COMMENT

# Vitamin C as a promoter of γδ T cells

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Human γδ T cells are a small $CD3^+$ subset exhibiting features of both innate and adaptive immune cell.[1] The major γδ T cell population in peripheral blood expresses the Vγ9 and Vδ2 chains and recognizes phosphoantigens (PAgs) that are overexpressed by tumor cells in the absence of genetic restriction. Activation and expansion of Vγ9Vδ2 T cells is also achieved by aminobisphosphonates (n-BPs), which promote the intracellular accumulation of PAgs. Because of their potent cytotoxic and antitumor activity, Vγ9Vδ2 T cells are particularly indicated for use in cancer immunotherapy, even against tumors with low mutational burdens.

There are two strategies to induce the modulation and activation of Vγ9Vδ2 T cells: (1) in vivo administration of PAgs or n-BPs with IL-2 and (2) adoptive transfer of ex vivo expanded Vγ9Vδ2 T cells.

Several observational and phase I clinical studies exploiting γδ T cells in cancer have been conducted over the last 15 years and have provided evidence for good safety but variable efficacy because of problems related either to the variation in the types of cancer treated or the heterogeneity in the protocols used to expand γδ T cells.[2] In addition to these technical issues, other factors related to the biological properties of these cells may influence the success or failure of γδ T cell-based immunotherapy;[2] the progressive loss of Vγ9Vδ2 T cells has been observed upon injection of PAgs or n-BPs and IL-2, together with progressive reductions in their proliferative response, most likely depending on activation-induced terminal differentiation and exhaustion.[2] Other immune cells may contribute to Vγ9Vδ2 T cell exhaustion/anergy: peripheral blood neutrophils internalize n-BPs and produce hydrogen peroxide that inhibits Vγ9Vδ2 T cell proliferation,[3] and myeloid cells also downregulate γδ T cell effector functions through the PD-1/PD-L1 pathway.[4] These pitfalls of in vivo activation of γδ T cells may be, at least in part, overcome by adoptively transferring ex vivo-expanded Vγ9Vδ2 T cells. However, this approach also has several limitations, the most important being the difficulty in obtaining adequate numbers of good quality and viable Vγ9Vδ2 effector T cells due to the high mortality rate of the expansion procedure and the progressive unresponsiveness to subsequent in vitro stimulation with PAgs or n-BPs and IL-2. Moreover, another major problem is how to maintain adequate levels and functions of the transferred Vγ9Vδ2 T cells in recipient patients for as long as possible. For instance, studies in renal cell cancer have shown improved efficacy when Vγ9Vδ2 T cells were administered with the n-BP zoledronate and/or IL-2, compared with that of Vγ9Vδ2 T cells administered alone,[5] or the administration of Vγ9Vδ2 T cells from haploidentical donors, which persisted for 28 days and expanded in vivo following injection of zoledronate and IL-2.[6]

In the May 2020 issue of *Cellular and Molecular Immunology*, a study by Yin and colleagues proposed an alternative approach to obtain high quality expansion of Vγ9Vδ2 T cells.[7] The researchers investigated the effects of vitamin C (VC) and its more stable derivative, L-ascorbic acid 2-phosphate (pVC), on the proliferation and effector function of human Vγ9Vδ2 T cells stimulated with zoledronate or PAgs and IL-2 and provided proof-of-principle that the modulatory activities of VC and pVC may help to increase the efficacy of Vγ9Vδ2 T cell expansion for subsequent adoptive immunotherapy.[7]

VC, also known as ascorbic acid, is a vitamin found in various foods, including citrus fruits, kiwifruit, guava, broccoli, Brussel sprouts, bell peppersand strawberries. Several mammals, including humans, cannot independently produce VC because they lack L-gulonolactone oxidase, the last enzyme in the VC metabolic pathway.[8]

VC is important for immune system functions; in fact, VC deficiency (a clinical condition known as Scurvy) results in increased susceptibility to potentially fatal infections such as pneumonia.[8] While VC does not prevent infections, some studies indicate that the regular use of VC supplements at a dose of least at 200 mg/day may reduce the duration and severity of the common cold by 8% in adults and 14% in children.[8]

VC influences both innate and adaptive immune responses. VC promotes the antimicrobial function of epithelial barriers, accumulates in phagocytes and enhances their chemotaxis, phagocytosis, generation of reactive oxygen species, and finally microbial killing. The role of VC in lymphocytes is not clear, but VC has been shown to enhance the differentiation and proliferation of B and T lymphocytes.[8]

In the study by Yin and colleagues, VC and pVC did not increase the extent of Vγ9Vδ2 T cell expansion following stimulation of PBMCs with n-BP or PAg and IL-2 but instead increased the proliferation of Vγ9Vδ2 T cell lines expanded for 14 days in response to pAgs and IL-2 (see Fig. 1). For pVC, this effect was not due to preventing exhaustion in PAg-restimulated Vγ9Vδ2 T cells but to the enhancement of cell cycle progression and cellular expansion.[7] Another result of the study needs to be considered: VC- and pVC-treated Vγ9Vδ2 T cells produced increased amounts of IFN-γ, and in the presence of pVC Vγ9Vδ2, T cells upregulated the expression of the transcription factors T-bet and GATA-3.[7] These important biological effects led us to consider the use of VC and pVC as promising cofactors to enhance Vγ9Vδ2 T cell proliferation and expansion for subsequent ex vivo adoptive immunotherapy. Moreover, Vγ9Vδ2 T cells generated in the presence of VC in culture express high levels of the costimulatory molecules CD80 and CD86, a finding that highly suggests that Vγ9Vδ2 T cells also acquire antigen-presenting capacity and

