymphoid tissues.

Because of the pleiotrophic effects complicating the direct infection of cytokine-deficient mice, it is unknown whether defective IL-12 signaling in NK cells alone will influence the course of viral infection (without loss of the IL-12R on other cell types). Thus, we examined the contribution of IL-12R signaling specifically in NK cells toward protection against MCMV infection. We transferred an equal number of purified WT NK cells from WT or Il12rb2−/− mice into neonatal DAP12-deficient mice. As a negative control, one group of neonates was given PBS without cells. All three groups were challenged with MCMV. Within the first 2 wk, all of the mice receiving PBS or purified Il12rb2−/− NK cells had died (Fig. 3 e). During the same period, >50% of the mice given purified WT NK cells survived (Fig. 3 e). Together, the data suggest that IL-12 signaling in NK cells alone is crucial for protective immunity against viral challenge. Interestingly, the mice receiving Il12rb2−/− NK cells died with delayed kinetics (days 9–15 PI) compared with mice receiving PBS (days 5–12 PI), perhaps because the Il12rb2−/− NK cells could still mediate cytotoxicity against infected cells; however, their inability to undergo proliferation similar to WT NK cells might have prevented a more efficacious response.

**NK cell memory depends on STAT4 signals but not IFN-γ secretion**

We investigated the importance of effector molecules downstream of the IL-12R in the generation of long-lived memory
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NK cells produced less IFN-γ than WT NK cells, even though CD69 was similarly up-regulated in both subsets at day 1.5 PI (Fig. 4 e). Although WT and Stat4−/− NK cells similarly down-regulated CD27 and up-regulated CD11b at day 1.5 PI, phenotypic differences were evident at day 7 PI in spleen and liver, with WT NK cells strongly down-regulating CD27 and up-regulating KLRG1 (Fig. 4 e). Together, these findings suggest that IL-12–induced signaling in virus-specific NK cells primarily uses the downstream STAT4 to initiate vital signals for a complete cell maturation program during MCMV infection. Interestingly, both WT and Stat4−/− NK cells at day 7 PI highly expressed CD90 (Thy-1; Fig. 4 f), a marker described to be present on memory NK cells (O’Leary et al., 2006; Paust et al., 2010), and were predominantly Ly49C/I− (Fig. 4 f), consistent with previous data demonstrating that Ly49C/I− or “unlicensed” NK cells in C57BL/6 mice dominate the NK cell response to MCMV (Orr et al., 2010).

