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High-dose Vitamin C as a new strategy to enhance cancer immunotherapy

Tutor:
Prof. Alberto Bardelli

Candidato:
Alessandro Magrì

Coordinator:
Prof. Francesco Novelli

'Success is not final

failure is not fatal

it is the courage to continue

that counts'

W. Churchill

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1. ABSTRACT

Immune checkpoint therapy (ICT) is very effective in tumors with high mutational burdens such as a fraction of melanoma, urothelial and lung cancers as well as MMR deficient (MMRd, MSI) tumors. Despite this, only a small subset of tumors derives clinical benefit from ICT. On the other hand, the clinical efficacy of immune checkpoint blockade remains very limited in extremely aggressive cancers such as pancreatic or in some highly prevalent tumors such as breast and MMR proficient (MMRp, MSS) colorectal cancer. For all these reasons, there is a need to find safe combinatorial strategies that can boost the efficacy of ICT and expand the tumor types and number of patients who may benefit from cancer immunotherapy. Vitamin C (VitC) is an essential dietary nutrient and its chronic deficiency contributes to impaired immunity. Immune cells accumulate high levels of VitC and some reports suggest a possible effect of VitC on immune cells through modulation of epigenetics. Indeed, VitC can act as a co-factor of TET dioxygenases and histone demethylases that are involved in DNA and histone demethylation reactions thus modulating gene expression. VitC is known to impair cancer cell growth in preclinical models but there is little clinical evidence on its antitumoral efficacy. Importantly, whether and how VitC modulates anticancer immune responses is mostly unknown. We found that high-dose VitC requires a fully competent immune system to delay the growth of breast, colorectal, melanoma and pancreatic tumors in experimental mouse models. We also report here that VitC induces tumor infiltration by cells of the immune system and delays cancer growth in a T cell dependent manner. Furthermore, adoptive cell transfer experiments show that VitC enhances the anticancer activity of CD8 T lymphocytes. We found that VitC co-operates with CTLA-4 and PD-1 blockade in several experimental mouse models. Most importantly, our data indicate that the combination of VitC and ICT can be curative in MMRd murine breast and colorectal tumors with high mutational burden. Based on these findings, we propose the initiation of clinical trials combining ICT with high doses of VitC.

2. INTRODUCTION

The first evidence of cancer has been reported to date back to 1,200 B.C. in Egypt (*1*). Ancient Greeks considered cancer to be a pathologic biliary excess and utilized surgery to remove it. They also noticed that surgery wasn't always enough to eradicate it. Curiously, the words *karkinos* and *karkinoma* were firstly used by Hippocratic physicians to describe tumors or non-healing ulcerous formations. (*2, 3*). Since then, many advances in cancer management have been made. The last century was crucial for the development of the modern cancer treatments. Radiotherapy was introduced at the beginning of the XX century thanks to the work of Marie Curie (Nobel Prize in Physics in 1903 and Chemistry in 1911). Subsequently, the introduction of chemotherapy revolutionized cancer therapy in the 1940's. Recently, molecular targeted therapies have demonstrated to be an alternative option and introduced the 'precision medicine' era (*4*). In the last decades, immune-based therapies have revolutionized the treatment of several tumor types. Despite the advances, the efficacy of cancer therapeutic options is limited and surgery -when possible- remains the best curative strategy. For this reason, the scientific community concentrates its energies and resources to find new strategies to fight cancer.

2.1 Cancer immunotherapy

The origin of cancer immunotherapy dates back to the 1890 with the 'Coley's toxins' (*5*). The idea stems from the observation of spontaneous remissions of sarcomas in rare-cancer patients who had developed 'erysipelas' (beta-hemolytic group A infection of the skin). Dr. Coley intratumorally injected inactivated-*Streptococcus Pyogenes* and *Serratia Marcescens* and effective responses were obtained. His work was viewed with strong skepticism by the scientific community. In the following years, few clinical trials were conducted and the efficacy of the Coley's toxin was not clearly demonstrated (*6*). A direct evolution of the Coley's toxin immunotherapy concept is the *bacillus Camette Guerin* (BCG) that is still administered intravesically to treat superficial bladder cancer (*7*). Since then, major progress has been made in understanding how the immune system controls cancer development.

Several strategies exploiting immunotherapy have been discovered and are currently employed. One of the first was the administration of interferons such as interferon gamma (IFNγ) or alpha (IFNα). Interferons were discovered in 1957 and then found to be able to induce tumor remission in mouse models (*8*). The first clinical trials with administration of IFNγ were conducted in 1986 and a subset of patients achieved clinical benefit (*9*). The major limitation in IFN-based treatment was related to its pro-inflammatory functions and thus the development of treatment-related adverse events. Nowadays there are different clinical trials that combine IFNα or IFNγ to vaccine-based therapies and chemotherapies. Even though its crucial role in immune mediation, IFNγ has not been approved by FDA to treat patients with a variety of cancer types, probably because of its contribution to tumor evasion (*10*). In parallel, IFNα has been approved in 1995 for the treatment of malignant melanoma and AIDS-related Kaposi sarcoma.

Another milestone of cancer immunotherapy is represented by administration of interleukin-2 (IL-2). IL-2 was discovered in 1975 as a growth-promoting agent for bone marrow-derived T lymphocytes (*11*). It was FDA approved in 1992 for the treatment of metastatic renal cell carcinoma and in 1998 for metastatic melanoma (*12, 13*). Nevertheless, studies showed that IL-2 as a single agent is not sufficient to improve patients' survival probably due to its dual functional properties on T cells and severe adverse effects when administered at high doses. Finally, it was conceived that IL-2 might be more beneficial in combination with other forms of immune-based therapies at lower doses rather than as a high-dose single agent (*12-14*).

In parallel, cancer vaccines aim to prime the immune system against specific cancer associated antigens. Despite considerable efforts to develop cancer vaccines, the clinical translation of cancer vaccines into efficacious therapies has been challenging for decades. Two prophylactic vaccines are currently approved by FDA, including one for hepatits B virus that can cause liver cancer and another for human papillomavirus which is responsible for about 70% of cervical cancers. Sipuleucel-T, an immune cell based vaccine, for the first time, resulted in increased overall

survival in hormone-refractory prostate cancer patients. This led to FDA approval of this vaccine in 2010 (*15*).

In last decades cancer immunotherapy experienced a 'renaissance' (**Figure 1**). In 2001, Shankaran and colleagues experimentally demonstrated that T lymphocytes protect from tumor development (*16*). Immunocompromised animals treated with carcinogens developed tumors faster in comparison to immunocompetent animals. This evidence supported the concept that the immune system contrasts the development of tumor cells by eliminating cells harboring non-self-mutations (*17*). Cancer cells that grow are those able to evade the immune system and are the result of a selection exerted by this process. This assumption makes the immune system an important guardian of cancer development. Immune cells eliminate tumor cells that harbor non-self-mutations but, tumor cells that are able to grow must develop immune evasion mechanisms. These studies highlighted the importance of cytotoxic T lymphocytes in cancer progression and suggested that T cell modulation might have therapeutic application (*16, 18*).

The evidence that non-self-mutations generate T cell mediated immune responses led to the development of adoptive cell therapies (*19*). This approach starts from patients' immune cells, that need to be expanded or engineered and then infused back. An example of adoptive T cell therapy approach, is the one based on chimeric antigen receptor (CAR) (*20*). CAR T cells history begins in 1989 when Dr. Zelig Eshhar engineered the first T lymphocytes (*21*). CAR T cells are engineered to specifically recognize cancer related neoantigens in a patient-specific manner. Recent evidences show that CAR T cells are able to reach 80-92% response rates in patients (*20, 22*). Two CAR T cell-based therapies have been approved by FDA in 2017 and 2018 for the treatment of different types of B cell malignancies.

Then, at the end of the XX century, James Allison and Tasuku Honjo discovered that cytotoxic T lymphocyte antigen 4 (CTLA-4) and the programmed cell death 1 (PD-1) receptors are involved as immune checkpoints (*23-25*). Modulation of this immune checkpoints leads to reactivation of suppressed immune responses and in some case leads to re-establishment of cancer immune surveillance. These findings, brought to

the development of immune checkpoint therapy and eventually to Dr. Allison and Dr. Honjo receiving the 2018 Nobel Prize for Medicine.

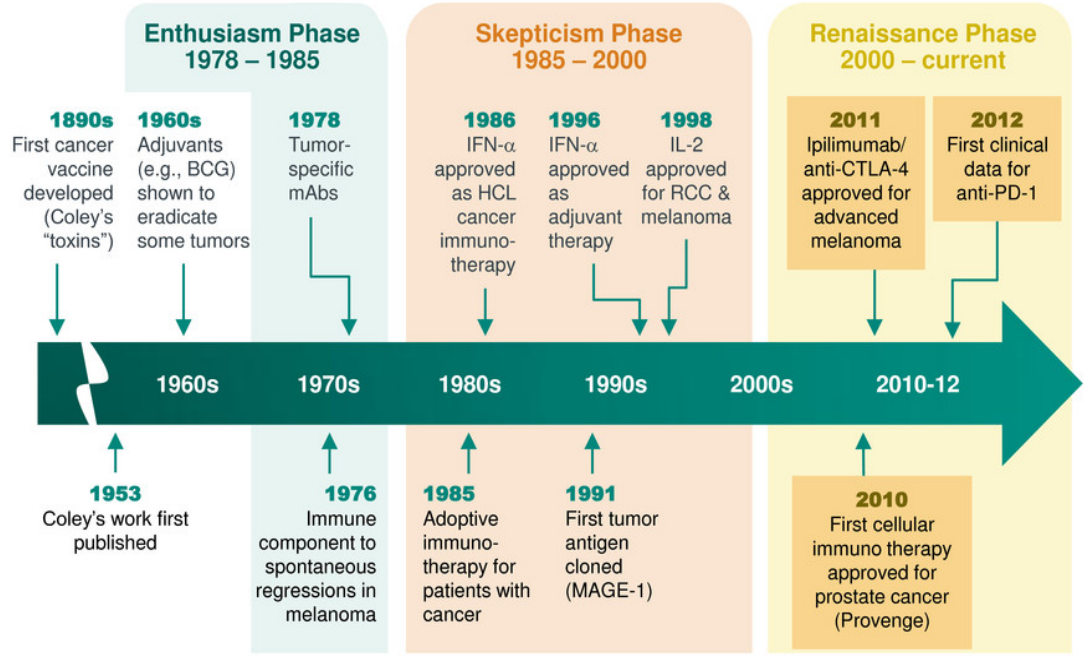

Figure 1. Brief timeline of cancer immunotherapy. Recently, with the discovery of immune checkpoints, cancer immunotherapy lives a 'renaissance' (from Merck.com).

2.2 Immune checkpoint therapy

Cytotoxic T lymphocytes require two distinct and concurrent activation signals in order to mount an effective immune response. In addition to the T cell receptor (TCR) activation signal, a co-stimulation signal is required. The co-stimulatory CD28 molecule on T lymphocytes binds B7 molecules (CD80 and CD86) on antigen presenting cells (APCs) and promotes T cell activation. The CTLA-4 receptor is involved in inhibition of the co-stimulatory CD28 molecule (**Figure 2**) (*26-28*). CTLA-4 is present on T cells and by affinity with CD80 and CD86, prevents ligation with CD28 thus limiting co-stimulatory signals to T lymphocytes. Given its molecular involvement with antigen presentation, the CTLA-4 receptor is thought to be prevalently involved during T cell priming and expansion phases in lymphoid tissues (*24, 29, 30*).

In parallel, the PD-1 receptor is gradually expressed upon TCR activation on exhausted lymphocytes and elicits inhibitory function if bound to its ligand, PD-ligand 1 (PD-L1) present on tumor cells and immune cells (**Figure 2**) (*31*). The activation of the PD-1 pathway negatively influences the metabolic and effector properties in cytotoxic T lymphocytes. In this case, the PD-1 signal is primarily involved on exhausted T cells within the tumor immune microenvironment (*24, 29*).

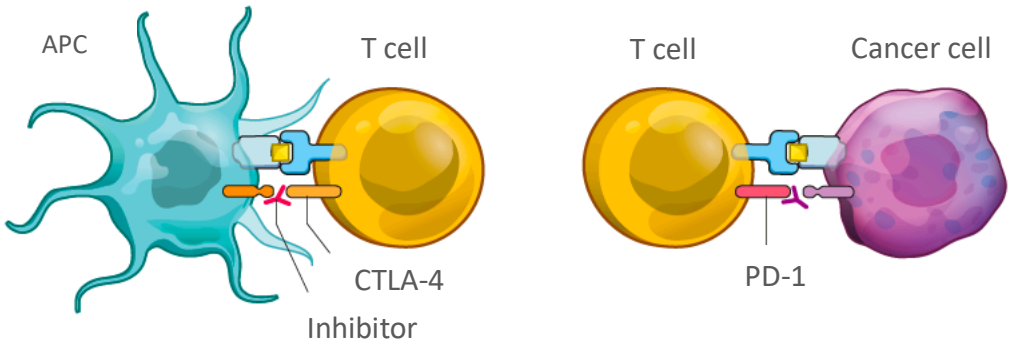

Figure 2. Left, the CTLA-4 receptor inhibits co-stimulatory signals between antigen-presenting cells and T cells. **Right**, the PD-1 receptor is expressed on exhausted lymphocytes that interact with the PD-ligand 1 expressed on cancer cells or immune cells (modified from Ledford et al, Nature 2018).

The blockade of this inhibitory signals with monoclonal antibodies (mAbs) is able to unleash T lymphocytes and re-establish immune responses in cancer. Immune checkpoint inhibitor-based therapies that target the CTLA-4 or the PD-1 pathway have achieved remarkable success in the treatment of selected malignancies. ICT based on anti-PD-1/PD-L1 and/or anti-CTLA-4 antibodies elicit prominent and long-lasting responses in tumors with high mutational and neoantigen burdens such as a fraction of melanoma, urothelial and lung cancers as well as MMR deficient (MMRd or Microsatellite Instable, MSI) tumors (*32-39*). Indeed, a variety of mAbs that target immune checkpoints have been FDA-approved in the last decade (**Figure 3**). The first was Ipilimumab (Bristol-Myers Squibb), an anti-CTLA-4 mAb which received approval in 2011 for the treatment of unresectable or metastatic melanoma. Pembrolizumab (Merck) targets the PD-1 receptor and was approved for the treatment of a large variety of cancers in 2014. Nivolumab (Bristol-Myers Squibb) is another mAb against PD-1 and has been approved the first time in 2015 for the

treatment of unresectable or advanced melanoma and then for a variety of additional cancer types. Anti-PD-1 Cemiplimab (Regeneron Pharmaceuticals) was approved in 2018 for the treatment of cutaneous squamous cell carcinoma. Tremelimumab (AstraZeneca) is an anti-CTLA-4 mAb that received an orphan drug designation in 2015 by FDA for the treatment of mesothelioma. A subset of mAbs that target the PD-L1 receptor such as Atezolizumab (Genentech), Avelumab (Merck and Pfizer) and Durvalumab (MedImmune and AstraZeneca) was approved recently for the treatment of a minority of cancer types. (*24*).

Immune checkpoint blockade is based on the administration of mAbs that target specific cancer-related pathways on immune cells. Thus, cancer- or patient-specific engineering of molecules or T cells is not required. Intriguingly, since immune checkpoint inhibitors target the host's immune system, they are supposed to generate a patient-specific immune response. Thus, CTLA-4 and PD-1 blockade will stimulate a repertoire of T cells that will potentially trigger an heterogeneous immune response and eventually target several cancer antigens. In parallel, since ICT targets non-specifically the immune system, has a higher potential of inducing immune-related toxicities in patients.

The CTLA-4 and the PD-1 receptors are only some of the immune checkpoints that have been discovered until today. Evaluation of therapeutic targeting of other immune checkpoints such as IDO-1, OX-40, TIM-3 and LAG-3 is still under investigation (*40-42*).

Different studies showed that immune checkpoint inhibition modulates the lymphoid and myeloid compartment and that targets specific cancer neoantigens (*43, 44*). These studies highlighted the importance of neoantigens and of the genomic landscape of tumors in the responsiveness to ICT.

Tumor type	Therapeutic agent	FDA approval year
Melanoma	Ipilimumab	2011
Melanoma	Nivolumab	2014
Melanoma	Pembrolizumab	2014
Non-small cell lung cancer	Nivolumab	2015
Non-small cell lung cancer	Pembrolizumab	2015
Melanoma (BRAF wt)	Ipilimumab + nivolumab	2015
Melanoma (adjuvant)	Ipilimumab	2015
Renal cell carcinoma	Nivolumab	2015
Mesothelioma (as orphan-drug)	Tremelimumab	2015
Hodgkin lymphoma	Nivolumab	2016
Urothelial carcinoma	Atezolizumab	2016
Head and neck squamous cell carcinoma	Nivolumab	2016
Head and neck squamous cell carcinoma	Pembrolizumab	2016
Melanoma (any BRAF status)	Ipilimumab + nivolumab	2016
Non-small cell lung cancer	Atezolizumab	2016
Hodgkin lymphoma	Pembrolizumab	2017
Merkel cell carcinoma	Avelumab	2017
Urothelial carcinoma	Avelumab	2017
Urothelial carcinoma	Durvalumab	2017
Urothelial carcinoma	Nivolumab	2017
Urothelial carcinoma	Pembrolizumab	2017
MSI-H MMR deficient metastatic CRC	Pembrolizumab	2017
MSI-H MMR deficient metastatic CRC	Nivolumab	2017
Pediatric melanoma	Ipilimumab	2017
Hepatocellular carcinoma	Nivolumab	2017
Gastric and gastroesophageal carcinoma	Pembrolizumab	2017
Non-small cell lung cancer	Durvalumab	2018
Renal cell carcinoma	Ipilimumab + nivolumab	2018
MSI-H MMR deficient metastatic CRC	Ipilimumab + nivolumab	2018
Liver cancer	Pembrolizumab	2018
Metastatic squamous cell skin cancer	Cemiplimab	2019
Triple negative breast cancer	Atezolizumab	2019
Kidney cancer	Avelumab	2019
Merkel cell carcinoma	Pembrolizumab	2019
Oesophageal squamous cell cancer	Pembrolizumab	2019

Figure 3. Tumor types summary for which immune checkpoint blockade therapies are FDA-approved. Ipilimumab and Tremelimumab are anti-CTLA-4 mAbs; Pembrolizumab, Nivolumab and Cemiplimab are anti-PD-1 mAbs. Atezolizumab, Durvalumab and Avelumab are anti-PD-L1 mAbs (modified from Wei et al, Cancer Discovery 2018).

