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REVIEW

IMMUNOMODULATORY EFFECTS OF LOW DOSE CHEMOTHERAPY AND PERSPECTIVES OF COMBINATION WITH IMMUNOTHERAPY

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IMMUNOMODULATORY EFFECTS OF LOW DOSE CHEMOTHERAPY AND PERSPECTIVES OF COMBINATION WITH IMMUNOTHERAPY

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Abstract

Nowadays cancer is one of the main causes of death and many efforts worldwide have been driven to find out new treatments and approaches in order to extinguish or reduce this group of disorder. Chemotherapy is the main treatment for cancer, however, conventional schedule based on maximum tolerated dose (MTD) show several side effects and frequently allow the development of drug resistance. In this review we present the evidence that metronomic chemotherapy, based on the frequent administration of low or intermediate doses of chemotherapeutics is as efficient as MTD and works better in some situations. Finally, we present some data indicating that noncytotoxic concentrations of antineoplastic agents are able to both up-regulate the immune system and increase the susceptibility of tumor cells to cytotoxic T lymphocytes. Taken together, data from the literature provide us the evidence that low concentrations of selected chemotherapeutics agents, rather than conventional high doses, should be chosen for combination with immunotherapy.

1. Introduction

Cancer was the third main cause of death worldwide, just after cardiovascular diseases and
infectious and parasitic diseases [1]. About 12.7 million new cases were reported in 2008 and
7.6 million deaths [2]. Conventional treatment of cancer includes surgery, chemotherapy and
radiotherapy, which can be used alone or in combination with each other.

Chemotherapy is the main kind of treatment being administrated both in cases of
inoperable diseases and as adjuvant treatment following surgery. In fact, more than half of all
people diagnosed with cancer receive chemotherapy as neo-adjuvant or adjuvant modality for
preoperative or postoperative treatment, respectively [3]. In the present review we report the
effects of different antineoplastic chemotherapeutics agents on the immune system, emphasizing
the immunomodulatory effects of moderate and low concentrations of drugs.

2. Conventional chemotherapy

Conventional chemotherapy is based on the maximum tolerated dose (MTD), which correspond
to the highest dose of a drug with tolerable side effects. This strategy has being shown to be
effective to cure or control disease in a great number of patients but it is associated with
significant short-term and long-term toxicity and side effects, including myelosuppression,
neutropenia, trombocytopenia, increased risk of infection and bleeding, gastrointestinal
dysfunctions, arthralgia, liver toxicity and the cardiac and nervous system damage [3-5].
Chemotherapy can also be associated with lymphodepletion as well as it modifies DCs function
and feasibility [6]. Despite an initially impressive tumor regression or even remission, regrowth

or recurrence is quite common in metastatic cancer [7]. As a consequence, it is estimated that 45% of cancer patients eventually succumb to their disease [8].

Calculation of MTD is done by the body surface area-dosing (BSA) method, which does not take into account the complex processes of cytotoxic drug elimination. Such calculation can lead to an unpredictable variation in the therapeutic effect, due to overdosing or underdosing. Overdosing is easily recognized due to the increased toxicity, but it is possible that unrecognized underdosing is more common and may occur in 30% or more of patients receiving standard regimen. Consequently, those patients who are inadvertently underdosed are at risk of a significantly reduced anticancer effect of the medication [9]. For instance, it was observed that breast cancer patients under adjuvant BSA-based dosing therapy with cyclophosphamide, doxorubicin and 5-fluorouracil (CAF) may show almost a 20% relative reduction in survival [10-11]. It is possible that a large proportion of these patients where underdosed, which may have facilitate the development of drug-resistance.

Antineoplastic chemotherapy agents work on highly proliferating tumor cells. However, proliferation of immune cells might be also affected by a variety of cytotoxic drugs and suppression of the immune response by conventional high-dose chemotherapy may support tumor escape, allowing the proliferation of chemoresistant variants of tumor cells [6, 12]. Based on this, oncologists have a great interest in minimizing toxicity while increasing drug efficacy [13], suggesting different approaches. Decreasing the dose of chemotherapeutics has been suggested as an alternative approach, which might decrease the disadvantages of conventional cytotoxic chemotherapy [6, 12].

3. Metronomic chemotherapy

The alternative schedule for chemotherapy is called low-dose metronomic therapy (LDM), which uses much lower doses of chemotherapeutics. The difference between this approach and simply underdosing is that the former involves more frequent administration [14-16] in order to maintain the serum levels of antineoplastic activity, and to avoid myelosuppression and other side effects. It has attracted attention as it may reduce the risk of drug resistance development while reducing the toxic effects [17]. Metronomic schedules are used for up to 3 years with significant success and attempts to shorten these maintenance regimens result in loss of efficacy [18].

