NUS National University of Singapore

Life Sciences Institute

Antibody Engineering Programme

Antibody Core Facility

PROJECT REPORT

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Client Name:

Project name and PPMS number: Defining the Impact of Orally-delivered KDV-111 on the Immune Cell Phenotype of Healthy Asians.
(PPMS 00000035)

Project Type:

Comprehensive Immune cell profiling of individuals taking the oral supplement KDV-111.

Prepared by:

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Background:

Nutrition is a key determinant in the development and health of the human immune system, and malnutrition is the most common cause of immunodeficiency worldwide. Micronutrients such as zinc; selenium; iron; copper; vitamins A, C, E, and B-6; and folic acid combined with an adequate intake of vitamins B(6), folate, B(12), C, E, and of selenium, zinc, copper, and iron are critical for the strength and regulation of both innate and adaptive immune responses. In this study, we analyze the effects of a supplement (KDV-111) on the principal Lymphoid and Myeloid cell subsets that constitute the human immune system.

KDV-111 is a natural compound that helps restore homeostasis and controls inflammation and has been promoted as a low-cost candidate for the regulation of infectious diseases. As the compound consists of purely food grade compounds with no toxins or allergens, it is classified as a dietary supplement. Although its current purpose is not to treat, diagnose, prevent or cure any disease, the compound is thought to have immunomodulatory properties. KDV-111 is manufactured using a proprietary reactor - a “biochemical cavitation mixer” that creates a “smart small molecule”. The principal device belongs to the cavitation technology family and is used for the intensification of technological processes in liquid media (liquid processing, splitting of complex molecules, "cold" pasteurization, destruction of solid inclusions). This process technology enables compression of a set of 12 molecules into the size of a single molecule. Its components are: Phosphoric Acid (58%), Zinc, Copper, Iron Pyrophosphate, Potassium, Calcium, Manganese, Glycyrrhizic Acid (8.4%), Silica (0.1%). The microelement is made up of a homogenized complex with special indication, pH = 0.0008-0.4, waterless in the final composition.

The complex molecules scan for the presence of pathogenic (bacterial, viral, fungal) etiologies at the cellular level by detecting changes in the characteristics or function of the electron-proton (KNa) pump on the membrane. The compounds are not reactive to healthy cells or tissues but may lead to apoptosis of infected cells in a process involving mitochondria activity. Specifically, the complex molecules trigger a cascade of biochemical events such as switching to mitochondria aerobic oxidation, restarting the methyl group with the “epigenetic” effects on DNA, and cellular apoptosis. It is unclear how and to what extent these mechanisms contribute to innate immune activation by cellular damage and stress, or how it contributes to the regulation of innate or adaptive immune responses in Asians.

The primary objective of this study is to analyse changes in the immune cell types and subsets in a cohort of healthy Singaporean donors after six weeks of KDV-111 intake. The secondary objective is to analyse the safety and tolerability of KDV-111. The data derived from this study can be viewed as a reference

dataset from Healthy individuals that can be employed for future comparisons with in clinical studies with disease cohorts.

Methods:

A detailed high-dimensional immune profiling analysis was conducted in 20 healthy subjects who were given KDV-111 supplementation over a duration of 8 weeks. In the initial screening visit, eligible patients provided informed consent, and their baseline investigation and eligibility (inclusion and exclusion criteria) documented. The baseline investigation data collected included demographics, medical/treatment history, physical examination, vital signs including blood pressure and pulse rate plus blood tests of immune cell subsets and phenotypes. During the screening visit, blood samples were collected from the 20 healthy subjects prior to being given the supplement (visit 1). Subjects were then given KDV-111 supplement at 1 oral drop (0.05ml) per 10kg of body weight (max 8 drops), every 8 hours (3 times a day) for 14 days.

10mL of blood per visit was collected weekly from the subjects over the course of 6 weeks (visits 2 - 7). Peripheral blood mononuclear cells (PBMCs) were obtained from the blood samples by Density centrifugation (Ficoll-Paque). The PBMCs were resuspended in freezing medium, stored in cryovials at −80°C and subsequently transferred to liquid nitrogen. For immunotyping, the PBMCs were thawed at 37°C, washed with PBS and resuspended in FACS staining buffer.

The PBMCs from each individual were divided equally for *ex vivo* staining with the T/NK and B cell/monocyte panels of antibodies for characterization of immune cell phenotypes. Staining of the PBMCs with antibodies from the T/NK and B cell/monocyte panels was carried out on ice for 20 mins in the dark. The PBMCs were then fixed with 4% PFA and resuspended in FACS staining buffer prior to analysis by high-dimensional flow cytometry (Cytek® Aurora). Flowjo was employed for quantitation of immune cell markers and detailed lymphoid /myeloid subset analyses.