2.3 Neoantigens in cancer immunotherapy

Trials with the anti-PD-1 molecule have shown remarkable clinical activity in tumor types such as melanoma, lung, bladder, stomach, renal cell, head and neck cancers, and Hodgkin's lymphoma (*32, 34, 36, 38*). Based on the relationship between pre-treatment CD8 positive T cell infiltrates and response to PD-1 blockade in melanoma, cytotoxic T cell activity appears to play a central role. An implicit conclusion from these clinical data is that in a fraction of patients, the endogenous T cell compartment is able to recognize neoepitopes that are displayed on the Major Histocompatibility Complexes (MHCs) on the surface of the malignant cells. As a direct test of the antitumor potential of the T cell compartment, work by Rosenberg and colleagues demonstrated that infusion of autologous *ex vivo* expanded tumor infiltrating lymphocytes can induce objective clinical responses in metastatic melanoma, and that at least part of this clinical activity is due to cytotoxic T cells (*45*).

A study published in 2014 showed that neoantigens are required and sufficient to mount tumor rejection in mouse models (*43*). In this study, the authors show that tumor rejection caused by ICT is mediated by few cancer neoantigens. They also found that administration of vaccines built on these neoantigens is equally efficient as PD-1 and CTLA-4 blockade. These findings indicate that the most effective immune response that guides tumor rejection upon ICT is mediated by cancer neoantigens (*43*).

The importance of neoantigens in human samples was confirmed by many studies that clearly showed that the neoantigenic landscape is fundamental to achieve clinical benefit in non-small cell lung cancer and in MMR deficient colorectal cancer (*32, 35, 46*).

In particular, cancer rejection epitopes may derive from two classes of antigens. A first class originates from non-mutated proteins to which T cell tolerance is incomplete, because of their restricted tissue expression pattern. A second class of potential cancer rejection antigens is formed by peptides that are entirely absent

from the normal human genome, so-called neoantigens. So, neoantigens are newly formed antigens that have not been previously recognized by the immune system. For the vast majority of human tumors without a viral etiology, such neo-epitopes are solely created by tumor-specific DNA alterations that result in the formation of novel protein sequences. For virus-associated tumors, such as cervical cancer and a subset of head and neck cancers, neoepitopes derive also from viral open reading frames (*47*).

Large-scale analyses of neoantigen specific T cell reactivity have now been carried out for a substantial number of patients, mostly in melanoma (*48-50*). With the caveat of a potential selection bias toward patients with a clinical benefit upon immunotherapy, these analyses provide a first estimate of the frequency with which the immune system recognizes the neoantigens that are formed as a consequence of mutations. The first conclusion that can be drawn from these analyses is that the T cell–based immune system reacts to both MHC class I–restricted (*49, 50*) and MHC class II–restricted neoantigens in a large fraction of melanoma patients. The second conclusion is that only a very small fraction of the nonsynonymous mutations in expressed genes in these tumors leads to the formation of a neoantigen for which CD4 or CD8 positive T cell reactivity can be detected within tumor-infiltrating lymphocytes.

Based on these observations, the presence of neoantigens that can potentially be recognized by autologous T cells is expected to correlate with cancer immunotherapy response. Indeed, tumors that harbor a high mutational burden are those that elicit the most promising responses to T cell-based immunotherapies (**Figure 4**). This group contains a sizable fraction of high-prevalence tumor types such as skin and lung cancer.

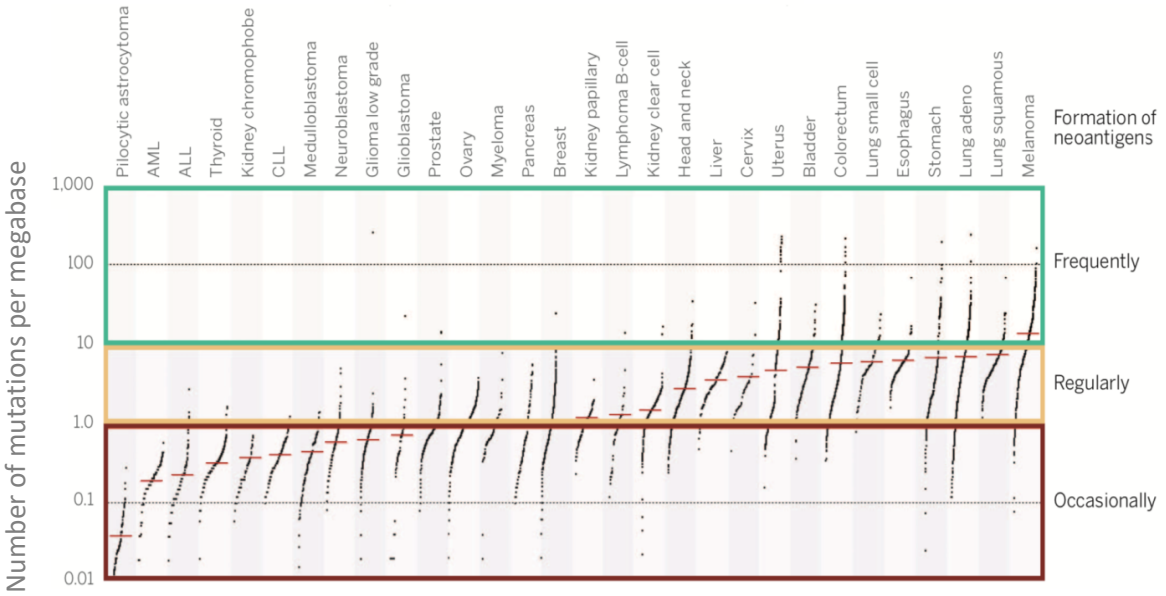

Figure 4. Estimate of the neoantigen repertoire in human cancers (modified from Schumacher and Schreiber, Science 2015)

2.4 DNA Mismatch Repair and tumor neoantigens

As mentioned, clinical evidence show that immune checkpoint modulators are particularly effective in hyper-mutated and MMRd/MSI colorectal cancer patients (*32, 35, 46*). The MSI phenotype is present in colorectal (15%) as well as in endometrial (30%), gastric (20%), ovarian, hepatocellular and renal clear cell carcinoma (all below 5%). This phenotype is often associated to MMR deficiency (*51, 52*). The MMR is responsible for the repair of DNA mismatches that occur during DNA replication. Tumors that harbor alterations at this level, often display increased mutational burdens, higher heterogeneity, tumor immune infiltration and curiously favorable clinical outcome (*53, 54*).

In mammalian cells, post-replicative DNA Mismatch Repair is performed by protein complexes consisting of MutL homologue 1 (MLH1), MutS protein homologue 2 (MSH2), MutS homologue 6 (MSH6) and PMS1 homologue 2 (PMS2). Other elements of the MMR machinery include the exonuclease 1 (EXO1) and polymerases capable of proofreading activity, such as Polymerase epsilon and delta (POLE and POLD)(**Figure 5**). MMR activity is required for the detection of single-nucleotide mismatches that potentially escape proofreading during replication. In addition,

MMR is essential for correcting small insertions and deletion that may occur when replication complexes move across repetitive sequences, so called microsatellites. Loss of MMR activity due to the lack of function of any of its key players is associated with tumor development and microsatellite instability. The most recurrent alteration in MMR found in cancer is epigenetic silencing of the MLH1 promoter, followed by genetic alterations in the MSH2 and MSH6 genes.

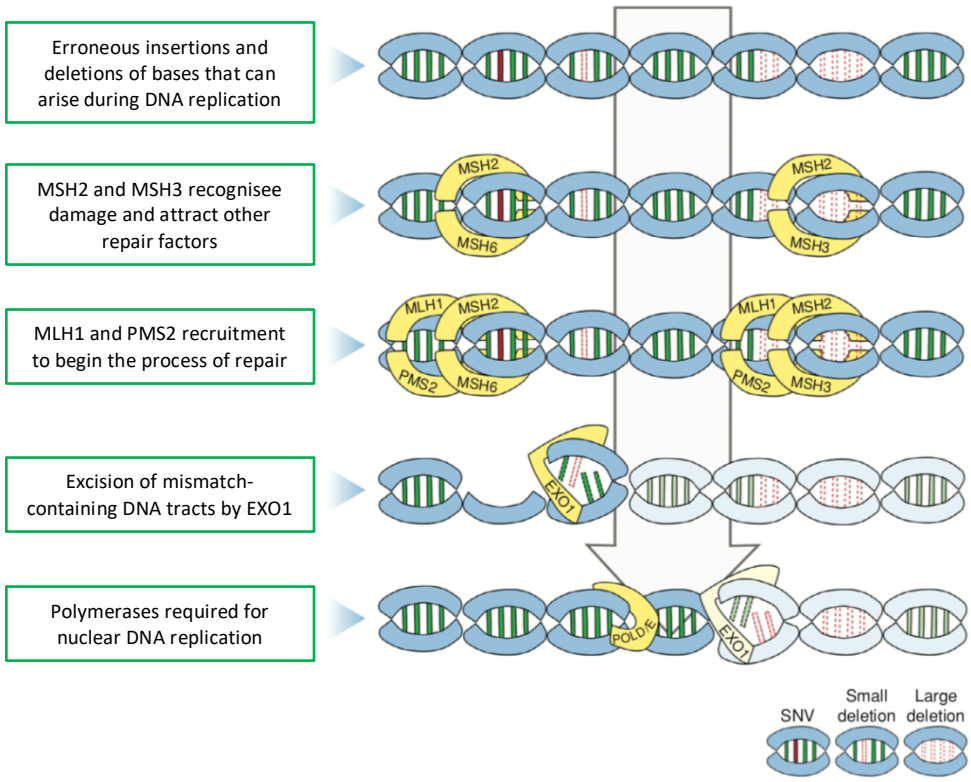

Figure 5. Molecular mechanisms of DNA mismatch repair (modified from Germano et al., Cancer Discovery 2018).

Germline mutations in MMR genes are associated with cancer disorders such as Lynch syndrome, when only one allele is inactivated (*55*), and constitutional MMRd, a rare disease caused by biallelic inactivation of MMR genes. Individuals affected by Lynch syndrome have an increased lifetime risk of developing colorectal tumors and other neoplasms of the gastrointestinal system, and usually develop tumors earlier than patients with corresponding sporadic tumors. Furthermore, Lynch syndrome is associated with the development of endometrial, urinary tract, ovarian, pancreatic,

breast tumors, gliomas, and skin neoplasms (*55*). In addition, constitutional MMRd confers a strong increase in the incidence of lympho-proliferative malignancies and childhood cancers.

The first comprehensive evidence that MSI colorectal tumors were correlated with higher mutational profiles arrived from a study conducted in 2012 by The Cancer Genome Atlas where 276 colon and rectum cancer patients were exome-sequenced and analyzed. From this study it was evident that MSI tumors display alterations at the level of the DNA MMR machinery and a significantly higher number of mutations and neoantigens (*51*).

2.5 DNA Mismatch Repair and immune checkpoint therapy

The evidence that MMRd/MSI tumors are more responsive to immune checkpoint modulation was reported in a clinical trial with the PD-1 inhibitor Pembrolizumab on colorectal, cholangiocarcinoma, endometrial, gastric and small intestine cancer patients (clinical trial NCT01876511) (*33*). Stratification of treated patients according to the status of the MMR genes clearly showed that MMRd cancers were more likely to respond to PD-1 inhibition, while MMRp tumors were refractory (**Figure 6**). Whole-exome sequencing revealed that MMRd tumors harbored a mean of 1,782 somatic mutations per tumor, in contrast to the 73 of MMR proficient tumors and confirmed that the hyper-mutated phenotype was a recurrent feature of immunotherapy responsive tumors (*33*).

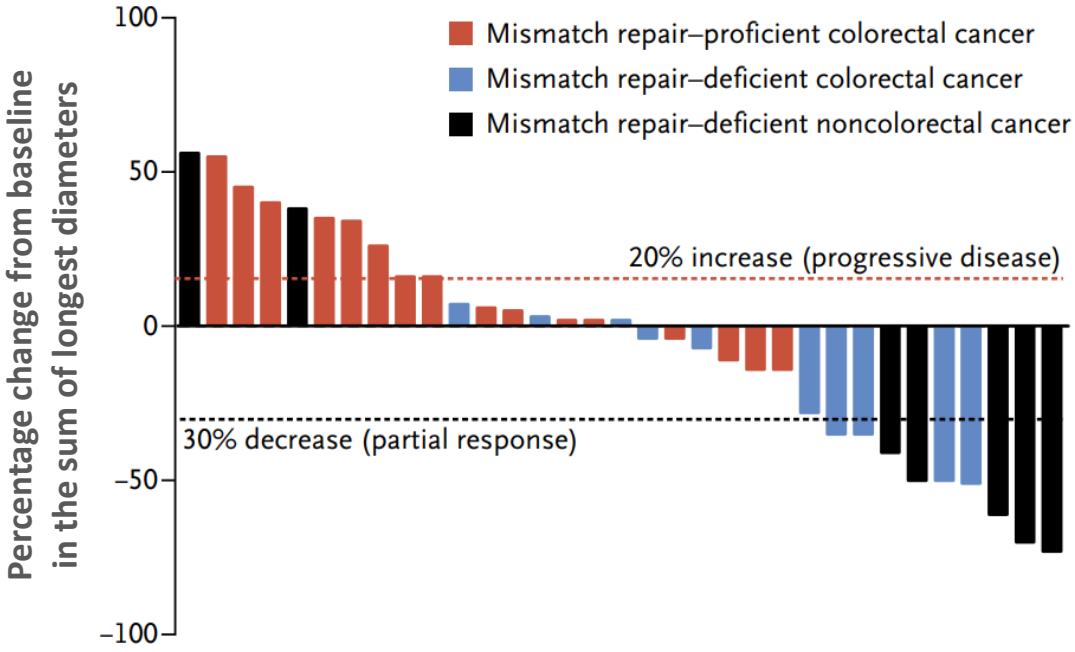

Figure 6. Original waterfall plot depicting the best change from baseline in target lesion size. A total of 41 patients were enrolled to receive Pembrolizumab every 14 days (modified from Le et al., NEJM 2015).

Subsequently, also the anti-PD-1 Nivolumab was approved for the treatment of MMRd/MSI colorectal tumors. Unfortunately, clinical data showed that only a small fraction were able to achieve a durable benefit from the treatment. In fact, only 30% of patients showed objective response to the treatment (*56*). For this reason, the combinatorial treatment of PD-1 and CTLA-4 inhibitors was tested and finally approved by FDA. The Check-mate 142 trial (clinical trial NCT02060188) in fact showed that the combination of Ipilimumab and Nivolumab increased the objective response rate in MMRd patients to 55% of patients (**Figure 7**)(*57*).

As discussed above, even within tumors with high mutational and neoantigen levels, only a subset of patients derives clinical benefit from ICT. For instance, approximately half of MMRd tumors do not achieve benefit to immune checkpoint modulators and among those that respond only a fraction achieve durable remissions (**Figure 7**) (*56, 57*).

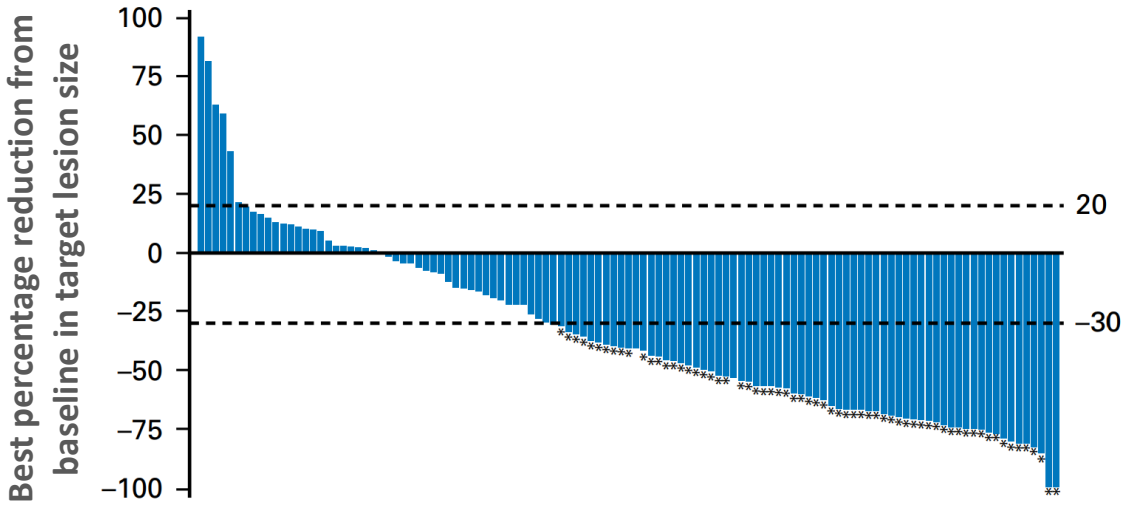

Figure 7. Original waterfall plot depicting the best change from baseline in target lesion size per investigator assessment. A total of 119 patients were enrolled to receive Nivolumab + Ipilimumab in the Check-mate 142 trial (modified from Overman et al., JCO 2018).

