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REVIEW

Empowering γδ T-cell functionality with vitamin C

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Vitamin C (ascorbic acid) is a potent antioxidant and a cofactor for various enzymes including histone demethylases and methylcytosine dioxygenases. Vitamin C also exerts direct cytotoxicity toward selected tumor cells including colorectal carcinoma. Moreover, vitamin C has been shown to impact immune cell differentiation at various levels including maturation and/or functionality of T cells and their progenitors, dendritic cells, B cells, and NK cells. γδ T cells have recently attracted great interest as effector cells for cell-based cancer immunotherapy, due to their HLA-independent recognition of a large variety of tumor cells. While γδ T cells can thus be also applied as an allogeneic off-the-shelf product, it is obvious that the effector function of γδ T cells needs to be optimized to ensure the best possible clinical efficacy. Here we review the immunomodulatory mechanisms of vitamin C with a special focus on how vitamin C enhances the effector function of γδ T cells. We also discuss future directions of how vitamin C can be used in the clinical setting to boost the efficacy of adoptive cell therapies.

Keywords: Adoptive cell therapy · γδ T cells · Immunotherapy · Vitamin C

Introduction

Vitamin C (VitC), also known as ascorbic acid, is a pleiotropic molecule with many diverse functions in cell biology and immunity. It is an essential vitamin that cannot be synthesized by primates including humans. Therefore, VitC needs to be taken up from dietary sources with a recommended daily amount of 90–105 mg [1]. The plasma concentration of VitC (normal range 26–84 μM) is regulated at the level of intestinal uptake and renal excretion [2]. VitC deficiency with persistent plasma levels below 11 μM results in scurvy disease which is at least partially due to diminished collagen stability because of reduced cofactor activity for enzymes involved in collagen synthesis [3]. Moreover, lower-than-normal plasma levels are also present in patients with advanced cancer, likely due to reduced intestinal uptake [4]. While the plasma concentration of VitC is in the micromolar range, millimolar concentrations are present in tissues, with the highest concentrations (2–10 mM) found in the adrenal gland and brain. Circulating immune cells in the blood contain 1–4 mM VitC [1].

VitC exists in two redox states: the reduced active form is ascorbate which enters into cells via sodium-dependent Vitamin C transporters (SVCT1/2). The oxidized form, dehydroascorbic acid (DHA), can be internalized via facilitated glucose transporters

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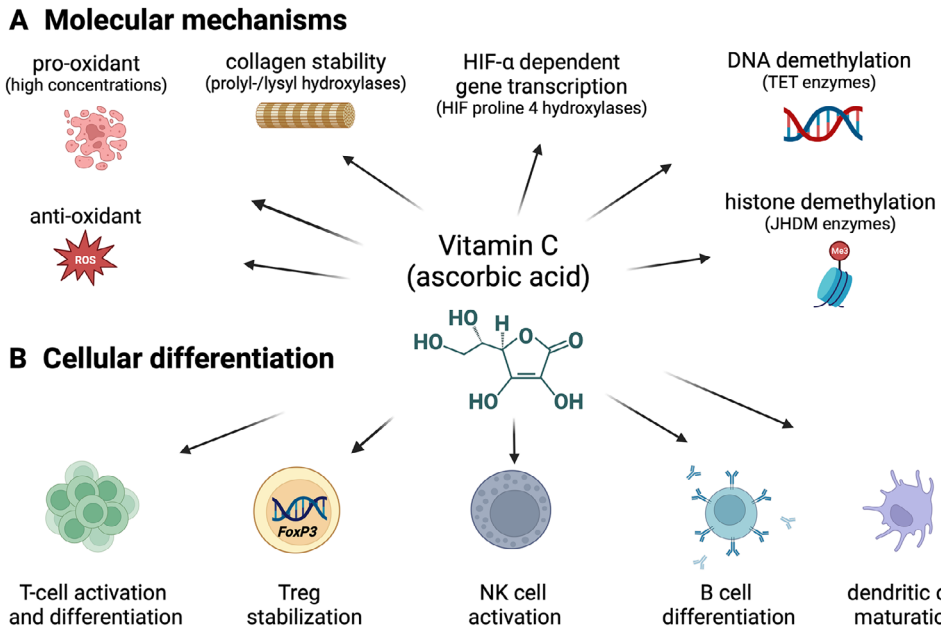


