Article

Leptin Signaling Protects NK Cells from Apoptosis During Development in Mouse Bone Marrow

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Increasing evidence indicates a role of leptin in immune response, but it remains largely unclear whether leptin signaling is involved in regulating NK cell development in the bone marrow (BM). In this study, we have characterized NK cell differentiation and maturation in the BM of leptin-receptor deficient *db/db* mice at a prediabetic stage. Although the BM cellularity was similar to the control value, the total number of NK cells was severely reduced in mutant mice. Flow cytometric analysis of *db/db* BM cells revealed significantly decreased frequencies of developing NK cells at various stages of differentiation. BM *db/db* NK cells displayed markedly increased apoptosis but maintained normal cell cycling status and proliferative capacity. Moreover, recombinant leptin could significantly enhance the survival of NK cells from wild-type mice in cultures. Further examination on NK cell functional activity showed that *db/db* NK cells exhibited normal intrinsic cytotoxicity with significantly increased IL-10 production. Taken together, our findings suggest that leptin signaling regulates NK cell development *via* enhancing the survival of immature NK cells in mouse BM. *Cellular & Molecular Immunology*. 2009;6(5):353-360.

Key Words: NK cell, bone marrow, lymphocyte development

Introduction

NK cells play a crucial role in the elimination of virus-infected cells and transformed cells (1). Derived from the common lymphoid progenitors, NK progenitors undergo a sequential developmental process including acquisition of receptors specific during their differentiation into mature NK cells in the BM (2). At the very early stage during their ontogeny, NK cell progenitors (NKP) express CD122 but neither the pan-NK cell marker NK1.1 nor DX5 (3). Subsequently, NKP develop into immature NK cells that are featured with upregulation of surface molecules such as LY49 and CD94-NKG2 , as well as extensive proliferation before their final differentiation into mature NK cells expressing DX5 and Mac-1 (2). Although considerable progress has been recently made in understanding the phenotypic changes during the process of NK cell development, much less is known about the regulatory requirement of NK cell ontogeny. Gene-targeting studies have demonstrated that transcription factors such as Ets-1 and Id2 are critical for NK cell development (4, 5). Emerging evidence also indicates that the complete phenotypic differentiation and functional maturation of NK cells require an intact BM microenvironment that provides not only direct interaction between stromal cells and developing NK cells but also soluble factors such as IL-15 (6). However, it remains largely unclear about what other factors derived from the BM may support NK cell development and further characterization of microenvironmental requirement will benefit our understanding of the regulatory mechanisms for NK cell ontogeny.

Leptin, a 16 kDa non-glycosylated polypeptide product of the obese (*ob*) gene, is a hormone primarily derived from adipocytes and BM stromal cells that has long been recognized as a key factor in regulating a wide range of biological responses including nutrient intake, energy

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Received Sep 25, 2009. Accepted Oct 12, 2009.

homeostasis, neuroendocrine function, angiogenesis, bone formation and reproduction (7-9). Early studies on *db/db* mice, in which the leptin receptor that transduces signals through leptin binding is truncated, have revealed that B and T cell development are severely affected with reduced numbers of lymphocytes in the peripheral lymphoid organs (9). Moreover, leptin has been shown to modulate the adaptive immunity *via* enhancing the survival of T cells and their production of pro-inflammatory cytokines such as IFN-γ and IL-2 (10). Recently, we have demonstrated an essential role for leptin signaling in the survival and maturation of BM-derived DCs, along with induction of CD40 expression *via* the activation of Akt (11, 12). Previous studies by Zhao et al have revealed that NK cells express the leptin receptor (13). In addition, reduced numbers of NK cells in various peripheral organs such as spleen and liver have been observed in *db/db* mice, but it remains unclear whether impaired leptin signaling affects NK cell development in the BM (14). While BM is a rich source of leptin and the functional development of NK cells largely depends on stromal cell-derived factors, it is plausible that leptin may potentially play a role in modulating NK cell development.

In the present study, we have performed phenotypic analysis on NK cell development at various differentiation stages in the BM of *db/db* mice compared with age-matched wild-type controls. We detected severely impaired NK cell development in the *db/db* mice at a prediabetic stage with developing NK cell populations significantly reduced in both frequencies and absolute numbers at various differentiation stages. In short-term culture, BM *db/db* NK cells displayed markedly increased apoptosis while their proliferative capacity in response to IL-15 stimulation remained unimpaired. Taken together, our results demonstrate that leptin signaling plays an indispensable role in NK cell development *via* enhancing the survival of immature NK cells in the BM.

