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REVIEW ARTICLE

The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy

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Abstract Cyclophosphamide is an alkylating agent belonging to the group of oxazaphosporines. As cyclophosphamide is in clinical use for more than 40 years, there is a lot of experience using this drug for the treatment of cancer and as an immunosuppressive agent for the treatment of autoimmune and immune-mediated diseases. Besides antimitotic and antireplicative effects, cyclophosphamide has immunosuppressive as well as immunomodulatory properties. Cyclophosphamide shows selectivity for T cells and is therefore now frequently used in tumour vaccination protocols and to control post-transplant allo-reactivity in haploidentical unmanipulated bone marrow after transplantation. The schedule of administration is of special importance for the immunological effect: while cyclophosphamide can be used in high-dose therapy for the complete eradication of haematopoietic cells, lower doses of cyclophosphamide are relatively selective for T cells. Of special interest is the fact that a single administration of low-dose cyclophosphamide is able to selectively suppress regulatory T cells (T_{reg}s). This effect can be used to counteract immunosuppression in cancer. However, cyclophosphamide can also increase the number of myeloid-derived suppressor cells. Combination of cyclophosphamide with other immunomodulatory agents could be a promising approach to treat different forms of advanced cancer.

Keywords T cells · Oxazaphosphorines · Immune reactivation · Immunosuppression

Introduction

Cyclophosphamide is one of the oldest anticancer drugs. It was discovered as early as 1958 and introduced into cancer therapy in 1959 [1]. It remains a mainstay in the therapy of haematological malignancies including lymphoma and leukaemia as well as of various epithelial tumours including breast, ovarian and small-cell lung carcinomas [2]. In Germany, the drug is approved for the therapy of ALL, CLL, Hodgkin and non-Hodgkin lymphoma, plasmocytoma, breast cancer, ovarian carcinoma, small-cell lung cancer, Ewing-, osteo- and rhabdomyosarcoma and neuroblastoma [3].

The drug is applied in many conditioning regimens before bone marrow transplantation for haematological malignancies (AML, MDS) as well as for aplastic anaemia. Besides cancer chemotherapy, other approved indications are life-threatening events in autoimmune and immune-mediated diseases as lupus nephritis, Wegener's granulomatosis and multiple sclerosis.

Important side effects are leuco- and thrombocytopenia, anaemia, cardio- and bladder toxicity. To avoid haemorrhagic cystitis, MESNA (sodium 2-sulfanylethanesulfonate) must be administered before cyclophosphamide administration to neutralise the toxic metabolite acrolein in the urine. In addition, nephrotoxicity, cardiotoxicity and liver toxicity occur. All these effects are highly dose dependent. Cardiotoxicity is dose-limiting in high-dose schedules of cyclophosphamide [4].

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Cyclophosphamide is an alkylating agent. The active metabolite phoshoramide mustard forms a highly reactive cyclic aziridinium cation, which can react with the N(7) of the guanine and with cytidine from the DNA [5]. Due to the two reactive moieties in the molecule, interstrand and inter-strand cross-links can be formed [6]. The effects of cyclophosphamide are therefore cell cycle independent. However, as with all alkylating agents, rapidly proliferating cells are most sensitive to cyclophosphamide [7].

Although the drug is in therapeutic use for many decades, new therapies were developed such as metronomic and high-dose therapy. Currently, new schedules are developed with the aim to overcome immunosuppression in advanced cancer.

The immune system usually prevents cancer by identifying cancer cells as "strange" through MHC proteins on the cell surface and are attacked by immunocompetent cells such natural killer cells, cytotoxic T cells and others [8]. Solid tumours can only be established after evasion from the immune system. Therefore, immuno-suppression in advanced cancer has come into the focus of cancer research in pharmaceutical industry and led to the approval of the very promising immune checkpoint inhibitors [9].

According to the classical textbook knowledge, the anticancer effect of cytostatic drugs is based on solely destroying cancer cells due to their high proliferation rate. Immunocompetent cells are also formed by fast growth, and therefore, cytotoxic therapy cause immunosuppression. However, the "old" cytotoxic drugs not only suppress immune response, but also interact in several ways with the immune system, especially with T cells [10].

The purpose of this review is—after giving a short introduction of the pharmacology—to summarise the actual knowledge on the effects of cyclophosphamide on the immune system. The interactions of this drug with the immune system are extensively investigated. Proposed mechanisms of the effects are discussed and critically appraised with the aim to identify promising approaches for further preclinical and clinical research.

