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Differential Effects of Vitamin D Supplementation on Natural Killer Cell Activity in Normal and Obese Mice

비타민 D의 보충이 자연살해세포의 기능에 미치는 영향: 비만 여부에 따른 차별적 효과

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Abstract

Differential Effects of Vitamin D Supplementation on Natural Killer Cell Activity in Normal and Obese Mice

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Vitamin D has immunoregulatory effects on both innate and adaptive immunity. Vitamin D has been reported to induce differentiation of monocytes into macrophages, inhibit maturation and differentiation of dendritic cells, and suppress adaptive immunity. There have been in vitro studies showing contradictory results regarding the role of vitamin D on NK cell functions, but little is known about this in vivo. The present study investigated whether vitamin D supplementation (50, 1000, or 10000 IU/kg of diet: DD, DC, or DS) affects NK cell functions in mice fed a control or high fat diet (10% or 45% kcal fat: CD or HFD) for 12 weeks. NK cell activity was assessed using radioisotope \( ^{51} \)Cr release assay against YAC-1 target cells and splenocyte subpopulation was measured by FACS analysis. Intracellular expression of IFN-\( \gamma \) by NK cells, CD4\(^+\) T cells, and CD8\(^+\) T
cells, and surface expression of NKG2D and CD107a were determined by FACS analysis upon stimulation of splenocytes with PMA (50 ng/mL)/ionomycin (0.5 μM) for 4 hours. The splenic NK cell activity was significantly higher in the CD-DS group than the HFD-DS group, and the CD-DS group showed significantly higher NK cell activity compared with the CD-DD and CD-DC groups, but no difference in NK cell activity was observed among the HFD groups fed different vitamin D levels. Of note, the splenic population of NK cells was significantly higher in the CD-DS group than the HFD-DS group. However, no significant differences were observed in the intracellular expression of IFN-γ and the surface expression of NKG2D and CD107a in NK cells by both dietary fat and vitamin D content. The splenic mRNA expression of Ifng and Ccl5 were significantly lower in the HFD groups compared with the CD groups, but there was no difference in the mRNA levels of Vdup1 and Vdr among the groups. Taken together, these results suggest that dietary vitamin D supplementation can modulate innate immunity by increasing NK cell activity in control mice but not in obese mice, which is presumably mediated through alternation of the splenic NK cell population.

KEY WORDS: NK cell, vitamin D, obesity, IFN-γ, CD107a

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Contents

Abstract --------------------------------------------------------------- i

Contents --------------------------------------------------------------- iii

List of Tables ---------------------------------------------------------- vi

List of Figures ---------------------------------------------------------- vii

List of Abbreviations ---------------------------------------------------- ix

I . Introduction ---------------------------------------------------------- 1

II. Literature Review

1. NK cells --------------------------------------------------------------- 4

1-1. NK cell development

1-2. NK cell receptors

1-3. Cytokine-induced activation and cytokine production by NK cells

1-4. NK cell-mediated killing mechanisms

1) Cell apoptosis mediated by secretion of cytotoxic granules

2) Fas/Fas ligand-mediated apoptosis

1-5. NK cells and adaptive immunity

2. Obesity and NK cells -------------------------------------------------- 13

3. Vitamin D and NK cells ----------------------------------------------- 15
Ⅲ. Materials and Methods

1. Animals  
2. Diets  
3. Methods  
   3-1. Tissue collection  
   3-2. Splenocyte isolation  
   3-3. NK cell activity assay  
   3-4. Serum leptin measurement  
   3-5. FACS analysis of subpopulation of splenocytes  
   3-6. FACS analysis of surface expression of NKG2D and CD107a, and intracellular expression of IFN-γ  
   3-7. Quantitative real-time PCR analysis  

4. Statistical analysis

Ⅳ. Results

1. Body weight, weight change, body fat, and dietary intake  
2. Effects of vitamin D and obesity on NK cell activity  
3. Effects of vitamin D and obesity on the subpopulation of splenocytes  
4. Effects of vitamin D and obesity on serum leptin levels  
5. Effects of vitamin D and obesity on the surface expression of NKG2D and CD107a, and intracellular expression of IFN-γ by
6. Effects of vitamin D and obesity on the surface expression of NKG2D and CD107a, and intracellular expression of IFN-γ by T cells

7. Effects of vitamin D and obesity on the expression of genes involved in NK cell functions

8. Effects of vitamin D and obesity on the expression of genes involved in vitamin D metabolism

V. Discussion

VI. Summary

VII. References

국문초록
List of Tables

Table 1. Activating and inhibitory NK cell receptors, and their ligands in mice and human ----------------------------------- 7

Table 2. Composition of the experimental diets -------------------------- 20

Table 3. Primer sequences used in real time PCR ----------------------- 29

Table 4. Body weight, weight change, body fat, and dietary intake of the mice ----------------------------------------------- 32
List of Figures

Figure 1. The experimental design ----------------------------- 18

Figure 2. Effects of vitamin D and obesity on NK cell activity------34

Figure 3. The subpopulation of splenocytes of mice fed with 6
different diets -----------------------------------------------37

Figure 4. Correlations between NK cell activities and splenic NK
cell populations -----------------------------------------------39

Figure 5. Effects of vitamin D and obesity on serum leptin
concentrations -----------------------------------------------41

Figure 6. Effects of vitamin D and obesity on the intracellular
expression of IFN-γ, and extracellular expression of
NKG2D and CD107a by NK cells ----------------------------------43

Figure 7. Effects of vitamin D and obesity on the intracellular
expression of IFN-γ, and extracellular expression of
NKG2D and CD107a by T cells ----------------------------------45

Figure 8. Effects of vitamin D and obesity on mRNA levels of NK
cell-related markers (Ifng, Il12b, Il15, Il18, Ccl5) -----------47

Figure 9. Effects of vitamin D and obesity on mRNA level of
vitamin D-related markers (Vdr, Vdup1)-------------------------49

Figure 10. Differential effects of vitamin D supplementation on NK
activity in control and obese mice-------------------------
List of Abbreviations

1,25-\((\text{OH})_2\)D₃, 1,25-dihydroxyvitamin D

25-(OH)D, 25-hydroxyvitamin D

Abs, antibodies

CD, control diet

FACS, fluorescence-activated cell sorting

HFD, high fat diet

IFN-\(\gamma\), Interferon-\(\gamma\)

JAK, Janus Kinase

LAK cells, lymphokine-activated killer cells

LSD, least significant difference

NK cells, natural killer cells

PBMCs, peripheral blood mononuclear cells

STAT, signal transducer and activator of transcription

TNF, tumor necrosis factor

VDR, vitamin D receptor

VDUP1, vitamin D3 up-regulated protein 1

WAT, white adipose tissue
I. Introduction

Natural killer (NK) cells are a subset of lymphocytes that are important in host defense during the early stage of cancer development, metastasis, or virus infection (Moretta et al. 1992; Lanier 1998; Vivier et al. 2008). Upon activation through surface receptors such as NKG2D, NK cells release cytotoxic granules and secrete cytokines such as Interferon-γ (IFN-γ) that can induce target cell death (Moretta et al. 2002; Vivier et al. 2011b; Vivier et al. 2012).

Obesity is known to be associated with immune dysfunction (Krishnan et al. 1982; Lamas et al. 2004), and impaired NK cell function with obesity has been reported in both animal and human (Jeffery et al. 1997; Smith et al. 2007; J. et al. 2015). With obesity, decreased NK cell numbers and activity (O'Shea et al. 2010; Laue et al. 2015), and downregulation of the expression of activating receptors (Nave et al. 2008) have been reported. Although the exact mechanisms for the decline of NK cell functions remain unclear, it has been suggested that obesity-induced alternation of circulating levels of adipokines such as leptin and adiponectin can affect NK cell functionality (Wrann et al. 2012; Hubner et al. 2013). In obese individuals, plasma leptin concentration is higher than in control weight subjects (Caro et al. 1996). While leptin receptor Ob-Rb, which mediates the leptin signal pathway, is expressed by immune cells including NK cells, the expression of leptin receptors and Janus kinase (JAK) and signal transducer and
activator of transcription (STAT) post-receptor transduction has been found to be impaired in obese individuals (Nave et al. 2008), indicating NK cell dysfunction in obesity.

