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BRIEF COMMUNICATION

Vitamin C Is an Essential Factor on the Anti-viral Immune Responses through the Production of Interferon-α/β at the Initial Stage of Influenza A Virus (H3N2) Infection

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L-ascorbic acid (vitamin C) is one of the well-known antiviral agents, especially to influenza virus. Since the in vivo anti-viral effect is still controversial, we investigated whether vitamin C could regulate influenza virus infection in vivo by using *Gulo (-/-)* mice, which cannot synthesize vitamin C like humans. First, we found that vitamin C-insufficient *Gulo (-/-)* mice expired within 1 week after intranasal inoculation of influenza virus (H3N2/Hongkong). Viral titers in the lung of vitamin C-insufficient *Gulo (-/-)* mice were definitely increased but production of anti-viral cytokine, interferon (IFN)-α/β, was decreased. On the contrary, the infiltration of inflammatory cells into the lung and production of pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-α/β, were increased in the lung. Taken together, vitamin C shows in vivo antiviral immune responses at the early time of infection, especially against influenza virus, through increased production of IFN-α/β.

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INTRODUCTION

Vitamin C is known as an essential anti-oxidant (1,2) and enzymatic co-factor for physiological reactions such as hormone production, collagen synthesis (3) and immune potentiation (4-6). Naturally, an insufficiency of vitamin C leads to severe injuries to multiple organs, especially to the heart and brain, since they are both highly aerobic organs that produce more oxygen radicals. In fact, studies of *in vivo* effect on vitamin C are difficult since most animals, except human and some primate, are capable of synthesizing vitamin C endogenously (7). However, *Gulo (-/-)* mice were recently developed by the L-gulono-γ-lactone oxidase (Gulo) gene deletion like human, thus they should be supplied with dietary vitamin C (8). It already has been reported that vitamin C concentration was decreased by 10~15% in plasma of the *Gulo (-/-)* mice without supplementation of vitamin C for 2 weeks (8). We also reported that vitamin C level was remarkably decreased in the most organs in the *Gulo (-/-)* mice without supplementation of vitamin C for 3 weeks (9).

In addition, we found that numbers of T cell was decreased in the spleen of vitamin C-insufficient *Gulo (-/-)* mice (10). Even though it is thought that vitamin C shows its anti-viral or anti-tumor effects through the up-regulation of the

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Abbreviations: IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; Gulo, L-gulono-gamma-lactone oxidase; BAL, bronchoalveolar

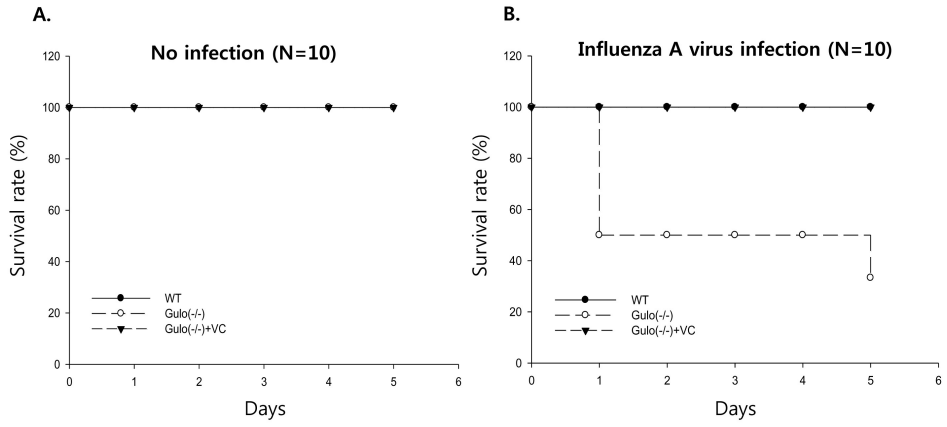

Figure 1. Increase of mortality of vitamin C-insufficient *Gulo (-/-)* mice by the infection of influenza A virus. Twenty hemagglutination units (HAU) of influenza A virus (H3N2/1/68/HongKong) was intranasally inoculated into wild type (n=10), vitamin C-sufficient *Gulo (-/-)* mice (n=10) and vitamin C-insufficient *Gulo (-/-)* mice (n=10) was as described in Materials and Methods. And then the survival of mice was monitored for 7 days after virus inoculation. (A) Mice without H3N2 infection, (B) Mice with 20 HAU of H3N2 infection.