Figure 1. Key molecular and cellular mechanisms of vitamin C action. (A) VitC is a potent antioxidant but also has pro-oxidant activity at high concentrations. It is a co-factor for a large family of Fe^{2+} and oxygen-dependent 2-oxoglutarate-dependent dioxygenases. Lysyl- and prolyl hydroxylases are important for the assembly of collagen fibers, while TET and JHDM enzymes are epigenetic regulators of DNA and histone demethylation. The HIF transcription factors regulate a large number of genes in an oxygen-dependent manner. Under normoxic conditions, HIF-α is hydroxylized by VitC-dependent hydroxylases and further degraded and thus inactive. (B) The numerous molecular effects of VitC influence the activation and differentiation of various immune cells including T cells, Treg, NK cells, B cells/plasma cells, and dendritic cells.

(GLUT). Upon internalization, DHA is rapidly converted back to ascorbic acid by DHA reductase [3, 5]. It appears that both mouse and human T cells preferentially use SVCT2 for the uptake of VitC [6, 7] while SVCT1 is largely used for intestinal uptake [8]. The overall tissue accumulation of VitC depends on local and systemic parameters including plasma availability and oxidative stress [9].

Pleiotropic molecular effects of VitC

At the molecular level, VitC regulates multiple biological activities including the redox system, various enzymes involved in oxygen-dependent transcription and epigenetic regulation, and kinases regulating signal transduction. As a consequence, VitC modulates the activation, differentiation, and survival or death of many cell types. In Figs. 1A and B, we summarize the key molecular mechanisms and how VitC affects the differentiation of immune cells, respectively.

Under physiological pH conditions, VitC is highly acidic and reduces free radicals by electron donation. Micromolar concentrations of VitC can thus act as free radical scavengers and reduce dangerous ROS thereby protecting cells from oxidative stress [10]. Higher concentrations, on the other hand, can also have pro-oxidant activity [3]. High concentrations of VitC can initiate ROS-induced tumor cell death [11]. Extracellular oxidation of VitC in the presence of labile iron (Fe^{3+}) ions generates hydrogen peroxide (H_2O_2) and extracellular DHA which imposes intracellular oxidative stress and glutathione (GSH) depletion upon internalization via GLUT1 [12, 13]. Tumor cells expressing high levels of GLUT1 are therefore susceptible to cell death triggered by high concentrations of VitC [14]. In this regard, a specific role for *KRAS* and *BRAF* mutations in rendering colorectal cancer cells susceptible to high-dose VitC treatment has been demonstrated by Yun et al. [15]. It was observed that the increased uptake of DHA via GLUT1 and subsequent intracellular ROS accumulation and depletion of GSH was associated with the inactivation of glyceraldehyde 3-phosphate dehydrogenase. The inhibition of glyceraldehyde 3-phosphate dehydrogenase in highly glycolytic *KRAS* and *BRAF* mutant cells induced cell death in mutant but not wild-type cells [15]. These studies have reinforced the interest in exploring high-dose intravenous application of VitC as a treatment modality in selected types of cancer [13, 16-18].

An important function of VitC is its cofactor activity for numerous enzymatic reactions. VitC itself and its derivatives semidehydroascorbate and DHA are substrates for enzymatic reactions. Such enzymatic reactions are involved in the recycling of ascorbate but also in many other physiological processes as summarized in [3]. Perhaps the most important group of enzymes where VitC acts as an essential cofactor are hydroxylases belonging to the group of 2-oxoglutarate-dependent dioxygenases (2ODDs). 2ODDs comprise a large family of enzymes that act on different substrates but all require divalent iron (Fe^{2+}), oxygen, and 2-oxoglutarate (also termed α-ketoglutarate) as co-substrates [19]. The role of VitC in the enzymatic reaction is to reduce Fe^{3+} to Fe^{2+} during oxidation to DHA, thereby enabling the 2ODDs

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to hydroxylize their substrate in the presence of oxygen and 2-oxoglutarate, giving rise to the formation of the hydroxylized substrate, succinate, and CO_2 [3]. As mentioned above, chronic VitC deficiency results in scurvy disease due to improper assembly of the extracellular matrix. The correct triple helical folding and cross-linking of collagen fibers (and thus the functioning of the extracellular matrix) strictly depends on the hydroxylation of proline and lysine residues by 2ODDs which require VitC as a co-factor [3].