Interestingly, looking back at the history of pediatric cancer therapy, several successful approaches could well be called "metronomic chemotherapy" but instead are known as "maintenance therapy" [13]. For instance, pediatric oncologists have shown that daily mercaptopurine and weekly methotrexate is a successful combination in leukemia [6].

Moreover, LDM has anti-angiogenic effects [19-20], which can be enhanced when combined with anti-angiogenic agents [21-24]. Recent studies comparing the efficacy of chemotherapy at high and low doses in combination with anti-angiogenic drugs documented the longest survival in animals that received low-dose chemotherapy and anti-angiogenic treatment. This was associated with no side effects and a visible decrease in tumor size [21, 25-26]. MTD and LDM chemotherapy have opposite effects on the mobilization and viability of circulating endothelial precursors (CEP) cells, which are interesting candidates for the treatment of ischemic diseases and for tumor targeting [27]. Nowadays cyclophosphamide (CYC) is clinically the most advanced "metronomic" drug [28] and it was observed that high-dose CYC mobilizes viable CEPs, whereas LDM CYC does not mobilize CEPs and, moreover, increases the frequency of

apoptotic CEPs. These cells may probably make a significant contribution to the growth of lymphoma models that were studied under the effect of this drug [29].

The combination of weekly vincristine and low-dose daily CYC is effective in neuroblastoma [30]. LDM vinblastine or CYC and celecoxib in heavily pretreated patients contributed with an impressive duration of stable disease with no toxicity [21]. CYC LDM and celecoxib in refractory high-grade lymphoma are efficient [31] and the combination of low-dose CYC with weekly vinblastine and rofecoxib is effective in a subset of Hodgkin's disease patients [32]. Garcia and colleagues have recently reported remarkable findings in their interim analysis of a phase II study of low-dose CYC and bevacuzimab in recurrent, refractory ovarian cancer [33]. Low-dose CYC has also been used with low-dose methotrexate in the treatment of early breast cancer and it has shown to be effective, through a possible angiogenesis inhibition caused by a decrease in the vascular endothelial growth factor [34]. *In vitro* results show the anti-angiogenic properties of LDM docetaxel, while *in vivo* this schedule is effective against gastric tumor and microvessel growth without toxic effect on nude mice [35]. Moderate doses of CYC works via the elimination of regulatory T cells (Treg) [36-38] whereas gemcitabine attenuates the tumor-suppressive environment by eliminating myeloid-derived suppressor cells [39-40].

Another strong candidate for LDM therapy is the drug paclitaxel (PAC) given its *in vitro* ability to inhibit endothelial cell functions relevant to angiogenesis at extraordinarily low concentrations and its broad-spectrum antitumor activity [41]. PAC LDM has a stronger anti-angiogenic and anti-lymphangiogenic activity than MTD for breast tumor/metastases and induced fewer toxic effects. Indeed, PAC LDM shows less degree of body weight loss and reduction of white blood cells count than MTD. However, MTD is more effective to induce tumor apoptosis, and there is a significant difference between these two schedules concerning the

apoptosis index of tumors treated with the two therapies [42]. Given the fact that breast cancers spread predominantly via the lymphatic system [43], the anti-lymphangiogenic activity of PAC LDM is a feature of this drug that could be of great value in the treatment of this type of disease. Moreover, this reduced toxicity makes it possible to use LDM as an adjuvant therapy for prolonged periods for advanced metastatic breast cancer [44]. Yet, the benefits of LDM and anti-angiogenic therapies are in general limited by acquired resistance [45]. LDM-based treatment regimens might lead to selection of hypoxia-resistant tumor cell populations as a mechanism of resistance [46].

4. Immunomodulatory effects of low noncytotoxic concentrations of chemotherapeutics

It was observed that ultra-low noncytotoxic concentration of selected antineoplastic agents is able to modulate the immune system. Recent findings suggest that some chemotherapeutic drugs can improve the efficacy of immunotherapy by different means and many efforts have been directed to the development of immunotherapeutic approaches, allowing the immune system to be able to specifically respond against cancer [6, 47].

Dendritic cells (DCs), the main antigen presenting cells (APCs) of the immune system, can also be influenced by chemotherapeutic agents. The dying tumor cells can release "danger" signals (damage-associated molecular pattern, DAMP) that lead to the activation of DCs and may facilitate tumor antigens engulfing and processing [48-49].