Vitamin D has been shown to affect both adaptive and innate immunity (Mora et al. 2008). Mice lacking vitamin D3 upregulated protein 1 (VDUP1) showed reduced NK cytotoxicity and number of CD3\(^+\)NK1.1\(^+\) cells (Lee et al. 2005), suggesting that vitamin D is critical for NK cell functions. However, the relationship between VDUP1 and NK cells has still not been completely uncovered. With in vitro treatment of 1,25-dihydroxyvitamin D \([1,25-(OH)\_2D\_3]\), enhanced lytic activity of NK cells has been observed against target tumor cells (Al-Jaderi et al. 2013). Additionally, inhibitory effects of vitamin D on NK cell activity and lymphokine-activated killer (LAK) cellular differentiation have been reported as well (Leung 1989; Weeres et al. 2014). Overall, the effects of vitamin D on NK cell activity are not well characterized and still remain contradictory. While, most of the previous studies have focused on the effect of in vitro vitamin D treatment on the functions of NK cells derived from human peripheral blood, few studies have been conducted to examine the in vivo effect of vitamin D on NK cell function.

Abnormality of serum vitamin D levels and dysfunction of vitamin D metabolism have been reported in obesity (Wortsman et al. 2000; Konradsen et al. 2005).
A number of clinical studies have shown that serum 25-hydroxyvitamin D [25-(OH)D] concentration (the major circulating form of vitamin D) is lower in obese individuals (Earthman et al. 2012), and body mass index has an inverse correlation with serum 25-(OH)D concentration (Konradsen et al. 2008). It has been proposed that this is partially due to decreased bioavailability of vitamin D and greater sequestration in adipose tissue (Wortsman et al. 2000; Ding et al. 2012). Both obesity and vitamin D can affect NK cell functions and vitamin D levels have been shown to be altered with obesity, therefore, there is a possibility that the differential impact of vitamin D on NK cell activity is observed with obesity.

Given the immunomodulatory capacity of vitamin D, the present study investigated the effect of in vivo vitamin D supplementation on NK cell function by measuring NK cell activity as well as expression of cytokine and activating receptor, and degranulation capacity. Effects of dietary supplementation of vitamin D on NK functions was evaluated in both control and high-fat diet (HFD)-induced obese mice.
II. Literature review

1. NK cells

Naturel killer (NK) cells are a subset of large granular lymphocyte that play a key role in innate immune system by exerting effector functions against various tumors and virus infections (Vivier et al. 2008). NK cells recognize infected cells which have altered or absent MHC class I molecules and directly induce killing of transformed cells such as tumor cells or virus-infected cells without antigen-specific immunization (Vivier et al. 2011a). This suggest the importance of NK cells in the host’s immune system in the early stage of cancer development and metastasis or virus infection (Moretta et al. 1992; Lanier 1998).

1-1. NK cell development

NK cell development occurs mainly in the BM from the common lymphoid progenitor cells with T cells and B cells (Lumeng 2012; Murphy 2012). NK cell commitment takes place through upregulation of CD122 and NK1.1 in B6 mice (Kim et al. 2002) and interactions with stromal cells situated in the BM regulate gene expression of surface molecules such as cytokine receptors, integrins, and a family of NK cell receptors (Chiossone et al. 2009).

NK cell maturation can be determined by surface phenotype as well as functional capacity (Clinthorne et al. 2013). Following acquisition of CD49 (DX5) in the early maturation, NK cells express CD11b and CD43, which are strongly
associated with the capacity to secrete IFN-γ (Effros et al. 1991). After maturation, NK cells emigrate from BM to peripheral tissues via the blood and are distributed to various lymphoid organs, such as spleen and LN, and non-lymphoid organs, including liver and lung, and peripheral blood (Hayakawa et al. 2006b). In the peripheral tissues, NK cells continue maturation to adapt the environment by upregulating killer cell lectin-like receptor G1 (KLRG1) and downregulating CD27 and TRAIL (Hayakawa et al. 2006b). By applying the marker CD27, NK cells can be further divided into 3 subsets in mice from early mature NK cells CD27+CD11b− NK cells to CD27+CD11b+ NK cells, followed by CD27−CD11b+ NK cells (Clinthorne et al. 2013).

1-2. NK cell receptors

NK cells have two distinct kinds of receptors, activating and inhibitory receptors, which engage in regulation of NK cell effector functions (Vivier et al. 2008). NK cell activating and inhibitory receptors and their ligands present in mice and humans are shown in the Table 1. By integrating the signals from the activating and inhibitory receptors, NK cells regulate their functions against target cells.

NK cell activation takes places after recognition of alternations in glycoprotein compositions from the cell surface (Murphy 2012). The triggering of activating receptors is accompanied by reduced signaling through inhibitory receptors and
this induces target cell death making it vulnerable to attack by NK cells (Smyth et al. 2005). The typical activation receptor is NKG2D that is overexpressed upon cellular stress or infection status, while expression is remained at low levels in normal conditions (Vivier et al. 2008; Raulet et al. 2009).

On the contrary, after identification of ‘self’, bearing normal levels of MHC class I molecules, NK cell activation is inhibited following recognition of negative signals through protein tyrosine phosphatases (Murphy 2012).
Table 1. Activating and inhibitory NK cell receptors, and their ligands in mice and human (Vivier et al. 2008)

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptors</strong></td>
<td><strong>Ligands</strong></td>
</tr>
<tr>
<td>CS1 (CRACC, CD319)</td>
<td>CD1 (CRACC, CD319)</td>
</tr>
<tr>
<td>αβ integrin</td>
<td>VCAM-1 (CD106)</td>
</tr>
<tr>
<td>β2 integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18)</td>
<td>ICAM-1 (CD54)</td>
</tr>
<tr>
<td>CD226 (DNAM-1)</td>
<td>CD112 (Nectin-2), CD155 (Necl-5)</td>
</tr>
<tr>
<td>CRTAM</td>
<td>Necl2</td>
</tr>
<tr>
<td>CD27</td>
<td>CD70</td>
</tr>
<tr>
<td>CD16</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>NKP46</td>
<td>Viral hemagglutinins, ?</td>
</tr>
<tr>
<td>KIR-S</td>
<td>HLA-C, ?</td>
</tr>
<tr>
<td>CD94 NKG2C</td>
<td>HLA-E</td>
</tr>
<tr>
<td>CD94 NKG2E</td>
<td>HLA-E</td>
</tr>
<tr>
<td>NKG2D</td>
<td>ULBP (RAET), MICA, MICB</td>
</tr>
<tr>
<td>NTB-A</td>
<td>NTB-A</td>
</tr>
<tr>
<td>PEN-5</td>
<td>L-selectin</td>
</tr>
<tr>
<td>CD96 (Tacile)</td>
<td>CD155 (Necl5)</td>
</tr>
<tr>
<td>Nkp80</td>
<td>ALCL</td>
</tr>
<tr>
<td>CD100</td>
<td>CD72</td>
</tr>
<tr>
<td>Nkp30</td>
<td>Pp65, BAT-3, ?</td>
</tr>
<tr>
<td>Nkp44</td>
<td>Viral hemagglutinins, ?</td>
</tr>
<tr>
<td>CEACAM1 (CD66)</td>
<td>CEACAM1 (CD66)</td>
</tr>
<tr>
<td>CD160 (BY55)</td>
<td>HLA-C</td>
</tr>
<tr>
<td><strong>Activating receptors</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Inhibitory receptors</strong></td>
<td></td>
</tr>
<tr>
<td>KIR-L</td>
<td>HLA-C,B and A</td>
</tr>
<tr>
<td>LAIR-1</td>
<td>Collagen</td>
</tr>
<tr>
<td>CD94-NKG2A</td>
<td>HLA-E</td>
</tr>
<tr>
<td>SIGLEC 3,7,9</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Cadherins</td>
</tr>
<tr>
<td>NKR-P1A</td>
<td>LLT-1</td>
</tr>
<tr>
<td>LILRB1 (CD85j, ILT2)</td>
<td>HLA class I</td>
</tr>
<tr>
<td>CD244 (2B4)</td>
<td>CD48</td>
</tr>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Cytokine-induced activation and cytokine production by NK cells

Cytokines play crucial roles in NK cell activation. Interferons or cytokines derived from macrophages are released upon viral infection and tumor cells and function as activator of NK cells (Murphy 2012). It is shown that cytolytic activity and IFN-γ production of NK cells are affected by a variety of cytokines and chemokines (Vivier et al. 2012). Cytokines related with NK cell activation are IL-2, IL-12, IL-15, IL-18, IFN-α, IFN-β and CCL-5 (Biron et al. 1999; Smyth et al. 2005).