Pharmacokinetics and metabolism

Cyclophosphamide can be applied both orally and intravenously. After oral administration, the absorption rate is very high. Bio-activation is necessary to exert cytotoxic effects. Cytochrome P450, mainly CYP 2B6 and 3A4 (besides CYP 2A6, 2C8, 2C9, 2C19 and 3A5), oxidises cyclophosphamide to 4-hydroxy-cyclophosphamide forming an equilibrium with aldophosphamide (Fig. 1) [11]. Both forms can enter the cell and are subsequently decomposed to phosphoramide mustard, the agent able to interfere with DNA forming cross-links. When aldophosphamide is cleaved, acrolein is also formed which concentrates in the bladder and can result in serious toxicity. MESNA (sodium 2-sulfanylethanesulfonate) must be administered before cyclophosphamide administration to neutralise acrolein in the bladder. The sulfhydryl group reacts with the vinyl group of acrolein.

Side-chain oxidation of cyclophosphamide to dechloroethyl-cyclophosphamide is also mediated by cytochrome P450, mainly by the CYP 3A4 isoenzyme and CYP 2B6 being also involved [12]. Thus, as both activation and inactivation of the drug are catalysed predominantly by the same enzyme, attempts to enhance the therapeutic index by adding inhibitors of P450 failed. The by-product chloroacetaledhyde has shown cytotoxic effects in vitro and may contribute to the neurotoxicity of cyclophosphamide [13, 14]. Another pathway of inactivation is cleavage with glutathione catalysed by GSTM1, GSTP1 and GSTT1. Polymorphisms in the genes encoding for the enzymes appear to play a role for toxicity and efficacy [15].

In vitro and in vivo investigations indicate that the activation to 4-hydroxy-cyclophosphamide is saturable, whereas the side-chain oxidation as an inactivating step is not [12]. This finding is also supported by pharmacokinetic modelling approaches [16].

Cyclophosphamide, as well as other oxazaphosporines, induces their own metabolism (autoinduction) [17]. Consequently, the clearance of cyclophosphamide is increased with repeated administrations and continuous infusions. Autoinduction and saturable metabolism of certain pathways result in nonlinear pharmacokinetics of cyclophosphamide [18]. Dose individualisation based on plasma concentration measurements of cyclophosphamide in the high-dose setting is possible and feasible [19]. Patients with individualised cyclophosphamide dosing showed a reduced incidence of nephrotoxicity as well as less liver toxicity with unchanged response rates compared with patients receiving conventional dosing.

Oxidation of the active aldophosphamide by ALDH to carboxyphosphamide is the main step of intracellular detoxification [20]. As cellular aldehyde dehydrogenase (ALDH), especially ALDH1A1, is the main mechanism of detoxification and ALDH1A1 is poorly expressed in lymphocytes, there is some selectivity for immunocompetent cells. In contrast, haematopoietic stem cells show high activity of ALDH1A1 and can tolerate higher concentrations of activated cyclophosphamide [21].

Pharmacogenetics

As cyclophosphamide requires bioactivation by P450 enzymes, the effect of genetic polymorphisms was investigated in several studies [22]. However, given the fact