The combinatorial strategy increases response rates on one hand, but exposes patients to a higher risk of immune-related adverse events on the other. In fact, around 30% of patients treated with ipilimumab were not able to complete the treatment schedule because of drug-related toxicities (*58*). Immune-related adverse events can be severe colitis, pneumonitis, encephalitis, toxic epidermal necrolysis, myocarditis, stomatitis, acute kidney injury, duodenal ulcer and autoimmune type I diabetes mellitus (*57*).

Importantly, the clinical efficacy of immunotherapy remains very limited in extremely aggressive cancers such as pancreatic or in some of the most prevalent tumors, such as breast or MMRp/MSS colorectal cancer (*24, 59-62*). For all these reasons, there is need to find new therapeutic strategies to improve response rates of responder patients, to increase the fraction of those who are not eligible at the moment and to find new strategies to decrease the exposure to potentially toxic therapies.

2.6 Vitamin C

Ascorbic acid (VitC) is a water-soluble vitamin. The term 'vitamin' indicates that is essential for human life. Mammals produce their own VitC through the activity of the L-Gulonolactone Oxidase (GULO) enzyme, but primates, guinea pigs and some bats have a defective version of the GULO gene and are not able to produce ascorbate (*63*). Therefore, VitC needs to be taken with the diet. Since VitC is water-soluble, it is not stored in large amounts in the body, so any extra amount is excreted through the urine. For this reason, humans should intake every day with the diet an indicated dose of 75-90 mg depending from the gender. The aliments that contain the most significant amount of VitC are peppers, guava, kiwi, broccoli and papaya (*64*).

Chronic VitC deficiency is a pathologic condition named 'scurvy' (*64*). Scurvy patients often develop weakness, personality changes, decreased red blood cells, bleedings, impaired immunity and infections. Symptoms arise usually within one month of lack of VitC. People with scurvy often die because of infections or bleeding. The therapy is oral administration of VitC. Curiously, in perhaps the first ever kind of clinical trial, James Lind showed that scurvy could be cured by citrus fruits in 1747 (*64*). Ascorbic acid was first isolated by Dr. Albert Szent-Gyorgyi in 1928 and shown to be the antiscorbutic factor in 1932 (*65, 66*). Subsequently, he sent an ascorbate sample to Walter Norman Haworth that was able to synthetize vitamin C in 1934. They were both awarded the Nobel Prize in 1937 in Chemistry and Medicine, respectively.

VitC is absorbed in the small intestine, it is transported by specific transporters termed Sodium-dependent Vitamin C Transporters SVCT 1 and 2 and achieves peak plasma concentrations approximately 120-180 minutes after ingestion. VitC can also be oxidized in the plasma and transformed in dehydroascorbic acid (DHA) which exists in several different forms and is transported by Glucose Transporters (GLUTs) (*64*). Several GLUTs have higher affinity for DHA than for glucose. As soon as DHA is transported, it undergoes immediate intracellular reduction to ascorbate. The roles of DHA and GLUTs in VitC economy in the intact organism is unclear, especially for humans. Data suggest that it is unlikely that DHA transport is the major pathway for

ascorbate transport in most tissues. In the physiological state, the amount of DHA in plasma is estimated as <1-2% of that of ascorbate.

To understand the role of the GULO enzyme, a knock out mouse has been generated. The mice are healthy but they show decreased voluntary locomotor activity, diminished physical strength and weight loss. Supplementation with ascorbate re-equilibrates mice weight in 24h (*67*).

2.7 Vitamin C and cancer

The first indication that VitC might have a therapeutic application in cancer was reported by Cameron and Pauling in 1976. They supplemented 100 cancer patients with 10 mg of intravenous and oral VitC and compared them to 1000 control patients (**Figure 8**). They concluded that VitC was able to increase the survival time by the factor of about 3 for most of them and by at least 20 for a few (10%) speculating that higher dosages may elicit greater effect. The clinical trial was not controlled neither randomized, in fact patients received different cytotoxic therapies. But clearly, in the data shown in the study, patients that received VitC lived longer than the untreated ones (*68*).

Since the initial clinical trial was not controlled, in 1985 the Mayo clinic conducted three different controlled and double-blind clinical trials on 367 patients that were administered with 10 mg VitC only by oral administration (**Figure 8**). These trials failed to replicate Pauling's findings. They found that oral administration of VitC as a single agent did not elicit any evident benefit to patients (*69*).

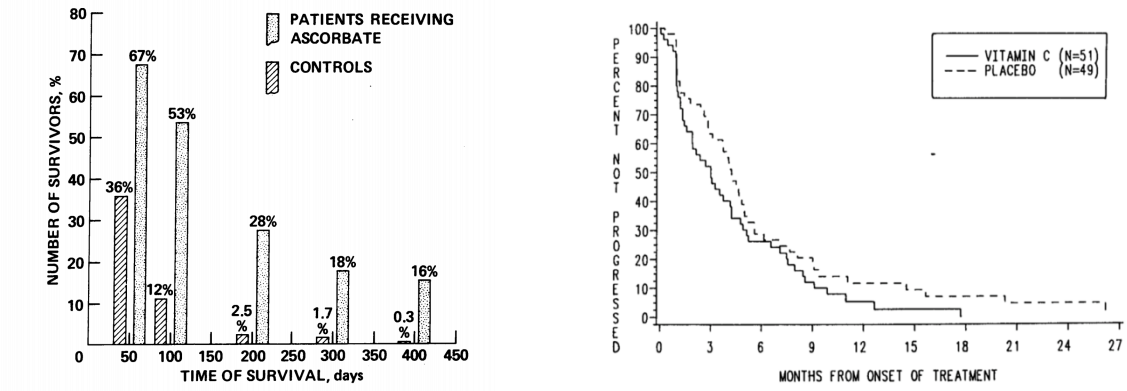

Figure 8. **Left**, original results from Cameron and Pauling clinical trial conducted in 1975. Patients were supplemented with 10 mg intravenous plus oral vitamin C. **Right,** Original results from the Mayo clinic in 1985. Patients were administered with 10 mg oral vitamin C.

For this reason, findings by Dr. Linus Pauling were criticized and the clinical application of VitC was not taken into account any further. Linus Pauling was awarded two Nobel Prizes in Chemistry and Peace respectively in 1954 and 1962.

The clinical evidences shown by Dr. Pauling and the Mayo clinic were controversial and confounding but the possibility that VitC may found therapeutic application in cancer management still elicited great interest.

Subsequently, in 1991 Dr. Padayatty and Dr. Levine demonstrated that the route of administration strongly affects pharmacokinetics of VitC in the plasma of healthy individuals (**Figure 9**). This study thus suggested that the possible discrepancy between Pauling's and Mayo's clinical investigations was due to the route of administration. In fact, they showed that the intravenous administration is able to reach the millimolar level in the plasma, while the oral route only leads to micromolar concentrations (*70, 71*).

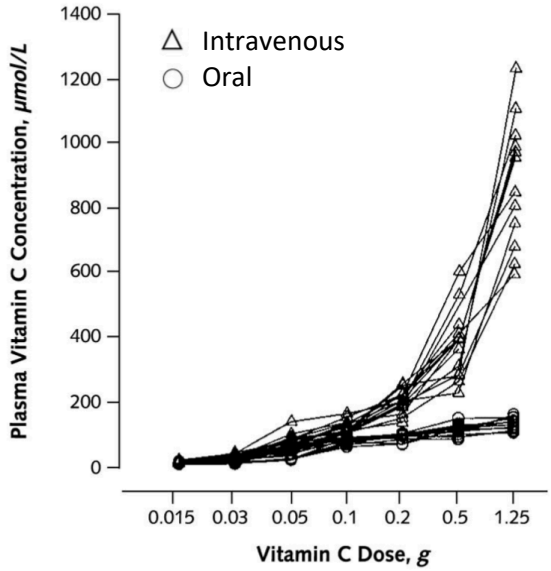

Figure 9. High-dose VitC reach millimolar levels in plasma if administered intravenously, while only micromolar levels if administered per os (modified from Padayatty et al., 2004).

Since then, many studies tried to investigate the molecular properties of VitC and its applications in cancer. Chemically, VitC is an electron-donor, or reducing agent, and since VitC can reduce oxidized species, or oxidants, VitC is often termed an antioxidant, but this terminology is misleading. Electrons from ascorbate can reduce metals such as copper and iron, leading to formation of superoxide and hydrogen peroxide and subsequent generation of reactive oxygen species. Thus, under some circumstances ascorbate, through its action as a reducing agent, will generate oxidants. This occurs when millimolar levels of ascorbate are reached in the plasma and in extracellular fluids (*72*, *73*).

In 2015, Cantley and colleagues showed that high-dose VitC selectively kills cancer cells harboring KRAS or BRAF mutations through reactive oxygen species (ROS) generation and disruption of metabolism (*74*). In their study they treated human colorectal cancer cells *in vitro* and *in vivo* and demonstrate that cells harboring KRAS or BRAF mutations are more sensitive to VitC treatment. They also showed that

APC-KRAS genetically engineered immunocompetent mice develop less tumors in comparison with APC mice if treated with high doses (4 g/kg) of VitC (*74*).

Some years later, Schoenfeld and colleagues showed that alterations in cancer cell mitochondrial oxidative metabolism result in increased levels of O2- and H2O2 that cause disruption of intracellular iron metabolism, selectively sensitize non-small-cell lung cancer (NSCLC) and glioblastoma (GBM) cells to ascorbate through pro-oxidant chemistry (**Figure 10**). In their study, VitC-induced ROS promote ferroptosis, which is a type of apoptosis triggered by iron metabolism crisis (*75*).

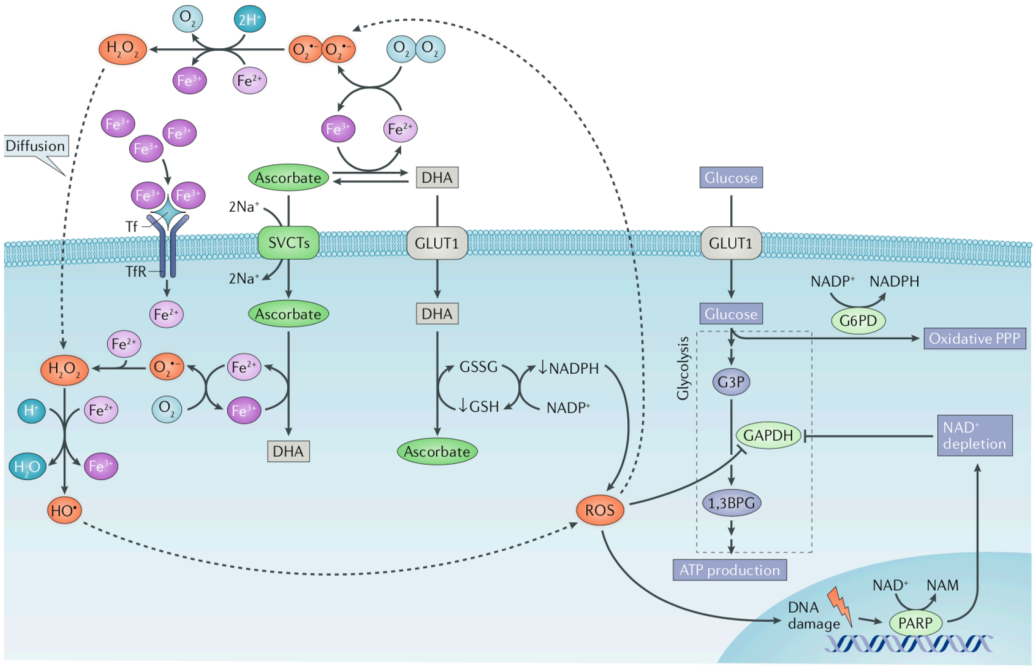

Figure 10. Pro-oxidant mechanism of VitC and cancer cell cytotoxicity (modified from Ngo et al., Nat Rev Can 2019).

In parallel, other studies suggested that VitC acts through a new mechanism that involves demethylating processes which is independent from ROS generation. VitC acts as a co-factor of a variety of dioxygenases and histone demethylases (*72, 73*). In particular, studies have shown that VitC stimulates the activity of Ten Eleven Translocation (TET) proteins. TET dioxygenases catalyze conversion of 5-methylcytosine to 5-hydroxymethylcytosine and then to other intermediate forms of

cytosines present in the DNA (**Figure 11**). This process causes demethylation of DNA and modulation of gene expression (*76, 77*).

The first study that demonstrated that VitC promotes TET activity, was published in 2013. In this study, Blaschke and colleagues demonstrate that VitC but no other anti-oxidant agents are able to induce TET-dependent demethylation of DNA in mouse embryonic stem cells. This demethylation activity was shown to modulate the expression of several stem cell associated genes (*78*).

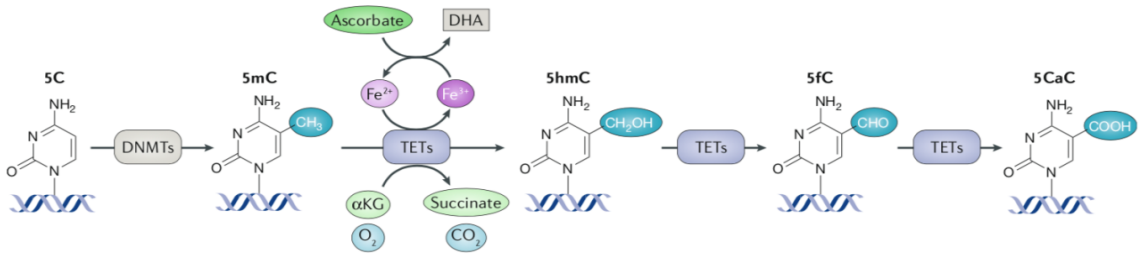

Figure 11. Regulation of TET enzymes by ascorbate (modified from Ngo et al., Nat Rev Can 2019).

A more recent study showed that VitC is able to mimic TET2 restoration in leukemic cells, increase DNA hypo-methylation and thus contrast malignant progression. Since TET2 alterations are found to be driver in leukemia, the importance of VitC in limiting leukemia progression elicited great interest (*79*).

Moreover, it has also been shown that through these demethylation programs, VitC is able to induce the activation of an IFNγ-inducing cellular response in combination with DNMT (DNA Methyltransferase) inhibitors. Treatment with VitC and DNMT inhibitors of human cancer cells induced the expression of an antiviral-like response thus increasing the effectiveness of treatment with DNMT inhibitors (*80*).

2.8 Vitamin C and immune function

The evidence that chronic deficiency of VitC contributes to impaired immunity suggests that VitC is strongly involved in the health of the immune system. Indeed, immune cells accumulate very high intracellular concentrations of VitC (50-100 fold

compared to plasma) suggesting that this cofactor is crucial for the function of these cells (*72, 73, 81-84*).

Some reports have suggested a possible effect of VitC on innate and adaptive immune responses in infectious diseases, but few studies provide clear demonstration of how VitC modulates immune cells (*81, 83*).

Some reports show that VitC may promote the activity of T lymphocytes. There is evidence that VitC can promote T cell maturation and differentiation (*84-86*). A paper published in 2013 showed that VitC is able to increase the proliferation of T lymphocytes *in vitro (85)*. In the study the Authors claim that the molecular mechanism by which VitC stimulates T lymphocytes might be through demethylation processes. In fact, supplementation of methylation inhibitors (DNMT inhibitors) with VitC is able to increase the diversity and proliferation of these cells *in vitro*.

Indeed, VitC can act as a cofactor of TET dioxygenases and histone demethylases thus modulating gene expression. A study published in 2016 showed that VitC induces proliferation of T regulatory (Treg) cells *in vitro* in a TET-dependent manner (*87, 88*). In these studies, VitC is able to enhance Treg proliferation by promoting the expression of genes that are crucial for the differentiation of these cells, such as FoxP3 (*88*). This effect is impaired in Treg cells where TET2 has been inactivated suggesting that VitC modulates Treg cells proliferation in a TET2 dependent manner.

Additionally, a study published in 2017 showed that VitC stimulates differentiation and proliferation of hematopoietic stem cells in GULO deficient mice in a TET-dependent manner (*89*). Engraftment and differentiation of adoptively transferred hematopoietic stem cells isolated from VitC deficient mice (GULO deficient) was more efficient in the presence of VitC rather than in its absence suggesting that VitC is an essential factor for the proliferation and activity of these cells *in vivo*.

These studies highlighted the importance of VitC on the proliferation and differentiation of a subset of immune cells, but interestingly, non-cell autonomous effects of VitC in cancer have not yet been extensively investigated. In fact, whether VitC may modulate the tumor immune microenvironment and influence anticancer immune responses is unknown.