2ODDS are also critically involved in the regulation of oxygen-dependent transcription factors, notably hypoxia-inducible transcription factors (HIFs). Adaptation to hypoxic conditions is important under physiological and pathophysiological conditions, and consequently, HIFs regulate a large number of genes [20, 21]. Under normoxic conditions, the α chain of HIF1/2/3 is hydroxylized due to the activation of HIF-proline-4-hydoxylases, where VitC plays again an important role as co-factor [21, 22], and is subsequently degraded in the proteasome. As a consequence, the transcriptional activity of HIFs is repressed. Under hypoxic conditions and in the absence of VitC co-stimulator activity, the HIF α chains are not hydroxylized and degraded, and the HIFs can exert their transcription factor activity and thus regulate gene expression in a context-dependent manner [3, 21].

In recent years, it was discovered that VitC is also a co-factor for 2ODDs involved in epigenetic regulation of cellular differentiation. Prominent mechanisms of epigenetic regulation include the methylation/demethylation of DNA and histones. The oxidation (and thus demethylation) of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5mhC) is mediated by ten-eleven-translocation (TET) proteins which are again 2-OG, oxygen, and VitC-dependent 2ODDs [1, 23]. There are three TET proteins with different roles in embryonic and hematopoietic development [1]. TET genes are frequently mutated in various human hematological malignancies and solid tumors and reduced levels of 5hmC are consequently found in various human cancers [24, 25]. VitC catalyzes TET activity and thus promotes DNA demethylation which is an important event in the regulation of various immunologically relevant genes (see below). Another class of 2ODD involved in epigenetic regulation is the Jumonij-C domain-containing histone demethylases (JHDM) [26]. The methylation of histones is catalyzed by histone methyl transferases. This process is reversible and demethylation can be accomplished by JHDM. The activity of JHDM is again regulated by VitC, illustrating yet another level of how VitC can potentially impact on epigenetic regulation [27].

Given the pleiotropic activities of VitC, it is reasonable to assume that VitC also affects signal transduction pathways. In fact, both activating and inhibitory activity of VitC on some kinases have been reported. It emerges that the overall outcome depends both on the cellular context but also on the stimulus. In cytokine-activated endothelial cells, VitC inhibited NF-κB activation which was mediated through inhibition of IκBα kinase by DHA [28]. Low-dose VitC was reported to inhibit the phorbolester-driven differentiation of myeloid HL-60 cells, associated with the inhibition of ERK phosphorylation [29]. On the other hand, VitC activates the JAK2/STAT2 pathway when VitC is taken up via the SVCT2 transporter [30]. A summary of the molecular effects of VitC is presented in Fig. 1A.

Immune modulation by VitC

In view of its numerous molecular effects, it is not surprising that VitC modulates multiple pathways of immune cell activation and differentiation as recently reviewed in several excellent reviews [18, 31, 32, 33]. A simplified overview is presented in Fig. 1B. VitC promotes the generation of mouse and human induced pluripotent stem cells, the development of double-positive T cells from the hematopoietic progenitor, and the maturation of T cells [34, 35, 36]. In general, VitC was found to preferentially drive T cells into the Th1 differentiation pathway [37, 38]. However, VitC also enhances the growth and effector activity of cytomegalovirus-specific human CD8 CTL [39]. These effects are likely due to the antioxidant and epigenetic activity of VitC. In several instances, the effect of VitC on DNA and histone (de)methylation has been specifically investigated. Through activation of TET enzymes, VitC induces hypomethylation in specific CpG sites of *FoxP3* thereby leading to increased stability of FoxP3 protein and suppressive function of regulatory T cells (Treg) [40, 41, 42]. VitC also stabilizes the regulatory function of induced CD8 Treg (iTreg) [43]. The stabilizing effect of VitC on *FOXP3* demethylation and suppressive function of Treg might be relevant for the optimization of manufacturing protocols of human Treg for therapeutic application [44]. On the other hand, histone demethylation by Jmjd2 (a member of the JHDM family) drives the differentiation of CD4 T cells toward IL-17 production [45]. Beyond T cells, VitC also modulates the differentiation of other immune cells with relevance for cancer immunotherapy. VitC enhances the generation and expansion of NK cells from hematopoietic progenitor cells and regulates the methylation status of *KIR* genes on human NK cells [46, 47]. Recently, it was also shown to enhance plasma cell differentiation in mouse and human B cells through TET2/3-dependent epigenetic remodeling and thereby increase antibody responses [48, 49]. VitC supports the generation of type I interferon-producing plasmacytoid dendritic cells from hematopoietic progenitor cells [50] and augments the maturation of dendritic cells through TET-dependent epigenetic regulation [51]. While not discussed further here, VitC also affects innate immune cells including macrophages and neutrophils [18].