Table 1: B cells and monocytes panel

Marker-conjugate	Function
CD19-BV510	B cells
CD14-PerCP-5.5	Monocytes
CD16-efluor450	Monocytes
CD3-APC-Cy7 (dump)	T cells
CD10-PE-Dazzle594	Immature B cells
CD21-APC	Memory B cells subsets
CD27-BV650	Memory B cells subsets
CD38-PE-Cy7	Plasmablasts
IgD-BUV737	B cell class switching
PD-1-BV711	Exhaustion marker

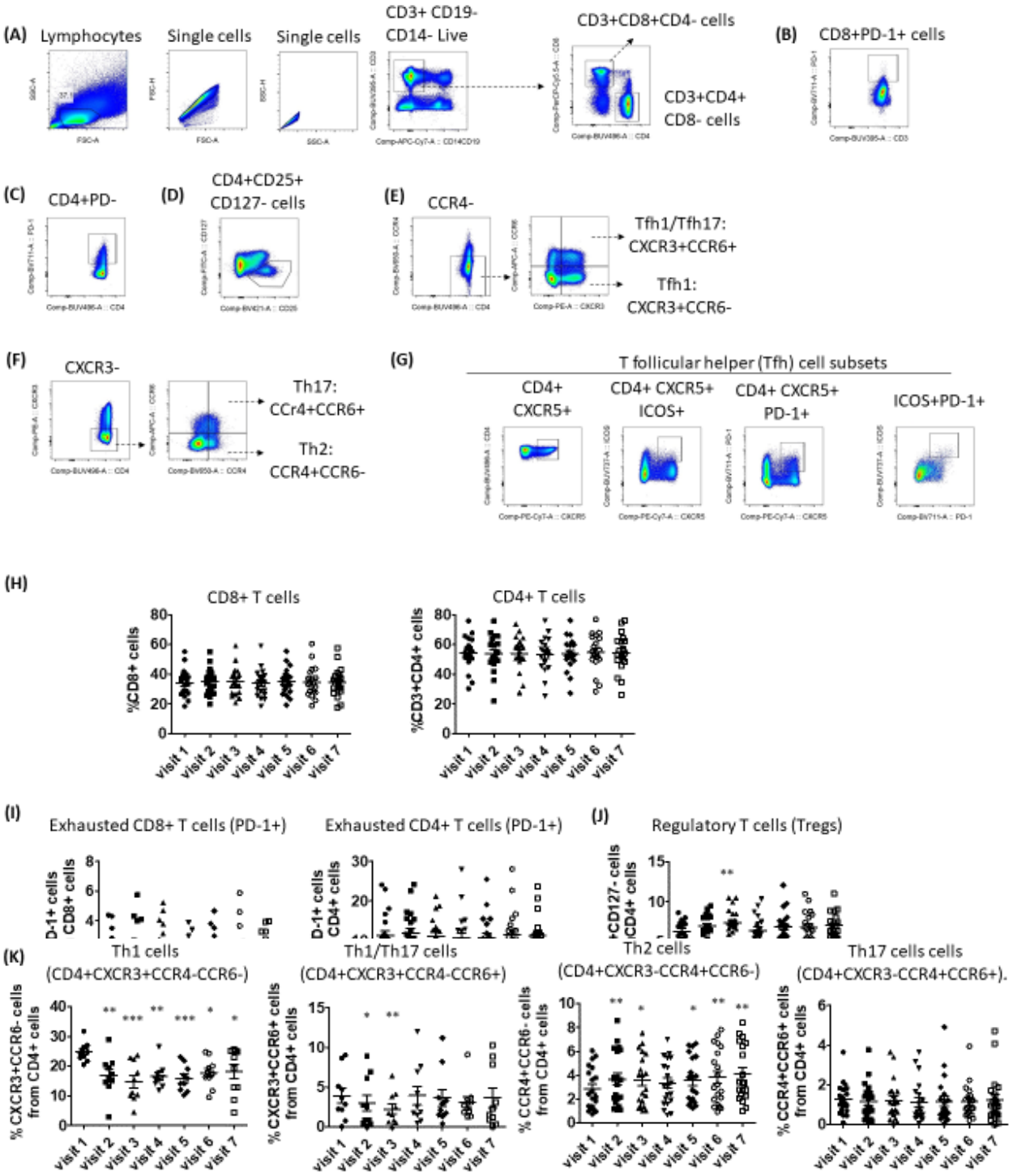
CD56-BV786	NK cells

Table 2: T and NK cells

Marker-conjugate	Function
CD14-APC-Cy7 (dump)	Monocytes
CD19-APC-Cy7 (dump)	B cells
CD3-BUV395	T cells
CD4-BUV496	CD4+ T cells
CD8- PerCP-5.5	CD8+ T cells
CD56-BV786	NK cells
CD16-efluor450	NK cells
CXCR5-PE-Cy7	T follicular helper cells (Tfh)
ICOS-BUV737	Tfh co-stimulatory molecule
PD-1-BV711	Tfh co-stimulatory molecule, exhaustion marker
CD25-BV421	T regulatory cells marker
CD127-FITC	Treg marker
CXCR3-PE	Th1
CCR4-BV650	Th2
CCR6-APC	Th17

Results:

A total of 20 participants completed the study. The mean age±SD was 32.4 ± 5.4 years and body mass index 21.9 ± 2.4 kg/m2. The screening systolic blood pressure was 114.7 ± 8.8 mmHg and diastolic blood pressure 67.9 ± 9.3 mmHg, with no significant change at the end of the study. Participants tolerated KDV-111 well with no adverse event and all blood profiles (blood urea, serum sodium, serum creatinine, serum albumin, aminotransaminases) did not change significantly at the end of the study.