We investigated the extent to which signaling through STAT4 influenced the generation of memory NK cells during MCMV infection. We transferred an equal number of Ly49H+ NK cells purified from WT (CD45.1) and Stat4−/− (CD45.2) mice into a Ly49H-deficient host (CD45.2). Transferred WT Ly49H+ NK cells proliferated robustly after MCMV infection, in contrast to the minimal expansion of transferred Stat4−/− Ly49H+ NK cells in the same host (Fig. 5 a). The percentage of WT NK cells at day 7 PI was ~10-fold higher than that of the co-transferred Stat4−/− counterparts (Fig. 5 b), highlighting NK cells during MCMV infection. Because Il12rb2−/− NK cells do not efficiently phosphorylate STAT4 or secrete as much IFN-γ as WT NK cells during MCMV infection (Fig. 2 e), we examined the contribution of both STAT4 and IFN-γ on the production of effector and memory Ly49H+ NK cells. To elucidate the importance of downstream effector molecule STAT4 on in vivo NK cell activation and response against MCMV, we generated mixed bone marrow chimeric mice. Reconstitution of all immune populations, including the NK compartment, was found to be equally distributed between WT and Stat4−/− (Fig. 4 a and not depicted). After MCMV infection, WT NK cells preferentially expanded over 7 d to constitute the main subset in the spleen and liver, exhibiting fivefold greater numbers compared with Stat4−/− NK cells (Fig. 4 a, b, and not depicted). After MCMV infection, WT NK cells preferentially expanded over 7 d to constitute the main subset in the spleen and liver, exhibiting fivefold greater numbers compared with Stat4−/− NK cells (Fig. 4 a, b, and not depicted). After MCMV infection, WT NK cells preferentially expanded over 7 d to constitute the main subset in the spleen and liver, exhibiting fivefold greater numbers compared with Stat4−/− NK cells (Fig. 4 a, b, and not depicted). 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Figure 4. WT NK cells outcompete Stat4−/− NK cells during MCMV infection. (a) Mixed bone marrow chimeric mice were infected with MCMV and percentages of WT (CD45.1+) and Stat4−/− (CD45.2+) NK cells in spleen and liver are shown for uninfected mice and various time points PI. (b) The graph shows absolute numbers of WT and Stat4−/− NK cells in spleen and liver on day 0 and 7 PI. Error bars show SEM (n = 3–5). (c) Percentages of Ly49H+ cells within the WT and Stat4−/− NK cell population in spleen and liver are shown for uninfected mice and various time points PI. (d) The graph shows absolute numbers of WT and Stat4−/− Ly49H+ NK cells in spleen and liver on day 0 and 7 PI. Error bars show SEM (n = 3–5). (e) Expression of CD69, CD27, CD11b, and CD90, and production of IFN-γ are shown for WT and Stat4−/− NK cells (compared with uninfected mice) at day 1.5 PI. (f) Expression of KLRG1, CD90, Ly49C/I, and CD27 are shown for WT and Stat4−/− Ly49H+ NK cells (compared with uninfected mice) at day 7 PI. All data are representative of three experiments with three to five mice per time point.
the importance of signaling through STAT4 in the expansion of virus-specific NK cells after MCMV infection and in the attainment of a long-lived memory NK cell population.

Lastly, we examined a possible autocrine role for IFN-γ produced by NK cells during infection. Because WT NK cells produced more IFN-γ than Ifngr−/− or Stat4−/− NK cells early during MCMV infection, could this IFN-γ feed back on the NK cells themselves and drive stronger proliferation and generation of memory cells? We first generated mixed bone marrow chimeric mice consisting of a 1:1 ratio of WT:Ifngr−/− hematopoietic cells. After MCMV infection of chimeric mice, WT and Ifngr−/− NK cells proliferated similarly over the first 7 d (Fig. 6 a), with nearly equal ratio of WT to Ifngr−/− NK cells. On day 7 PI, similar percentages of Ly49H-bearing cells existed in total WT and Ifngr−/− NK cell populations (Fig. 6 a), suggesting that IFN-γ does not influence the ability of NK cells themselves to undergo expansion. On day 1.5 PI, expression of CD69 and secretion of IFN-γ were also independent of IFN-γ receptor expression on the NK cell (Fig. 6 b). Similarly, no phenotypic differences were observed between WT and Ifngr−/− NK cells at day 7 PI (unpublished data). When we co-transferred equal numbers of enriched WT and Ifngr−/− NK cells into recipient mice followed by MCMV infection, we observed a similar expansion at day 7 PI and equal percentages of memory NK cells at day 50 PI (Fig. 6 c). Altogether, these data suggest that the robust generation of NK cell memory during MCMV infection requires STAT4-dependent but IFN-γ independent signals.

The recent discovery that primed NK cells can become long-lived memory cells during infection begs the question of what the mechanisms are behind such a phenomenon. Past studies using neutralizing antibodies and cytokine-deficient mice did not specifically address whether there was an in vivo role for IL-12 on NK cells independent of global effects on other cell types (Magram et al., 1996; Orange and Biron, 1996; Wu et al., 1997, 2000; Cousens et al., 1999; Pien and Biron, 2000). Although these previous studies found a role for IL-12 in activating NK cells, our current study demonstrates the absolute requirement for both IL-12 and STAT4 in the clonal expansion of antigen-specific NK cells and the generation of memory NK cells during MCMV infection.