Materials and Methods

Mice

Male C57BLKS/J *db/db* and C57BLKS/J wild-type control mice between 4 and 5 weeks old were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free animal facility at the University of Hong Kong. All *db/db* and age-matched control mice were used at age of 6-7 weeks before the onset of diabetes as revealed by normal blood glucose levels (11). All experiments were performed according to the guidelines of the Institutional Review Committee.

Preparation of cell suspensions

BM and splenic cell suspensions were prepared as described (15). Cell suspensions were treated with ACK buffer to lyse erythrocytes before cell enumeration.

Immunostaining and flow cytometry

FITC-, PE- and allophycocyanin (Cy5)-conjugated anti-mouse mAbs were purchased from BD PharMingen (San Diego, CA). Cells were labeled with the following mAbs: FITC-conjugated anti-CD122 (TM-β1), anti-NK1.1 (PK136), anti-NKG2ACE (20D5), anti-CD11b (Mac-1, M1/70), and Annexin V, PE-conjugated anti-NK1.1, anti-DX5, anti-CD94 (18d3), anti-Ly49AD (12A8), and Cy5-conjugated CD3 (145-2C11). Cells were analyzed with a FACSCalibur (Becton Dickinson, Mountain View, CA). Cell sorting was performed with an EPICS-Altra flow cytometer (Beckman Coulter, Fullerton, CA) and the purity of collected cells was routinely > 95%.

Proliferation assay

Freshly prepared BM cell suspensions from both *db/db* and control mice were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) at 37°C for 10 min before being cultured in complete medium supplemented with 20 ng/ml rmIL-15 (R&D Systems, Minneapolis, MN) for up to 7 days. After culture, the number of viable cells was determined by trypan blue staining. Frequencies of proliferating NK cells were defined by the newly dividing peaks on the histogram of CFSE gated on the $NK1.1^+$ cells as measured by flow cytometry.

Apoptotic assay

BM cell suspensions from *db/db* and control wild-type mice were cultured in serum-free RPMI 1640 medium for 4-10 h. Separately, BM cells from normal control mice were cultured in 2% FCS- RPMI 1640 media in the presence of either 200 ng/mL recombinant murine leptin (Peprotech, Rocky Hill, NJ, USA) or 1 μg/mL recombinant mouse leptin R/Fc chimera (Ob-R:Fc; R & D systems). After incubation, cells were stained with anti-NK1.1 mAb and Annexin V for flow cytometric analysis (15).

Cell cycle analysis

Freshly prepared BM cell suspensions from *db/db* and WT mice were stained with anti-DX5 and fixed for PI nuclear staining and flow cytometric analysis (15).

Quantitative RT-PCR analysis

Sorting-purified $DX5^+CD3^-$ NK cells from the BM and spleen of *db/db* and wild-type control mice were lyzed for RNA extraction and cDNA synthesis, as previously described (11). Quantitative PCR was performed using pre-designed and pre-validated probes from Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) for Bcl-2, Bax and Bcl-x_L. FAM-labeled Taqman probes were used as reporter dye while the GAPDH expression was detected with VIC/MGB labeled probe using the pre-developed Taqman Assay Reagents (Applied Biosystems). PCR reactions were set up with the 2X Taqman Universal Master Mix (Applied Biosystems). SYBR Green 2X PCR mix was used to set up the reaction according to manufacturer's instructions (Invitrogen). Gene expressions of perforin and granzyme B were analyzed using gene-specific primers with sequence as previously described (16). Primers used were: IFN-γ, sense: 5'-AAG CGT CAT TGA ATC ACA CC-3', and anti-sense:

5'-CGA ATC AGC AGC GACT CCTT-3'; TNF-α, sense: 5'-TGG CCT CCC TCT CAT CAG-3', anti-sense: 5'-GGC TGG CAC CAC TAG TTG-3'; TGF-β, sense:5'GCG GCA GCT GTA CAT TGA-3', anti-sense:5'-CCG GGT TGT GTT GGT TGT-3'; IL-10, sense:5'-GGC CCA GAA ATC AAG GAG-3', anti- sense:5'-CCT TGT AGA CAC CTT GGT-3'; IL-13, sense:5'-GCC GGT GCC AAG ATC TGT-3', anti-sense: 5'-GCC ATG CAA TAT CCT CTG-3' and the sequence for the endogenous control β-actin (195bp) is as follows: sense: 5'-GCG TGA CAT CAA AGA GAA GCT-3' and anti-sense: 5'-ATG CCA CAG GAT TCC ATA CC-3'. Relative gene expression was analyzed as described (11).