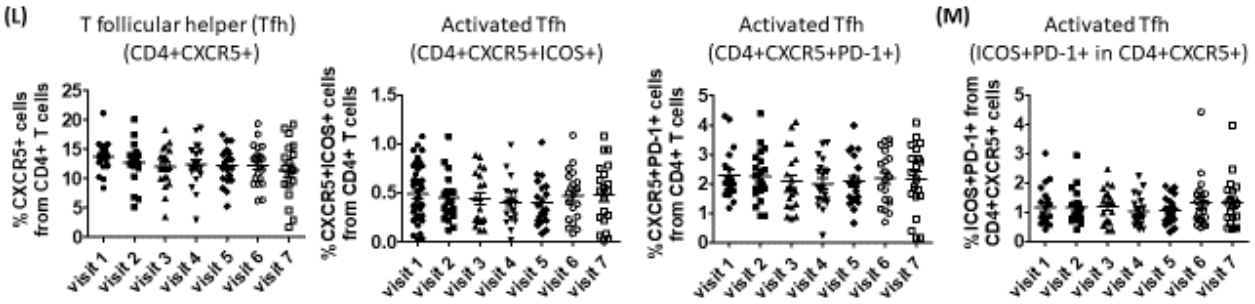


Figure 1. **(A)** To identify T cells subsets, a lymphocyte gate was defined based on high CD3 expression and exclusion of B cells (CD19), monocytes (CD14) and dead cells. The CD3+ population were divided into CD4+ and CD8-lymphocytes. PD-1 was used to identify exhausted **(B)** CD8 and **(C)** CD4 T cells. **(D)** Regulatory T cell (Tregs) subsets were defined as CD4+CD25+CD127low/-. Analysis of the CD4+ lymphocytes for the expression of CXCR3, CCR4, and CCR6 was done to identify **(E)** Th1 (CD4+CXCR3+CCR4-CCR6-), Th1/Th17 (CD4+CXCR3+CCR4-CCR6+), **(F)** Th2 cells (CD4+CXCR3-CCR4+CCR6-) and Th17 cells (CD4+CXCR3-CCR4+CCR6+). (G) Finally, Tfh cells were identified as CD4+CXCR5+ T cells . Expression of activation/Co-stimulatory markers, ICOS and PD-1, were also defined in the Tfh cells. Proportion of **(H)** CD8+ and CD4+ cells gated from CD3+ cells **(I)** PD-1+ cells from CD8+ and CD4+ cells **(J)** Tregs from CD4+ T cells **(K)** T helper (Th) cell subsets from CD4+ T cells **(L)** T follicular helper (Tfh) cell subsets from CD4+ T cells and **(M)** ICOS+PD-1+ cells from CD4+CXCR5+ cells.

CD4+ T helper cells are responsible for secretion of specific cytokines, and activation of cells of the innate immune system, B-lymphocytes and cytotoxic T cells [1]. CD8+ Cytotoxic T cells mediate their effector functions through production of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α and/or by cytolytic mechanisms [2]. The frequencies (numbers) of total CD4+ and CD8+ populations were not changed before and after the subjects had taken the KDV-111 supplement (Fig 1H).

Programmed death 1 (PD-1) is a marker of exhausted T cells and is induced in response to continuous stimulation as occurs in chronic infections and cancer [3]. PD-1 plays an immunosuppressive role in effector T cells via inhibition of TCR and CD28 signals, and consequently inhibits T-cell proliferation, cytokine production, and cytolytic function [4, 5]. There were no significant changes in the frequencies of PD-1-expressing CD4+ and CD8+ T cells (Figure 1I).