γδ T cells at the forefront of cellular cancer immunotherapy

γδ T cells expressing the γδ T-cell receptor (TCR) rather than the conventional αβ TCR are a numerically minor T-cell subset in blood but account for a substantial proportion of intraepithelial T cells in tissue, notably the intestine [52]. The γδ TCR consists of the variable (V) region-containing TCRγ and TCRδ chains

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associated to the invariant CD3 subunits [53, 54]. Depending on the TCR Vγ and Vδ use, the γδ T cells might have different functions [55]. Thus, γδ T cells are usually categorized according to their V gene usage. The majority (50–95%) of γδ T cells circulating in blood express a Vγ9Vδ2 TCR (termed Vδ2 in the following) while Vδ1 T cells co-expressing less restricted Vγ elements usually comprise less than 30% of the γδ T-cell pool. In recent years, γδ T cells have raised great interest for their potential application in cancer immunotherapy for the following two reasons: (1) γδ T cells do not depend on HLA molecules for antigen recognition; the HLA-independence opens the perspective for their potential application across HLA barriers, that is, in an allogeneic setting. (2) In contrast to CD4 and CD8 T cells with the conventional αβ TCR, γδ T cells recognize stress-associated molecules, such as those being upregulated upon cellular transformation or infection. For example, Vδ2 T cells recognize phosphorylated intermediates of the mevalonate (eukaryotes) or non-mevalonate (bacteria, some parasites) pathway of isoprenoid biosynthesis. Prototypic pyrophosphates ("phosphoantigens", pAg) are isopentenyl pyrophosphate, an intermediate of eukaryotic cholesterol synthesis [56], and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, an intermediate of the prokaryotic non-mevalonate pathway [57]. The activation of Vδ2 T cells by pAg requires the expression of butyrophilin (BTN) family members, specifically BTN2A1 and 3A1. Endogenous pAg binds to the cytoplasmatic B30.2 domain of BTN3A1 leading to interaction with BTN2A1 and most likely additional BTN proteins. This conformational change allows the binding to Vγ9 and subsequent TCR activation (see ref. [58] for review). Importantly, the accumulation of pAg in cell culture can be easily induced by aminobisphosphonates such as zoledronic acid (Zol) which are in clinical use for the treatment of bone diseases. In addition to its effects on bone resorption, Zol induces a potent and selective activation of Vδ2 T cells because it interferes with the mevalonate pathway and induces accumulation of γδ T-cell stimulating isopentenyl pyrophosphate [59]. Zol together with IL-2 is frequently used to expand γδ T cells in vitro for subsequent adoptive transfer. Small-scale clinical studies with transfer of autologous Zol-expanded γδ T cells in cancer patients performed since early 2000; however, have shown only limited efficacy [60]. A potential problem in this setting is the fact that γδ T cells from cancer patients frequently do not expand in vitro as well as γδ T cells from healthy donors. Due to their HLA independence, however, it is possible to consider the use of allogeneic γδ T cells (derived from healthy donors) for adoptive transfer into cancer patients. If successful, this strategy would also allow to develop γδ T cells as an off-the-shelf product which would certainly drastically broaden the applicability of γδ T-cell immunotherapy. In this regard, Yin et al. [61] have already performed a clinical phase I study where 132 patients with late-stage solid cancers received a total of 414 infusions of allogeneic Zol-expanded γδ T cells. This proof-of-principle showed that the allogeneic γδ T-cell transfer is safe and can be developed into clinical protocols for Vδ2 but also Vδ1 γδ T cells [61, 62].