Expression of CD40 on DCs is essential for their interaction with T lymphocytes and development of efficient Th1 responses [50]. Because expression of CD40 on DCs, as well as CD40-mediated DC function are suppressed during tumor progression [51], its up-regulation by

nontoxic chemotherapy should support the development of antitumor immunity in tumor-bearing hosts. In addition, CD40 ligation protects human and murine DCs from tumor-induced apoptosis by inducing expression of anti-apoptotic proteins from the Bcl-2 family [52-54].

The molecular pathways of certain "immunogenic" forms of cancer cell death that cause DC activation have recently been defined. In this aspect it was reported that anthracyclins (idarubicin, doxorubicin and mitoxantrone), that are DNA-damaging compounds, have been shown to induce the translocation of calreticulin to the surface of dying tumor cells promoting phagocytosis by DCs. The surface exposure of calreticulin is an event that occurs only in immunogenic death and it has shown to highly contribute to the immunogenic tumor cell death, making calreticulin an important mediator of the tumor cells uptake by DCs [55]. Some "danger" signals, as the high-mobility group box 1 (HMGB1) alarmin protein, are secreted by dying tumor cells, which interact with toll-like receptor 4 (TLR4) expressed by DCs. During chemotherapy or radiotherapy, DCs require signaling through TLR4 for efficient processing and cross-presentation of antigen from dying tumor cells, then activating specific T-cell immunity [56]. In addition, many chemotherapeutic agents, as CYC, induce production of uric acid caused by tumor cell death, which may activate DCs, thereby promoting tumor rejection [57]. Moreover, the ability of vinblastine-treated mature DCs to induce CD8+ T cell responses is higher, when compared with untreated DCs [58].

DCs have different sensitivities to conventional cytotoxic agents. In this aspect it was reported that PAC can up-regulate DC function *in vitro* [59] and so can other chemotherapeutic agents. Studies from our group showed that low noncytotoxic concentration of doxorubicin, methotrexate, mitomycin C and PAC directly up-regulate the ability of DCs to present antigens

to Ag-specific T cells *in vitro*. These drugs significantly up-regulate expression of CD80, CD86, CD40 and MHC class II molecules on DCs [60].

In fact, under such conditions, chemotherapeutic agents up-regulate APC function of DCs. Pretreatment of these APCs with doxorubicin, methotrexate, mitomycin C, PAC, vinblastine and vincristine displays an increased potential to present ovalbumin (OVA) to Ag-specific CD8+ T cells. OVA is a known model of antigen used due to its well characterized epitopes [60]. In addition, by analyzing the effect of low concentrations of chemotherapeutics on the expression of antigen processing machinery (APM) components, it has been determined that increased Ag-presenting abilities of chemotherapy-treated DCs is associated with the up-regulation of MHC class I APM components, including immunoproteasome, constitutive proteasome and chaperone proteins [60].

Furthermore, IL-12 might also play an important role in this phenomenon as a signal 3 molecule [61-62] and, indeed, doxorubicin, methotrexate, PAC and vinblastine increase spontaneous IL-12 expression in treated DCs [60]. In fact, in a murine model, the increased DC's ability to present antigens to Ag-specific T cells after treatment with such drugs is abolished in DCs generated from IL-12 knockout mice. It indicates that up-regulation of this phenomenon is IL-12-dependent and mediated by the autocrine or paracrine mechanisms. At the same time, IL-12 knockout and wild type DCs demonstrate similar capacity to up-regulate antigen presentation after their pretreatment with low concentrations of mitomycin C and vincristine, suggesting that these agents do not utilize IL-12-mediated pathways in DCs for stimulating antigen presentation [60]. Comparative analysis of these results suggests that these drugs utilize different chemomodulatory mechanisms to stimulate antigen presentation by DCs, as previously commented [60].

There is a direct evidence that the effect of doxorubicin, methotrexate and PAC in low noncytotoxic concentrations stimulated a strong DC activity [60], directly up-regulating phenotypic maturation of human DCs *in vitro* as demonstrated by the increased MLR function [63], while mitomycin C, vinblastine and vincristine show moderate to low effects on DCs [60].

Interestingly, mitomycin C, when used in concentrations that are significantly high (up to 6.0 μM) induce the generation of tolerogenic DCs, which express low levels of CD80 and CD86 and display low activity in the MLR assay [64]. Several other agents stimulate antigen-presenting function of DCs in the MLR assay. DCs treated with 5-aza-2-deoxycytidine (10 nM), methotrexate (5 nM) and mitomycin C (50 nM) show increased potential to stimulate T cell proliferation [63].