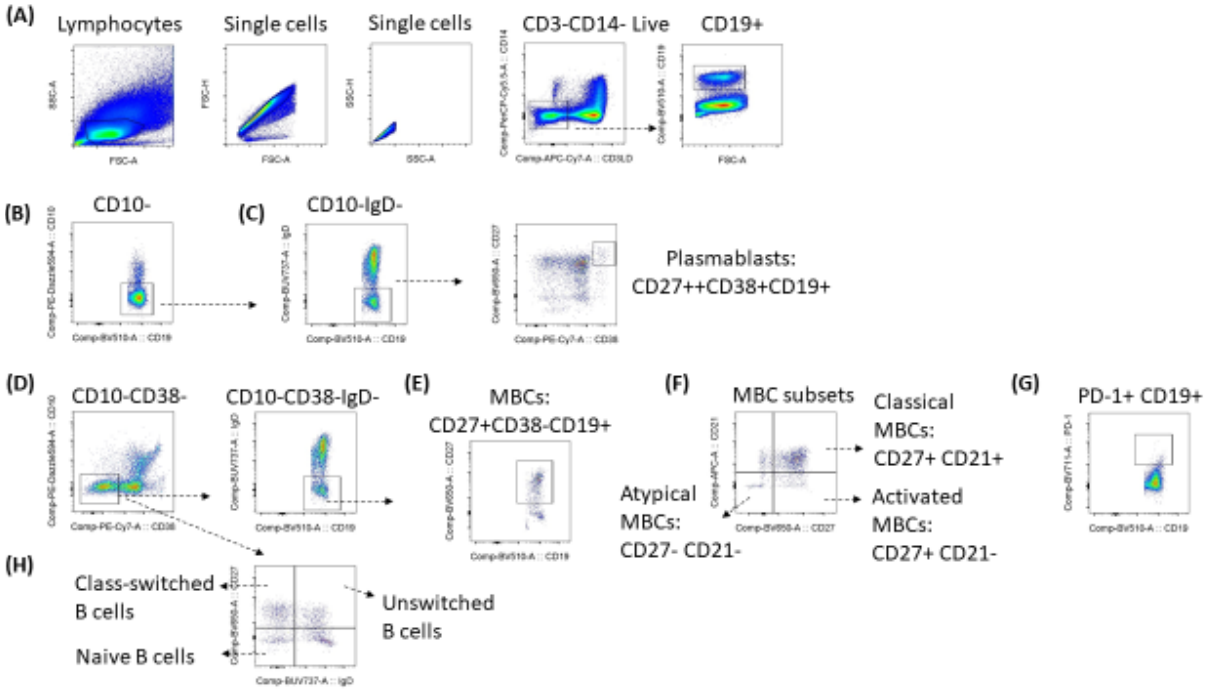
Regulatory T cells (Tregs) are characterized by the expression of CD25 and absence of CD127 and have been demonstrated to maintain immunotolerance by suppressing the antigen-specific or antigen non-specific T cell responses [6]. These cells are critical for the maintenance of immune homeostasis and reducing unwanted inflammatory reactions in both chronic and acute infection, autoimmunity and atopy. The frequency of Tregs was upregulated in the subjects at visit 2 after taking the supplement which peaked at visit 3 before returning to baseline (visit 1) levels (Fig 1J).

Activation of naive $CD4^+$ T cells leads to their differentiation into functional subsets (including Tregs mentioned above). The two principal subsets that have been most widely studied are termed T helper type 1 (Th1) and T helper type 2 (Th2) cells, based on their production of the cytokines interferon (IFN)-γ or interleukin (IL)-4, respectively [7]. Th1 cells secrete the cytokines IFN-γ, and tumor necrosis factor (TNF)-α, which function in protecting against intracellular infections by viruses and bacteria, as well as eliminating cancerous cells [8, 9]. Th2 cells secrete IL-4, -5, -10 and -13, which up-regulate antibody production and target parasitic organisms. Th2 cells activate B cells, which are adapted for defense against parasites that are vulnerable to IL-4-switched immunoglobulin (Ig)E production, IL-5-induced eosinophilia, and IL-3-

and IL-4-stimulated mast cell proliferation and degranulation [9]. However, Th2 cell may also mediate anti-inflammatory responses via the secretion of IL-10 and counteract the pro-inflammatory actions of Th1 cells [10]. A third subset of CD4+ T-helper cells, known as Th17 cells, produce IL-17, IL-17F, IL-6, IL-22 and TNF-α and play an integral role in both tissue inflammation and activation of neutrophils to combat extracellular bacteria [9]. A population of Th1Th17 cells that produce both type 1 cytokines (IFN-γ, TNF-α) and IL-17 has also been described [11]. These Th1Th17 lymphocytes play a well-established pathological role during autoimmune diseases [12],

In this study on a Singaporean volunteer cohort, consumption of KDV-111 led to a small but significant and sustained increase in the frequency of Th2 (CD4+CXCR3-CCR4+CCR6-) cells and a corresponding decrease in the Th1 subpopulation (CD4+CXCR3+CCR4-CCR6-) (Fig 1K). An acute (visits 2 and 3) reduction of the Th1/Th17 (CD4+CXCR3+CCR4-CCR6+) subpopulation was also observed (Fig 1K).

T follicular helper (Tfh) cells are known to be specialized providers of help to B cells and persistently express C-X-C chemokine receptor type 5 (CXCR5), which drives Tfh migration into B cell follicles in response to the specific ligand CXCL13 [13, 14]. Tfh cells express PD-1, inducible T cell costimulator (ICOS), CD40 ligand (CD40L) and IL-21, which serve as markers for Tfh cells, and interact with B cell surface ligands to promote the formation of germinal centers (GC), the differentiation of B cells and antibody production [14]. Although there was significant decrease in the frequency of total Tfh cells (CD4+CXCR5+) in the subjects after taking the supplement, there were no significant changes in the activation state of the Tfh cells as indicated by the expression of costimulatory and activation markers, ICOS and PD-1 [15, 16] (Fig 1L and M).