Conversely, activated NK cells produce various cytokines, including IFN-γ, tumor necrosis factor (TNF)-α, IL-13, and IL-10 in response to stimulation (Hayakawa et al. 2006a). In addition, chemokines such as CCL2, CCL3, CCL4, CCL5 are known to be secreted by NK cells (Walzer et al. 2005), which can influence cytolytic activity of NK cells (Robertson 2002).
1-4. NK cell-mediated killing mechanisms

There are diverse mechanisms that NK cells induce target cells death. Upon activation through the integration of signals from activating and inhibitory receptors, NK cells kill targets by releasing cytotoxic granules and by interacting between Fas and Fas ligand (Arase et al. 1995; Lee et al. 1996; Moretta et al. 2002).

1) Cell apoptosis mediated by secretion of cytotoxic granules

The major NK cell-mediated killing mechanism is by exocytosis of cytotoxic granules containing perforin, granzymes, and granulysin (Okada et al. 2003; Li et al. 2008). After target cell recognition, NK cells bound via surface receptors, and their contents penetrate the cell membrane of target cells to induce programmed cell apoptosis (Murphy 2012).

The representative cytotoxic effector proteins are perforin and granzyme. Perforin creates pore in target cells to disturb endosomal trafficking (Smyth et al. 2005) and assist penetration of cytotoxic granules such as granzyme B into the cytoplasm of target cells (Murphy 2012). Granzymes are a kind of serine proteases that trigger target cell apoptosis (Murphy 2012). Once they enter into the cytoplasm of the targets, target cell death is induced by the release of cytochrome c from disturbed mitochondria, DNA degradation following activation of caspase 3, and by caspase independent pathway (Smyth et al. 2005;
Vivier et al. 2011a; Murphy 2012).

When lytic granules including granzymes and perforins are released from effector cells, a degranulation marker CD107a appears on the cell surface (Bae et al. 2013), indicating that increased expression of CD107a reflects secretion of granzyme and perforin (Diefenbach et al. 2000).

CD107a is a surface marker of degranulation of NK cells as well as cytotoxic T cells, and is significantly up-regulated on the surface following stimulation with MHC-deprived target cells and with PMA/Ionomycin (Alter et al. 2004). There are some studies which presented that CD107a is a functional marker of NK cell activity, but the precise role of CD107a in NK cell biology still remains unclear, suggesting the need for further studies.

2) Fas/Fas ligand-mediated apoptosis

Another well-known mechanism of target cell death by NK cells is through cell membrane-associated Fas (CD95)-FasL (CD178) interaction (Arase et al. 1995). Fas is a member of TNF superfamily that is expressed in a surface membrane of cytotoxic lymphocytes including NK cells and cytotoxic T cells (Suds et al. 1993). FAS ligands are expressed by NK cells in response to tumor target cells and binding of FAS ligand (CD178) to FAS (CD95) creates signal which induces death of target cells (Smyth et al. 2005). After binding of Fas ligand to the death receptor Fas, caspases activation is induced, which initiates apoptotic
mechanisms in CD95-expressing target cell (Okamoto *et al.* 2000; Scott *et al.* 2009).
NK cells and adaptive immunity

NK cell functions are not confined to cytolytic effector capacity, but they also act as regulatory cells interacting with other innate immune cells, including DC, macrophage, and neutrophils (Moretta et al. 2005). IFN-γ and TNF produced by NK cells are shown to promote maturation of DCs and their production of IL-12, which in turn activates NK cells (Walzer et al. 2005).

NK cells are also act as an important connection between innate and adaptive immune responses. Interaction between NK cells and antigen-presenting cells, particularly DCs, regulates adaptive immune responses (Moretta 2002) by enhancing antigen presentation to cytotoxic T cells. In addition, a large amount of cytokines and chemokines secreted by NK cells in response to target cells can form and induce adaptive immune response (Moretta 2002). Priming of CD4+ T helper type 1 cells also can be induced by IFN-γ, a representative cytokine produced by activated NK cells (Krebs et al. 2009). Above this, NK cell-mediated killing impacts T cell responses by reducing antigenic load, whereas target cell debris produced by NK cell-mediated killing might promote antigen cross-presentation to cytotoxic T cells (Robbins et al. 2007).
2. Obesity and NK cells

A number of studies have shown that immune dysfunction is observed in obesity (Lamas et al. 2004; Mancuso et al. 2006). A decreased circulating number of NK cells and impairment of their function have been reported in obese subjects (Lynch et al. 2008).

Obesity with excessive body fat mass is known to lead to an elevation of serum adipokines, such as leptin and adiponectin (Krishnan et al. 1982; Hubner et al. 2013). The alternation of adipokine levels can modulate NK cell function including NK cell activity (Hubner et al. 2013; Sabrina Wilk et al. 2013). It is suggested that the alternation of adipokine levels might be due to decreased activation of post-receptor signaling components such as JAK-2 (Nave et al. 2008; Laue et al. 2015). Since obesity is related with immune dysfunction, it is convincing that obese individuals are susceptible to infection of pathogens. This suggest the importance of NK cells in obesity in consideration that NK cells are the first-line in immune defensive system mobilized during an infection and tumor genesis (Smith et al. 2007).

Decreased NK cell activity and intracellular expression of IFN-γ in NK cells of obese human were significantly improved after body fat mass reduction by decreasing daily energy intake and adjusting appropriate exercise program (J. et al. 2015). In addition, reversibility of NK cell activity was observed after body
weight and fat mass reduction through bariatric surgery (Moulin et al. 2011).

Nevertheless, studies on the change of NK cell function in obesity have been reported to be contradictory and tissue dependent. NK cell numbers in visceral fat are shown to be decreased or unchanged with obesity (S. Caspar-Bauguila et al. 2005; Carine Duffaut et al. 2009). However, the cytotoxicity of NK cells in the splenocytes was decreased but the difference were not significant (Zhigang Tian et al. 2002).
3. Vitamin D and NK cells

Vitamin D performs its function by the interaction with vitamin D receptor (VDR) acting as a transcription factor in many target cells (Ding et al. 2012). Since VDR is found in variety of cells including immune systems, there have been efforts to examine the biological role of vitamin D on the immune system. It was demonstrated that vitamin D has an impact on both innate and adaptive immunity (Mora et al. 2008). 1,25(OH)_{2}D_{3} has been shown to have an inhibitory effect in the maturation and differentiation of DC (Etten et al. 2005) and a regulating effect in adaptive immunity by suppressing induction of Th1 cytokine from T cells, particularly IFN-γ, while promoting Th2 immune responses by downregulating expression of IFN-γ expression through increasing IL-4 production (Mora et al. 2008).

However, despite the importance of NK cells as the first-line of defense cells, effects of vitamin D on NK cells have not been studied well and conflicting reports are found.

It is reported that NK cell functions are shown to be impaired by in vitro treatment of vitamin D. NK cell activity from CD16^{+} peripheral blood NK cells are reported to be inhibited in a time- and dose-dependent manner by 1,25(OH)_{2}D_{3} (Merino et al. 1989). In addition, there was a report that 1,25(OH)_{2}D_{3} inhibited NK cell activity and both IFN-γ and IL-2 activation of NK
cell activity (Leung 1989). 1,25(OH)_{2}D_{3} also showed a decrease in cytotoxicity of NK cells derived from purified hematopoietic stem cells and in LAK cellular differentiation (Leung 1989; Weeres et al. 2014).