NK cytotoxicity assay

NK cell activity was measured by a non-radioactive lytic assay of CFSE-labeled target cells. Consistently reliable correlation between non-radioactive and ^{51}Cr release assays had been confirmed in our laboratory before using this non-radioactive method. YAC-1 cells, the target cells for cytotoxicity assay, were labeled with CFSE at 37°C for 10 min. CFSE-labeled target cells were mixed with either splenocytes from both *db/db* and WT controls containing same amount of NK cells or sorting-purified $DX5^+CD3^-$ NK cells (the effector cells) and at effector : target (E : T) ratios of 160 : 1, 80 : 1, 40 : 1, 20 : 1 and 10 : 1. PI was used to stain lysed cells after incubation at 37°C for 20 h. Cytotoxicity was assessed by measuring the frequency of lysed CFSE-labeled target cells with flow cytometry and calculated as follows: Cytotoxicity (%) = (Specific lysis - Spontaneous lysis) / (100% - Spontaneous lysis) × 100%.

Detection of cytokine production

Sorting-purified $DX5^+CD3^-$ NK cells from the spleens of *db/db* and WT control mice were cultured in complete medium supplemented with IL-2 for 3 days. Culture supernatants were measured for the production of IFN-γ, TNF-α and IL-10 by ELISA analysis (11).

Statistical analysis

Analyses were performed with Student's *t*-test. The p values of ≤ 0.05 were considered as statistically significant.

Results

NK cell development is severely impaired in the BM of db/db mice

The *db/db* mice remained normal until around 8 weeks of age and then started to show increased blood glucose levels and body weight. To evaluate the effect of leptin receptor deficiency on NK cell ontogeny, all mutant mice were used around 6-7 weeks old at a pre-diabetic stage. In *db/db* mouse BM, the total number of nucleated cells was moderately decreased as compared with wild-type controls ($16.9 \pm 6.3 \times 10^6$/femur *vs*. $20.6 \pm 5.6 \times 10^6$/femur, respectively). However, the NK cell population size was significantly reduced, exhibiting approximately a 3-fold reduction in mutant BM compared with control values ($6.5 \pm 0.9 \times 10^5$ /femur *vs*. 2.2