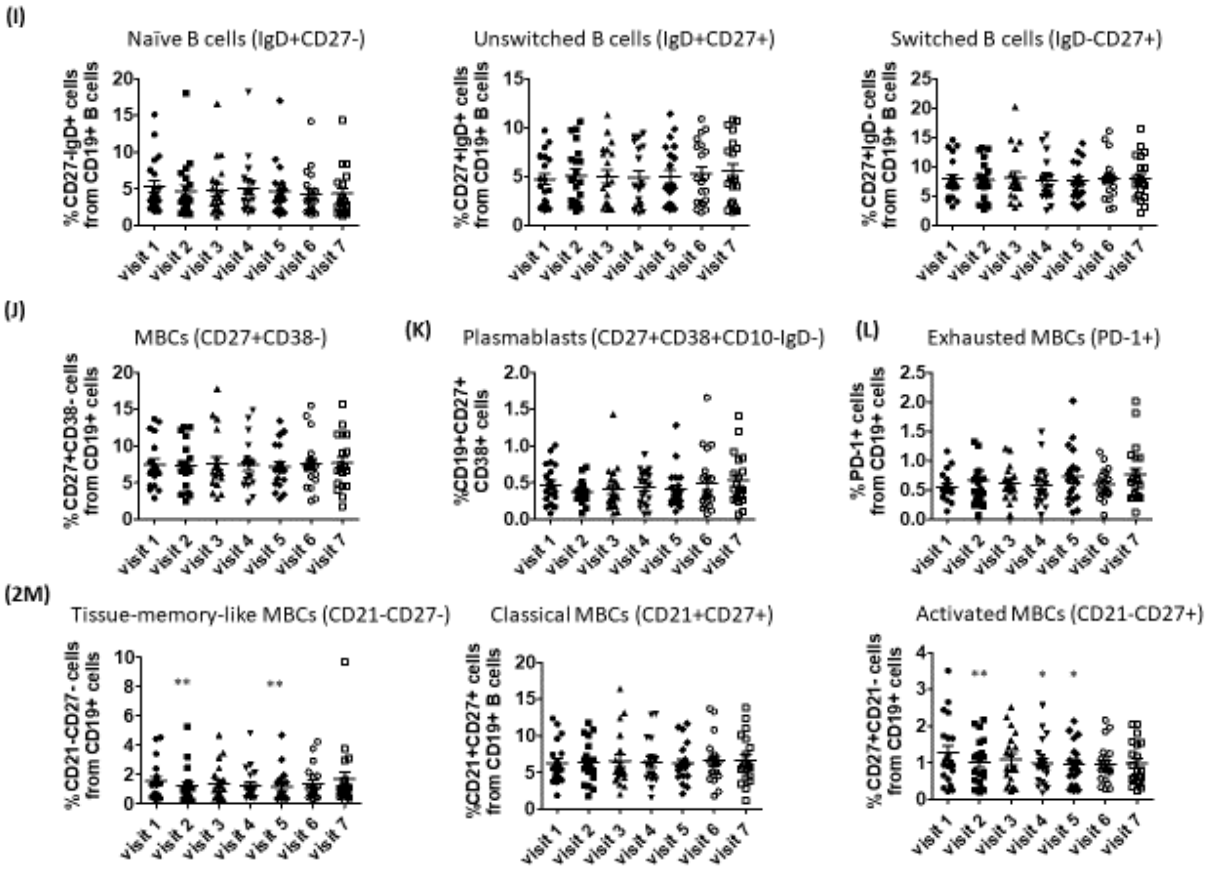


Figure 2. Characterization of B cell subsets by high-dimensional flow cytometry. **(A)** Single cells were defined based on FSC-A and SSC-A and doublet discrimination based on FSC-A/FSC-H and SSC-A/SSC-H. B cells were defined from a gate excluding T (CD3), monocytic (CD14) and dead cells, and positive selection of CD19-positive cells. **(B)** Mature B cells were selected from a CD10- gate and comprise **(C)** Plasmablasts ($CD27^{+}CD38^{high}IgD^{-}$) and **(D and E)** Memory B cells (CD27+CD38-IgD-). **(F)** Memory B cells can be further divided in subsets based on their maturation state by expression patterns of CD21 and CD27. **(G)** PD-1 is used to define exhausted B cells **(H)** $CD10^{-}$ mature B cells can also be divided in several subsets based on the expression of IgD and CD27. Naïve B cells express high levels of IgD but lack CD27. In contrast, $IgD^{+}CD27^{+}$ cells consist primarily of unswitched and marginal zone (MZ) B cells while , $IgD^{-}CD27^{+}$ represent the class switched B cell population. Proportion of **(I)** class-switched B cells **(J)** total memory B cells **(K)** plasmablasts **(L)** Exhausted B cells **(M)** memory B cell subsets in CD19+ B cells