On the other hand, there are observations which report stimulatory effect of *in vitro*-treated vitamin D on NK cells. It was reported that NK cell-mediated cytotoxicity was enhanced by 1,25(OH)_{2}D_{3} treatment from control subjects (Quesada et al. 1995). There was a study that the cytotoxicity of lymphokine-activated killer (LAK) cells was increased by 1,25(OH)_{2}D_{3} (Ravid et al. 1993). Moreover, patients with chronic renal failure accompanied by defective NK cell-mediated activity was corrected by 1,25(OH)_{2}D_{3} administration (Quesada et al. 1995).

Overall, the relationship between NK cells and vitamin D still remains inconclusive, suggesting the need for further investigation.
Ⅲ. Materials and methods

1. Animals

Five week-old male C57BL/6 mice (Central Animal Laboratory, Seoul, Korea) were housed in an specific pathogen free room under controlled temperature (23 ± 1°C), relative humidity (50 ± 10%), and light/dark cycle (12-h dark/12-h light 6:00AM-6:00PM). After 5 days of acclimation on the control diet, mice were randomly divided into 6 groups and fed diets differing in fat amount and vitamin D content for 12 weeks.

The experimental design and breeding process were shown briefly in Figure 1. Dietary intake was measured 4 times per week and body weight was measured once a week. At the end of the experimental period, mice were fasted for 12 h and euthanized by CO₂ asphyxiation. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (approval No. SNU-160329-1).
Figure 1. The experimental design

- **Control diet**
  - CD-DD group (n=8): 10% kcal fat + 50 IU vitamin D/kg diet
  - CD-DC group (n=8): 10% kcal fat + 1000 IU vitamin D/kg diet
  - CD-DS group (n=7): 10% kcal fat + 10000 IU vitamin D/kg diet

- **High fat diet**
  - HFD-DD group (n=8): 45% kcal fat + 50 IU vitamin D/kg of diet
  - HFD-DC group (n=8): 45% kcal fat + 1000 IU vitamin D/kg of diet
  - HFD-DS group (n=8): 45% kcal fat + 10000 IU vitamin D/kg of diet

Feeding period

C57BL/6 male mice
n=47

Group assignment

5 days of acclimation

Euthanization

0 week

12 week
2. Diets

There were six types of experimental diets differing in fat amount (10% or 45% kcal fat, CD or HFD) and vitamin D content (50, 1000, or 10000 IU/kg of diet, DD, DC, or DS, respectively) (CD-DD, #119320; CD-DC, #103816; CD-DS, #119334; HFD-DD, #119318; HFD-DC, #103818; HFD-DS, #119333; Dyets, Inc., Bethlehem, PA, USA). The specific composition of diets is shown in Table 2. All diets were stored at 4°C and provided as solid pellet form. Animals were fed experimental diets *ad libitum* for 12 weeks and were allowed free access to distilled water.
Table 2. Composition of the experimental diets

<table>
<thead>
<tr>
<th></th>
<th>CD-DD (10% kcal fat + 50 IU/kg of diet)</th>
<th>CD-DC (10% kcal fat + 1000 IU/kg of diet)</th>
<th>CD-DS (10% kcal fat + 10000 IU/kg of diet)</th>
<th>HFD-DD (45% kcal fat + 50 IU/kg of diet)</th>
<th>HFD-DC (45% kcal fat + 1000 IU/kg of diet)</th>
<th>HFD-DS (45% kcal fat + 10000 IU/kg of diet)</th>
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<td>Vitamin Mixc (g)</td>
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<td>Vit D3 Premix (100IU/g)</td>
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<td>Choline Bitartrate (g)</td>
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<td>Total (g)</td>
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<td>Kcal/g of diet</td>
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aResource: Dyets, Inc., Bethlehem, PA, USA
b35 g of mineral mix (Dyets, #200000) provides 5.2 g calcium, 4 g phosphorus, 3.6 g potassium, 1 g sodium, 1.6 g chloride, 0.3 g sulfur, 0.5 g magnesium, 35 mg iron, 6 mg copper, 54 mg manganese, 30 mg zinc, 2 mg chromium, 0.2 mg iodine, 0.1 mg selenium, and 4.2 g sucrose.
c10 g of vitamin mix (Dyets, #300050) provides 4000 IU vitamin A, 1000 IU vitamin D3, 50 IU vitamin E, 30 mg niacin, 16 mg pantothenic acid, 7 mg vitamin B6, 6 mg vitamin B1, 6 mg vitamin B2, 2mg folic acid, 0.8 mg menadione, 0.2 mg biotin, 10 μg vitamin B12, and 9.8 g sucrose.
3. Methods

3-1. Tissue collection

After euthanizing mice by CO2 asphyxiation, blood samples were collected by cardiac puncture and serum was isolated by centrifugation at 2000 rpm for 20 min, after which serum was stored at –80°C following coagulation at room temperature for 2 hrs. White adipose tissue (WAT), including perirenal, intraperitoneal, epididymal, and subcutaneous fat were collected, weighed, and stored at –80°C. Spleen was dissected out and splenocytes were isolated.
3-2. Splenocyte isolation

After aseptic removal, spleen was put into sterile RPMI 1640 (Lonza, walkersville, MD, USA) medium supplemented with 100,000 U/L of penicillin (GibcoBRL, Grand Island, NY, USA), 100 mg/L of streptomycin (GibcoBRL), 25 mmol/L of HEPES (Sigma Aldrich, St. Louis, MO, USA) and 2 mmol/L of L-glutamin (GibcoBRL) (complete RPMI). A single-cell suspension of splenocytes was prepared by homogenization of the spleen with sterile frosted glass slides. Splenocytes were separated via centrifugation (700 rpm) to remove tissue debris, and red blood cells were lysed using Gey’s solution. Isolated splenocytes were washed twice and their viability was determined by the trypan blue exclusion test, after which they were suspended in complete RPMI medium supplemented with 10% FBS at $1 \times 10^7$ cells/mL for further analysis.
3-3. NK cell activity assay

NK cell activity was measured using the radioisotope $^{51}$Cr release assay against YAC-1 target cells. YAC-1 cells (a molony leukemia virus-induced murine lymphoma cell line) were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were incubated with $^{51}$Cr (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) (25 μCi /10^6 cells) for 90 min at 37°C and 5% CO₂ by gently swirling every 15 min, and were washed twice and incubated for 30 min at 37°C. The labeled YAC-1 cells were added to diluted splenocytes in a 96-well round bottom plate to make final effector cell:target cell (E:T) ratios of 100:1, 50:1, 25:1, and 12.5:1. Spontaneous release (SR) was measured by the amount of $^{51}$Cr released from the target cells alone and maximum release (MR) was measured as the amount of $^{51}$Cr after incubation with 5% Triton-X solution. 100 μL of cell supernatant was collected and radioactivity was measured with an automatic gamma counter (2470 WIZARD 2, Perkin Elmer, Shelton, CT, USA). NK cell activity was shown as lysis percentage: percent specific release = (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100.
3-4. Serum leptin measurement

Serum leptin concentrations were measured by enzyme linked immunosorbent assay (Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.
3-5. FACS analysis of subpopulation of splenocytes

For the analysis of the splenocyte subpopulations, isolated splenocytes were resuspended in fluorescence-activated cell sorting (FACS)-staining buffer (0.09% sodium azide, 1% FBS, 1 x PBS based) at a concentration of 1 x 10^7 cells/mL. A total of 2 x 10^6 cells/sample were stained with antibodies (Abs) against cell-surface markers for 30 min at 4°C. The Abs used for staining the subpopulations were as follows: FITC-conjugated anti-mouse CD3 Molecular Complex, PE-conjugated anti-mouse NK-1.1, APC-conjugated anti-mouse CD11b, APC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD19, and PE-conjugated anti-mouse F4/80, all from BD Pharmingen, San Diego, CA, USA. After incubation, cells were washed and resuspended in FACS-staining buffer and then analyzed using FACSCalibur II (BD Biosciences, SA, USA) and FlowJo software version 10 (Tree Star Inc., Ashland, OR).
3-6. Surface expressions of NKG2D and CD107a, and intracellular expression of IFN-γ