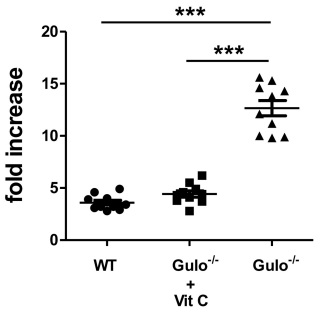

Figure 2. Increase of influenza A virus replication in the lung of vitamin C-insufficient *Gulo (-/-)* mice. To analyze the effect of vitamin C on the suppression of viral replication in the lung, the lungs were excised from sacrificed mice (n=10 per each group) and total RNA was purified from lung homogenate as described in Materials and Methods. The virus replication in the presence or absence of vitamin C was determined real time RT-PCR by using of specific primers for influenza virus M2 gene and β2m.

activity of natural killer (NK) cells and tumor specific cytolytic T lymphocytes (CTLs), its related evidences *in vivo* are still unclear. The reason why it is impossible to investigate *in vivo* effect of vitamin C is that all of animals could synthesize vitamin C from glucose thorough the action of L-glunolactone-γ-oxidase (*Gulo*), as described above (7). However, we confirmed that vitamin C up-regulates NK cell activity through the regulation of activating/inhibitory receptors on the surface of NK cell (our unpublished data). Since it is commonly known that vitamin C and NK cells are closely related to the prevention of common cold and the flu (11-13), we evaluated *in vivo* anti-viral effect of vitamin C and its related mechanism in *Gulo (-/-)* mice against influenza virus (H3N2/Hongkong/1/68). First, wild type, vitamin C-sufficient *Gulo (-/-)* mice and vitamin C-insufficient *Gulo (-/-)* mice were subjected to intranasal inoculation of 20 hemagglutination units (HAU) of influenza virus, and then their survival was monitored. Interestingly, we observed that vitamin C-insufficient *Gulo (-/-)* mice expired within 1 week, but all of wild type and vitamin C-sufficient *Gulo (-/-)* mice survived (Fig. 1B). However, the supplementation of vitamin C on a day after virus inoculation could not prevent the death of vitamin C-insufficient *Gulo (-/-)* mice (Fig. 1B). It suggests that a sufficient amount of vitamin C is needed to prevent *in vivo* pathogenesis of influenza virus. Also, considering that H3N2 influenza virus shows a good circulation in humans and pigs as well as a slow antigenic drift in swine (14), we believe that the antigenic divergence between human and swine influenza virus might be increased. Therefore, our results shown in Fig. 1 suggest that vitamin C may effectively prevent severe or fatal damages in humans by the infection of influenza virus as well. To clarify the underlying mechanisms on the survival by the presence of the sufficient amounts vitamin C in the mice, we examined the viral titers in the lung of each experimental group. As shown in Fig. 2, viral titer in the lung from vitamin C-insufficient *Gulo (-/-)* mice was 10 to 15-fold increased, when it was compared with viral titer in wild type and vitamin C-sufficient *Gulo (-/-)* mice. However, when *Gulo (-/-)* mice were supplemented with vitamin C after virus inoculation, we could not observe a definite suppression of viral replication. This provides the importance of the vitamin C concentration at the initial stage of influenza virus infection. That is to say, damages through the replication of influenza viruses can be effectively prevented, when vitamin C concentration is sufficiently high at the initial stage of viral infection. If it is insufficient, however, the pathogenesis of influenza virus could not be prevented.

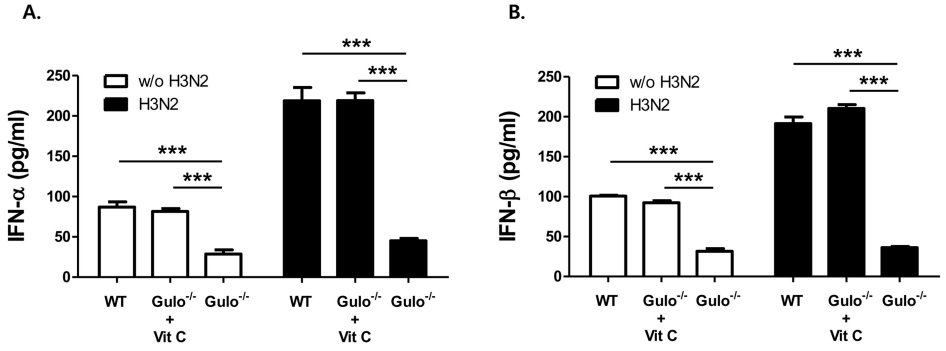

Figure 3. Defect on the production of IFN-α/β vitamin C-insufficient *Gulo (-/-)* mice. The levels of IFN-α (A) and IFN-β (B) in BAL fluids from wild type (n=6), vitamin C-sufficient *Gulo (-/-)* mice (n=6) and vitamin C-insufficient *Gulo (-/-)* mice (n=6) were measured by ELISA as described in Materials and Methods, after 1 day of influenza A virus infection. Results are representative of three independent experiments and each performed in triplicates. Values are the mean±SD.