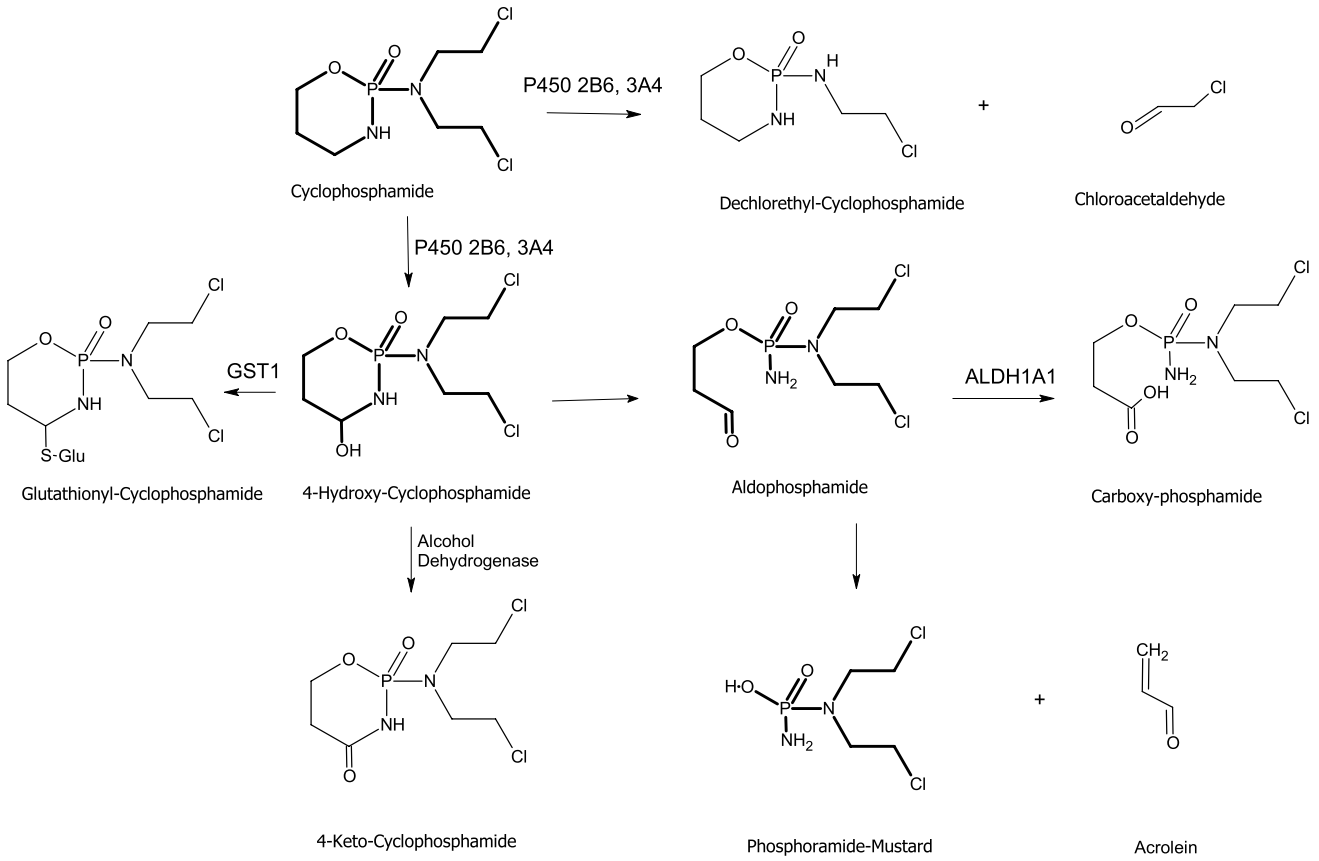

Fig. 1 Metabolism of cyclophosphamide. Structures in *bold* show the pathway leading to the active metabolites. *ALDH* aldehyde dehydrogenase, *GST* glutathione-S-transferase, *P450* cytochrome P450

that most of the metabolic pathways of cyclophosphamide are catalysed by several enzymes, there are no extreme effects of certain polymorphisms in genes coding for P450 enzymes. As GST's are important for detoxification of cyclophosphamide, polymorphisms in GSTT1, GSTM1 and GSTP1 were found to have an effect on clinical outcome measured as survival or relapse rate as well as on toxicity [23]. ALDH1A1 and 3A1 are important for intracellular detoxification of 4-hydroxy-cyclophosphamide and pharmacogenetic studies showed an influence of these polymorphisms on the toxicity of cyclophosphamide. There is currently no clear recommendation for genotyping before cyclophosphamide therapy [22]. However, protocols involving genotyping for polymorphism in GST`s as well as in ALDH genes appear to be meaningful in order to improve the therapeutic index of cyclophosphamide.

Schedules of administration

In conditioning regimens before bone marrow transplantation, cyclophosphamide is administered in a dose of 40–60 mg/kg on two to four consecutive days in combination with busulfan, melphalan or other cytostatics. For the treatment of solid tumours and ALL, conventional doses are 400–1000 mg/m^2 alone or in combination therapies with 5-FU, etoposide, methotrexate or anthracyclines [3]. Metronomic therapy protocols used for the treatment of solid tumours and multiple myeloma administer daily doses of 25–100 mg cyclophosphamide, often in combination with cytostatics (methotrexate, etoposide and vinblastine) or other drugs like thalidomide, celecoxib or corticosteroids [24]. Up to now, metronomic therapy schemes are only applied in clinical therapy optimisation studies and are not considered as standard therapy.

