

Erythropoietin and Cyclophosphamide Combination Treatment Additively Enhances Antibody Production in Mice

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Erythropoietin (EPO), a key therapy for several types of anemia, e.g. cancer-related anemia [1], is also known to display non-erythroid effects, such as heart failure improvement and neuroprotection [2]. It should be noted that there are ongoing safety concerns regarding EPO treatment [3]. Although EPO receptor (EPO-R) expression has been reported in various solid tumors, its functionality is still controversial [4].

Among the non-erythroid effects of EPO, accumulating data show that EPO can affect the immune system, including both cellular and humoral responses. We have previously shown that EPO displays anti-neoplastic activity in patients with multiple myeloma [5]. This EPO activity was also manifested in murine multiple myeloma models [6] and was related to stimulatory effects on both humoral and cellular immune responses [7, 8]. In addition, we demonstrated that administration of high doses of EPO [180 U recombinant human EPO (rHuEPO) thrice/week] to dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH)-injected BALB/c mice resulted in an increase in anti-DNP IgG1 production [8]. Thus far, our reports have focused on EPO as the only treatment. However, in the context of clinical treatment for cancer-related anemia, EPO is usually administered along with chemotherapy. In view of the fact that chemotherapy agents may act on the immune system, we decided to de-

termine the possible effects of such combination treatments on the production of anti-DNP antibodies.

Cyclophosphamide (CP) is a known cytotoxic alkylating agent widely used in cancer chemotherapy. While it functions as an immunosuppressive agent at high doses, the anti-neoplastic activities of CP at low doses are attributed to enhancement of cellular and humoral immunity. Augmentation of the immune response following low-dose CP treatment was observed in both murine models and humans injected with various tumor antigens [9]. These effects were attributed to the ability of low-dose CP to specifically inhibit the activity and proliferation of suppressive Th CD25+ cells [10].

Here, we chose to focus on the humoral immunomodulatory effects of lower doses of EPO combined with low-dose CP, thus simulating the routine clinical conditions, yet under experimentally controlled conditions, in the absence of a malignant disease.

The present study was designed to compare serum levels of anti-DNP immunoglobulin in DNP-KLH-injected C57BL/6 mice that were treated with either low doses of EPO or with CP alone, or with the combination of both

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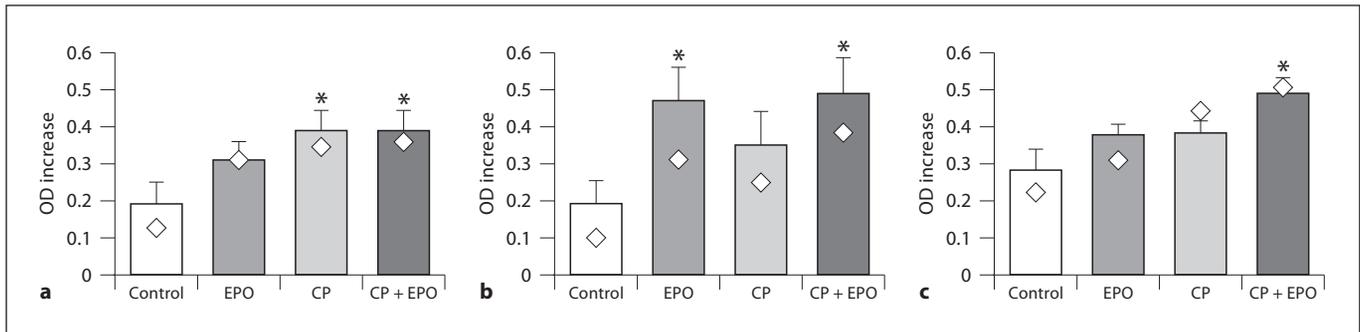