Both Vδ2 and Vδ1 γδ T cells have a broad antitumor activity and are therefore currently considered as promising effector cells for cancer immunotherapy [63, 64, 65]. However, there is a considerable need to optimize the efficacy of γδ T-cell therapy. In addition to various concepts of γδ T-cell products (unmodified Vδ2 or Vδ1, drug-resistant, chimeric antigen receptor (CAR) modified), there are also many antibody constructs (e.g. bispecific engagers) in development or clinical studies that selectively target tumor-reactive γδ T cells (see refs. [52, 65] for recent reviews). Clearly, there is a need for optimization of the in vitro manufacturing process of cellular products but also for the in vivo activation of γδ T cells by selected antibodies [66] or bispecific engagers [67]. Based on the multitude of reports in the literature on immunomodulatory and enhancing effects of VitC we have studied its role in the in vitro activation of human γδ T cells.

VitC enhances the functionality of human γδ T cells

Supplementation of the culture medium with VitC increased the proliferative expansion of γδ T cells, both when purified γδ T cells were stimulated with pAg or when γδ T cells within PBMC were selectively activated with Zol [61, 68]. For cell culture, we routinely use L-ascorbic acid 2-phosphate (pVC) which is more stable and frequently used in stem cell research [69]. However, appropriately buffered regular VitC (as used for i.v. application in humans) can also be used. In addition to proliferation, VitC enhanced several other parameters like IFN-γ production, cell cycle progression, and metabolic activity. Strikingly, VitC has a strong rescuing effect on the re-stimulation of expanded γδ T cells with pAg where a large fraction of activated T cells usually undergoes activation-induced cell death [70]. The presence of VitC led to a massive expansion of the restimulated γδ T cells which we think is an important observation when optimizing expansion protocols for therapeutic application of γδ T cells. γδ T cells expanded for 12 days in the presence of VitC also exerted higher antitumor activity in a preclinical humanized mouse model and in vitro against human tumor target cell lines [61]. Even when γδ T cells were cultured in the presence of VitC for only several days, they showed increased antitumor activity (unpublished). Ongoing studies investigate in more detail the mechanisms behind the boosting effect of VitC on γδ T-cell functionality. We observed increased Ca^{2+} influx when Zol-expanded γδ T-cell lines were short-term cultured (4–24 h) in the presence of VitC and stimulated by anti-CD3 antibodies. This was associated with increased nuclear translocation and activity of the NF-κB and NFAT transcription factors in the presence of VitC (unpublished results). Not unexpectedly, VitC also altered the transcriptional profile of Zol-expanded γδ T cells (unpublished results), and a detailed analysis is currently underway. The altered transcriptional profile might also explain the increased Ca^{2+} influx in short-term cultures with VitC.

In order to improve cancer immunotherapy, we have developed a new format of a CAR, in which the antitumoral single-chain Fv fragment is directly fused to the αβ TCR [71, 72] and γδ TCR [73]. This new construct is called the TCR fusion construct (TRuC). We tested whether VitC could improve tumor killing by

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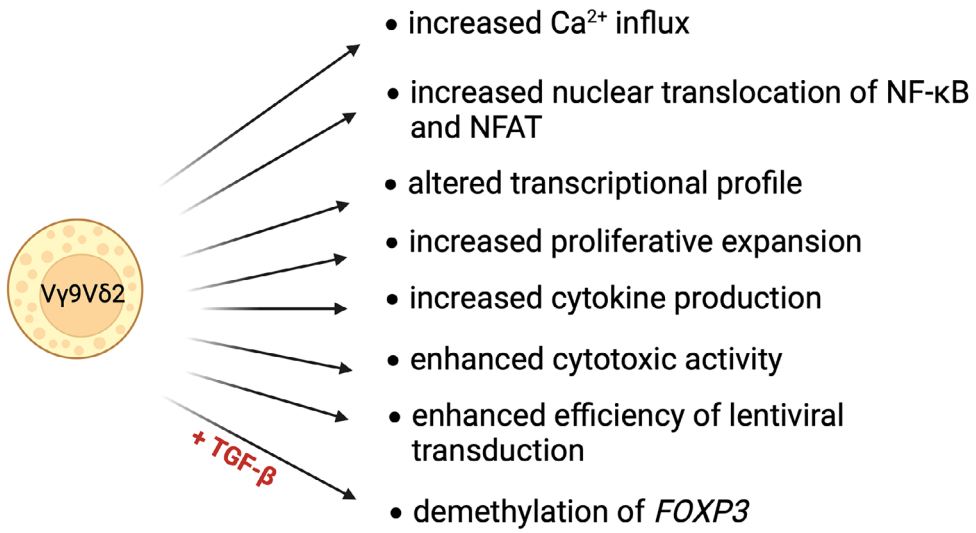