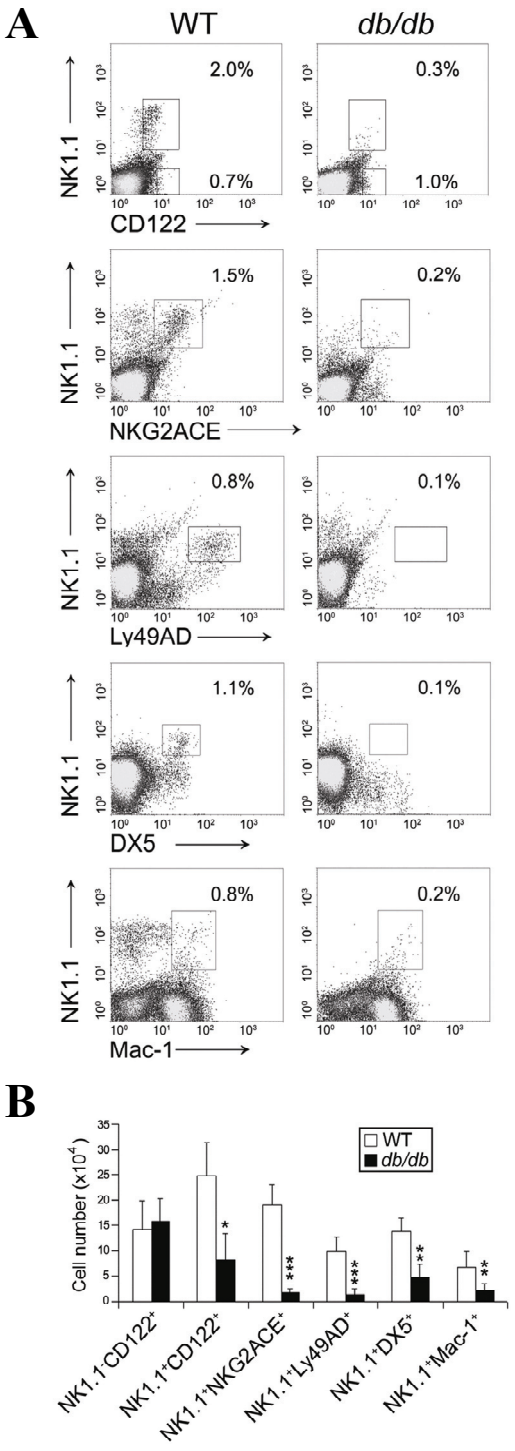

Figure 1. Impaired NK cell development in the BM of *db/db* mice. (A) Phenotypic analysis of BM NK cells was performed for both *db/db* and WT control mice. The $CD3^-$ cell population was gated for analyzing NK developmental stages by flow cytometry. For different NK cell subsets, percentages of the relevant subsets are indicated in the representative profiles. (B) Absolute numbers of BM NK cell subsets were measured and compared with age-matched wild type controls. Data are derived from ten separate experiments (mean ± SD; $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$).