2.9 Clinical trials exploiting Vitamin C for cancer treatment

Despite the controversial evidences from the clinical investigations conducted by Cameron and Pauling and subsequently by the Mayo clinic, other clinical investigations have been completed or are actually ongoing (**Figure 12**) (*72, 73, 82*).

The studies conducted by Cantley and colleagues led to clinical trials that evaluated the addition of VitC to chemotherapeutics that might exploit ROS-induced cytotoxicity. Treatment with VitC as a single agent is ongoing in pancreatic, colorectal and lung cancer patients (clinical trial: NCT03146962). A total of 50 patients have been enrolled to receive 1.25 g/kg VitC for 4 days/week. One cohort will follow the schedule for 2-4 weeks and a second cohort will follow it for 6 months. Treatment with VitC combined with other therapies is ongoing in colorectal cancer patients (clinical trial: NCT02969681) where 1.5 g/kg VitC for 3 days every 2 weeks is added to FOLFOX with or without bevacizumab. These clinical trials are ongoing.

In addition, Schoenfeld conducted two clinical trials where high-dose VitC was supplemented to standard treatment. One (clinical trial NCT02344355), in glioblastoma patients treated with temozolomide and radiotherapy, where a total of 87.5 g VitC were added three times per week during radiation therapy. The second one, is conducted on lung cancer patients (clinical trial: NCT02420314) treated with carboplatin and paclitaxel. In this study 75 g of VitC were infused twice per week for a total of 12 weeks. These clinical trials are still ongoing but promising preliminary results have been published in 2017 (*75*).

More recently, studies conducted on the role of VitC in hematopoietic stem cells differentiation and leukemogenesis re-opened the possibility of deeper clinical investigations on VitC.

Evidence supporting the existence of an anticancer effect of intravenous ascorbate is not clear, whether it is given a single agent or in combination with other concurrent standard therapies. However, in single-arm trials that used intravenous ascorbate in combination with concurrent standard therapies, it is unclear which agent delivered which effects. Overall, clinical trials data indicate that high-dose intravenous VitC is well tolerated by patients in combination with different therapeutic regimens.

As described, clinical trials are exploring the efficacy of VitC as a single agent in combination with chemotherapy or targeted agents. But interestingly, the potential of combining VitC with immune modulators for anticancer purposes has not been yet explored (**Figure 12**).

Phase	Trial name	Institution	Trial design	Cancer type	Drugs
Phase I	Gemcitabine, ascorbate, radiation therapy for pancreatic cancer	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Single arm	Pancreatic neoplasms	Gemcitabine; radiation + IV ascorbate
Phase I	High-dose ascorbate in glioblastoma multiforme	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Single arm	Glioblastoma	Radiation + temozolomide + IV ascorbate
Phase I/II	High-dose ascorbate + nanoparticle paclitaxel protein bound + cisplatin + gemcitabine in patients who have had no prior therapy for their metastatic pancreatic cancer	Piedmont Cancer Institute (USA)	Single arm	Metastatic pancreatic cancer	Paclitaxel, cisplatin, gemcitabine + IV ascorbate
Phase I/II	High-dose ascorbate + nanoparticle paclitaxel protein bound + cisplatin + gemcitabine in patients who have no prior therapy for their metastatic pancreatic cancer	HonorHealth Research Institute, University of California–San Diego Moores Cancer Center and Piedmont Cancer Institute (USA)	Single arm	Pancreatic cancer	Paclitaxel protein-bound, cisplatin + IV ascorbate
Phase II	High-dose ascorbate in stage IV non-small-cell lung cancer	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Single arm	Non-small-cell lung cancer	Paclitaxel; carboplatin + IV ascorbate
Phase II	Therapeutic use of IV vitamin C in allogeneic stem cell transplant recipients	Virginia Commonwealth University–Massey Cancer Center (USA)	Single arm	Hodgkin lymphoma, lymphoid leukaemia and multiple myeloma	IV and oral vitamin C
Phase II	Pharmacological ascorbate combined with radiation and temozolomide in glioblastoma multiforme: a phase II trial	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Single arm	Glioblastoma multiforme	Radiation + temozolomide + IV ascorbate
Phase II	High-dose vitamin C IV infusion in patients with resectable or metastatic solid tumor malignancies	Weill Cornell Medicine (USA)	Single arm	Colorectal, pancreatic and lung cancer with *KRAS* or *BRAF* mutation	IV ascorbate
Phase II	Pharmacological ascorbate with concurrent chemotherapy and radiation therapy for non-small-cell lung cancer	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Single arm	Non-small-cell lung cancer	Radiation, paclitaxel, carboplatin + IV ascorbate
Phase II	Pharmacological ascorbate, gemcitabine, nab-paclitaxel for metastatic pancreatic cancer	Holden Comprehensive Cancer Center at the University of Iowa (USA)	Randomized two-arm	Pancreatic neoplasms	Paclitaxel, gemcitabine + IV ascorbate
Phase II	Ascorbic acid in combination with docetaxel in men with metastatic prostate cancer	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (USA)	Randomized two-arm	Hormone-resistant prostate cancer, metastatic prostate carcinoma and stage IV prostate cancer	IV ascorbate + docetaxel
Phase II	IV ascorbic acid as an adjunct to pazopanib in the first-line setting for metastatic or unresectable clear cell renal cell carcinoma (ccRCC)	Mayo Clinic in Florida and Minnesota, Illinois CancerCare-Peoria, Iowa-Wide Oncology Research Coalition NCORP and Sanford Medical Center Fargo (USA)	Randomized two-arm	Clear cell renal cell carcinoma	Pazopanib hydrochloride + IV ascorbate
Phase II	Ascorbic acid and combination chemotherapy in treating patients with relapsed or refractory lymphoma	Mayo Clinic in Arizona, Minnesota and Florida and Holden Comprehensive Cancer Center at the University of Iowa (USA)	Randomized two-arm	B cell lymphoma with MYC and BCL2/BCL6 rearrangement, and recurrent Hodgkin lymphoma	Carboplatin, cisplatin + IV ascorbate
Phase III	IV ascorbic acid in advanced gastric cancer	Sun Yat-Sen University Cancer Center (China)	Randomized two-arm	Gastric cancer	mFOLFOX6 + IV ascorbate
Phase III	IV ascorbic acid in combination with FOLFOX$^{+/-}$ bevacizumab versus treatment with FOLFOX$^{+/-}$ bevacizumab alone as first-line therapy for advanced colorectal cancer	Sun Yat-Sen University Cancer Center (China)	Randomized two-arm	Colorectal neoplasms	mFOLFOX6, bevacizuman + IV ascorbate

Figure 12: Overview of clinical trials with high-dose intravenous vitamin C (modified from Ngo et al., Nat Rev Can 2019).

3. AIM OF THE STUDY

Immune checkpoint therapy (ICT) achieved remarkable success in the treatment of several kind of malignancies but only a subset of patients derives complete clinical benefit. Additionally, in some cases, treatment-related adverse events limit ICT efficacy. For these reasons, there is a need to find new and safe combinatorial strategies that can enhance the efficacy of ICT and expand the tumor types and number of patients who may benefit from cancer immunotherapy.

Despite extensive investigations, whether and how VitC modulates the tumor immune-environment is mostly unknown and the relevance of VitC as a cancer therapy remains unclear. While several clinical trials are exploring the efficacy of combining VitC with chemotherapy or targeted agents, the potential of combining VitC with immune modulators for anticancer purposes has not been explored.

The aim of the present study was to utilize experimental mouse models to investigate whether VitC might elicit anticancer activity through adaptive immune responses and enhance cancer immunotherapy.

4. RESULTS

4.1 VitC delays tumor growth in immunocompetent syngeneic mice

We asked whether VitC could exert anticancer effects not only in a cancer cell-autonomous manner but also through modulation of anti-tumor immune responses. To address this, we studied several mouse cancer models including colorectal (CT26, MC38), breast (TSA and 4T1), melanoma (B16) and pancreatic (PDAC). To explore the impact of the immune system on the cancer cell growth, the growth of each tumor was monitored in immunocompromised (NOD-SCID) as well as in immunocompetent syngeneic mice. Breast cancer cells were orthotopically injected in the mammary fat pad, while the other tumor cell lines were injected subcutaneously. Once the tumors reached around 100 mm^3 in size (typically 5-10 days) immunocompetent and immunocompromised animals (**Figure 13**) were randomized to receive either control vehicle or high-dose VitC (4 g/kg per day i.p.).

We observed that in most cases tumor growth was significantly delayed by the addition of VitC only in the presence of a fully competent immune system (**Figure 13 A**).

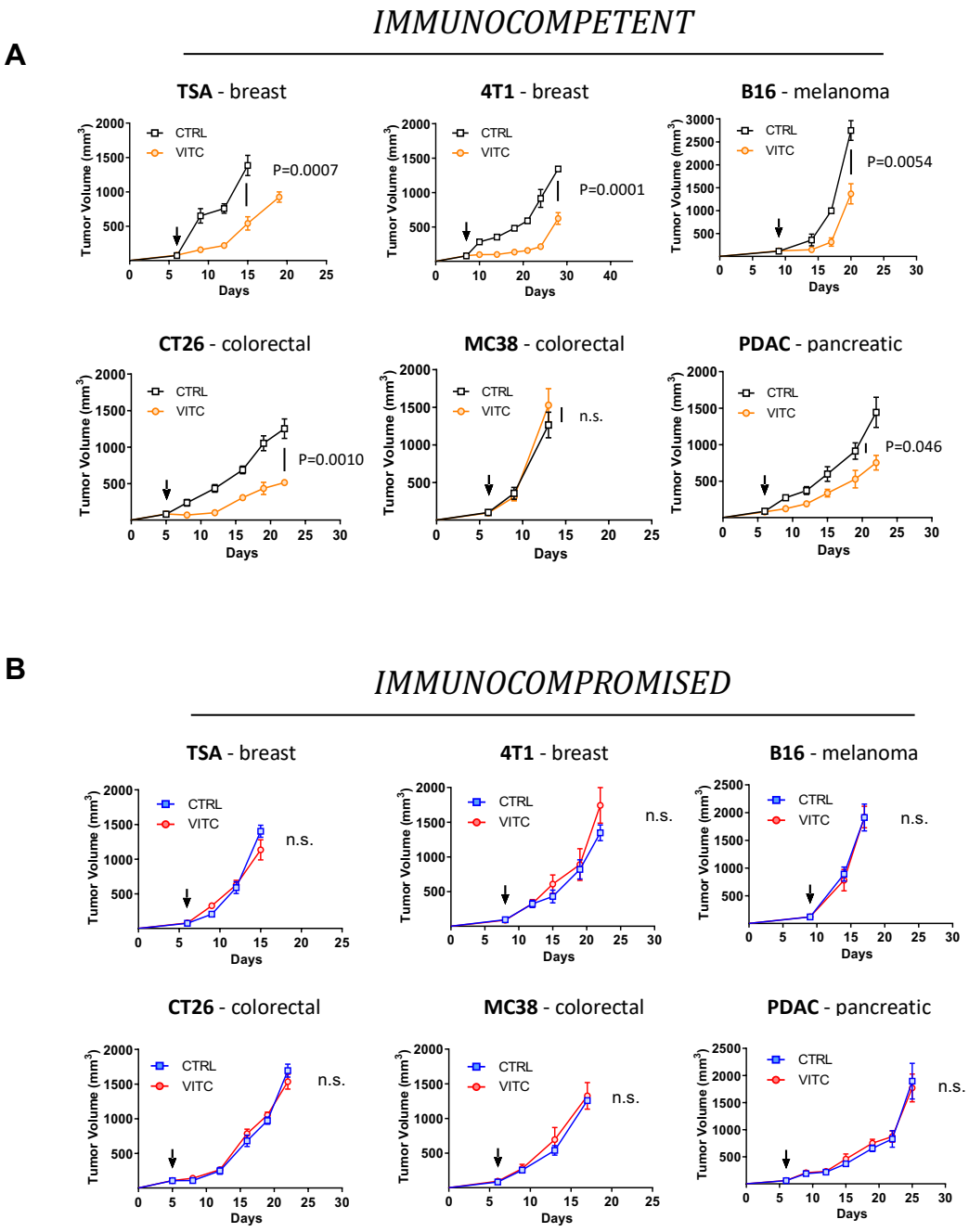

Figure 13. The indicated cell lines were injected orthotopically (100,000 cells for TSA and 4T1 models) or subcutaneously (500,000 cells for CT26, MC38, B16, and PDAC models) in **A**, immunocompetent syngeneic mice and in **B**, immunocompromised NOD-SCID mice. VitC (4 g/kg) was administered daily by i.p. injections and treatment was started when tumor volumes reached around 100 mm^3 (indicated by the black arrow). Every experimental group was composed at least of 6 mice. Data are shown as mean ± SEM. P values were calculated by two-tailed unpaired Student's t-test. NS, not significantly different. Ctrl, control. VitC, vitamin C.

Since high-dose VitC has been demonstrated to kill cancer cells through its pro-oxidant property, we wanted to assess if the VitC effects we observed in immunocompetent syngeneic mice were dependent or not from oxidative stress. Interestingly, the tumor anti-proliferative effect of VitC in immunocompetent mice was maintained in the presence of anti-oxidant N-acetyl cysteine (NAC) administration, which abrogated VitC pro-oxidative effects (**Figure 14**). The evidence that VitC exerts anticancer therapeutic effects in immunocompetent but not immunocompromised mice, suggests that VitC antitumor activity is primarily dependent on some immunomodulatory functions rather than its pro-oxidant effects.

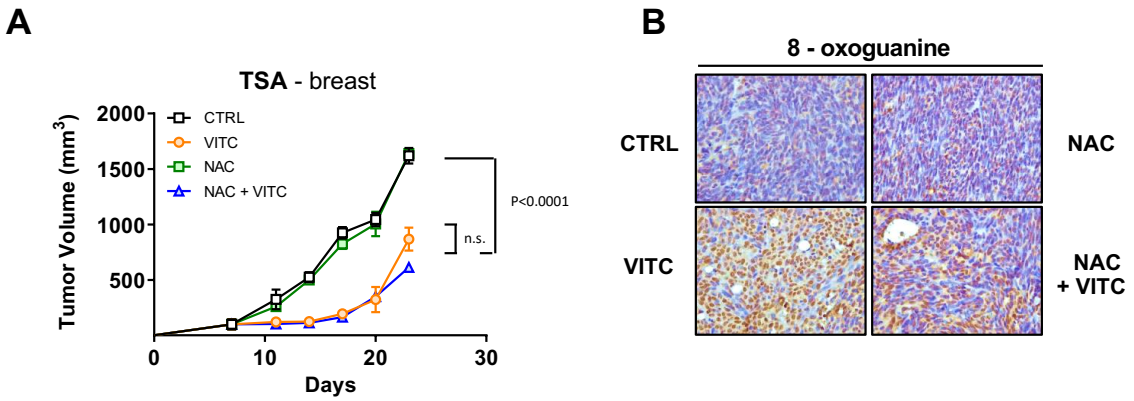

Figure 14. A, TSA cells were orthotopically injected in immunocompetent mice (100,000 cells) and N-acetyl cysteine was supplemented to the drinking water (30 mM, pH adjusted to 7.2) throughout the entire experiment where indicated. **B**, Tumors were explanted at the end of the experiment and FFPE sections were stained for 8-oxo guanine as a marker of ROS-induced DNA damage. Each experimental group was composed of at least of 6 mice. P values were calculated using one-way ANOVA at the indicated timepoint. NS, not significantly different. Ctrl, control. VitC, vitamin C. NAC, N-acetyl cysteine.

4.2 Vitamin C enhances the efficacy of immune checkpoint therapy

Immune checkpoint therapy (ICT) can unleash the immune system and induce prolonged remissions in several tumors, but their efficacy is still very limited in some of the most prevalent malignancies such as breast and colon cancer. Next, we assessed whether VitC could enhance the efficacy of ICT. Immune checkpoint modulators (anti-PD1 and anti-CTLA-4 mAbs, ICT) alone and in combination were administered to mice bearing syngeneic pancreatic, breast or colorectal tumors. In pancreatic PDAC and breast 4T1 models, the triple therapy combining VitC with anti-PD1 and anti-CTLA-4 (VitC + ICT) induced a significant tumor growth delay compared to single treatments, but without leading to tumor eradication (**Figure 15 A and B**). In the second breast cancer model (TSA), combinatorial VitC + ICT induced prolonged tumor growth impairment (**Figure 15 C**). Importantly, a subset of mice (8 out of 13) that received the triple therapy rejected TSA tumors, remained tumor free for up to a year and eventually died without evidence of cancer, suggesting that the treatment had been curative (**Figure 15 D**). In the subset of mice that displayed complete regression, no tumors developed even when they were later re-challenged with the same cancer cells (**Figure 15 D**), indicating that mice elicited complete immune responses and that effective antitumor memory T cells had been expanded.