Effects on the immune system

Preclinical studies

Very early reports already showed a strong effect of cyclophosphamide on antibody formation, leucocyte and lymphocyte formation in a rat model [25]. In this report, Potel and Brock demonstrated that a single dose did not cause a prolonged immunosuppression in comparison with

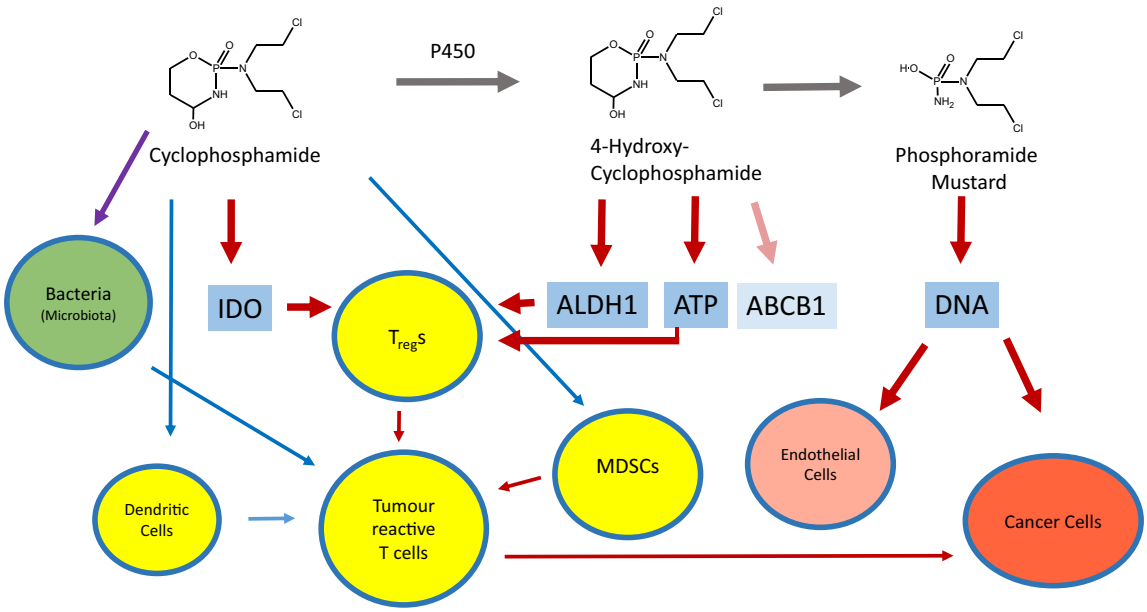

Fig. 2 Proposed mechanism for the effect of cyclophosphamide and its active metabolites on immune response. The *red arrows* indicate inhibition or consumption. The *blue arrows* indicate stimulation

repeated dosing. In another rat model, cyclophosphamide caused a stronger reduction in the number of leucocytes in comparison with similar doses of ifosfamide, trofosfamide or azathioprine [26]. The stronger immunosuppressive properties of cyclophosphamide in comparison with antimetabolites like azathioprine or 6-mercaptopurine was confirmed in another set of experiments with rats [27]. In the cotton-pellet model for inflammation in the rat, cyclophosphamide displayed a stronger inhibition than the thiopurines. In the same report, a reduction in the number and activity of lymphocytes in the spleen was seen in a mouse model with cyclophosphamide [28]. In comparison with busulfan, cyclophosphamide showed a pronounced reduction in the number of leucocytes and granulocytes in the first days after administration in a mouse model. However, recovery occurs within 10 days with the number of granulocytes increasing even higher than before treatment [29]. With the exception of this unexpectedly high increase in granulocyte, all these effects did not indicate any selective effect on certain subpopulations of immunocompetent cells.

As early as 1974, it was suggested that cyclophosphamide reverses immune tolerance in guinea pigs by inhibition of a so far unknown suppressor cell population. At this time, the authors could not determine whether these cells belong to the subset of B or T lymphocytes. The inhibition of the suppressor cell population was temporarily. After 2 weeks the suppressor cells recovered [30]. A few years later, Röllinghoff et al. [31] described a cyclophosphamide-sensitive fraction of T cells capable of suppressing antigen-specific cytototxic T lymphocytes (CTL`s) in a mouse model.

The first clear evidence of the immunotherapeutic activity of cyclophosphamide was demonstrated by Awwad and North in mice [32]. The authors choose the L5178Y lymphoma model as this tumour is resistant to the direct action of cyclophosphamide. While the injection of 150 mg/kg cyclophosphamide alone or the transfer of immune cells had no effect on the tumour, the combination of both showed prolonged response in T cell deficient mice. The experiments clearly showed that cyclophosphamide could eliminate suppressor T cells (T_{reg}s) which can neutralise the sensitised donor T cells injected after administration of cyclophosphamide. With a growing interest in immunotherapy for cancer, a great number of investigations were conducted in the last years showing an association of a high number of T_{reg}s ($CD4^+$ $CD25^+$ forkhead box $p3^+$) with a poor prognosis in solid tumours [33–36].