Figure 2. Modulation of γδ T-cell activation and differentiation by vitamin C. Exposure to VitC has been reported to promote the activation and differentiation of human γδ T cells at various levels. VitC also enhances lentiviral transduction of γδ T cells by increasing the proliferation of the cells after transduction. Furthermore, VitC also induces *FOXP3* hypomethylation in human γδ T cells which, however, is strictly dependent on additional presence of TGF-β.

the anti-CD19 TRuC γδ T cells. We found that VitC improved the recovery of Vδ2- and Vδ1/Vδ2-expanded γδ T cells after lentiviral transduction, most likely by improving the proliferation of the γδ T cells and reducing their death [73]. Furthermore, the anti-CD19 TRuC-expressing γδ T cells, both Vδ2- and Vδ1/Vδ2-expanded, showed increased antitumor activity when cultured in the presence of VitC after lentiviral transduction (unpublished). Similarly, a better expansion and maintenance of a stem cell phenotype with reduced exhaustion of CD19-CAR transduced $CD3^+$ T cells have been also observed by others [74]. Based on these results and in view of the various ongoing studies with CAR γδ T cells targeting different tumor-expressed antigens [74] we suggest including VitC in the manufacturing process.

As VitC is a potent epigenetic modifier, it can be assumed that at least some of the above-mentioned enhancing effects on human γδ T cells are due to epigenetic rewiring which we have not yet studied in detail, with the exception of analyzing FOXP3. γδ T cells isolated from blood do not express FOXP3 but can be induced so by TGF-β which also modulates the cytotoxic function of γδ T cells [75]. Well in line with FoxP3-expressing CD4 T cells [74—74] we found that VitC strongly demethylates specific CpG regions in the *FOXP3* locus. Importantly, however, *FOXP3* hypomethylation required both TGF-β and VitC, no demethylation was observed in TGF-β-treated FOXP3-positive γδ T cells in the absence of VitC [76]. Of course, γδ T cells expanded in vitro for cancer immunotherapy should not express functionally active (demethylated) FOXP3 (and thus be immunoregulatory). In the absence of TGF-β, this is not the case. A summary of the in vitro effects of VitC on human γδ T cells is shown in Figure 2.

While we have focused here on VitC, it should be emphasized that other vitamins may also have a major impact on activation of T cells including γδ T cells. As an example, vitamin D (VitD) has pleiotropic but generally anti-inflammatory effects on the immune system which form the basis for its therapeutic use in autoimmune diseases like multiple sclerosis [77]. At the molecular level, the bioactive form of VitD, $1\alpha,25(OH)_2D_3$ (calcitriol) switches off pro-inflammatory programs in Th1 cells and induces IL-10 production [78]. In line, VitD was reported to inhibit IFN-γ production also in human Vδ2 γδ T cells [79, 80]. In contrast, however, enhancing effects of VitD on the cytokine production and cytotoxic activity of Vδ2 T cells were reported by Li et al. [81]. While the underlying reasons for the seemingly contradictory results are unclear and require further investigation, it will be interesting to consider how two widely therapeutically applied vitamins, that is, VitC and VitD in concert modulate immune responses and γδ T-cell activation [82].