It is known that type I interferons (IFNs), IFN-α and -β, play an important role in prevention of viral pathogenesis as their amounts increase within 1 or 2 days after virus infection (15,16). We then measured the amounts of IFN-α and -β in bronchoalveolar (BAL) fluid and plasma in each mice after intranasal inoculation of influenza virus. As we expected, the levels of IFN-α and -β in BAL fluid and plasma from vitamin C-insufficient *Gulo (-/-)* mice were quiet lower than those in wild type and vitamin C-sufficient *Gulo (-/-)* mice (Fig. 3A and B). This result proves that vitamin C is an essential factor for the production of anti-vital immune response during the early phase of virus infection through the production of type I IFNs. The phosphorylation of signal transducers and activators of transcription (STATs) is the critical signaling process after the dimerization of its receptors, when type I IFNs are increased (17,18). Even though it is not presented this reports, we found the defects on the phosphorylation of STAT3 in T cells from *Gulo (-/-)* mice upon vitamin C insufficiency (10). Therefore, the activation of STATs in vitamin C-sufficient *Gulo (-/-)* mice upon virus infection should be further investigated.

The defects on the production of type I IFNs followed by the activation of STATs are closely related to the inflammatory responses due to the failure of controlling virus replication at the initial stage of its infection (19). To investigate the inflammatory response in vitamin C-sufficient *Gulo (-/-)* mice by influenza virus infection, we compared the levels of pro-inflammatory cytokines, IL-1α/β and TNF-α in the BAL fluids from wild type, vitamin C-sufficient *Gulo (-/-)* mice and vitamin C-insufficient *Gulo (-/-)* mice after virus infection. As a result, the production of IL-IL-1α/β and TNF-α was definitely increased in vitamin C-insufficient *Gulo (-/-)* mice (Fig. 4A~C). At the same time, the infiltration of inflammatory cells into the lung is increased approximately 2.5-fold in vitamin C-insufficient *Gulo (-/-)* mice than in others (Fig. 4D). In our previous report regarding the preventive role of vitamin C on the pathogenesis of acute liver inflammation, we have observed that the increase of the infiltration of immune cells into the liver and slight increase of TNF-α production in vitamin C-insufficient *Gulo (-/-)* mice (10). And then the excessive inflammatory responses were occurred by the *in vivo* challenge of concanamycin A (10). Taken together, it seems that *in vivo* vitamin C insufficient status induces hyper-reactive immune responses against virus/bacterial infection or chemicals.

It is generally known that plasma concentration of vitamin C in the mice is approximately 80~100 μM. When the amount of vitamin C is present less than 20 μM in the plasma, we called it as vitamin C insufficiency or "sub-scurvy" (9). Considering that systemic concentration of vitamin C in *Gulo (-/-)* mice at 3 weeks after vitamin C withdrawal was 10~20 μM, we presented the effect of vitamin C insufficiency, not vitamin C deficiency. In conclusion, vitamin C plays a critical role *in vivo* anti-viral immune responses against influenza virus through the increase of IFN-IL-1α/β production. Therefore, it might be possible that the maintaining sufficient levels of vitamin C in the plasma by the continuous uptake through the diet or supplement could effectively prevent *in vivo* pathogenesis of influenza virus at the initial stage of viral infection.

MATERIALS AND METHODS

Mice and viruses

The *Gulo (-/-)* mice were obtained from the Mutant Mouse Regional Resource Center (University of California, Davis, USA). C57BL/6J wild-type and the *Gulo (-/-)* mice were maintained in a specific pathogen-free condition at an animal fa-