To analyze the surface expression of NKG2D and CD107a and the intracellular expression of IFN-γ, splenocytes were stimulated in complete medium with 50 ng/mL of PMA (Sigma Aldrich) and 0.5 μM of ionomycin (Sigma Aldrich) for 4 h in the presence of BD GolgiStop™ protein transport inhibitor containing monensin (BD Biosciences). Cells were harvested and resuspended in FACS-staining buffer, and a total of $2 \times 10^6$ cells/sample were labeled with surface-staining Abs, which were as follows: FITC-conjugated anti-mouse CD3 Molecular complex, APC-conjugated anti-mouse NK-1.1, APC-conjugated anti-mouse CD4, and PE-conjugated anti-mouse CD8a, all from BD Pharmingen. Cells were incubated at 4°C for 30 min with the surface-staining Abs, fixed, then permeabilized with BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit. Cells were stained with Abs or corresponding isotype-matched Abs, which were as follows: PE-conjugated Rat IgG1, κ isotype control; PE-conjugated Rat IgG2a, κ isotype control; PE-conjugated anti-mouse IFN-γ; PE-conjugated anti-mouse CD314 (NKG2D); and PE-conjugated anti-mouse CD107a; all from BD Pharmingen, San Diego, CA, USA. After incubation at 4°C for 30 min, cells were fixed with fixer containing 4 % formaldehyde and washed to preserve them until analysis with FACSCalibur II and FlowJo software.
version 10 (Tree Star Inc).
3-7. Quantitative real-time PCR analysis

Splenocytes were used to extract total RNA with RNAiso Plus (Takara, Otsu, Shiga, Japan) following stimulation with 50 ng/mL of PMA (Sigma Aldrich) and 0.5 μM of ionomycin (Sigma Aldrich) for 4 h. RNA sample quality was tested with a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA) and concentration of the samples was determined using a spectrophotometer (DU530, BECKMAN, Fullerton, CA, USA) by measuring their absorbance at 260 and 280 nm. Isolated RNA was reverse transcribed into cDNA using PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio Inc., Otsu, Shiga, Japan) and a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was conducted with a StepOne™ Real-time PCR system (Applied Biosystems). Each PCR reaction mixture included reverse transcribed cDNA, SYBR Premix Ex Taq, ROX reference dye (Takara Bio Inc), and specific forward and reverse primers. Relative expression levels of the genes were calculated by the 2-ΔCt method and adjusted with Gapdh used as a housekeeping gene. Primer sequences used for RT-PCR are shown in Table 3.
Table 3. Primer sequences used in real time PCR

<table>
<thead>
<tr>
<th>GENE</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ifng</td>
<td>TGGACCTGTGGGTTGTTGAC</td>
<td>GAACTGGGAAAGGATGGTG</td>
</tr>
<tr>
<td>I112b</td>
<td>CGGGTCTGGTTGATGATGT</td>
<td>AGCAGTAGAGTTCCCCTGA</td>
</tr>
<tr>
<td>I115</td>
<td>GAAGGGCAACTGAGAGCAGA</td>
<td>TCTATGGGGGAAGCCAAACTG</td>
</tr>
<tr>
<td>I118</td>
<td>TGGAAATACAGGCCAGGTCA</td>
<td>TGCCAAAAGGAGATGATGC</td>
</tr>
<tr>
<td>Ccl5</td>
<td>CTTGAACCCACTTCTTCTCTTG</td>
<td>TGCTGCTTTGCTACCTC</td>
</tr>
<tr>
<td>Vdr</td>
<td>ATGTCCAGTGAGGGGTGTA</td>
<td>TGCTGAGGAGCAACAGCAC</td>
</tr>
<tr>
<td>Vdup1</td>
<td>TACTGATTGCCACCACATCTTG</td>
<td>CCCACCCACTACACTGAGG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GGAGAAACCTGCCAGTA</td>
<td>AAGAGTGGGAGTGGCTGTTG</td>
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</table>

*a* Ifng, interferon gamma; I112b, interleukin 12p40; I115, interleukin 15; I118, interleukin 18; Ccl5, chemokine ligand 5; Vdr, vitamin D receptor; Vdup1, vitamin D3 up-regulated protein 1; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
4. Statistical analysis

All statistical analyses were carried out using SPSS statistical software version 21.0 (IBM SPSS Statistics, Chicago, IL, USA). All data were represented as means ± SEM. Significant differences ($P < 0.05$) were determined using a two-way ANOVA test to evaluate the overall effect of vitamin D and fat amount, followed by Fisher’s least significant difference (LSD) post-hoc test to compare differences between the individual groups. Pearson’s correlation test was performed to analyze any correlations between the variables.
IV. Results

1. Body weight, weight change, body fat, and dietary intake

   After 12 weeks of feeding, body weight (P < 0.001), weight change (P < 0.001), and WAT weights (P < 0.001) were significantly higher in HFD groups compared with CD groups, but there was no significant effect of vitamin D. The average dietary intake for 12 weeks (g/day) was significantly lower in the HFD groups (P < 0.001), whereas the average energy intake was significantly higher in the HFD groups compared with the CD groups (P < 0.001) because of a higher percentage of fat amount in the high fat diet. However, no significant differences in food intake were observed regarding vitamin D levels (Table 4).
Table 4. Body weight, weight change, body fat, and dietary intake of the mice $^{a,b}$

<table>
<thead>
<tr>
<th></th>
<th>CD</th>
<th>HFD</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DD (n=8)</td>
<td>DC (n=8)</td>
<td>DS (n=7)</td>
</tr>
<tr>
<td><strong>Body weight at week 0 (g)</strong></td>
<td>21.0 ± 0.3</td>
<td>21.1 ± 0.5</td>
<td>20.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Body weight at week 12 (g)</strong></td>
<td>30.3 ± 0.9$^b$</td>
<td>31.0 ± 0.7$^b$</td>
<td>31.5 ± 0.6$^b$</td>
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<tr>
<td><strong>weight change (g)</strong></td>
<td>9.3 ± 1.0$^b$</td>
<td>9.9 ± 0.8$^b$</td>
<td>11.0 ± 0.5$^b$</td>
</tr>
<tr>
<td><strong>Average dietary intake for 12 week (g/d)</strong></td>
<td>2.94 ± 0.04$^{ab}$</td>
<td>2.96 ± 0.05$^{ab}$</td>
<td>3.11 ± 0.14$^a$</td>
</tr>
<tr>
<td><strong>Average energy intake for 12 week (kcal/d)</strong></td>
<td>10.9 ± 0.2$^c$</td>
<td>10.9 ± 0.2$^c$</td>
<td>11.5 ± 0.5$^{bc}$</td>
</tr>
<tr>
<td><strong>WAT (g)$^c$</strong></td>
<td>2.37 ± 0.20$^b$</td>
<td>2.36 ± 0.31$^b$</td>
<td>2.36 ± 0.34$^b$</td>
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</table>

$^a$Values are means ± SEM, n = 6 to 8 per group (n = 47)

$^b$Two-way ANOVA was used to determine the effect of fat and vitamin D intake. Different superscripts indicate significant difference ($P < 0.05$) by Fisher's LSD multiple comparison test.

$^c$WAT includes perirenal, intraperitoneal, epididymal, and subcutaneous fat.

CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
2. Effects of vitamin D and obesity on NK cell activity

There was an interaction between fat amount and vitamin D levels regarding NK cell activity at E:T ratios of 100:1 ($P = 0.006$) and 50:1 ($P = 0.033$). NK cell activity was significantly enhanced by vitamin D supplementation in the CD groups at E:T ratios of 100:1 ($P = 0.006$) and 50:1 ($P = 0.042$). However, these differences were not found in mice fed the HFD. The CD-DS group showed significantly higher NK cell activity compared with the HFD-DS group at E:T ratios of 100:1 ($P < 0.001$), 50:1 ($P < 0.001$), and 12.5:1 ($P = 0.036$), and tended to be higher at E:T ratios of 25:1 compared with the HFD-DS group ($P = 0.053$) (Figure 2).
Figure 2. Effects of vitamin D and obesity on NK cell activity

Values are means ± SEM, n = 6 to 8 per group. Means with different letters indicate significant differences (P < 0.05) by Fisher's LSD multiple comparison test.

CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
3. Effects of vitamin D and obesity on the splenocyte subpopulations

Percentages of total splenic NK cells (CD3−NK1.1+), immature NK cells (CD3−NK1.1+CD11b−), and mature NK cells (CD3−NK1.1+CD11b+) were significantly lower in the HFD groups compared with the CD groups ($P = 0.017$, $P = 0.001$ and $P = 0.001$, respectively), whereas there was no statistical difference regarding vitamin D levels. Mice in the CD-DS group had significantly higher percentages of CD11b− NK cells, CD11b+ NK cells, and total NK cells compared to mice in the HFD-DS group ($P = 0.006$, $P = 0.001$, $P < 0.001$, respectively) (Figure 3-A, C).

The percentages of other immune cells including NKT cells, CD4+ T cells, CD8+ T cells, and B cells were significantly different among the groups (Figure 3-A, B). There was an interaction between fat amount and vitamin D levels in the percentage of splenic NKT cells ($P = 0.045$) and the percentage of NKT cells in the spleen was significantly lower in the HFD groups compared with the CD groups. Furthermore, mice in the CD-DS group had a significantly higher percentage of NKT cells compared to mice in the HFD-DS group ($P = 0.002$). The percentages of CD4+ T ($P = 0.042$) and CD8+ T cells ($P = 0.001$) in the spleen were significantly lower in the HFD groups compared with the CD groups, while the percentage of B cells ($P < 0.001$) was significantly higher in HFD
groups compared with CD groups. There were no significant differences in percentages of splenic CD4$^+$ T, CD8$^+$ T, and B cells according to different vitamin D levels, and no significant difference in the percentage of splenic macrophages was observed regardless of different dietary treatments.

Positive correlations were observed between the percentage of total splenic NK cells and NK cell activity at E:T ratios of 100:1 ($r = 0.457, P = 0.002$) and 50:1 ($r = 0.417, P = 0.004$). The percentages of splenic CD11b$^+$ NK cells and NK cell activity at E:T ratios of 100:1 ($r = 0.514, P < 0.001$) and 50:1 ($r = 0.496, P = 0.001$) showed significantly positive correlations. In addition, the percentage of splenic CD11b$^-$ NK cells correlated positively with NK cell activity at E:T ratios of 100:1 ($r = 0.300, P = 0.045$) (Figure 4).
A

Percentage (%)

CD  HFD
NK cell  CD11b+ NK cell  CD11b+ NK cell  NKT cell  macrophage

Cell types

B

Percentage (%)

CD  HFD
CD4+ T cell  CD8+ T cell  B cell

Cell types
Figure 3. The subpopulation of splenocytes of mice fed with 6 different diets: (A) splenic subpopulations of CD11b⁺/CD11b⁻ NK cells (CD3⁺NK1.1⁺CD11b⁺/CD3⁺ NK1.1⁺CD11b⁻), total NK cells (sum of CD11b⁻ and CD11b⁺ NK cells), NKT cells (CD3⁺NK1.1⁺), and macrophage (F4/80⁺CD11b⁺) from C57BL6 mice fed with 6 different diets regarding fat amount and vitamin D content; (B) splenic subpopulations of CD4⁺ T cells (CD3⁺CD4⁺), CD8⁺ T cells (CD3⁺CD8⁺), and B cells (CD3⁻CD19⁺) from C57BL6 mice fed with 6 different diets according to fat amount and vitamin D content; (C) splenic subpopulations of CD11b⁺ NK cells and CD11b⁻ NK cells presented as dot plots of the FlowJo analyses.

Values are means ± SEM, n = 6 to 8 per group. Means with different letters indicate significant differences (P < 0.05) by Fisher's LSD multiple comparison test.

CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
Figure 4. Correlations between NK activities and splenic NK cell populations

Correlations between NK activities at E:T ratios of 100:1 and 50:1 and splenic percentage of total NK cells (A) and CD11b⁺ NK cells (B); correlation between NK activities at E:T ratio of 100:1 and CD11b⁻ NK cells (C).

Pearson’s correlation coefficient ($r$) and P-value are indicated for each region.

CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
4. Effects of vitamin D and obesity on serum leptin levels

Serum leptin concentrations were significantly higher in mice fed the HFD compared with those fed with control diets ($P < 0.001$). The level of vitamin D in the diet did not have a significant effect on leptin levels (Figure 5). Serum leptin level showed significant positive correlations with weight change ($r = 0.878, P < 0.001$) and WAT weight ($r = 0.939, P < 0.001$) (data not shown).
Figure 5. Effects of vitamin D and obesity on serum leptin concentrations

Values are means ± SEM, n = 6 per group. Means with different letters indicate significant differences (P < 0.05) by Fisher's LSD multiple comparison test.
CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
5. Effects of vitamin D and obesity on the surface expression of NKG2D and CD107a, and intracellular expression of IFN-γ by NK cells

To investigate the effect of obesity and vitamin D on NK cell functions, the surface expression levels of NKG2D and CD107a (a degranulation marker), and the intracellular expression level of IFN-γ by NK cells were determined. Neither fat amount nor vitamin D levels had a significant impact on the intracellular expression of IFN-γ and the surface expression of NKG2D and CD107a in NK cells (Figure 6).
Figure 6. Effects of vitamin D and obesity on the intracellular expression of IFN-γ, and extracellular expression of NKG2D and CD107a by NK cells
Values are means ± SEM, n = 6 to 8 per group.
CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
6. Effects of vitamin D and obesity on the surface expression of NKG2D and CD107a, and intracellular expression of IFN-γ by T cells

The intracellular expression of IFN-γ and extracellular expression of NKG2D and CD107a were determined to examine the effect of HFD-induced obesity and vitamin D on the cytolytic function of T cells. The surface expression of CD107a by CD8+ T cells was significantly lowered by vitamin D ($P = 0.023$). The surface expression of CD107a by CD8+ T cells was significantly higher in CD-DD group compared with CD-DS group. In addition, the surface expression of CD107a tended to be lower in HFD groups compared with CD groups ($P = 0.057$) (Figure 7-A). However, the intracellular expression of IFN-γ by CD4+ T cells, CD8+ T cells, and NKT cells and the surface expression of NKG2D by CD4+ T cells, CD8+ T cells, and NKT cells were not significantly different among the groups (Figure 7-A, B).
Figure 7. Effects of vitamin D and obesity on the intracellular expression of IFN-γ, and extracellular expression of NKG2D and CD107a by T cells: (A) intracellular expression of IFN-γ by CD4⁺ T cells, CD8⁺ T cells, and NKT cells, and surface expression of CD107a by NKT cells and (B) surface expression of NKG2D by CD4 T⁺ cells, CD8⁺ T cells, and NKT cells.

Values are means ± SEM, n = 4 to 6 per group. Means with different letters indicate significant differences (P < 0.05) by Fisher's LSD multiple comparison test.

CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet; CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet; HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.

45
7. Effects of vitamin D and obesity on the expression of genes involved in NK cell functions

In order to examine the effect of obesity and vitamin D on cytokines and chemokines involved with the functions of NK cells, expression of Ifng, Il12b, Il15, Il18, and Ccl5 genes were determined from PMA/Ionomycin-stimulated splenocytes. The HFD groups had lower mRNA levels of Ifng ($P = 0.030$) and Ccl5 ($P = 0.017$) compared with the CD groups, whereas there was no significant difference in terms of vitamin D levels. The CD-DS group had significantly higher mRNA expression of Ccl5 compared with the HFD-DS group. However, the mRNA levels of Il12b, Il15, and Il18 were not significantly different among the groups (Figure 8).
Figure 8. Effects of vitamin D and obesity on mRNA levels of NK cell-related markers (Ifng, Il12b, Il15, Il18, Ccl5)

Values are means ± SEM, n = 5 to 8 per group. Means with different letters indicate significant differences (P < 0.05) by Fisher's LSD multiple comparison test.