In a mouse model, Peng et al. demonstrated that cyclophosphamide enhanced antitumor effects of a HPV vaccine. Comparison of different schedules of administration identified the strongest effect on tumour shrinkage with a single-dose cyclophosphamide of 50 mg/kg. According to the allometric rule [37], this would translate into a dose of about 8 mg/kg or 600 mg in a typical male adult. A transient reduction of $CD4^+$ forkhead box $p3^+$ (Foxp3) T_{reg} population in the tumour microenvironment could be observed [38]. Daily administration of 10–20 mg/kg cyclophosphamide resulted in a sustained reduction of T_{reg}s; however, the tumour-specific $CD8^+$ T cells were also

reduced. No difference in response was observed between the different dosing schedules. Overall, the authors recommend a single-dose cyclophosphamide for further evaluation because of the easier administration. With the daily administration, the reduction of $CD8^+$ T cells indicates immunosuppression and appears not to be the best choice.

In a colon cancer mouse model, Son et al. showed that cyclophosphamide in combination with an immunotherapeutic approach improved survival. Animals were treated with ex vivo cultured autologous dendritic cells (DC`s) injected directly into freshly irradiated tumour tissue in order to provoke an immune response. Treatment with 30 mg/kg cyclophosphamide before injecting the DC`s in a weekly schedule resulted in improved tumour control in comparison to DC`s alone [39].

A similar approach was applied by Tongu et al., and instead of irradiation, they used doxorubicin in order to improve the presentation of tumour antigens to dendritic cells in a mouse model, BALB/c mice with two tumour lesions induced by CT-26 cell lines. Low-dose cyclophosphamide administration after injection of doxorubicin in one of the tumour resulted in growth suppression of the remote tumour associated with an increase in interferon-γ, tumour necrosis factor (TNF)-α mRNA expression and a decrease in Foxp3 [40].

Besides the T_{reg}s, cyclophosphamide has also effects on other cells of the immune system. According to investigations of Salem et al. [41] in a mouse model, cyclophosphamide can enhance the relative number and activation status of myeloid dendritic cells. This activation is not a secondary effect of T_{reg} depletion, as the number of dendritic cells increased in bone marrow before their expansion preferentially in the spleen and liver after injection of cyclophosphamide [42].

More than 15 years ago, it was shown that cyclophosphamide also induces the release of an immature myeloid cell fraction characterised by the surface markers CD11, Ly-6G (also termed Gr-1) and CD31 [43]. These cells were later termed myeloid-derived suppressor cells (MDSCs). MDSCs display immunosuppressive properties like deactivating dendritic and reactive T cells and activation of T_{reg}s. Gemcitabine is able to selectively eliminate MDSCs in animal models [44]. Consequently, the combination of low-dose cyclophosphamide and gemcitabine caused tumour regression in a mouse model associated with a reduction in the number of both T_{reg}s and MDSCs [45], (Fig. 2) .

Clinical results

In patients suffering from condylomata acuminata, a disease caused by human papilloma virus (HPV), treatment with cyclophosphamide prevented recurrence after destruction of the warts by laser therapy. Analysis of peripheral blood showed a dramatic decrease in T_{reg}s after treatment with 50 mg cyclophosphamide daily for 1 week. T_{reg}s appear to be attracted by chemokines produced in the wart by HPV-infected cells and thus reduce immune response to HPV [46]. While relapsed patients could be resolved by treatment with another week of daily cyclophosphamide, a higher dose of 200 mg cyclophosphamide showed almost no effect. Although not conducted in cancer patients, this example demonstrated that T_{reg} suppression by cyclophosphamide can translate into clinical benefit for patients in situations with very limited therapy options.

Ge et al. treated 12 patients with metastatic breast cancer with daily 50 mg cyclo-phosphamide for 3 months and observed the number of different T cell types in peripheral blood. The lowest number of T_{reg}s was found after 14 days of treatment. Subsequently, the number of T_{reg}s gradually increased and reached pretreatment numbers at day 56 [47]. This finding clearly demonstrates that continuous administration of cyclophosphamide (metronomic therapy) causes only a transient depletion of T_{reg}s. Accordingly, Ellebach et al. [48] found no decrease in T_{reg}s during treatment with metronomic cyclophosphamide in patients with metastatic melanoma receiving dendritic cell vaccination and interleukin 2.