Increased expression of CD40 molecules on DCs treated with methotrexate and mitomycin C is in agreement with their increased ability to stimulate T cell proliferation in the MLR assay. However, this correlation was not seen for other tested drugs, suggesting the importance of other mechanisms involved in up-regulation of antigen-presenting function of DCs by chemomodulation [63].

Besides the use of LDM, other different combinations of therapies have been considered as there might be other alternatives to overcome tumor resistance to conventional treatments. For instance, rationale for combining chemotherapy with immunotherapeutic strategies has been widely discussed and a few reports provide evidence that combining cytotoxic agents with immunostimulation may result in low tumorigenicity and higher immunogenicity when compared with either therapy alone [6, 47, 65-67].

4.1. Paclitaxel

In a murine lung carcinoma model the combination of low-dose PAC followed by intratumoral injection of DC vaccine is much more efficient for the treatment than either DC vaccine or PAC alone in terms of the inhibition of tumor growth, tumor infiltration by CD4+ and CD8+ T cell and induction of a tumor-specific immune response in regional lymph nodes. In addition, the chosen concentration of PAC is not toxic to bone marrow cells and even stimulated DC maturation and function. Furthermore, tumor-induced inhibition of DC maturation and motility was reversed by pretreatment of tumor cells with low-dose PAC. The observation that PAC stimulates DCs and protects them from tumor-mediated inhibition supports the design of intratumoral administration of DCs after pretreatment of tumor-bearing mice with LDM [59]. In the breast tumor model, PAC alone has very poor antitumor activity; but chemotherapy, combined with DC vaccines, also results in a substantial delay in tumor progression [68].

The incubation of tumor cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) induces surface modification in these cells [69]. GM-CSF has a variety of effects on the immune response, such as up-regulation of MHC II expression on macrophages, enhancement of DC maturation, stimulation of DC migration and production of a localized inflammatory response [70], being used in many immunotherapy schemes. The use of surface modified tumor cells as antitumor vaccines and its combination with PAC have potential clinical benefit for patients with prostate cancer. As DCs express high level of GM-CSF receptors [71], they take up this cytokine and process the whole tumor cell and its antigens. In a murine model, the vaccine is potent to induce the uptake of tumor antigens by DCs, more than the administration of free GM-CSF in the same concentration. This indicates that the increased expression of GM-CSF on the cell vaccine is critical to the synergetic effect of the combination therapy, enhancing the endocytosis of tumor vaccine by DCs [69].

Low-dose PAC combined with the GM-CSF-surface-modified tumor-cell vaccine is effective to enhance tumor regression when the vaccine is administrated after PAC. This drug mainly enhances the antitumor immune response at the priming time, which suggests that pre-vaccine PAC administration (i.e., at the time of priming) is more potent than post-vaccine PAC. At the lower doses (4 and 20 mg/kg), pre-vaccine administration was superior to post-vaccine administration. However, the sequence of administration depends on the dose of the drug, because at higher doses (40mg/kg) no significant differences are identified between the administration sequences [69].

The administration of the low-dose PAC followed by the vaccine induced a high degree of CD8+ T cell infiltration in tumor tissue, suggesting an induction of tumor-specific immune response. The combination treatment also induced higher number of tumor-specific IFN-γ CD8+ T cells than vaccine alone. Different doses of PAC enhance the antitumor effect by various immunomodulatory mechanisms. Low-dose PAC is potent enough to induce phagocytosis of tumor antigens by DCs and to enhance the maturation of DCs, which cause DCs to prime the antitumor immune response [69].

A decrease in the Treg subpopulation is only induced in high doses of PAC and this was not found when low-dose PAC was administrated. Since the effect of PAC on DCs is dose dependent, high concentrations of PAC obviously induce the apoptosis of DCs, whereas low concentrations not only avoid the apoptosis of DCs but also induce their maturation and cease the inhibition of maturation caused by the tumor. The effect of this type of treatment may be enhanced by selecting the proper sequence for various drug/ vaccine combinations based on the immunomodulatory mechanisms of each treatment [69].