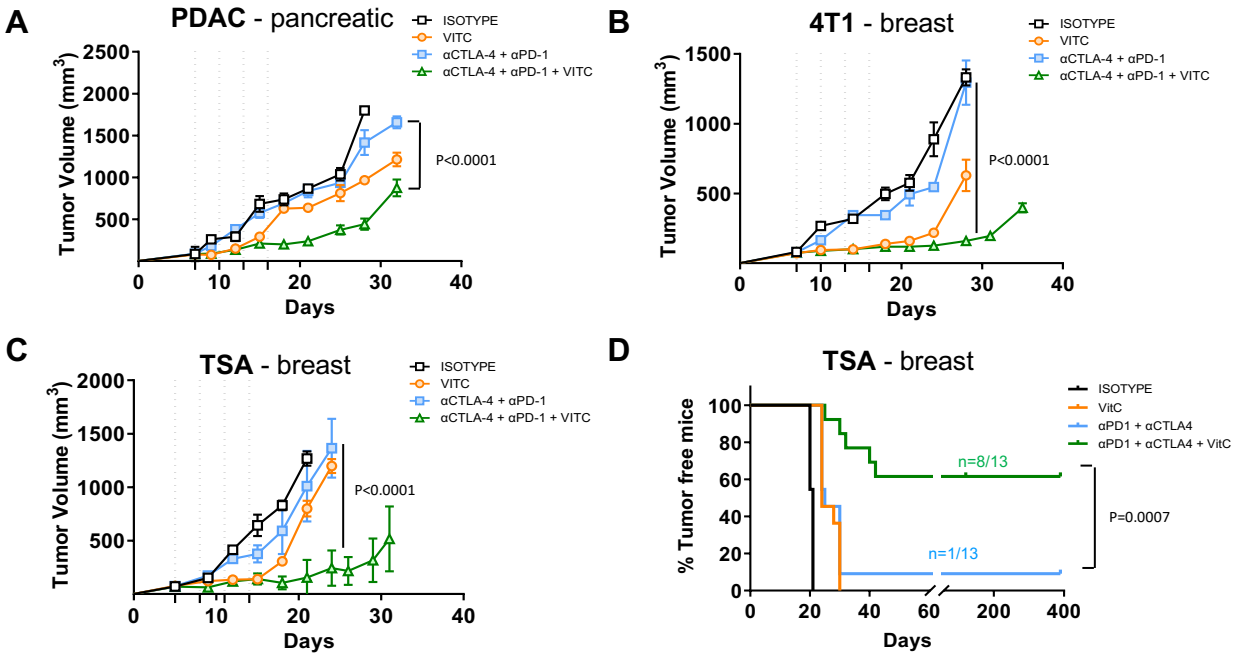

Figure 15. A, 4T1 breast cancer cells were injected orthotopically (100,000 cells) in syngeneic mice. **B**, PDAC pancreatic cancer cells were injected subcutaneously (500,000 cells) in syngeneic mice **C**, TSA breast cancer cells were injected orthotopically (100,000 cells) in syngeneic mice. **D**, Tumor relapse-free survival of mice treated with VitC, ICT or their combination and followed for over a year. Two-independent experiments performed on a total of n=13 mice are shown in survival curves. VitC (4g/kg) was administered i.p. daily starting when tumors reached a volume around 100 mm^3. Anti-CTLA-4 (200 µg/mouse) and anti-PD1 (250 µg/mouse) were given at the timepoints indicated by the dashed vertical lines in the charts. In combinatorial treatments, VitC was administered since the first cycle of immunotherapy. Every experimental group was composed at least of 5 mice. Statistical analysis used one-way ANOVA for tumor growth comparison at the indicated timepoints and log-rank test (Mantel-Cox) for survival analysis. NS, not significantly different. VitC, Vitamin C.

Treatment with VitC and immune modulators in the previous experiments were administered when tumors were approximately 100 mm^3 in volume. To study the impact of combined VitC and ICT on larger tumors, we injected CT26 colon cancer cells and started treatment nearly at 800-1,000 mm^3 in volume. Despite the significant disease burden, the triple therapy induced strong tumor impairment and remission in most of the animals (7 out of 13) (**Figure 16 A**). Also in this case, mice remained tumor free up to a year and no tumor developed even when they were later re-challenged with the same cancer cells indicating effective antitumor immune memory (**Figure 16 B**).

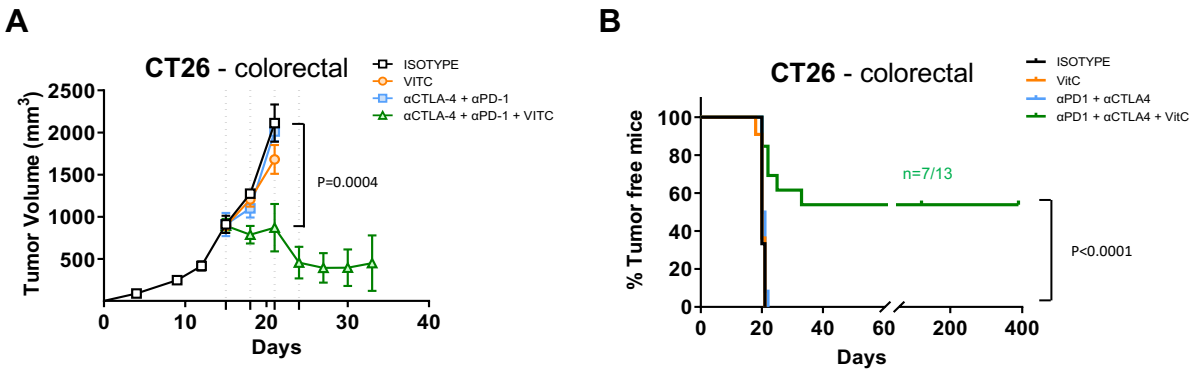

Figure 16. A, CT26 colorectal cancer cells were injected subcutaneously (500,000 cells) in syngeneic mice. **B**, Tumor relapse-free survival of mice treated with VitC, ICT or their combination and followed for over a year. Two-independent experiments performed on a total of n=13 mice are shown in survival curves. VitC (4g/kg) was administered i.p. daily starting when tumors reached a volume around 800-1000 mm^3. Anti-CTLA-4 (200 µg/mouse) and anti-PD1 (250 µg/mouse) were given at the timepoints indicated by the dashed vertical lines in the charts. In combinatorial treatments, VitC was administered since the first cycle of immunotherapy. Every experimental group was composed at least of 6 mice. Statistical analysis used one-way ANOVA for tumor growth comparison at the indicated timepoints and log-rank test (Mantel-Cox) for survival analysis. NS, not significantly different.

4.3 Vitamin C induces recruitment and activation of tumor infiltrating CD8 T lymphocytes

To characterize the immunological response observed combining VitC and ICT, we explanted orthotopically grown TSA breast tumors and isolated immune infiltrating cells fractions from control and treated mice. Flow cytometry analysis showed that the CD45 positive fraction was increased in VitC-treated cancers. In parallel, infiltration by natural killer cells, dendritic cells, macrophages and Treg cells was not significantly affected by VitC treatment (not shown).

To investigate whether VitC may influence T lymphocyte infiltration, we analyzed TSA fixed tumors using immune-fluorescence analyses. These analyses showed that VitC treatment induced tumor infiltration by CD8 T cells, which were further increased by combining VitC and ICT (**Figure 17 A and B**).

Flow cytometry analysis revealed also that combined treatment of VitC and ICT induced infiltration of effector lymphocytes, as shown by positive staining for T cell activation markers CD44 and CD69 (**Figure 17 C**). VitC as a single agent induced increased positivity for CD69 proliferation and activation marker on both CD4 and CD8 T cells; while VitC combined with ICT induced stronger expression of the CD44 antigen-priming marker (**Figure 17 C**).

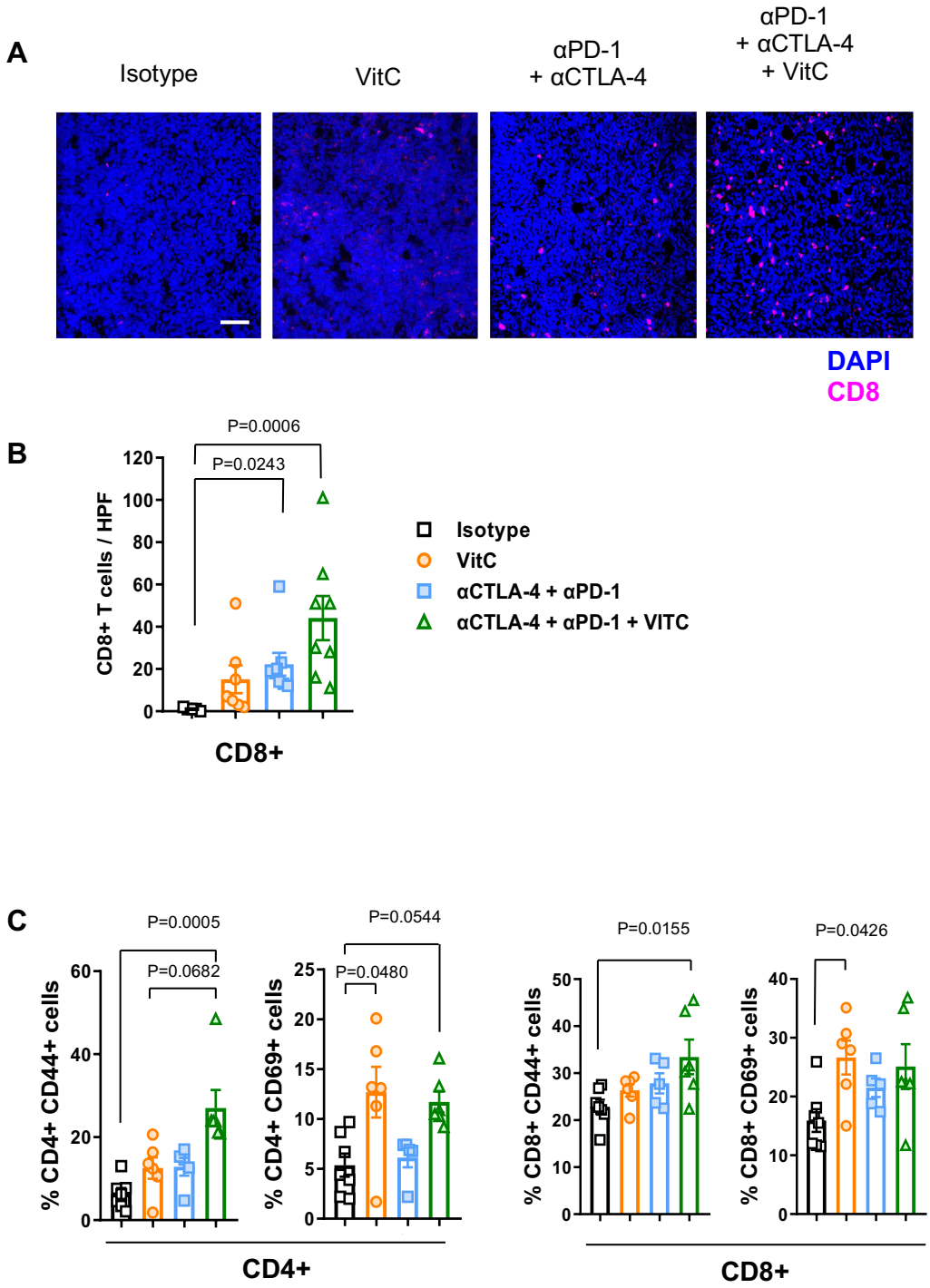

Figure 17. TSA cells were orthotopically injected (100,000 cells) in immunocompetent mice and tumor infiltrating immune populations were analyzed. **A**, Immunofluorescence analysis of CD8 tumor infiltrating lymphocytes. Scale bar is representative of 75 μm. **B**, Quantification of CD8 T cells from panel A. T cell counts per high-power field from six different mice. **C**, TSA orthotopic tumors were explanted, single cell suspended and analyzed by flow cytometry. Staining for memory/effector markers on CD4 and CD8 T cells. The fraction of positive cells was calculated respectively on CD4+ and CD8+ live events (500,000 events were acquired for each sample). P values were calculated using non parametric analysis for panels B; one-way ANOVA for panel C. NS, not significantly different. Ctrl, control. VitC, Vitamin C.

4.4 VitC enhances expression of IFNγ in spleen derived T lymphocytes

To understand how the VitC effect we observed was mediated by T cells, we isolated T lymphocytes from the spleen of VitC-treated immunocompetent animals and analyzed them for interferon gamma (IFNγ) positivity, which is a sensor of T cell activation.

We repeated the experiment shown in figure 13A in immunocompetent mice injected orthotopically with TSA breast cancer cells. VitC treatment (4 g/kg daily) was started when tumors reached nearly 100 mm^3. After 25 days since tumor injection we euthanized the mice, harvested the spleens and isolated the splenocytes from VitC-treated and control mice. Splenocytes were stimulated *in vitro* with T cell stimulation cocktails (phorbol 12-myristate 13-acetate and ionomycin) for 6 hours and then stained for extra- and intra- cellular markers.

Interestingly, we found that CD4+ and CD8+ T lymphocytes isolated from the spleen of VitC-treated immunocompetent animals, displayed higher levels of IFNγ in comparison with control mice (**Figure 18 A and B**) suggesting that VitC treatment influences immune responses and in particular T lymphocyte function.

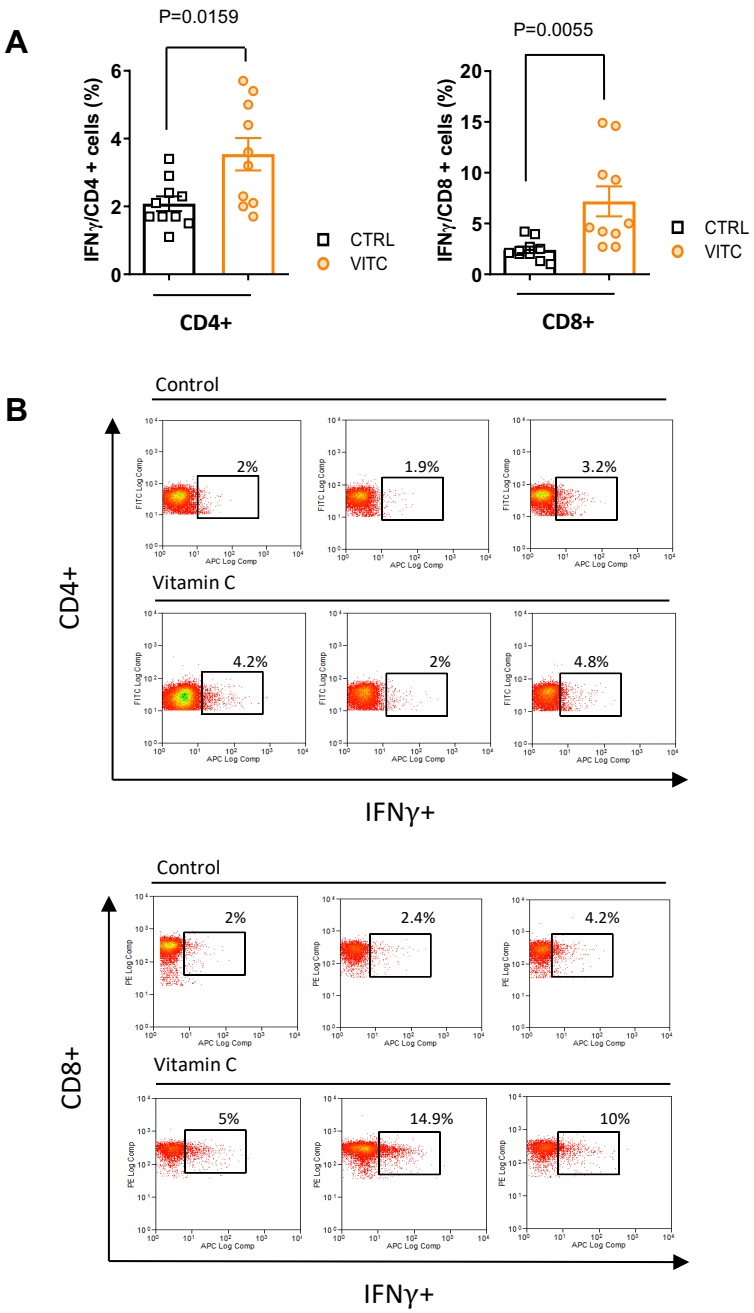

Figure 18. TSA cells were orthotopically injected in immunocompetent mice (100,000 cells). **A**, Flow cytometry analysis of IFNγ release on CD4 and CD8 spleen-derived lymphocytes isolated from untreated and VitC-treated mice injected orthotopically with TSA cancer cells. Spleens were harvested 30 days after tumor cell injection and T lymphocytes were stimulated in vitro. Percentage was calculated respectively on CD4 and CD8 live events. The indicated cell percentages are gated on: CD45+ live, CD4+/CD8+, IFNγ+ (500,000 events were taken for each sample). **B**, Representative IFNγ positivity on CD4 T cells and CD8 T cells shown in panel A. The fraction of positive cells was calculated respectively on CD4 and CD8 positive live events. Every experimental group was composed of at least of 10 mice. Data are shown as mean ± SEM. P values were calculated using two-tailed unpaired Student's t-test. NS, not significantly different. Ctrl, control. VitC, vitamin C.

4.5 Depletion of T cells impairs VitC anticancer activity

Thus, to address the impact of T cell lymphocytes in the anticancer effects of VitC we used two different approaches.

On the one hand, we repeated the experiment shown in Figure 13A in the presence of monoclonal antibodies targeting CD4 or CD8 positive T cells. Depleting and control isotype antibodies were administered from day 0 to mice and VitC treatments were started when tumors reached approximately 100 mm^3 in volume. Strikingly, we observed that anti-CD4 or anti-CD8 antibodies completely impaired the anticancer effect of VitC in breast TSA and colorectal CT26 tumors (**Figure 19**).