In a study of cyclophosphamide with the renal cancer cell vaccine IMA901, 68 patients were randomized to receive a single dose of cyclophosphamide 300 mg/m^2 3 days before the vaccine and G-CSF, or only the vaccine. Patients receiving cyclophosphamide showed a trend to prolonged survival. In the subgroup of immune responders, the difference was statistically significant in favour of the cyclophosphamide group [49]. Possibly, the response rate would be higher with a lower dose of cyclophosphamide.

In both animals and patients, it was shown that cyclophosphamide also induces differentiation of pro-inflammatory Th17 T cells. Formation of these cells was not induced by conversion from T_{reg}s but rather by differentiation from the $CD4^+$ T cell pool [50].

A randomized trial of peptide vaccination with or without daily 50 mg cyclophosphamide for 49 days underlined the effect of the daily metronomic schedule on the induction of MDSCs. While T_{reg}s slightly decreased during treatment in that study, the number of MDSCs increased [51]. MDSCs might compensate the positive effects of T_{reg} depletion. As a consequence, no clinical benefit of the treatment with cyclophosphamide was observed. This result underlines the findings that the daily metronomic schedule is not useful for immune reactivation.

Chemotherapy with doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 every 2 weeks results in an increase in the number of MDSCs in peripheral blood [52]. Of note, the increase in MDSCs was not pronounced with

paclitaxel 175 mg/m^2 in that study. All patients received pegfilgrastim on day 2 of chemotherapy in an attempt to reconstitute immune response.

Besides immunreactivation, cyclophosphamide can also be used to induce tolerance in patients receiving haploidentical bone marrow transplantation (BMT). After BMT, patients are at high risk of developing graft versus host disease (GvHD) due to the alloreactivity of T cells. Low-dose cyclophosphamide can be used in a narrow time window on day 3 and 4 post-transplantation in order to attenuate the activity of T cells [53].

Proposed mechanisms of immune reactivation by cyclophosphamide

ALDH1

As mentioned above, ALDH1A1 is the enzyme mainly responsible for the detoxification of 4-hydroxy-cyclophosphamide, the active metabolite of cyclophosphamide, and ALDH1A1 is poorly expressed in lymphocytes [20, 21]. In contrast, stem cells show high activity of ALDH1A1. Therefore, they can tolerate higher concentrations of activated cyclophosphamide. 4-Perhydroxy-cyclophosphamide and mafosfamide are derivatives which hydrolyse non-enzymatically to 4-hydroxy-cyclophosphamide under physiological conditions. They can be used for ex vivo bone marrow purging in transplantation, because in contrast to residual malignant cells, stem cells are protected by high ALDH1 activity [54].

Kanakry et al. investigated the expression of ALDH1 after treatment with mafosfamide in the in vitro model of mixed lymphocyte reaction as well as in patients after bone marrow transplantation. They found an upregulation of ALDH1 in T_{reg}s at day 7 after administering mafosfamide [55]. In the same investigation, similar observations were found in patients after bone marrow transplantation with cyclophosphamide conditioning. These results are somewhat contradictory to other findings mentioned above where it was demonstrated that high-dose cyclophosphamide can induce tolerance in patients after BMT [53]. However, immune tolerance does not require T_{reg} upregulation for immunosuppression but may be mediated by MDSC upregulation.

Taken together, it appears that differential ALDH1 expression in certain T cell subpopulation cannot sufficiently explain the effects of low-dose cyclophosphamide on immune response to solid tumours.

IDO

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in a cascade responsible for the conversion of tryptophan to kynurenine. Metabolites of tryptophan and/or tryptophan depletion result in immunosuppression by inhibiting antigen-specific T cell proliferation [56] and conversion of cytotoxic T cells to T_{reg}s. Clinical results in leukaemia and solid tumours demonstrate that kynurenine concentrations in plasma or tumour tissue are of prognostic value, although the relationship is not clear-cut [57, 58]. Therefore, inhibition of IDO is believed to be promising strategy applied in clinical research to overcome immunosuppression in advanced cancer [59]. Many clinical trials are ongoing with IDO inhibitors such as 1 Methyl-L-tryptophan (Indoximod, NLG 8189) [60]. There are patents pending on the use of single-dose oxazaphosphorines (cyclophosphamide or trofosfamide) as inhibitors of IDO. The authors claim that the IDO inhibition is not due to the active metabolites but rather the drugs itself [61]. Other researchers state that in vitro, cyclophosphamide in combination with fludarabine downregulates IDO expression, but not cyclophosphamide alone [62]. However, the authors used mafosfamide instead of cyclo-phosphamide in these cell culture experiments with the lymphoma cell line Jeko-1. This is contradictory to the patent application of Niemeyer and Pohl stating that cyclophosphamide itself and not its active metabolites inhibits IDO [61]. In summary, it appears possible that the effect of cyclophosphamide on T_{reg}s can be explained, at least in part, by direct IDO inhibition, but other mechanisms must also be involved.