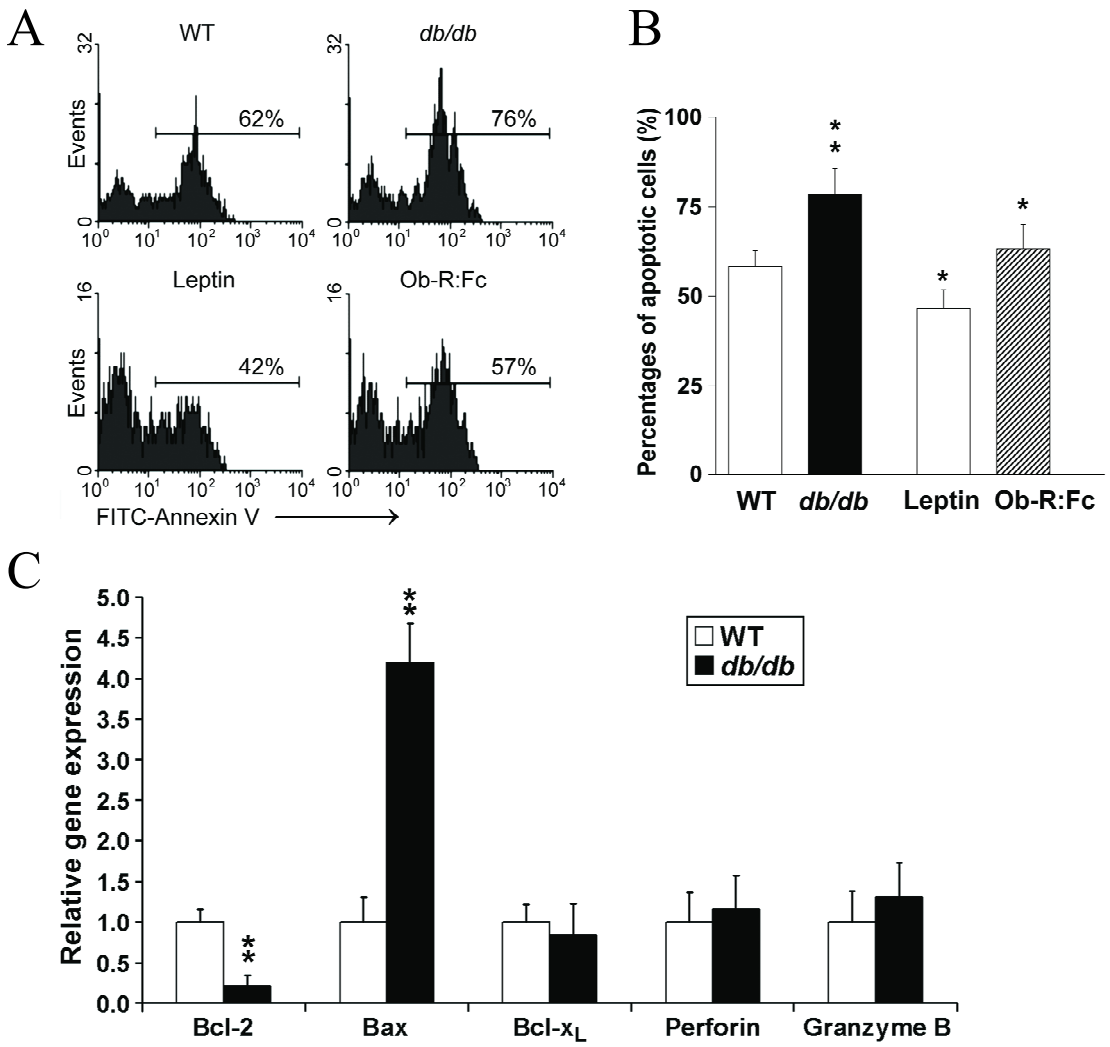

Figure 2. Increased apoptosis among *db/db* NK cells. (A) Enhanced apoptosis of *db/db* NK cells in short term culture. BM cells of *db/db* and WT mice were cultured in medium without FCS for 10 h. Separately, WT BM cells were cultured in 2% FCS-RPMI 1640 media with the presence of either leptin or Ob-R:Fc for 10 h. Frequencies of Annexin V^+ $NK1.1^+$ cells are indicated in representative histograms. (B) Data show the apoptotic incidences of BM NK cells after 10 h of culture with different conditions or treatments. Data are derived from three separate experiments (Mean ± SD; $*p < 0.05$ and $**p < 0.01$). (C) Total RNA was isolated from sorting-purified $DX5^+CD3^-$ NK cells from the BM of *db/db* and WT control mice and evaluated for gene expression levels of Bcl-2, Bax, Bcl-x_L, perforin and granzyme B by quantitative RT-PCR. Results are representative of triplicate samples normalized to GAPDH (for Bcl-2, Bax, Bcl-x_L) or β-actin (for perforin and granzyme B) from 2 separate experiments. (Mean ± SEM; $**p < 0.01$).

± 0.7×10^5/femur, respectively; $p < 0.01$). Accordingly, the population size of NK cells in the spleen of *db/db* mice showed a 45% reduction, consistent with previous findings (14). Similar patterns of reduction in NK cell populations were also observed in other peripheral organs of *db/db* mice (data not shown). Taken together, these results indicate a significant reduction of NK cell populations in the BM of *db/db* mice.

To determine whether leptin-receptor deficiency affects NK ontogeny at specific developmental stages, various BM NK cell subsets at different differentiation stages in *db/db* mice were characterized by flow cytometry. Our phenotypic analysis revealed that $NK1.1^-CD122^+CD3^-$ NKP remained unimpaired while developing NK cells ($NK1.1^+CD122^+CD3^-$) were significantly reduced by approximately 3 fold in both frequencies and absolute cell numbers in the mutant BM compared with WT controls (Figure 1). Notably, immature NK cells including $NK1.1^+NKG2ACE^+$ and $NK1.1^+$ $Ly49AD^+$ cell populations exhibited a striking reduction by 8-12 fold (Figure 1B). Moreover, $NK1.1^+DX5^+$ and $NK1.1^+Mac\text{-}1^+$ mature NK cell subsets were also markedly decreased in the mutant BM (Figure 1). Here, our data clearly show that leptin signaling is required for the development of NK cells in the BM.