The amount of inflammation in the host environment during CD8+ T cell priming has been suggested to dictate the production of effector and memory cells. In recent studies where the degree of inflammation was varied while antigen concentration was kept constant, memory CD8+ T cell potential was determined by a gradient of T-bet expression in which moderate inflammation correlated with robust memory cells, whereas high inflammation correlated with short-lived effector cells and reduced memory potential (Joshi et al., 2007;...
Pipkin et al., 2010). Consistent with this study, another group showed that IL-12–deficient mice produced a diminished effector CD8+ T cell response during Listeria monocytogenes infection but generated higher numbers of memory CD8+ T cells and greater protection against reinfection (Pearce and Shen, 2007). However, when mice receiving WT or IL-12R–deficient TCR transgenic CD8+ T cells were infected with viral pathogens, the conclusions were less clear. One study observed normal clonal expansion but diminished memory responses in CD8+ T cells that could not sense IL-12 during vaccinia virus infection (Xiao et al., 2009), whereas another study showed normal effector and memory responses during infection with vaccinia virus, LCMV, and VSV (Kepler et al., 2009). These discrepancies may be a result of different TCR transgenic mice and recombinant pathogens used in the studies, but they highlight the need to resolve these inconsistencies.

In contrast to the inconclusive role of IL-12 on T cells, the NK cell response in the absence of IL-12 signaling resulted in a severe detriment in both effector and memory NK cell numbers. During MCMV infection, the IL-12R–deficient Ly49H+ NK cells showed a remarkable 10- to 20-fold reduction in absolute number of effector cells in spleen and liver compared with WT NK cells, and a complete deficiency in memory cells. Even when effector NK cell numbers (isolated at day 7 PI) were normalized, only WT NK cells could be detected at later time points after adoptive transfer (Fig. 3 d), suggesting that IL-12 signaling programs NK cells during priming to demonstrate longevity after activation and expansion. Unlike CD8+ T cells that expanded less in the absence of IL-12 but generated higher memory cell numbers (Pearce and Shen, 2007), our results suggest an absolute requirement for IL-12 signals in the generation of memory NK cells. These findings are strengthened by the severe defect in clonal expansion and memory cell formation in STAT4-deficient NK cells. Interestingly, we do observe some low level phosphorylation of STAT4 in the IL12h2−/− NK cells compared with WT NK cells (Fig. 2 e), suggesting that other cytokines produced during MCMV infection may share the STAT4 signaling pathway. IFN-α/β has been shown to activate STAT4 (in addition to STAT1) in T cells (Nguyen et al., 2002); however, findings in IFN-α receptor–deficient mice that STAT4 is normally phosphorylated during MCMV infection suggest that IL-12 signaling is the predominant pathway for STAT4 phosphorylation leading to IFN-γ production in NK cells (Nguyen et al., 2002; unpublished data). Developing a system for NK cells where we can vary the inflammation while keeping the antigen dose constant will allow us to determine whether, like CD8+ T cells, there exist optional and suboptimal degrees of inflammation that drive generation of effector and memory NK cells. In addition, the molecular mechanisms behind NK cell programming by IL-12 during MCMV infection remain to be elucidated. Future studies will determine how IL-12 signaling influences histone acetylation and chromatin remodeling at promoter sites of genes important for the generation of NK cell memory.

Like NK cells, memory CD8+ T cells surprisingly possess the innate-like ability to produce IFN-γ and proliferate after exposure to proinflammatory cytokines alone, without TCR and co-stimulatory receptor ligation (Berg and Forman, 2006). Given their relatedness during ontogeny (both are derived from a common lymphoid progenitor) and possible shared evolutionary ancestry, perhaps it is not surprising that parallel mechanisms exist in NK cells and T cells for their activation, proliferation, and generation of memory during infection. The importance of environmental cues, in addition to antigen receptor triggering, is becoming more evident, and a greater understanding of these mechanisms will allow for development of therapeutics and vaccines against infectious disease.