ABCB1

Dimeloe et al. [63] recently proposed another mechanism for the effects of cyclophosphamide: they found that T_{reg}s lack the ABCB1 transporter. ABCB1 transports cytotoxic drugs such as doxorubicin, taxanes, etoposide or vinca alkaloids out of the cells [64]. Dimeloe et al. suggest that the lack of ABCB1 is the reason for the selective effect of cyclophosphamide on T_{reg}s. The authors observed a slight increase in toxicity when adding the ABCB1 inhibitor verapamil to cyclophosphamide and mafosfamide in $CD4^+$ T cells. This increase in toxicity was not observed with T_{reg}s.

However, there is only little evidence that cyclophosphamide or its active metabolite is substrate of ABCB1. In an extensive review by Zhang et al. [65], the authors conclude that active transport of cyclophosphamide and its active metabolites cannot be excluded, and ABCB1 plays only a small role in the transport of oxazaphosphorines.

Dimeloe et al. used both cyclophosphamide and mafosfamide for their experiments. The latter substance is more adequate given the negligible activity of P450 (necessary for the bioactivation of cyclophosphamide) in this setting and effects of cyclophosphamide are of very limited value given the low toxicity in comparison with mafosfamide.

If cyclophosphamide or its active metabolites would be substrates of ABCB1, similar effects with other cytotoxic drugs who are substrates of ABCB1 are expected. In fact, in a mouse model testing adoptive T cell transfer, treatment with single low-dose doxorubicin or paclitaxel resulted in reduced numbers of T_{reg}s. The authors explain the effects not via ABCB1. Instead, they argue that low-dose doxorubicin or paclitaxel may reduce the expression of the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) [66].

ATP

Zhao et al. [67] suggests that the depletion of T_{reg}s is due to the reduction in intracellular ATP. Increased glutathione synthesis, increased cyclic AMP formation as well as CD30 and CD73 catalysing the cleavage of ATP to adenosine may result in a high ATP consumption leading to a decrease in glutathione. High cytosolic glutathione levels are required for the detoxification of the active cyclophosphamide metabolites. The authors found very low levels of ATP in T_{reg}s compared to other T cells. Therefore, T_{reg}s may be depleted at concentrations of cyclophosphamide metabolites tolerable for other T cells.

Microbiome

Another interesting aspect of immunomodulation by cyclophosphamide is the effect on the intestinal microbiome. Some gram-positive bacteria cross the intestinal barrier after administration of cyclophosphamide and induce an inflammatory immune response. This non-specific immune activation contributes to the tumour control by the immune system [68]. There is increasing evidence that the tumour microenvironment is strongly influenced by the composition of the microbiome in the gut [69].

In a mouse model, cyclophosphamide induces the recruitment and accumulation of $CD3^+$ T cells, especially Th1 and Th17 helper cells. Bacterial translocation of gram-positive species such as *Lactobacillus*, *Enterococcus* and *Clostridium* after treatment with cyclophosphamide of tumour-bearing mice could be observed. In the same experimental setup, pre-treatment of mice with vancomycin, an antibiotic selectively killing gram-positive bacteria, substantially reduces the immuno-modulatory and anti-tumour effects of cyclophosphamide [70]. These very exciting results await confirmation in clinical studies.

Proliferation rate

The simplest explanation of the selectivity of cyclophosphamide for T_{reg}s is the proliferative nature of these cells in advanced tumours [71]. Cytostatic drugs like cyclophosphamide exert their effects on highly proliferating cells only. T_{reg}s depleted by cyclophosphamide are characterised by high expression of Ki-67 indicating a high proliferation rate. In a mouse model of mesothelioma, the T cell fraction preferentially depleted by cyclophosphamide was characterised by high expression of CD4, CD25, tumour necrosis factor (TNF) receptor 2, foxp3 and inducible costimulator (ICOS) [72]. The latter protein indicates a highly suppressive fraction of T cells [73].