VitC enhances cancer immunotherapies beyond γδ T cells

The initial studies of Cameron and Pauling on the beneficial effect of VitC in cancer patients [83] were followed by criticism and reduced interest, but the accumulated evidence today indicates that VitC is a powerful supplement to current cancer immunotherapies [13, 14, 16, 17]. Specifically, it seems promising to combine immune checkpoint therapies with VitC application. In fact, it has been already demonstrated in murine models that high-dose VitC enhances immune checkpoint therapy in several cancer models. It was shown that VitC modulated the tumor microenvironment and led to increased CD8 T-cell infiltration and cytotoxic activity of transferred T cells [84, 85]. Moreover, VitC was recently shown to downregulate PD-L1 expression in breast cancer cells through transcriptional regulation [86]. Last but not least, high-dose VitC also synergistically increased the antitumor effect of

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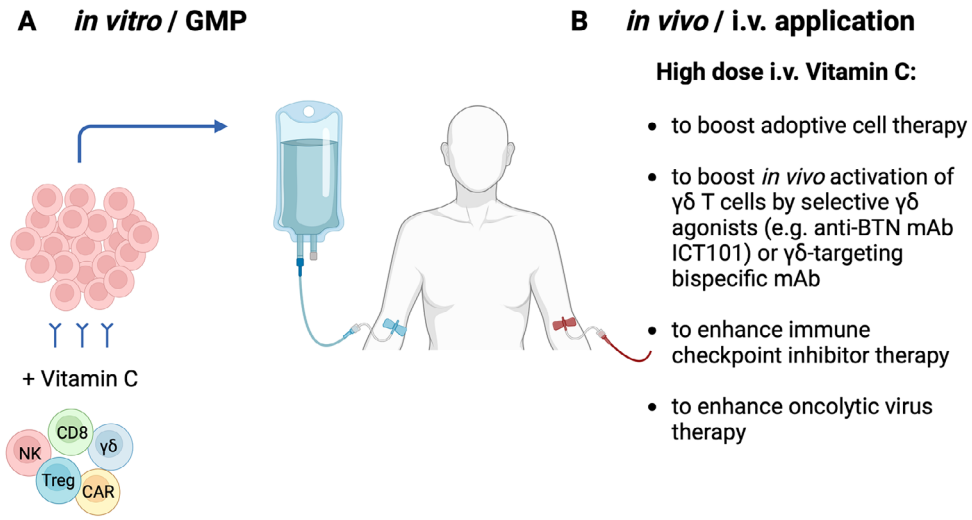


Figure 3. Potential use of Vitamin C in cancer immunotherapy. (A) Supplementation of culture medium with VitC increases the expansion and functionality of various cellular products, such as γδ T cells. VitC can be easily integrated into the GMP-conform manufacturing process. (B) High dose i.v. application of VitC might boost the efficacy of adoptive cell transfer and the in vivo activation of γδ T cells by antibody constructs. VitC could boost checkpoint inhibitor therapy and synergize with oncolytic virus therapies.

oncolytic adenovirus therapy by enhancing immunogenic tumor cell death [87]. Furthermore, VitC may also enhance the efficacy of adoptive cell therapies. VitC is available as a product licensed for high dose i.v. application and thus can be easily integrated into manufacturing processes of cellular products including CAR T-cells, NK cells, and γδ T cells in compliance with GMP requirements (Fig. 3A). In addition, VitC can be infused in high concentrations as a single agent or in combination with immunotherapy (Fig. 3B). It is important to note that oral uptake does not generate plasma levels (>10 mmol/L) required to mediate antitumor and/or immunomodulatory effects [9, 16].

Concluding remarks

As discussed in this review, VitC has multiple effects which should be beneficial for various cancer immunotherapies. Both the in vitro treatment of cellular products during the manufacturing process and the high-dose in vivo application can be considered. Furthermore, VitC might help to boost the in vivo activation of tumor-reactive γδ T cells by antibodies or bispecific engagers (Fig. 3B) [66, 67]. In conclusion, VitC is a cheap and readily available supplement for available (cancer) immunotherapies.

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Abbreviations: **2ODD**: 2-oxoglutarate-dependent dioxygenase · **DHA**: dehydroascorbic acid · **HIF**: hypoxia-inducible transcription factor · **JHDM**: Jumonij-C domain-containing histone demethylase · **pAg**: phosphoantigen · **TET**: ten-eleven-translocation · **VitC**: vitamin C · **Zol**: zoledronic acid

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