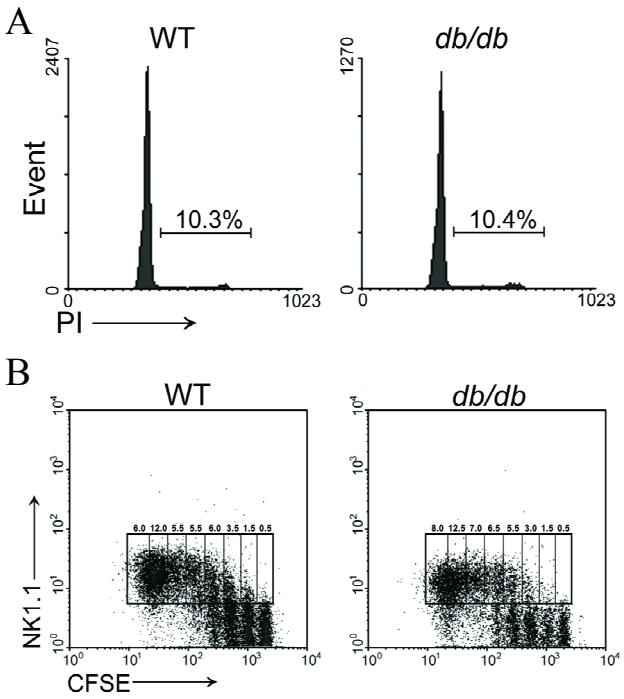

Figure 3. Normal proliferative capacity in *db/db* NK cells. (A) Cell cycle analysis by nuclear staining of $DX5^+$ NK cells in the BM of *db/db* and control WT mice. Representative histograms from three separate experiments are shown, in which the percentages of $DX5^+$ NK cells in the $S+G_2/M$ phase are indicated. (B) BM cells from *db/db* and WT control mice were labeled with CFSE and cultured with IL-15 for 7 days before flow cytometric analysis. The numbers beside the grids indicate percentage of cells expressing NK1.1 in each division. The profiles shown are representative of three separate experiments.

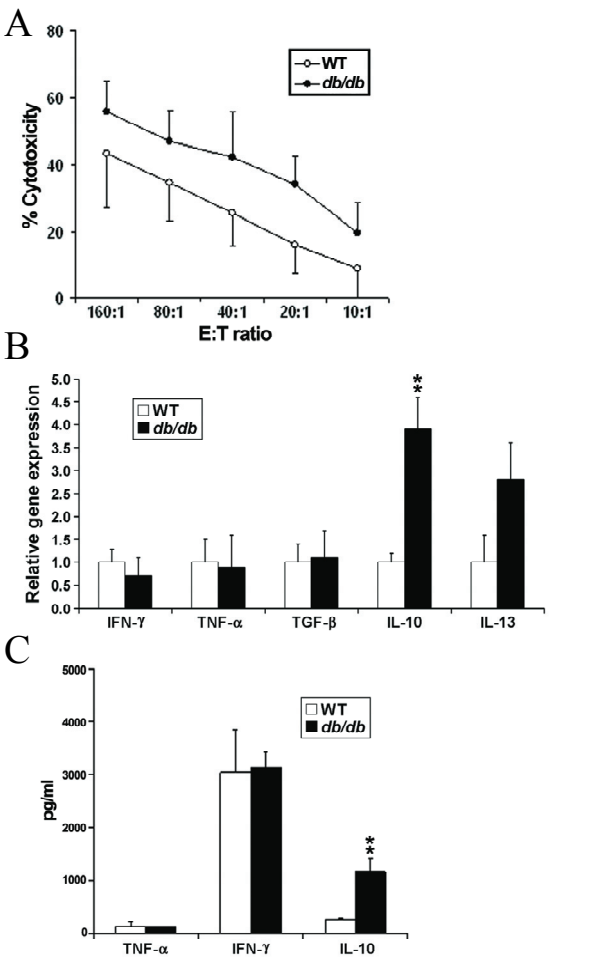

Figure 4. Normal cytotoxicity of mature *db/db* NK cells with increased IL-10 production. (A) CFSE-labeled YAC-1 target cells were mixed with either *db/db* or WT splenocytes containing same amount of NK cells at different effector/target (E/T) ratios. Data are derived from three separate experiments (Mean ± SD; $p > 0.05$). (B) Total RNA was isolated from sorting-purified splenic $DX5^+CD3^-$ NK cells from *db/db* and WT mice and evaluated for gene expression of IFN-γ, TNF-α, TGF-β, IL-10 and IL-13 by quantitative RT-PCR analysis. Results are representative of triplicate samples from 2 separate experiments (Mean ± SEM; ** $p < 0.01$). (C) Sorting-purified $DX5^+CD3^-$ NK cells from the spleens of *db/db* and WT control mice were cultured with IL-2 for 3 days and production of TNF-α, IFN-γ and IL-10 in the culture supernatant was analyzed by ELISA analysis. Results are representative of three separate experiments (Mean ± SEM; ** $p < 0.01$).