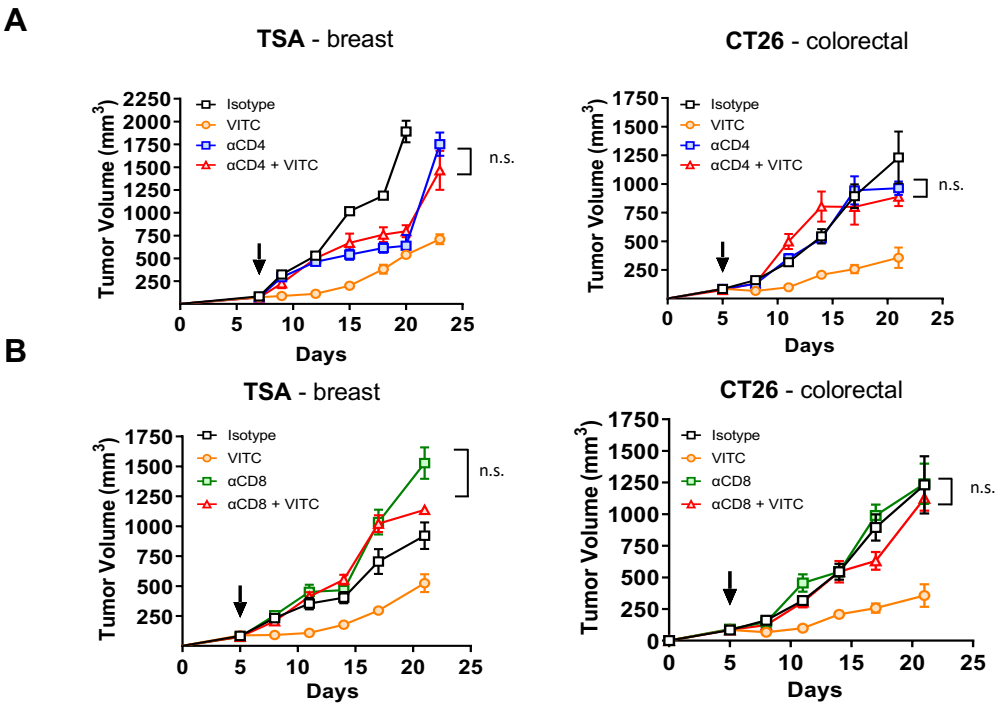

Figure 19 A, Depletion of CD4 T cells and **B**, depletion of CD8 T cells in the indicated cell models. Mice were treated with anti-CD4 and anti-CD8 depleting mAbs (400 µg per mouse at day 0, then 100 µg per mouse at day 1, day 2 and every 3 days through the entire course of the experiment). Control mice were administered the isotype antibody. Every experimental group was composed of at least of 5 mice. TSA cells were orthotopically injected (100,000 cells) and CT26 colorectal cancer cells were subcutaneously injected (500,000) in immunocompetent mice. Data are shown as mean ± SEM. P values were calculated using two-tailed unpaired Student's t-test; NS, not significantly different. Ctrl, control. VitC, vitamin C. αCD4, anti-CD4. αCD8, anti-CD8.

4.6 VitC enhances the anticancer potential of CD8 T cells

To further assess the role of T cells, we adoptively transferred T lymphocytes from immunocompetent to immunocompromised animals according to the experimental plan shown in Figure 20A. To this purpose, we first injected breast cancer cells (TSA) in the mammary fat pad of syngeneic mice and when tumors reached at least 100 mm^3 in volume, mice were treated with VitC or control vehicle. After 30 days, spleens were explanted and CD4 and CD8 T cells were isolated (**Figure 20 A**). In parallel, TSA cells were implanted in immunocompromised mice. Five and ten days after TSA cell implantation, CD4 and CD8 T cells isolated from VitC or control vehicle treated immunocompetent mice were injected i.v. in immunocompromised animals. We found that the adoptive transfer of CD4 T cells induced tumor growth delay, but no difference was detected when we transferred CD4 T cells from VitC treated mice or from mice that had received control vehicle (**Figure 20 B**). Instead, when we transferred CD8 T lymphocytes, only those originating from mice that were previously treated with VitC displayed an anti-tumor effect (**Figure 20 C**). Altogether these results show that treatment with high-dose of VitC delays tumor growth and indicate this depends on T lymphocytes, primarily on CD8 T cells.

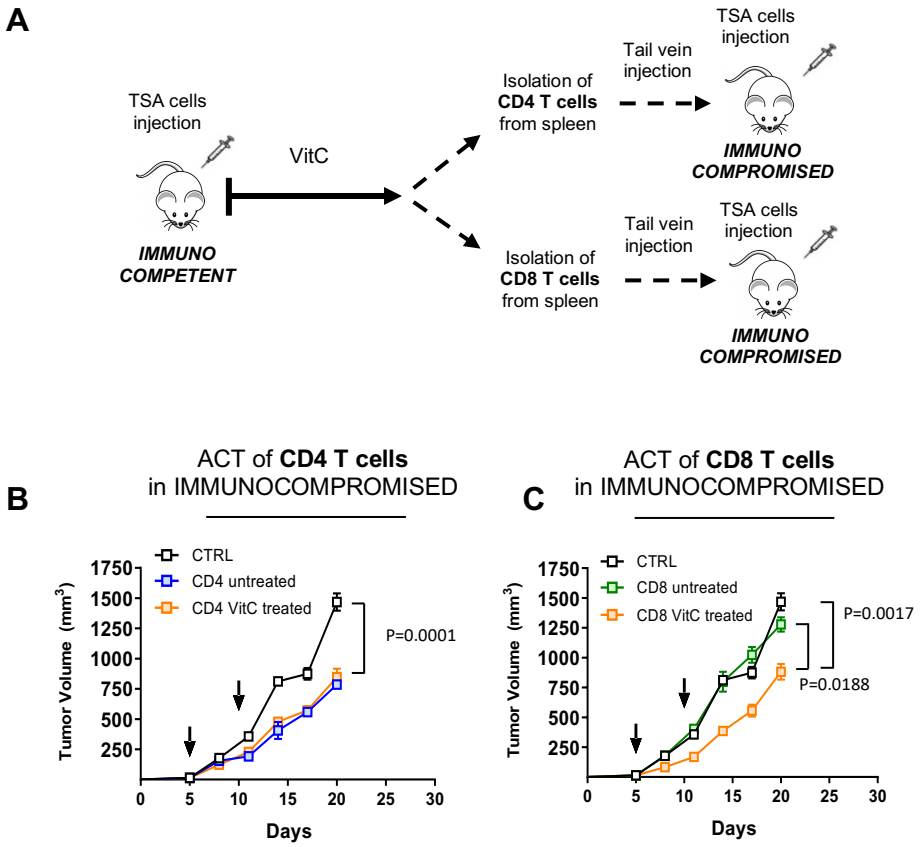

Figure 20. A, Adoptive cell transfer of untreated and VitC-treated was performed according to the experimental workflow. Isolated **B**, CD4 and **C**, CD8 T lymphocytes were administered by tail vein injection to immunocompromised NOD-SCID mice orthotopically injected with the same TSA tumors (n=4). Black arrows indicate the timepoints of T cell tail vein infusion. Five million T cells per injection were administered to each mouse. Every experimental group was composed of at least of 5 mice. Data are shown as mean ± SEM. P values were calculated using two-tailed unpaired Student's t-test. NS, not significantly different. Ctrl, control. VitC, vitamin C.

4.7 Inactivation of DNA repair in mouse cancer cells

Immune checkpoint modulation is approved for the treatment of any tumor type displaying microsatellite instability (MSI), which is the result of DNA Mismatch Repair (MMR) inactivation. Unfortunately, only a subset of MMR deficient (MMRd) tumors responds to immune checkpoint modulators and among those that respond only a fraction derive long lasting benefits. We wondered whether VitC could improve the magnitude and durability of clinical benefit from immune therapies on MSI tumors. To study the effect of VitC on MMRd we decided to generate mouse cancer cells with MMR deficiency to study cancer sensitivity to ICT. The most recurrent alteration in MMRd/MSI tumors is inactivation of the MLH-1 gene. For this reason, we employed the CRISPR/CAS gene editing technology to inactivate the MLH-1 gene. We utilized two MMR proficient (MMRp) mouse breast TSA and colorectal CT26 cancer cells lines. Cells were transfected with different sgRNA guides and then single cell diluted in order to resemble homogeneous clonal populations. Empty-vector guides were used to generate controls. Gene editing was confirmed at the protein level for different clones of TSA and CT26 cancer cells to avoid off-target events (**Figure 21A**).

We then next verified whether inactivation of MMR repair leads to MSI. Analysis showed that inactivation of MLH-1 led to MSI in MLH1 inactivated cancer cells (**Figure 21B**).

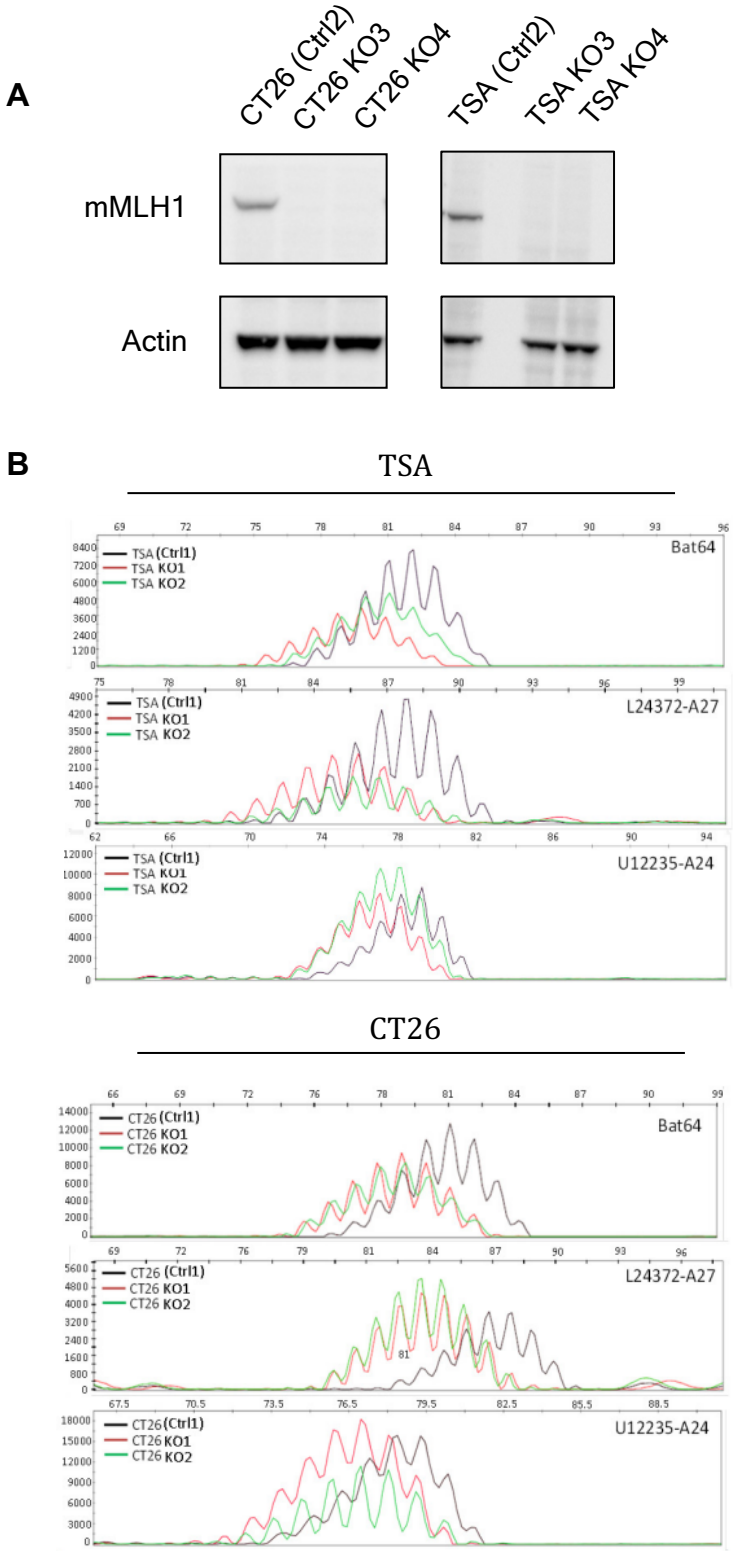

Figure 21. A, Western blot of MLH-1 edited cells. **B**, Effect of MLH-1 inactivation on microsatellite instability in mouse cancer cells. The MSI status was evaluated by comparing mononucleotide repeats of TSA (lower) and CT26 (upper). The mononucleotide regions Bat64, L24372-A27 and U12235-A24 were used to evaluate microsatellite instability. The shift of knock out diagrams (red and green) on the left in comparison to the control (black) indicates a shortening of fragments and thus impairment of the repair of mononucleotide repeats in the microsatellite regions.

4.8 Inactivation of MLH-1 impairs the growth of mouse tumors in immunocompetent mice

To assess whether the inactivation of the MLH-1 gene may have an impact on the growth of mouse cancer cells we subcutaneously injected them in immunocompromised and immunocompetent animals. We found that inactivation of MLH-1 did not affect the growth of TSA and CT26 cancer cells in immunocompromised NOD-SCID mice. In fact, after approximately 30 days mice had to be euthanized (**Figure 22 A**). In parallel, MLH-1 wild-type and knock out cells were injected in immunocompetent syngeneic mice after different times of cell culturing. No difference in tumor growth was detected after 65 days of cell culturing (not shown). While, after 157 days of cell culturing the cells were not able to form or formed only indolent tumors in immunocompetent syngeneic mice (**Figure 22 B**). These data suggest that MMRd cells require time to accumulate mutations that may trigger cancer immune surveillance.

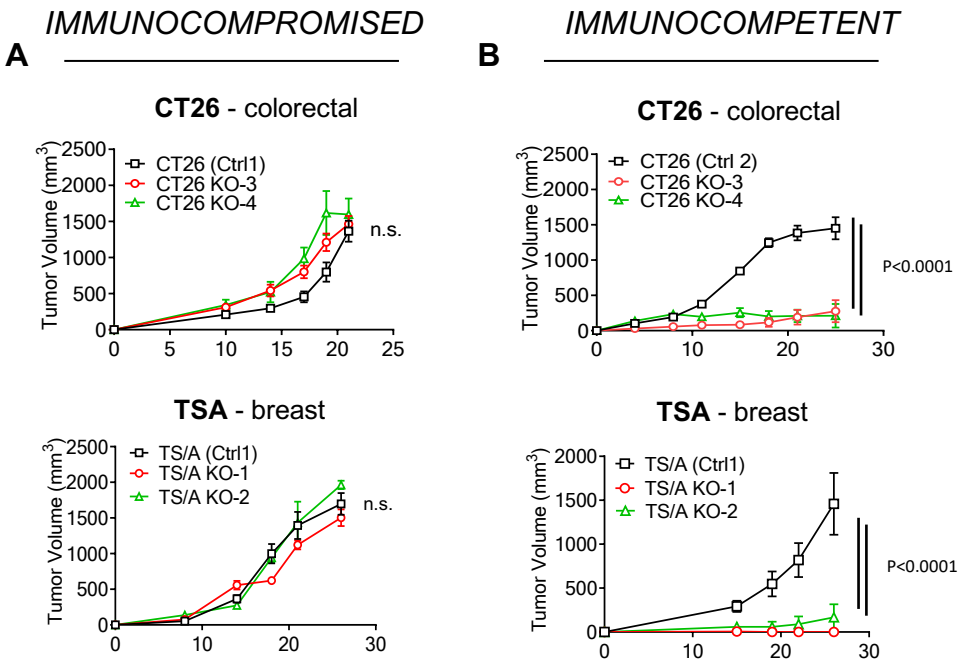

Figure 22. The indicated TSA and CT26 clones were injected subcutaneously (500,000 cells) in **A**, immunocompromised NOD-SCID and in **B**, immunocompetent syngeneic mice. Two independent guides were used to exclude off-target effects. A non-targeting vector was used to generate control cells (black lines). Mean ± s.e.m are shown. Every experiment included a minimum of 7 mice. For all experiments, statistical analysis was a two-tailed Student's t test. N.S, no significantly different.

Given the evidences that MMRd/MSI tumors have been demonstrated to correlate with and increased mutational burden and thus sensitivity to immune checkpoint modulation, we asked how inactivation of DNA repair affects the mutational and neoantigenic landscape of tumors. To this end, we performed exome sequencing of CT26 cells collected at different time points. In parallel, RNA sequencing was performed to filter only for expressed sequences and for peptides that have the potential to be presented on the major histocompatibility complex (MHC). We found that, in MMRp cells, the number of neoantigens remained essentially constant over time (**Figure 23**). In contrast, MLH-1 knock out tumors showed a dynamic increase of the mutational burden resulting in an accumulation of predicted neoantigens (**Figure 23**). This kind of analysis shows that time is necessary to acquire and accumulate mutations. This would explain why MLH-1 knock out cells require time to be rejected when injected in immunocompetent syngeneic mice.