**MATERIALS AND METHODS**

**Mice and infections.** C57BL/6 and congeneric CD45.1+ mice were purchased from the National Cancer Institute. IL-12Rβ2 chain–deficient (Wu et al., 2000; provided by J. DeKruis, University of California, San Francisco [UCSF], San Francisco, CA), STAT4-deficient (Kaplan et al., 1996), IFN-γ receptor–deficient, β2m-deficient, Ly49H-deficient (Foddl-Cornu et al., 2008; provided by S. Vidal, McGill University, Montreal, Canada), and DAP12-deficient (Bakker et al., 2000) mice on a C57BL/6 background were bred at the UCSF and Memorial Sloan Kettering Cancer Center and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Mixed bone marrow chimeric mice were generated as described previously (Sun et al., 2009). Mice were infected by intraperitoneal injections of Smith strain MCMV (5 × 104 PFU). Newborn mice were infected with 2 × 103 PFU of MCMV.

**Flow cytometry.** Fc receptors were blocked with 2.4G2 mAb before surface or intracellular staining with indicated antibodies or isotype-matched control immunoglobulin (BD, E Bioscience, or BioLegend). For measuring apoptotic, freshly isolated splenocytes were first stained with antibodies against NK1.1 or anti-Ly49H antibodies (provided by W. Yokoyama, Washington University, St. Louis, MO), and enriched NK cells or whole splenocytes were stained with PE-conjugated Annexin V (BD), according to the manufacturer’s protocol. Samples were acquired on an LSRII (BD) and analyzed with Flowjo software (Tree Star).

**NK cell enrichment, CFSE labeling, and adoptive transfer.** NK cells were enriched with an NK Cell Isolation kit (Miltenyi Biotech) or a method where spleen cells were incubated with purified rat antibodies against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119, followed by anti-rat IgG antibodies conjugated to magnetic beads (QIAGEN). Purified NK cells were injected intravenously into adult recipients or intraperitonally into neonatal recipients 1 d before viral infection. In some experiments, NK cells or unfractionated splenocytes were labeled with varying concentrations of CFSE before intravenous injection. Labeling of cells with CFSE was performed in accordance with the manufacturer’s instructions (Invitrogen).

**Ex vivo stimulation of NK cells and cytotoxicity assay.** Tissue culture plates treated with N-((1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulphate (Sigma-Aldrich) were coated with 10 µg/ml anti-NK1.1 or anti-Ly49H antibodies (provided by W. Yokoyama, Washington University, St. Louis, MO), and enriched NK cells or whole splenocytes were incubated for 5 h at 37°C in the presence of GolgiPlug (BD), followed by staining for intracellular cytokines. Uncoated or PBS-coated wells served as negative controls, and addition of 50 ng/ml PMA and 1 µg/ml ionomycin during incubation served as a positive control.

Enriched NK cells were used as effector cells ex vivo in a 6-h 51Cr release assay against Ba/F3 and m157-transfected Ba/F3 target cells. Percentage of Ly49H+ NK cells in each group was determined by flow cytometry and absolute numbers were normalized before incubation with targets. Large numbers of NK cells from Ly49H-deficient mice were included as a negative control to demonstrate specificity of receptor–ligand interactions.
Statistical methods. The Mann–Whitney nonparametric U test was used to compare survival between groups of mice. A value of 25 d was assigned to survivors living >25 d after MCMV infection. Student’s t test was used to compare groups in ex vivo stimulation experiments.

We thank the Sun and Lanier laboratories for comments and discussions. We thank Joseph DeRisi, Silvia Vidal, Wayne Yokoyama, Sasha Rudensky, and Gregoire Altan-Bonnet for providing mice and reagents critical to this study.

J.C. Sun was an Irvington Postdoctoral Fellow of the Cancer Research Institute, and is supported by the Searle Scholars Program grant and National Institutes of Health (NIH) grant AI085304. S. Madera is supported by a Tri-Institutional Medical Scientist Training Program grant. N.A. Bezman is supported by a fellowship from the American Cancer Society. The Juvenile Diabetes Research Foundation supported J.N. Beilke. M.H. Kaplan is supported by NIH grant AI045515. L.L. Lanier is an American Cancer Society Professor and supported by NIH grants AI068129.

J.N. Beilke is an employee of Nova Nordisk. The other authors have no financial conflicts of interest.

Submitted: 22 August 2011
Accepted: 7 March 2012

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