Tfh cells support B cell growth, class switching and differentiation of naïve B cells into memory B cells (MBCs) and high affinity antibody-secreting plasma cells [17, 18]. $CD10^{-}$ mature B cells are divided in three subsets based on CD27 and IgD expression, namely naïve B cells ($IgD^{+}CD27^{-}$), IgD^{-} class-switched B cells consisting plasmablasts (CD27++CD38+) and memory B cells (CD27+CD38-) and $IgD^{+}CD27^{+}$ unswitched B cells [19]. There were no significant differences in the frequencies of naïve (CD19+CD10-CD27-IgD+), unswitched (CD19+CD10-CD27+IgD+) or class-switched B cells (CD19+CD10-CD27+IgD-) before and after the subjects had taken the supplement (Fig 2I). Total MBCs (CD19+CD10-IgD-CD27+CD38-, Fig 2J), plasmablasts (CD19+CD10-IgD-CD27++CD38+, Fig 2K) or exhausted (CD19+CD10-PD-1+, Fig 2L) B cells were also relatively unchanged between the visits.

Analysis of B cell subsets based on CD21 and CD27 expression allows for the definition of the maturation state of memory B cells. (17,18). Conventional MBCs ($CD21^{+}CD27^{+}$) represent the majority of circulating B-cell subsets in healthy donors and are often referred to as resting memory B cells, consistent with their low levels of expression of activation markers [20-22]. Non-conventional MBCs, which are not seen in substantial numbers in healthy individuals, include exhausted tissue-like MBCs (TLM, CD21-CD27-) and activated MBCs (AM, CD21-CD27+) [20, 21]. There were significant reductions in the frequencies of TLM and AM B cells after the subjects took the supplement but the frequencies of resting memory B cells remained unchanged (Fig 2M).

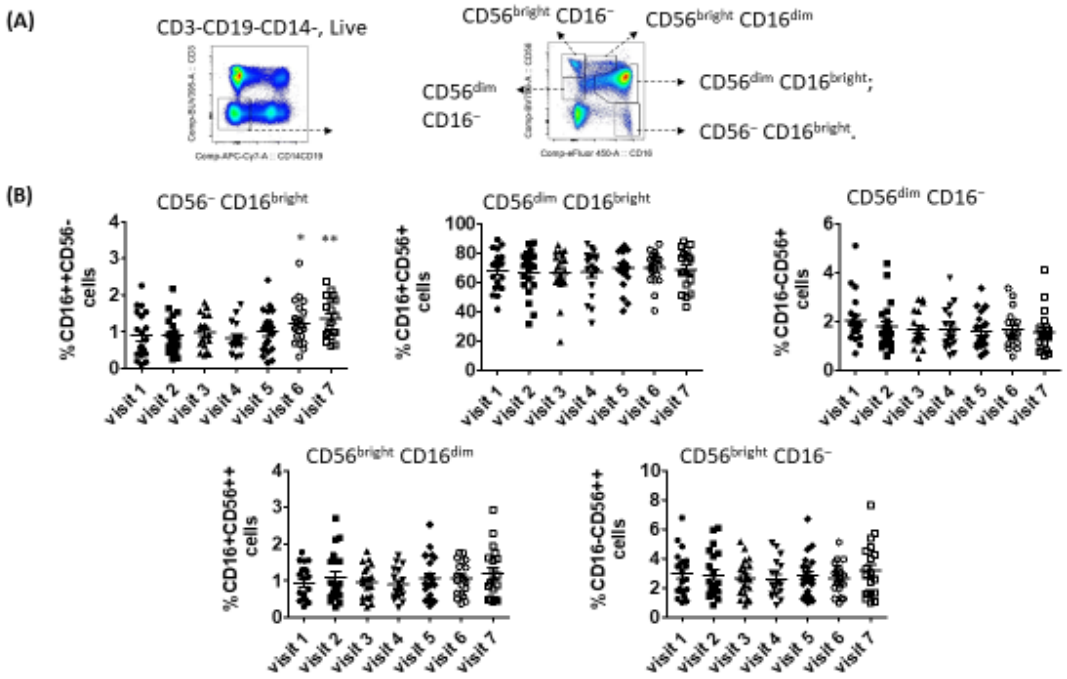


Figure 3. **(A)** Natural killer (NK) cell subsets in human PBMCs based on the relative expression of CD16 and CD56. PBMCs were stained with anti-CD3, anti-CD14, anti-CD16, anti-CD19 and anti-CD56 antibodies. T cells ($CD3^{-}$), B cells ($CD19^{-}$) and monocytes (CD14) were excluded. **(B)** NK cells subsets were defined by expression of CD16 and CD56. 1, $CD56^{bright}$ $CD16^{-}$; 2, $CD56^{bright}$ $CD16^{dim}$; 3, $CD56^{dim}$ $CD16^{-}$; 4, $CD56^{dim}$ $CD16^{+}$; 5, $CD56^{-}$ $CD16^{+}$.