BM NK cells from db/db mice display increased apoptosis but maintain normal proliferative capacity

To determine whether increased cell death contributes to the diminished NK cell populations in *db/db* mouse BM, we used a well established short-term culture assay to evaluate the kinetics of NK cell apoptosis by flow cytometry (15). At various time intervals of incubation (up to 10 h), the apoptotic incidences of $NK1.1^+$ NK cells from mutant BM were consistently higher than control values (Figure 2). After 10 h of incubation, $NK1.1^+$ NK cells from *db/db* BM showed significantly increased apoptotic frequencies (78.6 ± 7.2% *vs*. 58.2 ± 4.5%, $p < 0.01$). To further confirm the role of leptin in NK cell survival, freshly prepared BM cells from WT mice were incubated with either recombinant leptin or a soluble leptin blocker Ob-R:Fc. As shown in Figure 2B, leptin could significantly promote the survival of NK cells whereas the specific blocking of leptin activity with Ob-R:Fc treatment greatly increased NK cell apoptosis in short term culture. Thus, our current data suggest a role for leptin signaling in enhancing the survival of developing NK cells. Next, we sought to determine whether leptin-receptor deficiency affects the expression of Bcl-2 family gene transcripts in developing NK cells. Quantitative RT-PCR analysis was performed on sorting-purified viable $NK1.1^+CD3^-$ NK cells from BM cell suspensions of *db/db* and control mice. We found that *db/db* NK cells displayed markedly downregulated Bcl-2 expression by 5 fold whereas Bax was significantly upregulated (Figure 2C). These results suggest that leptin receptor deficiency impairs the survival of developing NK cells possibly by modulating the expression of Bcl-2 family

genes. Interestingly, *db/db* NK cells showed no obvious change in the levels of perforin and granzyme B gene expression (Figure 2C), indicating that leptin-receptor deficiency may not affect the intrinsic NK cytotoxicity.

To determine whether developing *db/db* NK cells show any defect in proliferation, we performed cell cycle analysis on $DX5^+$ NK cells, a population known for undergoing proliferative expansion during development. As shown in Figure 3A, the DNA content profiles of NK cells from freshly prepared *db/db* BM suspensions showed no difference in their cell cycle characteristics compared with WT controls. To verify whether *db/db* NK cells exhibit any impaired proliferative capacity in response to cytokine stimulation, BM cells were labeled with CFSE and cultured with IL-15 for up to 7 days. Flow cytometric analysis showed that *db/db* NK cells displayed no defects in their proliferative activities as compared with WT controls (Figure 3B), demonstrating that leptin receptor deficiency does not affect the proliferative capacity of NK cells.

Peripheral NK cells in db/db mice display normal cytotoxicity with increased IL-10 production

To evaluate the functional changes of mature NK cells in *db/db* mice, we next examined NK cytotoxicity against target YAC-1 cells. Interestingly, splenic NK cells from *db/db* mice displayed effective cytotoxicity against the target cells with even moderately increased lytic activity compared with WT NK cells (Figure 4A), which was further confirmed by using sorting-purified splenic NK cells as effector cells that gave similar results (data not shown). These data suggest that leptin receptor-deficiency does not impair the intrinsic cytotoxic function of mature NK cells, consistent with previous findings that the basal levels of splenic NK cell cytotoxicity in *db/db* mice did not differ significantly from wild-type controls (14).

We have previously observed that DCs derived from *db/db* mouse BM displayed markedly impaired immune functions with a Th-2 type cytokine profile (11). Next, we sought to determine the cytokine expression profile in sorting-purified $DX5^+CD3^-$ NK cells from the spleens of *db/db* and WT mice by quantitative RT-PCR analysis. In freshly-isolated *db/db* NK cells, IL-10 expression was significantly increased whereas levels of other cytokine genes did not show any significant changes (Figure 4B). The data were further supported by *in vitro* cultures of *db/db* NK cells in the presence of IL-2 for 3 days. Consistently, IL-10 production was significantly increased in culture supernatants of *db/db* NK cells as revealed by ELISA analysis (Figure 4C). These data suggest a previously unrecognized role of leptin-signaling in regulating the cytokine production by NK cells toward a Th2 phenotype. Further studies will help to clarify the functional significance of leptin-mediated cytokine production in NK cells.

Discussion

In this study, we have revealed that NK cell development is severely impaired in the BM of *db/db* mice. Our phenotypic analyses show that the population size of $NK1.1^-CD122^+CD3^-$ NKP is normal whereas developing NK cells ($NK1.1^+CD122^+CD3^-$) are significantly reduced in both frequencies and absolute cell numbers in the *db/db* BM. Upon further differentiation with the acquisition of cell surface CD94-NKG2 and LY49 receptors, the *db/db* NK cell subsets were even more severely impaired. Therefore, our data demonstrate that leptin signaling is, though not required for the NK lineage commitment, is indispensable for developing NK cells at the later differentiation stages.