CDDD, 10% kcal fat + 50 IU/kg of diet; CDDC, 10% kcal fat + 1000 IU/kg of diet;
CDDS, 10% kcal fat + 10000 IU/kg of diet; HFDDD, 45% kcal fat + 50 IU/kg of diet;
HFDDC, 45% kcal fat + 1000 IU/kg of diet; HFDDS, 45% kcal fat + 10000 IU/kg of diet.
8. Effects of vitamin D and obesity on the expression of genes involved in vitamin D metabolism

To determine whether the expression of vitamin D-related genes were affected by vitamin D and obesity, splenic mRNA levels of Vdr and Vdup1 were determined from splenocytes following stimulation with PMA/Ionomycin. Gene expression of Vdr and Vdup1 were not affected by obesity or vitamin D (Figure 9).
Figure 9. Effects of vitamin D and obesity on mRNA level of vitamin D-related markers (Vdr, Vdup1)

Values are means ± SEM, n = 5 to 8 per group.
CD-DD, 10% kcal fat + 50 IU/kg of diet; CD-DC, 10% kcal fat + 1000 IU/kg of diet;
CD-DS, 10% kcal fat + 10000 IU/kg of diet; HFD-DD, 45% kcal fat + 50 IU/kg of diet;
HFD-DC, 45% kcal fat + 1000 IU/kg of diet; HFD-DS, 45% kcal fat + 10000 IU/kg of diet.
V. Discussion

This study demonstrates that vitamin D supplementation has differential effects on NK cell activity depending on the adiposity. Vitamin D supplementation enhanced splenic NK cell activity in the control mice but not in the HFD-induced obese mice. This enhancement of NK cell activity might have been caused by the increased percentages of the splenocyte subpopulations of total NK cells, CD11b⁺ NK cells, and CD11b⁻ NK cells in control mice supplemented with vitamin D.

The effects of vitamin D regarding NK cell function has been investigated by other researchers, but contradictory results have been reported. In some in vivo studies, NK cell cytotoxicity and IFN-γ secretion by NK cells derived from hematopoietic stem cells and peripheral blood mononuclear cells (PBMCs) were inhibited by 1,25-(OH)₂D₃ in a dose-dependent manner (Leung 1989; Weeres et al. 2014). On the other hand, 1,25-(OH)₂D₃ has been shown to increase the activity of NK cells derived from PBMCs, LAK cells, and NK YT cells (Ravid et al. 1993; Balogh et al. 1999; Al-Jaderi et al. 2013). Different types of NK cells such as primary NK cells and an NK cell-line were used in the studies, which might explain the conflicting reports among the studies. Only few studies have examined the in vivo effect of vitamin D on NK cell activity. NK cells undergo maturation in the developmental stage from immature (CD11b⁻) to mature (CD11b⁺) NK cells (Clinthorne et al. 2013), and the present study showed that the
splenic population of CD11b$^-$ as well as CD11b$^+$ NK cells was significantly higher in the CD-DS group compared with the HFD-DS group. This result suggests that alternation in percentage of the NK cell subpopulation possibly contributed to the increased NK cell activity with vitamin D supplementation in lean mice. While vitamin D enhanced NK cell activity in the control mice, there was no significant effect of vitamin D supplementation on NK cell activity in the HFD-induced obese mice, and in line with previous studies, it has been reported that serum leptin levels were significantly higher in the obese mice compared to the control mice (Nave et al. 2008; Park et al. 2013; Bahr et al. 2017). NK cells incubated with leptin for a short time (18 h) has been reported to show increased NK cell activity, but a relatively long incubation period of over 72 h resulted in impaired NK cell proliferation and cytotoxicity through down-regulation of the JAK-STAT signaling pathway (Wrann et al. 2012; Naylor et al. 2016). Chronic exposure to higher leptin levels in obese mice could have contributed to the decreased percentage of splenic NK cells as well as NK cell reactiveness to vitamin D supplementation, resulting in different responses to vitamin D supplementation between the CD and HFD groups.

In this study, no significant differences were observed in the surface expression of NKG2D and CD107a, or the intracellular expression of IFN-γ by NK cells after vitamin D supplementation. Not many studies have been performed to
elucidate the relationship between vitamin D and the expression of IFN-γ, NKG2D, and CD107a by NK cells. Nevertheless, it has been proposed that the expression of IFN-γ by human NK cells is suppressed by 1,25-(OH)_{2}D_{3} in a dose-dependent manner (Leung 1989). The surface expression of NKp30, NKp46, and NKG2D by NK cells have been reported to be unaffected by supplementation with *in vitro* 1,25-(OH)_{2}D_{3} (Weeres *et al.* 2014). In one clinical study, the expression of CD107a in NK cells from the PBMCs in women was downregulated by 1,25-(OH)_{2}D_{3} when co-cultured with K562 cells (Merino *et al.* 1989). However, these studies are different from the present study due to the *in vitro* treatment of vitamin D.

Furthermore, the present study showed that the surface expression of CD107a by CD8^{+} T cells was significantly lowered by vitamin D. Since CD107a is a surface marker for the degranulation of cytotoxic T cells as well as NK cells (Alter *et al.* 2004), its decreased surface expression suggests impaired cytotoxic activity of CD8^{+} T cells against target cells. Alterations in adaptive immune responses by vitamin D treatment have been reported which is in line with findings from this study. It has been demonstrated that 1,25-(OH)_{2}D_{3} suppresses granzyme A expression via suppression of the Th1 cytokine response (Vidyarani *et al.* 2009). In a VDR knockout mouse model, CD8^{+} cytotoxic T cells produced less granzyme B (Yuzefpolskiy *et al.* 2014), suggesting decrease in degranulation.
and consequential release of the cytotoxic granules by CD8 T cells in the absence of vitamin D signal.

IFN-γ is known to be produced by both NK and T cells (Balogh et al. 1999), and CCL-5, which is known to increase the cytolytic activity of NK cell, is produced by activated NK cells (Robertson 2002), T cells, and endothelium platelets (Muthian et al. 2006). The results from the present study showed that the mRNA levels of Ifng and Ccl5 in splenocytes were significantly lower in HFD-induced obese mice. However, this result doesn’t represent the mRNA expression exclusively by NK cells since the population of NK cells was about 4.8% of the total splenocytes, and T cells (31.2%) and B cells (56.7%) comprise the major population. Rather, it can be explained that the difference in the mRNA expression level of Ifng with obesity was due to the impact of obesity on T cells. The sum of CD4+ and CD8+ T cell population in the spleen was 6.9 % lower in the HFD group than the CD group. Since both CD4+ T cells and CD8+ T cells express IFN-γ, lower splenic subpopulation of these cells in the HFD group could influence mRNA expression of Ifng in the obese mice resulting in lower Ifng expression.

The expression levels of Ifng and Ccl5 in visceral adipose tissue have been reported to be higher in obese mice compared with control mice (Sell et al. 2012). However, the expression of IFN-γ in splenic NK cells did not show any
significant differences between obese and lean mice (Lee et al. 2016). In the initial obesity-induced inflammatory phase, chemokines such as \( Ccl5 \) recruits T and B lymphocytes to sites of inflammation, and following the local inflammatory responses, proinflammatory Th1 cytokines such as \( Ifng \) are produced for M1 macrophage recruitment (Kintscher et al. 2008; Rocha et al. 2008; Winer et al. 2011). Therefore, the expression of \( Ifng \) and \( Ccl5 \) can be up-regulated through this lymphocyte infiltration. The expression of \( Ifng \) and \( Ccl5 \) in the immune cells might be different from the expression of these in response to the inflammatory reactions within and immune cell infiltration into adipose tissue.

There were no significant effects of vitamin D or fat amount on mRNA levels of \( Vdr \) and \( Vdup1 \). \( 1,25\text{-}(OH)_{2}D_{3} \) acts on immune cells to exert its effect by binding to VDRs (Haussler et al. 2011). It was demonstrated that the expression of \( Vdrs \) was higher in the WAT from obese patients compared to control subjects following the supplementation of vitamin D (Clemente-Postigo et al. 2015; Lee et al. 2016), but few studies have focused on \( Vdr \) expression in the splenocytes after supplementation of vitamin D. It has been suggested that the expression of \( Vdrs \) is induced by \( 1,25\text{-}(OH)_{2}D_{3} \) treatment of monocytes and macrophages (Mora et al. 2008), but there have been conflicting reports concerning whether it has a direct effect on the \( Vdr \) expression by T and B cells (Veldman et al. 2000; Chen et al. 2007; Mora et al. 2008). In regard to VDUP-1, reduced NK cell activity, number
of CD3-NK1.1\(^+\) cells, and IFN-γ expression of NK cells have been reported in VDUP-1 knock out mice (Lee et al. 2005), suggesting that vitamin D can affect NK cell functions. However, little is known and further investigation still needed into the effect of \textit{in vivo} supplementation of VDUP-1 and NK cell functions.