We also reported that PAC is able to increase the immunogenicity of tumor cells and that tumor cells pre-treated with ultra low concentrations of PAC are better targets for tumor-specific cytotoxic T lymphocytes (CTLs). In fact, DCs engulfing tumor cells under such treatment induce CTLs with a higher cytotoxic potential than DCs loaded with non-treated tumor cells and the CTLs induced by tumor lysate-pulsed DCs kill live tumor cells more efficiently if these tumor cells are pretreated with noncytotoxic concentrations (Fig 1) [72].
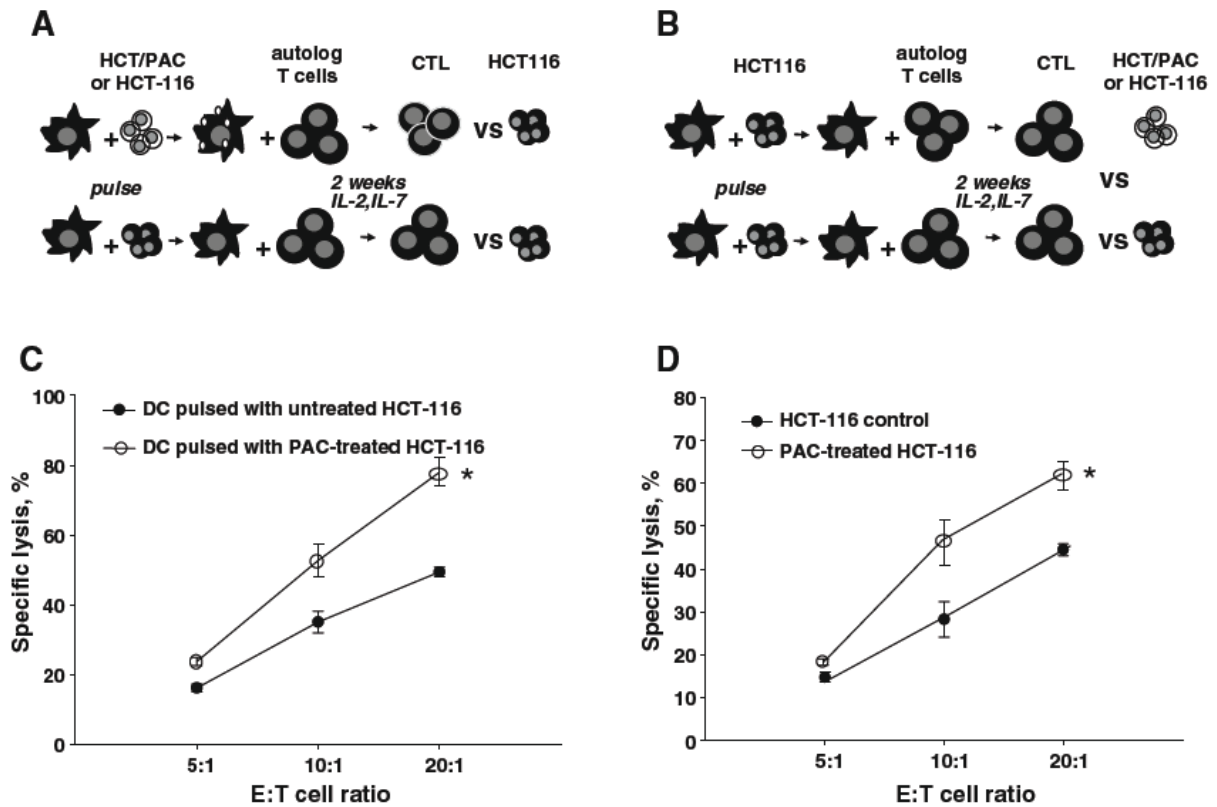

Figure 1: Analysis of cytolytic activity of tumor-specific CTLs induced by DCs loaded with paclitaxel-pretreated tumor cells (A.C) and CTLs targeting paclitaxel-pretreated tumor cells (B,D). A. Human monocyte derived DCs were pulsed with lysates from untreated HCT-116 cells (HCT-116) and HCT-116 cells pre-treated with 500 pM paclitaxel for 48 h (HCT/PAC). Autologous T cells were cultured with IL-2 and IL-7 for 2 weeks and stimulated with tumor antigen loaded DCs. Cytolytic activity of CTLs was tested against untreated HCT-116 target cells in a 4 h cytotoxic assay (C). B. Human monocyte-derived DCs were pulsed with lysates from untreated HCT-116 cells and used for generation of tumor-specific CTLs as above. Cytolytic activity of CTLs was tested against untreated and paclitaxel pre-treated HCT-116 target cells in a 4 h cytotoxic assay (D). The results represent the mean±standard error from three independent experiments. *$p < 0.05$ [73]