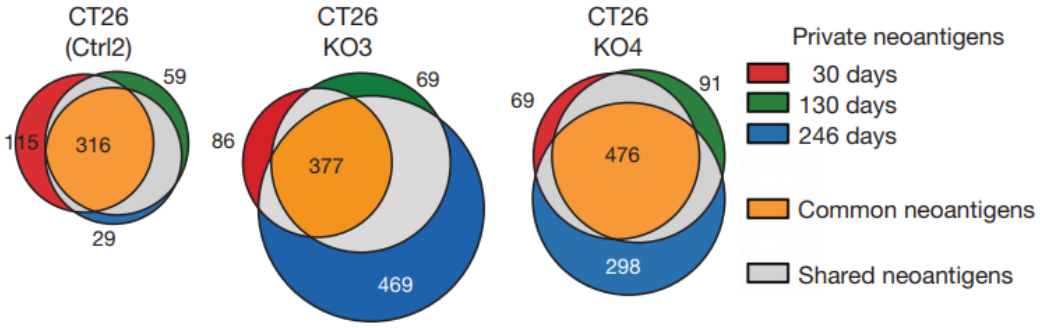

Figure 23. Neoantigen load of CT26 cancer cells. Exome data of the indicated cell models were compared. Coding variants identified by exome sequencing were used for calculating mutants and predicted neoantigens, as described in the Methods. Private events were defined as predicted neoantigens that were present only at one specific time point. Shared neoantigens were present at two time points. Common neoantigens were present at all time points. The numbers of predicted private and common neoantigens are indicated. The first time point corresponds to the level of mutations after the establishment of MLH1-knockout clones (30 days). The second time point corresponds to approximately 130 days. The third time point has been sequenced 246 days after knockout of MLH1.

4.9 VitC anticancer effect is more prominent on hyper-mutated MMR deficient tumors

Using the MMRd model we developed we assessed the impact of VitC on tumor with high mutational burden and the MSI phenotype. To this end we parallelly injected MLH-1 wild type and MLH-1 knockout TSA and CT26 cancer cells in immunocompromised NOD-SCID mice. Once again, independently of the MLH1 status (and neoantigen burdens) VitC had no effect on tumor growth in immunocompromised mice (**Figure 24**)

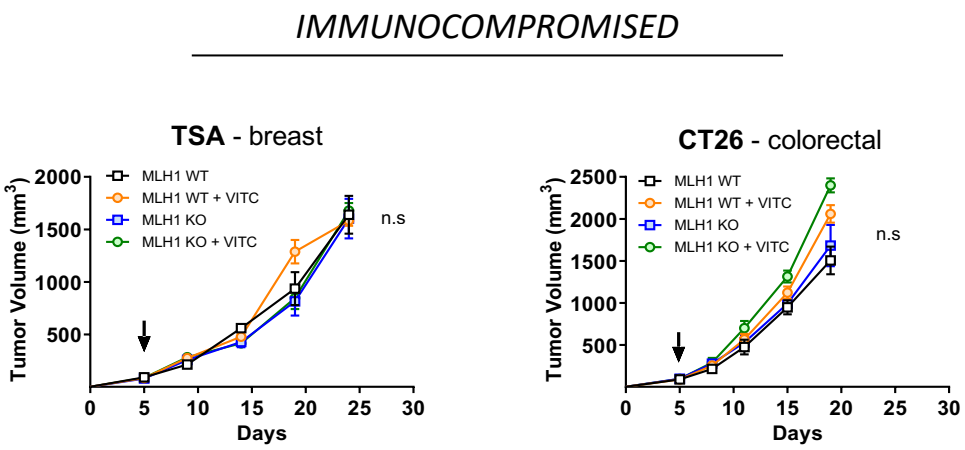

Figure 24. Effect of VitC on MLH1-WT and MLH1-KO tumors in immunocompromised mice. Both models were injected subcutaneously (500,000 cells) in immunocompromised NOD-SCID mice. VitC treatment started when tumors reached approximately 100 mm^3 in volume. Every experimental group was composed at least of 5 mice. Statistical analysis used student's t test at the indicated timepoints. NS, not significantly different. VitC, Vitamin C.

Since we previously found that MLH1-KO cells grow slower than their parental counterpart in syngeneic mice, we injected them initially in immunocompromised animals until large tumors were established. Next, tumor samples were explanted, fragmented and transplanted into multiple immunocompetent mice that were then randomized to receive either control vehicle or VitC when tumors reached nearly 150 mm^3 in volume as shown in **Figure 25 A**. In immunocompetent hosts, the

growth of MMRd was slower compared with MMRp tumors as expected (**Figure 25 B and C**).

Remarkably, the effect of VitC alone was more prominent in MMR deficient cancers than in their MMRp counterparts (**Figure 25 B and C**). These findings suggest that the antitumor effect of VitC is enhanced in mice with tumors harboring increased mutational/neoantigen burdens. These results further highlight that in the presence of larger tumors, such as those transplanted from immunocompromised animals, the efficacy of VitC alone is rather modest (**Figure 25 B**).

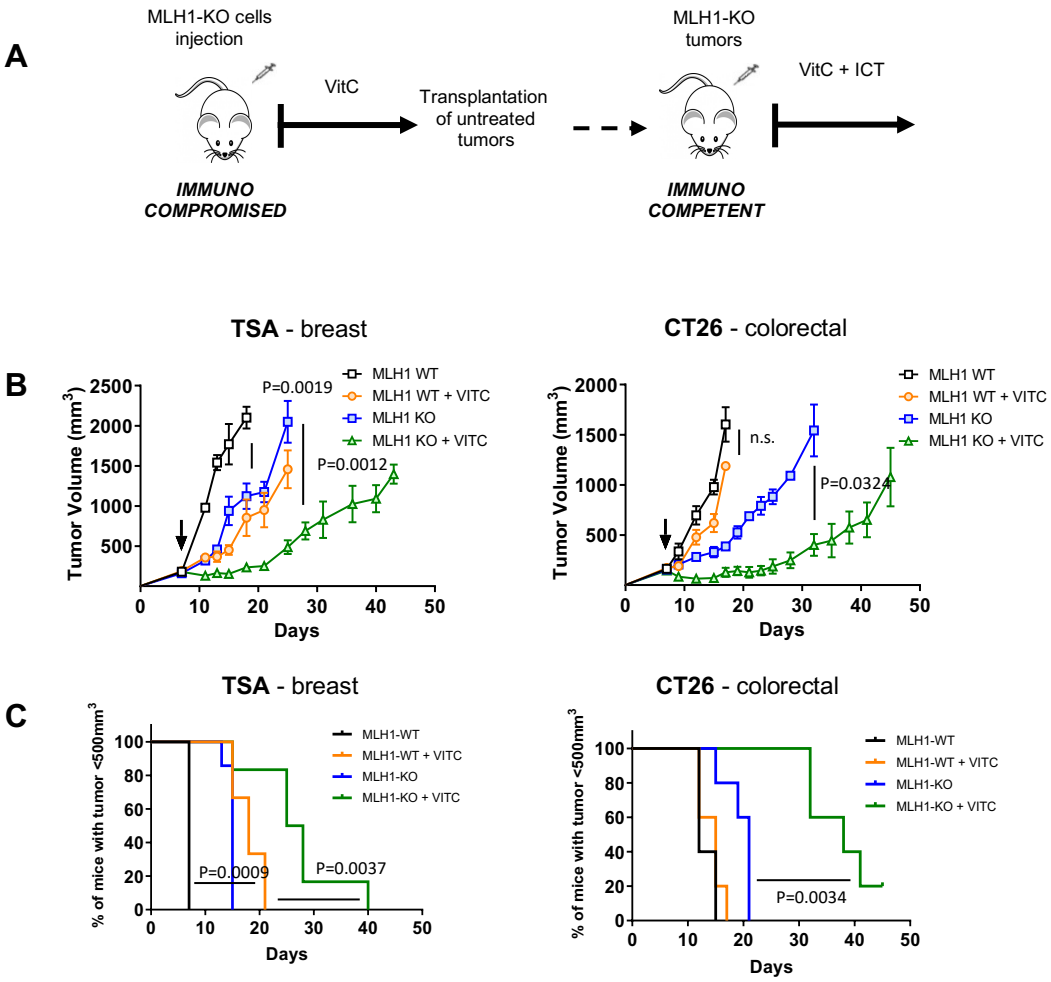

Figure 25. A, MLH1-WT and MLH1-KO cells were first subcutaneously injected (500,000 cells) in immunocompromised mice according to the indicated experimental design. **B**, Then, small fragments of untreated tumors bearing the indicated MLH1 genotype were transplanted in immunocompetent syngeneic mice; VitC (4g/kg) was administered by i.p. injection 5 days/week, starting when tumors reached a volume around 150-200 mm^3 (black arrow) to ensure tumor engraftment. **C**, Percentage of mice with tumors <500 mm^3 in volume referred to the experiment in panel B. Every experimental group was composed at least of 5 mice. Statistical analysis used student's t test at the indicated timepoints. Survival analysis in panel C used log-rank test (Mantel-Cox) analysis. NS, not significantly different.

4.10 Addition of VitC induces complete remission of MMR deficient tumors unresponsive to single immune checkpoint inhibitors

Prompted by the evidence that MMRd tumors may be more sensitive to VitC treatment, we assessed the impact of high-dose VitC addition to immune checkpoint inhibitors.

We performed the experiment according to the same transplantation experimental setting shown in figure 26A. When TSA and CT26 tumors reached approximately 800-1,000 mm^3 in volume, mice were randomized to receive VitC, ICT or their combination. The addition of individual immune modulators (anti-PD1 or anti-CTLA-4) to VitC improved anticancer response in mice bearing MMRd tumors (**Figure 26 A and B**). Remarkably, the combination of VitC with anti-CTLA-4 induced complete tumor regression in nearly all mice and no relapses were seen for up to a year**.** Notably, approximately after one month of treatment, the effect of VitC + anti-CTLA-4 was comparable to the effect induced by the combination of anti-PD1 and anti-CTLA-4 mAbs. Finally, no tumors developed when mice bearing MMRd tumors that achieved complete response on VitC and ICT combination were later re-challenged with the same cancer cells (**Figure 26 A and B**). This indicates that these mice had developed protective immunity and immunological memory.

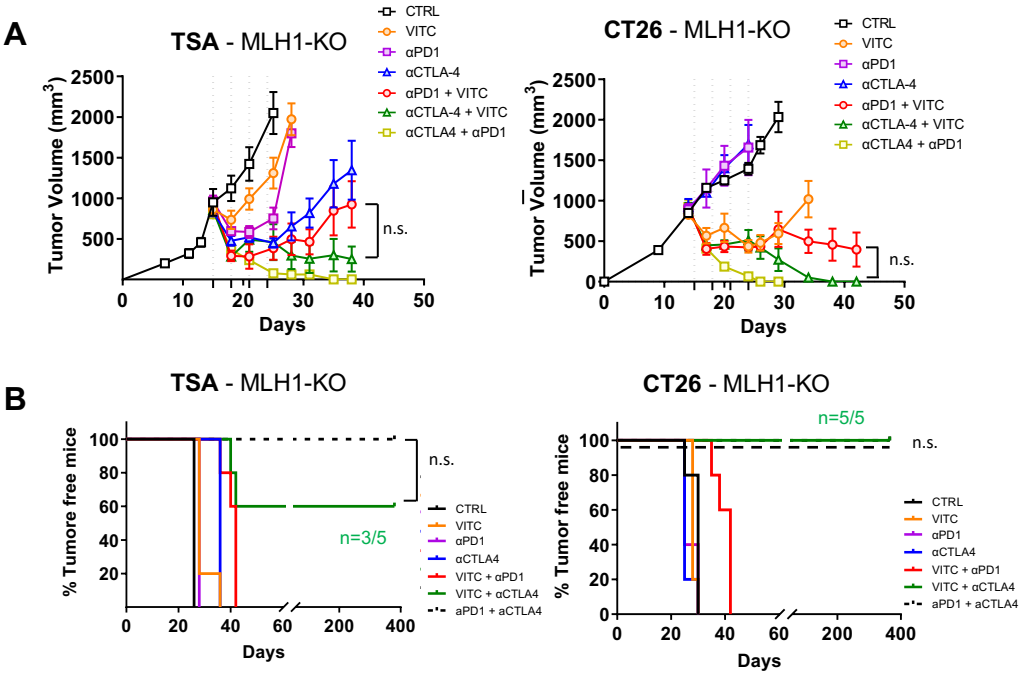

Figure 26. A, In the same setting as indicated in figure 25, MLH1-KO tumors were transplanted in immunocompetent syngeneic mice and treated with ICT and VitC (4g/kg) starting at a tumor volume of 800-1,000 mm^3. Anti-CTLA-4 (200 µg/mouse) and anti-PD1 (250 µg/mouse) were given at the timepoints indicated by the dashed vertical lines in the charts. **B**, Tumor relapse-free survival of mice treated with VitC, immune checkpoint inhibitors or their combination shown in panel. Every experimental group was composed at least of 5 mice. Statistical analysis used one-way ANOVA at the indicated timepoints. Survival analysis in panel B used log-rank test (Mantel-Cox) analysis. NS, not significantly different.

5. DISCUSSION

In this work we investigated whether and to what extent the anticancer activity of VitC relies on the host immune system. We found that in the majority of tumor types, VitC had the capacity to potentiate adaptive immune responses against cancer cells and synergize with immune checkpoint therapy (ICT). In experimental models these effects are therapeutically relevant, being effective in MMR proficient tumors and often curative in MMR deficient tumors.

Several studies have previously shown that administration of VitC in murine models can impair or delay tumor development (*72, 74, 75, 90*). However, none of them have systematically compared the anticancer efficacy of VitC in immunocompromised versus immunocompetent animals. Our study shows that VitC can delay tumor growth by stimulating adaptive immune responses. Importantly, we show that depletion of CD8 T lymphocytes in immunocompetent syngeneic mice severely impairs and often completely abolishes VitC effects.

VitC has previously been shown to enhance differentiation and proliferation of myeloid and lymphoid cells, likely due to its gene regulating effects (*88, 89*). Physiological concentrations of VitC were reported to preserve the immunosuppressive capacity of T regulatory cells and prevent autoimmunity. We found that high doses of VitC did not significantly affect the percentage of tumor infiltrating T regulatory cells. This study shows that *in vivo* administration of VitC increases the number of tumor infiltrating T cells and enhances activation of CD4 and CD8 effector T cells. This is in agreement with a very recent study that also showed increased intratumoral T cell infiltration when mice were treated with VitC (*90*). The findings in this study are also in line with another work that found a higher frequency of CD8 effector and memory T cells when mice were inoculated with tumor lysate-loaded dendritic cells that had been pre-treated *ex-vivo* with VitC (*91*).

In this work we did not investigate the molecular mechanisms leading to T cell activation following administration of VitC in mice, since this aspect has already been studied. In fact, several reports have previously shown that VitC leads to

epigenetic modulation of T cells and their activation, since VitC can act as a cofactor for both DNA and histone demethylases (*77, 85, 89*). In relation to this, we speculate that VitC may cause rejuvenation of T cells, favor their expansion and clonal diversity (*85, 92, 93*).

We further found that addition of VitC can potentiate the efficacy of combined anti-CTLA-4 and anti-PD-1 blockade in breast, pancreatic and colorectal MMR proficient murine models. Not only this combination delayed tumor growth in most cases, but in a few mice complete regressions were observed. We found that combining VitC and ICT further enhanced tumor infiltrating CD8 T cells compared to the increase observed with single treatments.

Immune checkpoint inhibitors are approved for the treatment of several malignancies (*24*). However, intrinsic unresponsiveness is seen in most cases. For instance, only a fraction of patients with MMRd tumors benefit from immunotherapy. Combinations of immune checkpoint inhibitors including anti-CTLA-4 and anti-PD-1 achieve responses in a larger fraction of MMRd patients, but at the price of higher toxicities (*57, 58*). While addition of VitC did not improve the activity of anti-PD-1 alone, VitC strongly enhanced the efficacy of anti-CTLA-4 as a monotherapy and their combination was sufficient to induce a complete response in almost all mice bearing MMR deficient tumors.

The mechanism underlying the remarkable synergy between VitC and immune checkpoint inhibitors deserves further studies. We found increased expression of interferon gamma (IFNγ) production from T cells extracted following VitC administration. This is in line with the previously reported VitC pro-inflammatory functions (*81*). It is possible that VitC improves T cell responses and tumor control during treatment with immune checkpoint inhibitors by reverting T cell exhaustion-associated DNA-methylation programs (*77, 92, 93*).

It is not excluded that VitC could exert its functions not only on immune cells but also on cancer cells; or even that only the presence of an intact immune system may unleash a functional synthetic lethality. In this regard, a recent study has shown that

VitC can stimulate TET2 activity in cancer cells and potentiate the efficacy of anti-PD-L1 immune therapy in mouse melanoma cells ectopically expressing the ovalbumin antigen (*90*). However, in our models VitC strongly enhanced the efficacy of anti-CTLA-4 as a monotherapy suggesting that the primary contribution to the VitC synergy with ICT is exerted on T lymphocytes and most likely during the priming or expansion phases. This is also in line with the observation that the combination of VitC with ICT is very effective in tumors with high mutational burden. In fact, the most striking effects were observed in the models with high neoantigenic burdens such as carcinogen-induced CT26 mouse cells and MMR deficient tumors. We speculate that a high tumor mutational burden may generate an expanded T cell cancer-specific repertoire which is fostered by the immune modulatory properties of VitC.