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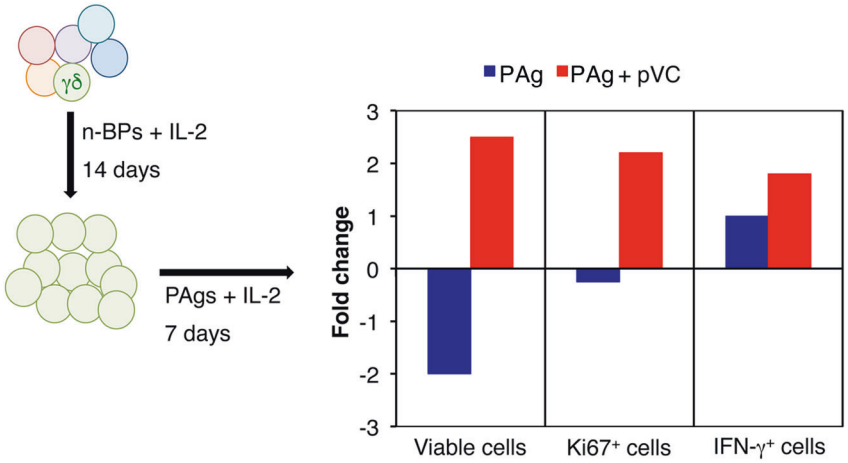


**Fig. 1** pVC promotes the proliferation of PAg-restimulated γδ T cell lines. Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 14 days with n-BPs (such as zoledronate) and IL-2 to generate short-term Vγ9Vδ2 T cell lines. Subsequently, the generated γδ T cells were restimulated for 7 days with PAgs (such as BrHPP) and IL-2 in the presence or absence of pVC. Thereafter, the absolute number of viable Vγ9Vδ2 T cells and the percentages of Vγ9Vδ2 T cells expressing Ki67 and IFN-γ were determined. The histogram bars represent the fold change in each tested parameter

indirectly exert antitumor effects by activating CD8 cytotoxic T lymphocytes.[9]

It is known that γδ T cells in the tumor microenvironment (TME) may play opposing functions depending on the specific γδ T cell subset present at the tumor site and the differential IFN-γ or IL17 expression, which reflects the dual functional activity of these cells.[10] Although VC and pVC preferentially promote the expansion of Vγ9Vδ2 T cells capable of producing IFN-γ but not IL17, there is a concern that needs to be investigated and could limit the use of VC for in vivo expansion of Vγ9Vδ2 T cells. In fact, the same authors demonstrated in another recent paper that in the presence of TGF-β, pVC potently increases FOXP3 expression and the suppressive activity of Vγ9Vδ2 T cells by epigenetic modifications of the FOXP3 gene.[11]

It is known that the TME drives the differentiation of Vγ9Vδ2 T cells toward immunosuppressive subsets, and the TME is extremely rich in TGF-β, which is produced by several cell types, such as cancer-associated fibroblasts, M2 macrophages, Tregs, and tumor cells.[10] Hence, in vivo VC administration could be detrimental, causing a switch from antitumoral to protumoral activity in Vγ9Vδ2 T cells. Moreover, in the study by Yin and colleagues, VC was effective in vitro at a concentration of 50 μg/ml/$10^6$ PBMCs, and ~$3 \times 10^{13}$ cells make up the human body, which corresponds to a VC dose of 1500 g, far beyond the tolerable upper limit of 2 g. Accordingly, a recent study in mice showed that high-dose VC (4 g/kg body weight administered intraperitoneally 5 days per week) enhanced immune checkpoint therapy but only in the presence of a fully competent immune system.[12]

While the above considerations may hamper the use of VC in vivo to promote the activation of Vγ9Vδ2 T cells, VC and its more stable derivative pVC could be excellent factors to be added to cell cultures for large-scale Vγ9Vδ2 T cell expansion, which would also take advantage of the ability of these factors to induce Th1 polarization, as demonstrated by IFN-γ production, thus yielding a high quality Vγ9Vδ2 T cell product to be administered to patients.

Optimization of culture conditions to obtain sufficient numbers of viable Vγ9Vδ2 T cells that may be equipped with antitumor effector functions (cytotoxic activity, IFN-γ production, etc.) is needed to generate high-quality Vγ9Vδ2 T cell products for adoptive immunotherapy. Current protocols are characterized by high exhaustion and anergy rates, probably due, in whole or in part, to the effects of IL-2, which is used in combination with PAgs or n-BPs to induce Vγ9Vδ2 T cell proliferation and expansion. The study by Yin and colleagues suggests the possibility that the addition of VC or pVC to cultures may promote optimal expansion of Vγ9Vδ2 T cells equipped with effector and antitumor functions; preclinical studies in immunodeficient mice reconstituted with Vγ9Vδ2 T cells generated in the presence or absence of VC may help to compare their antitumor activities and define the most appropriate and effective use of VC in immunotherapeutic protocols.

Finally, Yin and colleagues studied the effects of VC and pVC only on Vγ9Vδ2 T cells but not on other γδ T cell subsets. Since tissue-resident Vδ1 T cells have potent antitumor activity and it is now possible to achieve large-scale expansion of Vδ1 T cells,[13] it would be worth studying the effects of VC and pVC on this γδ T cell population.

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## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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