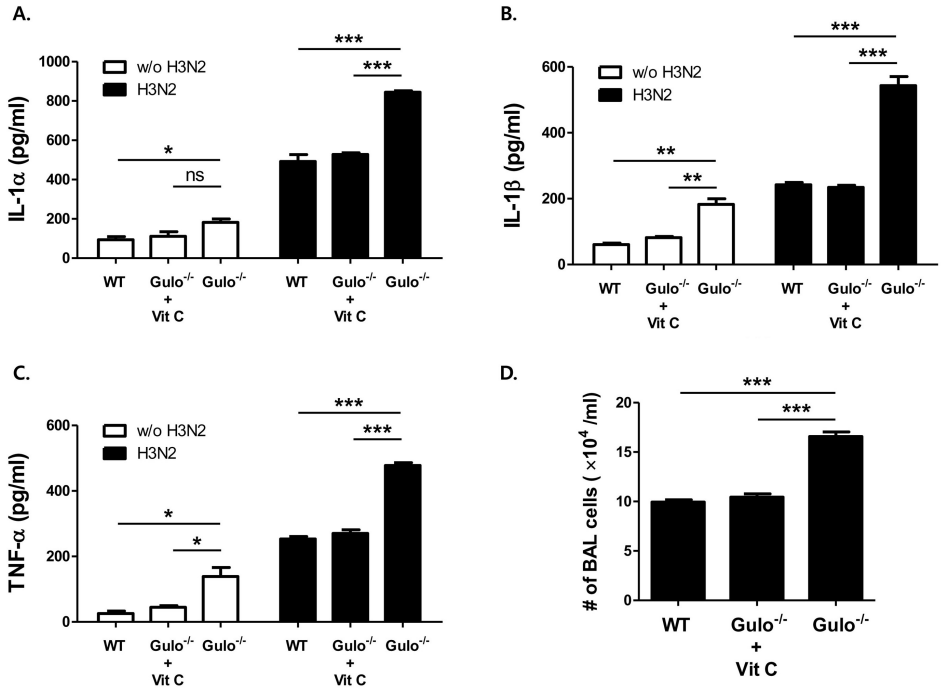

Figure 4. Increased production of IL-α/β and TNF-α in the lung of vitamin C-insufficient *Gulo (-/-)* mice by influenza A virus infection. The levels of IL-1α (A), IL-1β (B) and TNF-α (C) in BAL fluids obtained from wild type (n=6), vitamin C-sufficient *Gulo (-/-)* mice (n=6) and vitamin C-insufficient *Gulo (-/-)* mice (n=6) were measured by ELISA as described in Materials and Methods. Results are representative of three independent experiments and each performed in triplicates. Values are the mean±SD. (D) After centrifugation of BAL fluids, the numbers of infiltrated immune cells into the lung upon influenza A virus infection of in the presence or absence of vitamin C were counted under microscope.

cility in the Seoul National University College of Medicine. Male *Gulo (-/-)* mice (4∼5 weeks old) were maintained for about 3 weeks with or without vitamin C supplementation (Sodium L-ascorbate, 3.3 g/L, Sigma, St. Louis, MO, USA) in their drinking water. The influenza A virus (IAV) strain was kindly provided by Huan H Nguyen (International Vaccine Institute, Seoul, Korea). IAV (HongKong/1/68, H3N2) was grown for 7∼10 days in embryonated hens eggs, and then titrated by hemagglutination inhibition assay (20).

Intranasal inoculation of IAV

Mice were anesthetized and intranasally administered 30 μl PBS containing 20 HAU IAV (HongKong/1/68, H3N2). The illness and mortality of mice were then monitored daily for 7 day.

Blood and bronchoalveolar (BAL) fluids collection

After 1 day of IAV, blood was collected from the intra-orbital plexus with a heparinized capillary tube. The levels of IFN-α/β (PBL Interferon Source, Piscataway, NJ, USA), IL-1α/β and TNF-α (R&D system, Minneapolis, MN, USA) were measured using ELISA, according to the manufacturer's instructions. BAL fluids were collected by lavaging the trachea and lungs with a total of 2 ml PBS containing 1% bovine serum albumin and 0.1% NaN 3 (blocking solution). Total cell numbers per BAL was determined by counting under the microscope.

Analysis of viral replication by real time RT-PCR

Total RNAs of naive or IAV-infected mice were isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and were reverse- transcribed into cDNA using oligo (dT) primers and AMV reverse transcriptase (iNtRON, Daejeon, Korea). The real-time RT-PCR was processed with SYBR Green PCR master mix (MBI Fermentas, St. Leon-Rot, Germany) and performed using an ExiCycler^{TM} (Bioneer, Daejeon, Korea). The specific primers for IAV M2 gene (5'-AAGACCAATCCTGTCACCTC-TGA-3' and 5'-CAAAGCGTCTACGC TGCAG TCC-3') and mouse β2m gene (5'-TGTCTCACTGACCGGCCTGT-3' and 5'-GTT-CAGTATGTTCGGCTTCC-3'). The difference between expression of IAV M2 and β2m was calculated using the $2^{-\Delta(\Delta CT)}$ method.

Statistical analysis

Data were presented as the means±SDs. Unpaired two-tailed t test was used to compare the two groups (WT vs. *Gulo (-/-)* or *Gulo (-/-)* vs. *Gulo (-/-)*+vitamin C). p-values of <0.05 were considered statistically significant. Statistical tests were carried out using GraphPad InStat version 5.01 (GraphPad Software, Le Jolla, CA).

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CONFLICTS OF INTERESTS

The authors have no financial conflict of interest.

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