In addition to promoting DNA demethylation mediated by TET enzyme activation, high doses of VitC may kill cancer cells via oxidative stress and by disrupting iron metabolism (*74, 75*). It has been reported that also immune checkpoint inhibitors can kill cancer cells via ferroptosis. IFNγ released by cytotoxic T cells can deplete cysteine levels in cancer cells by down-regulation of its transporters, thus favoring tumor cell lipid peroxidation and ferroptosis (*94*). Accordingly, cysteine deprivation was able to synergize with checkpoint inhibitors to induce anti-tumor immune response and ferroptosis. It is possible that VitC may orchestrate a similar program making cysteine depleted cancer cells more vulnerable to the attack of the adaptive immune system unleashed by checkpoint inhibitors and lead to consequent ferroptosis. However, we found that concomitant administration of a ROS scavenger did not blunt VitC efficacy in immunocompetent mice, indicating that oxidative stress is unlikely to exert a major impact in the models described in this work. Nevertheless, the interplay between tumor and immune cells in the presence of high doses of VitC should be investigated throughout and elucidated in further studies.

5.1 Conclusive summary and final remarks

The evidence that a vitamin has such a remarkable impact on ICT in highly aggressive mouse cancer models prompted us to consider designing novel clinical trials, which must keep in mind the points highlighted below. Advanced cancer patients reportedly have compromised VitC status and it is likely that intravenous administration of VitC would be required to achieve pharmacologically relevant concentrations. On the bright side, VitC is known to be non-toxic even at high doses, and intravenous administration of VitC is generally considered to be safe. However, the optimal duration of VitC treatment has not been established. This is particularly relevant when planning combination studies with immune checkpoint inhibitors that are often administered for several months or years. Results from the adoptive cell transfer experiments suggest that VitC exposure could be critical in the priming and clonal expansion phases as well as during lymphocyte-mediated cancer cell killing. Based on our findings, we propose that VitC should be tested concomitantly to the first few cycles of immune checkpoint inhibitors. Although mice received only four cycles of ICT, no signs of immune-related adverse events or other toxicities were seen in animals co-treated with VitC and ICT, suggesting that combinatorial regimens may be tolerated by cancer patients. Nevertheless, this issue will require assessment in a formal phase I study in which escalating doses of VitC should be administered with concomitant ICT.

In summary in this study we found that VitC can stimulate anticancer adaptive immunity and enhance the efficacy of immune checkpoint inhibitors in mouse cancer models, including MMR proficient and deficient tumors, thus supporting the design of combination clinical trials testing immunomodulation by VitC.

6. METHODS

6.1 Mouse cell lines

The TSA breast cancer cell line was established from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a Balb/c mouse (*95*). TSA cells were provided by F. Cavallo (Molecular Biotechnology Center, University of Torino). CT26 is a mouse undifferentiated colon carcinoma, derived from Balb/c mice (*96*). CT26 were purchased by ATCC. MC38 is a mouse colon adenocarcinoma line derived from a C57/BL6 mouse and cells were kindly provided by M. Rescigno (European Institute of Oncology). 4T1 is a spontaneous mammary adeno-carcinoma derived from a Balb/c mouse and were purchased from ATCC (*97*). PDAC cells were isolated from FVB transgenic mice bearing pancreatic cancers with the following genotype: p48cre, KrasLSL-G12D, p53R172H/+, Ink4a/Arfflox/+(*98*). PDAC cells were kindly provided by D. Hanahan (ISREC, EPFL, Lausanne). B16 is a melanoma cell line derived from a C57/BL6 mouse purchased by ATCC (*99*). CT26, MC38, 4T1 and PDAC cells were cultured in RPMI 1640 10% FBS, plus glutamine, penicillin and streptomycin (Sigma Aldrich). TSA and B16 cells were cultured in DMEM 10% FBS plus glutamine, penicillin and streptomycin (Sigma Aldrich). All cell lines were tested for mycoplasma regularly. To ensure that the parental cell models were tumorigenic, before starting the experiments all the lines were injected into matched syngeneic mice. On tumor formation, we re-established in vitro cell cultures.

6.2 Animal studies

All animal procedures were approved by the Ethical Commission of the Candiolo Cancer Institute and by the Italian Ministry of Health, and they were performed in accordance with institutional guidelines and international law and policies. The number of mice included in the experiments and the inclusion/exclusion criteria were based on institutional guidelines. We observed tumor size limits in accordance with institutional guidelines. Six to eight week-old female and male C57BL/6J, BALB/c, FVB/N and NOD-SCID mice were employed according to the approved

protocol. Mice were obtained from Charles River. All experiments involved a minimum of five mice per group. Tumor size was measured every three/four days and calculated using the formula: V = (d2×D)/2 (d = minor tumor axis; D = major tumor axis) and reported as tumor mass volume (mm^3, mean ± SEM of individual tumor volumes). The investigators were not blinded; measurements were acquired before the identification of the cages. No statistical methods were used to predetermine sample size.

6.3 Mice treatments

Ascorbate (Sigma Aldrich) was prepared weekly by resuspending the powder in sterile water. Ascorbate was administered intraperitoneally 5 days/week at 4g/kg dosage. The anti-mouse PD-1 (clone RMP1-14), anti-mouse CTLA-4 (clone 9H10), anti-mouse CD4 (YTS191), anti-mouse CD8a (YTS169.4), Rat IgG2a, polyclonal Syrian Hamster IgG and rat IgG2b antibodies were purchased from BioXcell. Randomization was used for the experiments in which therapeutic effects had to be evaluated. Animals were treated i.p. with 250 μg anti-PD-1 antibody per mouse, and 200 μg anti-CTLA-4 antibody per mouse. Treatments were administrated at the timepoints indicated in the graphs after checking for tumor establishment. In combinatorial treatments, VitC was administered since the first cycle of immunotherapy. Isotype controls were injected according to the same schedule. Anti-mouse CD4 and CD8a were used for depletion of T cells in immunocompetent mice. Anti-mouse CD4, CD8a and matched isotype mAbs (400 μg per mouse) were injected intraperitoneally on the same day as tumor inoculation. Depleting antibodies were administered (100 μg per mouse) on day one and day two and every three days since tumor cells injection. Depleting antibodies and matched isotypes were administered continuously every three days along the entire course of the experiments. Flow cytometry analysis was performed every three days to assess the level of CD4+ and CD8+ cells in the bloodstream of mice. The fraction of CD4+ or CD8+ cells relative to CD45+ cells was around 20% before and 0.5% after the administration of depleting antibody. The low fraction of CD4+ and CD8+ cells (0.5%) was maintained throughout the entire experiment.

6.4 Flow cytometry cell analysis

Mouse tumors were cut into small pieces, disaggregated with collagenase (1.5 mg ml–1) and filtered through 70 μm strainers. Cells were stained with specific antibodies and Zombie Violet Fixable Viability Kit (BioLegend). Phenotype analysis was performed with the following antibodies purchased from BioLegend: anti-CD45-PerCp (30F11), anti-CD11b-APC (M1/70), anti-CD3-PE/Cy7 (17A2), anti-CD4-FITC (RM4-5), anti-CD8-PE or FITC (YTS156.7.7), anti-F4/80-APC (BM8), anti-CD49b-PE (DX5), anti-CD44-APC (IM7), anti-CD69-PE (H1.2F3), anti-CD62L-Pe/Cy7 (MEL-14), anti-CD11c-FITC (N418), anti-CD28-PE (37.51), anti-CD25-APC (PC61), anti-CD127-Pe/Cy7 (A7R34), anti-FoxP3-PE (MF-14). For FoxP3 staining, cells were isolated and stained with surface antibodies for 30 minutes, and then fixed and permeabilized using the FoxP3 Fix/Perm Buffer set (BioLegend). Cells were then stained with anti-FoxP3-PE (Bio-legend). For IFNγ staining, cells were *in vitro* stimulated with the cell stimulation cocktail (eBiosciences) and incubated with GolgiStop and GolgiPlug (BD Biosciences). After 6 hours of incubation, cells were washed and stained for extracellular markers. Then, cell permeabilization was performed by using the Cytofix/Cytoperm kit (BD Biosciences) and then stained for intracellular marker with anti-IFNγ-APC (XMG1.2 - BioLegend). All flow cytometry was performed using the FACS Dako instrument and FlowJo software.

6.5 Immunofluorescence analysis

Detection of T cells was performed with a modification of the method for immunofluorescence of fresh frozen tissues described previously . In brief, tumor samples were included in Killik (Bio-Optica), serially cut (10 μm) and fixed using cold acetone:methanol (1:1). Samples were incubated for one hour in blocking buffer (1% BSA and 2% of goat serum in PBS with 0.05% of Tween and 0.1% of Triton) and incubated overnight with anti-CD8 (clone YTS169 from Thermo Fisher Scientific). For detection, anti-rat Alexa Fluor 647 was used (Thermo Fisher Scientific).

6.6 Adoptive T Cell Transfer

Mice were euthanized and their splenocytes isolated as previously described (*100*). Briefly, spleens were minced and passed through a 70 μm cell strainer. Afterward, red blood cells were lysed with ACK lysis buffer (Gibco) and the remaining splenocytes washed with MACS buffer. Magnetic bead sorting using CD4+ (Miltenyi) and CD8a+ T cell isolation kit (Miltenyi) were used to acquire untouched CD4+ and CD8+ T cells. The purity of the enriched cells was greater than 94%. Cells were dissolved in 100 μl of PBS and intravenously injected in an orthotopic model of breast cancer. Mice were injected twice with 5 million T cells by tailvein injection at day 5 and 10 since cancer cells injection.

6.7 Anti-oxidant analysis

In experiments where anti-oxidants were administered, N-acetyl cysteine was supplemented to the drinking water of mice (30 mM, pH adjusted to 7,2) as previously described (*74*) and freshly renewed every three days. To check anti-oxidant effects on tumors, 8-oxo guanine (Abcam – N45.1) staining was performed by immunohistochemistry on FFPE sections (*101*).

6.8 Gene editing

To knockout Mlh1, we used the genome editing one vector system (lentiCRISPR-v2) (Addgene #52961). sgRNAs were designed using the CRISPR tool (http://crispr.mit.edu) to minimize potential off-target effects. The following sgRNA sequences were used: sgRNA2: TCACCGTGATCAGGGTGCCC; sgRNA3: CAACCAGGGCACCCTGATCA; sgRNA6: ATTGGCAAGCATAAGCCATG. Annealed sgRNA oligonucleotides targeting mouse Mlh1 were cloned into Bsmbl lentiCRISPR-v2 plasmid, as previously described (*102*). Lentiviral particles were generated by co-transfection of HEK293T with the viral vector and packaging plasmids pCMV-VSV-G (Addgene #8454) and psPAX2 (Addgene #12260). Supernatant from transfected HEK293T was collected, passed through a 0.22 μm filter to remove cell debris and frozen as 1-ml aliquots at −80 °C. Cells were infected with lentivirus at

approximately 60% confluence in the presence of 8 μg ml−1 polybrene (Millipore). Puromycin (Sigma Aldrich) was used to select CRISPR–Cas9-infected cells. To identify Mlh1-knockout clones, infected populations were single-cell cloned in 96-well plates. Approximately thirty clones for each cell models were selected from 96-well plates and the presence of MLH1 was verified by western blot analysis. To express the CRISPR–Cas9 system transiently, we transfected cells with lentiCRISPR-v2 vector plasmid (using the same guides as described above). Transfection was carried out using Lipofectamine 3000 (Life technologies) and Opti-MEM (Invitrogen), according to the manufacturer's instructions. After 48 hours, cells were incubated with puromycin (Sigma Aldrich) for four days and subsequently single-cell-diluted in 96-well plates. We selected clones that lacked Mlh1 and confirmed absence of Cas9 on the basis of western blot analysis.

6.9 Microsatellite instability analysis

Microsatellite instability in mouse cells was determined using a panel of three microsatellite markers as previously described (*103*). Amplification was performed with the following labelled primers: fluorescein mBat64, forward GCCCACACTCCTGAAAACAGTCAT and reverse CCCTGGTGTGGCAACTTTAAGC; AC096777 JOE, forward TCCCTGT ATAACCCTGGCTGACT and reverse GCAACCAGTTGTCCTGGCGTGGA; AA003063 Tamra, forward ACGTCAAAAATCAATGTTAGG and reverse CAG CAAGGGTCCCTGTCTTA; U12235 JOE, forward GCTCATCTTCGT TCCCTGTC and reverse CATTCGGTGGAAAGCTCTGA; L24372 fluorescein, forward GGGAAGACTGCTTAGGGAAGA and reverse ATTTGGCTTTCAA GCATCCATA. The PCR reaction was performed in 20 μl of PCR reaction using Platinum Taq Polymerase Kit from Invitrogen and 20 ng of DNA. The cycling profile was as follows: 1 cycle at 94°C for 4min; 94°C for 30s, 56°C for 45s and 72 °C for 30 s for a total of 35 cycles. A final extension at 72 °C for 6 min completed the amplification. PCR fragments were separated on a 3730 DNA analyser (Applied Biosystems) and raw data were analysed with GeneMapper software.

6.10 Mutational load and neoantigen prediction analysis

Genomic DNA was extracted using ReliaPrep gDNA KIT (Promega). Whole exome sequencing was performed at Integragen (Evry). Libraries were sequenced using Illumina HiSeq 4000. The bioinformatics analysis was performed at our institution on exome sequencing data provided by Integragen. Raw data in FastQ format were initially demultiplexed using CASAVA 1.8 software as paired-end 75-bp reads. On average, we observed a median depth of 70×, with more than 97% of the targeted region covered by at least one read. Before further analysis, pair-end reads were aligned to the mouse references (assembly mm10) using the BWA-mem algorithm. Next, PCR duplicates were removed from the alignment files using the 'rmdup' SAMtools command. An NGS pipeline previously developed by our laboratory was used to identify single nucleotide variants (SNVs) and indels. Somatic variations were called subtracting germline variations present in BALB/c (data downloaded from the 'Mouse Genomes Project'; http://www. sanger.ac.uk/science/data/mouse-genomes-project). Only positions present with a minimum depth of 5× and supported by at least 1% allelic frequency were considered. To calculate the significance of an allele's frequency, a Fisher's exact test was performed for each variant. The mutational burden (number of variants per Mb) was calculated considering only coding variants that were normalized on the targeted region for each data point. Predicted neoantigens were calculated starting from the file of coding variations, annotated and filtered for gene expression values (expected count >10) using RNA-seq data from the same sample (see below). For each variation, the mutant peptide sequence was obtained: for SNVs, we introduced the altered amino acid in the candidate peptide; for frameshifts, we took the newly generated frame. Mutated peptide sequences were trimmed and then processed using NetMHC 4.0, with k-mer of 8–11 length. Haplotypes for mouse samples were set to H2-Kd and H2-Dd for the BALB/c background. Predicted neoantigens were filtered by a rank threshold of 0.5. Alterations that produced more than one predicted neoantigen were clustered through a custom script (exploiting the Levenshtein distance) to create a consensus family. For each family the peptide with the best rank was considered.

6.11 RNA sequencing analysis

To extrapolate expressed neoantigens, we performed RNA-seq of the MMR-proficient and MMR-deficient clones. The RNA concentration and integrity was evaluated with the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit. Total RNA (800 ng) with RNA integrity number (RIN) score between 8 and 10 was used as input to the Illumina TruSeq RNA Sample Prep Kit v2-Set B (48Rxn), according to the manufacturer's protocol. The standard RNA fragmentation profile as recommended by Illumina was used (94 °C for 8 min for the TruSeq RNA Sample Prep Kit). PCR-amplified RNA-seq library quality was assessed using the Agilent DNA 1000 kit on the Agilent 2100 BioAnalyzer and quantified using Qubit 3.0 Fluorometer (LifeTechnologies). Libraries were diluted to 10 nM using Tris-HCl (10 mM pH 8.5) and then pooled together. Diluted pools were denatured according to standard Illumina protocol and 1.8 pM were run on NextSeq500 using high output Reagent cartridge V2 for 150 cycles. A single-read 150-cycle run was performed. The transcriptome profile of RNA-seq data was calculated using MapSplice v.2.2.041 and RSEM42 software package. Genes with at least 10 expected counts in the output file were considered to be expressed.

6.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism Software. To determine statistical significance for tumor growth curves, normality and Lognormality tests were performed for each experiment. In the case of a Gaussian-like distribution, student t test for two group comparison (P values were adjusted with Welch correction) and one-way ANOVA for more than two group comparison (P values were adjusted with Tukey correction) were performed. In case of a non-Gaussian distribution, non-parametric tests were performed (P values were adjusted with Welch correction). For immuno-phenotypic analysis, normality and Lognormality tests were performed. Statistical significance was calculated using one-way ANOVA (P values were adjusted with Tukey correction) in case of Gaussian-like distribution. Non-parametric analyses (P values adjusted with Welch correction) were conducted for datasets that failed to pass a normality test. The Kaplan-Meyer

method was used for survival analysis, and P values were calculated using the log-rank test (Mantel-Cox). All data are presented as mean ± SEM Sample sizes were chosen with adequate power, on the basis of our previous studies and literature surveys. The number of replicates and sample size for in vivo experiments were limited according to the requirements of the Italian Ministry of Health. Animal studies were performed in accordance with institutional guidelines and international law and policies. When therapy was applied, we performed randomization. In this case, tumor-free mice or mice with a tumor larger than 50% of the average were excluded from the experiment.

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