Kanakry et al. [74] also suggested that in advanced cancer, T_{reg}s are the most proliferative cells of the T cell fraction and are therefore most sensitive to cyclophosphamide. In contrast, by allogeneic stimulation, conventional T cells proliferate faster and may be more susceptible to cyclophosphamide, whereas T_{reg}s are less sensitive in this situation. However, all these effect are observed with high concentrations of cyclophosphamide (or mafosfamide in the in vitro experiments) and may not be applicable for low-dose schedules.

Discussion

More than 50 years after the introduction of cyclophosphamide into cancer therapy, there is increasing evidence that the paradigm of the maximal tolerated dose as the maximal effective dose is not correct. Indeed, intelligent selection of the schedule of administration in the treatment with older drugs may offer new perspectives, even for the treatment of advanced cancer where immunosuppression is critical.

It is obvious that the distinction of so-called targeted therapies like tyrosine kinase inhibitors versus non-targeted cytotoxic drugs like cyclophosphamide is not meaningful as both classes of drugs display both target and off-target effects. Intelligent protocols will use any drug in an optimal dose and schedule in order to utilise their effect not only on cancer cells, but also on all other cells interfering with the tumour.

Taken together, single low-dose cyclophosphamide (i.e. 25–100 mg in adults), not metronomic cyclophosphamide is an option for restoring immune response in patients with advanced cancer. An interval of about 10 days appears to be sufficient to restore immune reaction.

It must be kept in mind that an important interplay exists between the immune system and cancer chemotherapy, not only by growth inhibition of different cell fractions of the immune system, but also by immunostimulation through apoptosis of the cancer cells [75]. In addition, one has to be aware that cytotoxic therapy can trigger immunoresponse to cancer in that.

(a) Killing cancer cells result in the release of damage-associated molecular patterns (DAMPs) attracting myeloid and lymphoid cells and

(b) "Resetting" the immune system by destroying most of the leucocytes. This destruction leads to increased production of new immunocompetent cells afterwards. These newly formed immunoreactive cells may have an improved recognition of the cancer cells due to the release of specific proteins by apoptotic cancer cells [76].

As an example for this, it was shown that anthracyclines can promote tumour antigen presentation by dendritic cells through increased expression of calreticulin and the high -obility group box 1 (HMBG1) from cancer cells [77]. This finding underlines that the concept of cytotoxic chemotherapy being only immunosuppressive is not valid anymore.

Combination of cyclophosphamide with cancer vaccine strategies appears a promising approach [78]. At the moment, it is unclear whether the effect can be maintained with repeated administrations in a weekly or every second week schedule. Future studies should focus on such low and single-dose schedules.

Concluding remarks

The mechanism of immune reactivation by cyclophosphamide is not fully understood. So far, there is no convincing hypothesis explaining the effects of cyclophosphamide on different T cell subpopulations. Investigations addressing the mechanism of T_{reg} depletion are inconclusive and do not provide a clear explanation for the effects observed. One reason for this knowledge gap is that many in vitro studies lack a proper design of the experiments, for example the choice of the test substance and the applied amount of drug. Cyclophosphamide requires bioactivation by P450 enzymes. In cell culture without P450-containing cells, derivatives spontaneously forming 4-hydroxy-cyclophosphamide like 4-perhydroxy-cyclophosphamide- or mafosfamide must be used to reflect the situation in vivo.

Another problem is the claim in the literature stating that transport by ABCB1 out of cells substantially contributes to the detoxification of cyclophosphamide and/or its active metabolites [63]. In vitro experiments do not provide clear evidence for the involvement of ABCB1 in the efflux of cyclophosphamide or its metabolites [79, 80] In fact, there are no investigations measuring the transport of cyclophosphamide and its metabolites by ABCB1. All the investigations on cyclophosphamide and ABCB1 rely on indirect evidence such as increased toxicity of cyclophosphamide in combination with ABCB1 inhibitors [81]. Pharmacokinetic/genetic studies could not show an influence on ABCB1 polymorphism on the pharmacokinetics of cyclophosphamide [82, 83].

A high proliferation rate of T_{reg}s in advanced cancer, low expression of ALDH1 in T_{reg}s, inhibition of IDO, ATP depletion and interactions with the microbiome appear to play a role in the selective depletion of T_{reg}s by cyclophosphamide. For the development of treatment protocols, the effects of MDSCs must be kept in mind which may reduce the immune reactivation by cyclophosphamide. Sequential or combination therapy with drugs like gemcitabine or 5-FU able to supress MDSCs and/or anthracyclines to activate dendritic cells appear to be a meaningful approach. The optimal dose may need to be individualised based on drug concentrations and monitoring of the immune response.

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