Natural killer (NK) cells are a group of innate immune cells that display cytolytic activity against cells under stress such as tumor cells and virus-infected cells. In human peripheral blood, five NK cell subpopulations can be defined based on the relative expression of the markers CD16 and CD56: $CD56^{bright}$ $CD16^{-}$, $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{-}$, $CD56^{dim}$ $CD16^{bright}$, and $CD56^{-}$ $CD16^{bright}$[23,24].

In peripheral blood, the $CD56^{dim}CD16^{bright}$ NK cells represent at least 90% of all peripheral blood NK cells and are frequently described as the most cytotoxic subset, whereas $CD56^{bright}CD16^{dim/-}$ NK cells are abundant cytokine producers but the least cytotoxic [25, 26]. In healthy individuals, $CD56^{dim}$ $CD16^{-}$ and $CD56^{-}$ $CD16^{bright}$ are numerically in the minority. The role of $CD56^{dim}$ $CD16^{-}$ cells is largely unknown, but has been postulated to be an immature precursor of $CD56^{dim}$ NK cells (30). $CD56^{-}$ $CD16^{bright}$ NK cells are a dysfunctional subset of NK cells that have been found to be expanded in infectious diseases such as human immunodeficiency virus infection [24, 27] and HCV [28].

An increase during the later stages of the study (visit 6 and 7) of the $CD56^{-}$ $CD16^{bright}$ subpopulation was observed while there appeared to be a slight increase in the cytotoxic $CD56^{dim}CD16^{bright}$ NK cell subset and a decrease in the frequency of the $CD56^{dim}$ $CD16^{-}$ subpopulation although these were not statistically significant (Fig 3B).

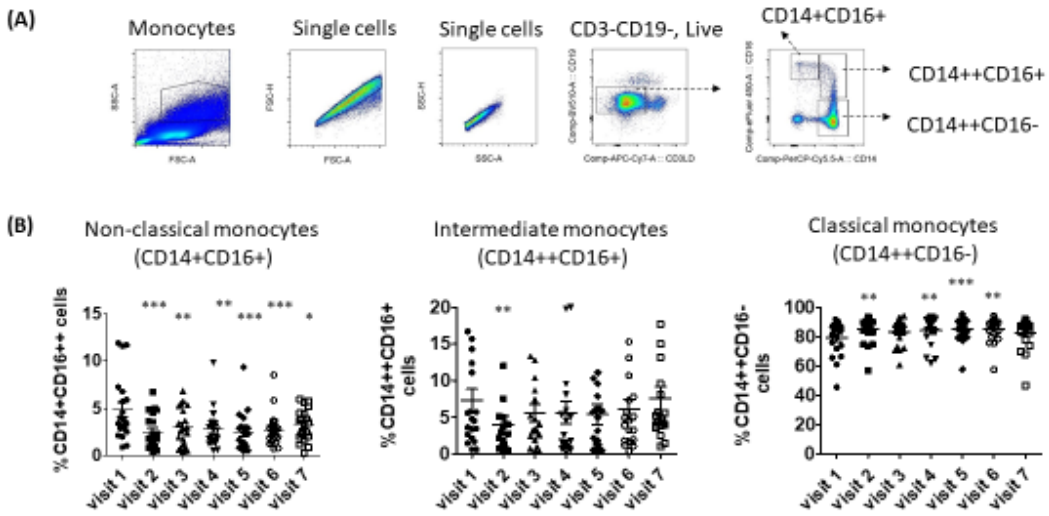


Figure 4. **(A)** Monocytes are defined by high forward and side scatter and subsequent selection from a gate which excluded T cells (CD3) and B cells (CD19). **(B)** Monocyte subsets were classified as classical ($CD14^{++}$, $CD16^{-}$), intermediate ($CD14^{++}$, $CD16^{+}$), and non-classical monocyte ($CD14^{+}$, $CD16^{++}$) monocytes.

Human monocytes are bone marrow-derived myeloid cells that circulate in the blood and can differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells that regulate innate and adaptive immune responses [29]. Monocytes can be distinguished based on CD14 and CD16 expression [30].

Classical monocytes comprise about 80% of circulating monocytes and express high levels of CD14 but not CD16. These monocytes function as phagocytic cells to remove microorganisms and dying cells [30, 31]. Intermediate monocytes express both CD14 and CD16, and are mainly associated with production of reactive oxygen species (ROS), antigen presentation, proinflammatory responses, wound healing, and parasite recognition [31, 32].

Non-classical monocytes are characterized by low expression levels of CD14 and high levels of CD16 [30, 31]. Non-classical monocytes exhibit greater pro-inflammatory activity and are also involved in antigen presentation and wound healing [30, 33].