Consistent with substantially increased apoptotic frequencies of BM *db/db* NK cells in culture, we show that leptin can significantly promote the survival of NK cells whereas the specific blocking of leptin activity with Ob-R:Fc treatment abrogates this effect. These results demonstrate a trophic effect of leptin signaling on the survival of developing NK cells in mouse BM. Importantly, leptin has been shown to exert protective effect on starvation-induced apoptosis of T lymphocytes *via* up-regulation of anti-apoptotic Bcl-xL gene (8, 17) as well as both spontaneous and induced apoptosis of DCs (18). Our current findings that *db/db* NK cells display markedly downregulated Bcl-2 expression with upregulated Bax transcripts further suggest that leptin signaling promotes NK cell survival possibly *via* modulating Bcl-2 family gene expression, consistent with previous findings that both leptin and leptin receptor activation can promote the survival of DCs and neutrophils associated with upregulated Bcl-2 gene expression (18, 19). Our results support the established notion that lymphopoiesis is a tightly regulated process which requires a delicate balance between cell production by proliferation and cell death by apoptosis (20). In our previous studies, DCs from *db/db* mice show increased apoptosis and display significantly downregulated activities of the PI3K/Akt pathway (11). As activated PI3K/Akt pathway induces Bcl-2 gene expression, further studies can help to delineate the leptin-activated signaling pathways underlying the enhanced survival of NK cells. It has been well recognized that NK cell development in BM relies on the expression of transcription factors including Id2 (5), Ets-1 (4), IRF-1 (21), and IRF-2 (22), and cytokines such as BM-derived IL-15 (6), in addition to interaction with the BM stroma (23). Since there is evidence that leptin deficiency reduces IL-15 expression in hepatic Kupffer cells and leads to NK cell depletion in *ob/ob* livers (24), further studies are warranted to examine whether *db/db* BM produces reduced level of IL-15 that contributes to impaired NK cell development. Nevertheless, our current data suggest that the previously observed NK cell reduction in the spleen of *db/db* mice might be possibly attributed at least in part to the impaired NK cell development in BM rather than selective reduction of mature splenic NK cells in the peripheral lymphoid system (13).

Interestingly, *db/db* NK cells show no obvious change in the levels of perforin and granzyme B gene expression (Figure 2C), indicating that leptin-receptor deficiency may not affect the intrinsic NK cytotoxicity. These results are in

agreement with previous findings that the basal levels of splenic NK cell cytotoxicity in *db/db* mice did not differ significantly from wild-type controls although markedly retarded cytotoxic function was observed in poly I:C-activated *db/db* NK cells (14). We have previously observed that DCs derived from *db/db* mouse BM displayed markedly impaired immune functions with a Th-2 type cytokine profile (11). In this study, we have found that IL-10 expression in freshly-isolated *db/db* NK cells is significantly increased whereas levels of other cytokine genes do not show any significant changes, which is further supported by findings from *in vitro* cultures of *db/db* NK cells on their cytokine secretion (Figure 4). IL-10 is known to suppress the antigen-dependent proliferation of T cells and inhibit DC-derived inflammatory mediators (25, 26). Although it remains currently unclear about the functional implication of leptin-signaling deficiency in skewing the NK cell cytokine profile toward a Th-2 phenotype, our findings suggest that IL-10-producting NK cells may provide a feedback mechanism for modulating immune responses (27, 28).

It has become clear that certain cytokines are critically involved in regulating the generation of NK cells because mice deficient in the common cytokine receptor γ chain for IL-2, IL-4, IL-7 and IL-15 are devoid of peripheral NK cells (29). Normal NK cell development depends upon functional interactions between hematopoietic progenitors and BM microenvironment *via* both adhesion molecules and soluble growth factors. Our current data clearly demonstrate that leptin signaling is important for the generation of developing NK cells in the BM. With the increasing efforts to explore leptin as a therapeutic option (10, 30), it will be of interest to investigate whether the modulation of leptin signaling in mature NK cells toward a polarized Th2 phenotype has potential implications in immunotherapy.

Acknowledgement

This work was supported by a grant to L Lu from the Research Grants Council of Hong Kong.

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