4.2. Cyclophosphamide

Recent clinical studies have shown that low-dose CYC also induces beneficial immunomodulatory effects in the immunotherapy context [37, 73]. The prevalence of Tregs increases in tumor bearing mice in the tumor site, peripheral blood and lymph nodes as compared with normal controls, whereas selective depletion of these cells may be accomplished following the administration of a low-dose CYC. The continuous nonfrequent administrations of low-dose CYC at 45-day intervals block the renewal of Tregs in mice with multiple myeloma (MM) and enable the restoration of an efficient immune response against the tumor cells, thereby leading to prolonged survival and prevention of disease recurrence. More frequent administrations of low-dose CYC at 7 or 21 day intervals did not improve the therapeutic effect, since the mice developed MM in a higher incidence. Hence, distinctive time-schedule injections of low-dose CYC are beneficial for breaking immune tolerance against MM tumor cells [74-75]. This effect induced beneficial immunomodulatory effects in the context of DC-based immunotherapy, which leads to an increase in survival that is further improved by depleting Tregs. These results anticipate that antitumor immune responses elicited by DC-based immunotherapy in humans might be improved by simultaneously depleting Tregs using low-dose CYC [76]. Moreover, the combination of DC-based immunotherapy and CYC administration significantly improved survival compared to DC-based immunotherapy or CYC administration alone. Therefore, CYC is a powerful tool to optimize suboptimal DC-based immunotherapy. Although CYC alone also improves survival, the combination of both was shown to be significantly better [76].

Chemotherapy causes the disruption of tumor stroma that allows more CTLs to penetrate into tumor site, so that single agents have short-lived effects [68]. It is generally believed that the

antitumor immunity induced by the combination of chemotherapy and immunotherapy depends on the homeostatic role of immunotherapy acting during the recovery phase that follows the chemotherapy mediated myelo-lymphodepletion [77].

Despite the well-described effects of CYC on Treg elimination, these cells are rapidly reconstituted in both secondary lymphoid tissues and tumor site, what could discourage new approaches in this field. Further attempts to prolong Treg depletion yield no additional benefit [78].

The ability of this drug to reverse the systemic and in situ DC paralysis in the tumor-bearing hosts and its ability to mobilize early proliferating DC progenitors are other crucial mechanisms for enhancing antitumor immunity. In addition, it was observed that CYC administration, through myelosuppression and rebound reconstitution of myelopoiesis, leads to reversal of systemic and in situ tumor-induced DC paralysis. Moreover, CYC mobilized proliferating early DC progenitors to the tumor site where they yielded new DCs [78].

These several findings are relevant for understanding multifaceted mechanisms behind the beneficial effects of CYC on augmenting antitumor immunity, since through its myelosuppressive action, CYC eliminates tumor infiltrating DCs and mobilizes early DC precursors. These precursors cells proliferate rapidly in periphery and increase the presence of new APCs that can prime de novo T cell responses [78].

Thus, CYC has a direct impact on DC-T cell interactions in tumor-bearing host that ultimately lead to a better priming of tumor-specific T cells. These observations increase the notion that mobilization of early DC precursors to sites of ongoing immune response and their differentiation into mature DCs can lead to optimal T cell priming [79-80]. Therefore, CYC can

influence the DC homeostasis both in situ and systemically through its unique ability to mobilize DC precursors [78].

Beneficial effects of CYC in augmenting antitumor immunity are not only a result of drug's ability to eliminate Tregs and/or increase the homeostasis-driven expansion of pre-existing tumor-specific effector T cells [36, 81-82]. CYC also influences antitumor immune responses by resetting the DC homeostasis in tumor-bearing host [78].

A single administration of low-dose CYC (50 mg/kg) in tumor-bearing mice prior to immunization with DCs increased the frequency of IFN-γ secreting antitumor CTLs [37]. This is in agreement with the fact that different chemotherapeutic agents in very low concentrations increase the ability of DCs to induce T cell proliferation [63].

5. Conclusion and perspectives

Low-dose chemotherapy, including the use of noncytotoxic concentrations, metronomic schedule and non-frequent low-dose administration of antineoplastic agents is an alternative for cancer chemotherapy. These different schedules minimize the side effects of conventional MTD, which are the main disadvantage of classical treatment. Up-regulating effects on DCs allow us to suggest that the use of low concentrations of selected chemotherapeutics, rather than high doses, should be used in combination with immunotherapy in order to overcome the immunosuppression induced by cancer.

6. Conflict of interest statement

None declared.

7. Acknowledgements

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