A significant and sustained reduction in the frequency of non-classical monocytes and a corresponding increase in the frequency of classical monocytes were observed in the subjects after taking the supplement (Fig 4B). The frequency of intermediate monocytes was also decreased at visit 2 after supplementation although the frequency of cells recovered to levels similar to that of visit 1 by visit 7 (Fig 4B).

Conclusions:

Nutirent supplements have been shown to have an impact in previous studies on treating and reducing the risk of infections and on regulating the inflammatory processes that contribute to pathology [34]. Under these circumstances, there is good rationale to test the effect of KDV-111 which is postulated to have immunomodulatory effects. From this study, we observed a general shift towards an regulated or anti-inflammatory phenotype as seen by the increase in frequency of Tregs and Th2 cells, and a reduction in pro-inflammatory cells such as Th1, Th1/Th17 and non-classical monocytes.

Tregs act to suppress immune response, thereby maintaining homeostasis and self-tolerance. Excessive Treg activity may lead to immunodeficiency, chronic infection and cancer, whereas dysfunctional Treg activity may result in excessive inflammation and autoimmune disorders [35, 36]. In this study, an increase in Treg frequency was observed with the levels returning to baseline levels by visit 4. At the same time points, a reduction in the frequency of Th1/Th17 cells was observed. Tregs have been reported to keep pro-inflammatory Th1/Th17 cells in check [37, 38]**.** Therefore, supplementation with KDV-111 might help reduce the pathogenic inflammatory effects of Th1/Th17 cells by upregulation of Tregs.

A decrease in the frequency of Th1 cells and a corresponding increase in Th2 cells was also observed after the subjects took the supplement. The Th1 and Th2 populations, and the cytokines they release, have an antagonistic relationship and one subtype may be dominant in response to a particular pathogen at any one time [9]. Th2 cells have been shown to have anti-inflammatory effects but are also known to potentiate the humoral immune responses by stimulating promoting B cell proliferation, antibody production, and class-switching [9]. Therefore, a possible enhancement of the humoral immunity may be another effect observed in the subjects after KDV-111 supplementation.

Among the B cell subsets, the only changes were reductions in the tissue-like MBCs and the activated MBCs. The tissue-like MBCs have been associated with an exhausted B cell phenotype and display impaired signalling, survival, antiviral cytokine production, and differentiation into Ab-producing cells [39-41]. Interestingly, the levels of PD-1, an exhaustion marker in immune cells, was not increased although this could be because the changes were relatively minor. The activated MBCs are predisposed to differentiate into plasma cells in response to diseases such as HIV infection, influenza, systemic lupus erythematosus and rheumatoid arthritis [42]. A reduction in the tissue-like MBCs suggests that KDV-111 might help promote humoral immunity in the healthy individuals. The reduction in both cell types may also reflect the absence of chronic immune activation/inflammation that drive expansion of the tissue-like MBCs or infections that trigger activated MBC expansion in the healthy subjects. The resting MBC population remained predominant in the healthy individuals but were relatively unchanged through the course of the study.

Although an increase in the frequency of $CD56^{dim}CD16^{bright}$ NK cells and a decrease in $CD56^{dim}$ $CD16^{-}$ NK cells was observed, the levels of the NK cell subsets were relatively stable through the course of the study. An increase in the CD56- NK population may be due to exposure to immunosuppressive milieu leading to downregulation of CD56, concomitant with abolition of their cytotoxicity [43]. Although the increase in CD56- NK cells is an indication of pathological inflammation, a longer term study may be required to track the changes to the CD56- population of NK cells since these changes were minor and occurred only close to the end of the study course.

A reduction in the CD16+ monocytes was observed after the subjects had taken KDV-111; especially the non-classical monocytes, which showed a sustained reduction through the course of the study. The $CD16^+$ monocytes have been shown to produce higher amounts of TNF and minimal IL-10 in response to toll-like receptor (TLR) stimulation *in vitro* compared to classical monocytes [44] and were the predominant subsets in conditions such as asthma, coronary artery diseases and Crohn's disease, and during infections such as sepsis and hepatitis B [45-47], indicative of a pro-inflammatory role of the $CD16^+$ subsets. In addition, obesity has been shown to induce monocytosis of the intermediate and non-classical subsets, and increased secretion of pro-inflammatory cytokines [48, 49]. In contrast, classical monocytes are reportedly involved in anti-inflammatory cytokine secretion [50] and have restorative functions in skeletal muscle [51] and in acute liver injury [52]. KDV-111 supplementation may thus suppress pro-inflammatory effects by downregulating non-classical monocytes and promoting expansion of classical monocytes.

In conclusion, KDV-111 in this study was shown to promote an non- or anti-inflammatory phenotype, potentially augment humoral immunity by significant upregulation of Th2 cells and downregulation of exhausted TL MBCs. KDV-111 may also help mitigate pathogenic inflammation by downregulating proinflammatory Th1/Th17 cells and non-classical monocytes. The findings from this study provide a platform for future studies on KDV-111 supplementation with an emphasis on diseases where inflammation play a role in the underlying pathology.

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