Taken together, vitamin D supplementation exhibited an increase in NK cell activity in control mice but there was no effect of vitamin D in obese mice. This enhancement in NK cell activity might be due to the higher splenic subpopulation of NK cells in control mice than obese mice when vitamin D was supplemented (Figure 10). Results from this study suggest that dietary vitamin D supplementation acts as a stimulator of NK cell functions contributing to the enhancement of innate immunity.
Figure 10. Differential effects of vitamin D supplementation on NK cell activity in control and obese mice
VI. SUMMARY

The present study investigated the effect of vitamin D and obesity on NK cell functions. After feeding mice for 12 weeks with diets differed in fat amount (10% or 45% kcal fat, CD or HFD) and vitamin D content (50, 1000, 10000 IU/kg of diet, DD, DC, or DS), body weight, weight change, WAT weights, and serum leptin level were measured. In addition, NK cell activity, subpopulation of splenocytes, surface expression of NKG2D, CD107a, intracellular expression of IFN-γ by NK cells and T cells, and markers involved in NK cell function and vitamin D metabolism were analyzed. The results of the study were as follows.

1) The HFD groups had significantly higher body weight, weight gain, and WAT weights compared with the CD groups. In addition, the HFD groups had significantly higher serum leptin level compared to the CD groups.

2) NK cell activity was significantly higher in the CD-DS group than the HFD-DS group, and the CD-DS group showed significantly higher NK cell activity compared with the CD-DD and CD-DC groups, but no difference in NK cell activity was observed among the HFD groups fed different vitamin D levels.

3) Splenic percentages of total NK cells, CD11b− NK cells, and CD11b+...
NK cells were significantly lower in HFD groups compared with the CD groups. Total NK cells and CD11b⁺ NK cells showed higher splenic NK cell percentages in the CD-DS group compared to the CD-DD group. Positive correlations were observed between splenic percentage of NK cells and NK cell activity.

4) The percentage of NKT cells in the spleen was significantly higher in the CD-DS group than the HFD-DS group, and the CD-DS group showed significantly higher splenic percentages compared with the CD-DD and CD-DC groups. The percentages of CD4⁺ T cells and CD8⁺ T cells in the spleen were significantly lower in the HFD groups compared with the CD groups, while the percentage of B cells was significantly higher in the HFD groups compared with the CD groups. No significant differences were shown in splenic percentages of CD4⁺ T cells, CD8⁺ T cells, and B cells according to different vitamin D levels.

5) Intracellular expression of IFN-γ and surface expressions of NKG2D and CD107a in NK cells were not influenced by different fat amount and vitamin D levels.

6) The surface expression of CD107a by CD8⁺ T cells was significantly lowered by vitamin D, and tended to have lower surface expression of CD107a in the HFD groups compared with CD groups.
7) The HFD groups had lower mRNA levels of *Ifng* and *Ccl5* compared with the CD groups, whereas there was no significant difference in terms of vitamin D levels. However, mRNA levels of *Il12b*, *Il15*, and *Il18* were not significantly different among groups. Gene expression of *Vdr* and *Vdup1* were also not influenced by obesity or vitamin D.

These results suggest that vitamin D supplementation increased NK cell activity in control mice but not in obese mice. This enhancement of NK cell activity might be mediated through alternation of the splenic NK cell population.
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국문초록

비타민 D의 보충이 자연살해세포의 기능에 미치는 영향: 비만 여부에 따른 차별적 효과

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비타민 D는 면역 조절 효과가 있으며 적응면역뿐만 아니라 내재면역의 기능에도 관여한다는 것이 알려져 있다. 비타민 D는 적응면역의 작용은 억제하며, 내재면역의 경우 단핵구가 대식세포로 분화하는 것을 촉진하고, 수지상세포의 성숙과 분화는 억제하는 것으로 보고되었다. 그러나 비타민 D가 자연살해세포의 기능에 미치는 영향에 관한 연구는 부족한 실정이고, 선행된 연구의 대부분이 비타민 D가 자연살해세포의 기능에 미치는 영향을 세포에 직접 비타민 D를 처리하여 효과를 살펴본 연구이며 상반된 결과를 보여주고 있다. 따라서, 본 연구에서는 식이를 통한 비타민 D의 보충에 따른 자연살해세포의 기능 변화를 살펴보고자 하였고, 비만이 자연살해세포의 살해능력에 미치는 영향을 확인하고자 하였다. 5주령의 C5BL/6 마우스를 지방의 함량 (10% 또는 45%의 총 지방 함량; CD, HFD)과 비타민 D의 수준 (식이 kg 당 50, 1000, 10000
IU: DD, DC, DS)에 따라 6 그룹으로 구분하여 12주 동안 식이를 공급 하였다. 자연살해세포의 살해능력, 사이토카인 분비, 활성 수용체의 발현, 탈과립성을 측정하였다. 제중, 섭취량, 백색지방량, 혈중 렌틴 농도, 자연살해세포의 살해능력, 비장면역세포의 비율, 자연살해세포 및 T 세포로부터 세포 내 IFN-γ의 발현 및 세포 표면 NKG2D와 CD107a의 발현을 측정하였고, 비장면역세포에서 자연살해세포의 기능 및 비타민 D의 대사와 관련된 지표를 확인하였다. 그 결과, 12주 후의 제중 및 제중 변화, 백색지방 무게는 고지방 식이 섭취군에서 높았고, 혈중 렌틴 농도도 고지방 섭취군에서 대조군 보다 더 높음을 확인하였다. 자연살해 세포의 살해능력은 대조군에서는 비타민 D의 보충군에서 높았으나 고지방 섭취군에서는 비타민 D의 보충 효과가 관찰되지 않았다. 비장면역세포의 비율을 분석한 결과, 성숙한 자연살해세포 (CD11b+)와 미성숙한 자연살해세포 (CD11b-)의 비율이 고지방 섭취군에서 대조군에 비해 낮았고, 대조군에서는 비타민 D 보충군이 비타민 D 결핍군보다 더 높은 비율을 나타냈다. 비장면역세포에서 자연살해 T 세포의 비율은 대조군 에서는 비타민 D에 의한 비중이 증가되었으나, 고지방 섭취군에서는 유의적인 차이가 없었다. PMA (50 ng/mL)/Ionomycin (0.5 μM)으로 4시간 동안 자극한 자연살해세포에서 발현된 세포 내 IFN-γ 및 세포표면 NKG2D와 CD107a 수준은 비만이나 비타민 D의 섭취 수준에 의한 영향이 없었으나, CD8⁺ T cell에서의 CD107a 발현은 비타민 D 보충군에서 낮게 나타났다. 또한, 비장면역세포에서 발현되는 Ifng와 Ccl5의 수
준은 대조군에 비해 고지방 섭취군에서 유의적으로 낮았으나, Vdr와 Vdup1의 발현은 비타민 D나 비만에 의한 영향이 없었다. 결론적으로, 본 연구에서는 비타민 D를 경구 보충하였을 때 대조군에서 자연살해세포의 살해능력을 상승시켰으나 고지방 섭취군에서는 영향을 주지 못함을 확인하였고, 이러한 대조군에서의 효과는 자연살해세포의 비율 변화가 영향을 주었을 것으로 생각된다. 본 연구는 비타민 D가 자연살해세포를 자극하는 요인으로 작용함으로써 선천면역 반응의 향상에 기여할 수 있다는 것을 확인한 점에서 의미가 있다고 사료된다.

주요어: 자연살해세포, 비타민 D, 비만, 인터페론 감마, CD107a
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