Fig. 1. EPO increases the levels of anti-DNP IgG2a antibodies and additively improves anti-DNP total Ig when administered with CP. **a** IgG1. **b** IgG2a. **c** Total Ig. **a–c** Female C57BL/6 mice, aged 8–12 weeks, were immunized with 10 µg DNP-KLH s.c. as described [8]. Mice were injected three times per week with 90 U rHuEPO (Epoetin-alfa, Eprex®; Janssen-Cilag) in 200 µl PBS or with diluent as a control, for 2 weeks, starting from the 2nd day following antigen injection. Mice received two intraperitoneal injections of 12.5 mg/kg CP (Endoxan®; Baxter Oncology) in 200 µl PBS, or just PBS alone (control) on days –2 and –1 before antigen injection. Blood samples were drawn before and 2 weeks after

immunization. ELISA was used to determine Ig levels in serum samples of pre-immune and immunized mice. Serum dilution (1:5,000) was chosen to ensure OD values for the immune sera in the range of 0.2–1 OD_{450 nm}. The OD values obtained for pre-immune sera of all the mouse groups were the same. Bars represent the mean increase in OD = (OD of immune mice) – (OD pre-immune mice) ± SEM of samples from 3 independent experiments (in total: 10–11 mice in each experimental group). Diamonds represent the median increase in OD values. * *p* < 0.05 vs. control (non-parametric Mann-Whitney test and Student's unpaired two-tailed *t* test).

(CP + EPO). Anti-DNP Ig levels were determined by ELISA, and are represented by the mean optical density (OD) increase ± SEM.

We first assessed anti-DNP Ig production in the DNP-KLH-injected mice that were treated with a lower dose of EPO alone (90 U rHuEPO thrice/week). The results showed a significant increase in anti-DNP IgG2a levels 2 weeks following antigen injection: EPO-OD_{450 nm} = 0.47 ± 0.09 versus control-OD_{450 nm} = 0.18 ± 0.07, *p* = 0.028 (fig. 1b). In addition, following EPO treatment, a slight non-significant increase in anti-DNP IgG1 was observed (fig. 1a). In parallel, mice treated with CP only were also immunized with DNP-KLH. In line with the reports on the ability of CP to enhance Ig production against various antigens [9], treatment with low doses of CP prior to DNP-KLH injection significantly increased anti-DNP IgG1 levels 2 weeks after antigen injection: CP-OD_{450 nm} = 0.38 ± 0.06 versus control-OD_{450 nm} = 0.18 ± 0.06, *p* = 0.036 (fig. 1a). This was accompanied by a non-significant increase in IgG2a (fig. 1b). It should be noted that in both cases of treatment with either EPO or CP alone there was a slight (non-significant) increase in total anti-DNP Ig levels (fig. 1c).

Combined treatment with both EPO and CP in the DNP-KLH-injected mice maintained the effects of each treatment alone on the specific IgG subtype, and showed an increase in both IgG1 and IgG2a levels (CP + EPO-

OD_{450 nm} = 0.38 ± 0.06 and 0.49 ± 0.1, respectively; fig. 1a, b). Moreover, the combined CP + EPO treatment resulted in elevated anti-DNP total Ig compared to the non-treated group: CP + EPO-OD_{450 nm} = 0.48 ± 0.05 versus control-OD_{450 nm} = 0.28 ± 0.07, *p* = 0.022 (fig. 1c). Since this effect was not manifested following treatment with either CP or EPO alone, we infer that the CP-EPO combination treatment results in an additive effect on enhanced Ig production.

By preferentially elevating IgG2a levels, it seems that lower doses of EPO are able to confer a Th1 pattern to the immune response in the DNP-KLH-injected mice. In our previous study, mice exposed to higher doses of EPO (180 U rHuEPO thrice/week), as well as EPO-overexpressing transgenic mice, produced a differential increase in IgG subtypes in response to injection of different antigens. The IgG subtype increase was dependent on the antigen that the mice had received. Mice injected with hepatitis B surface antigen had increased levels of IgG2a, reflecting a Th1 response, while mice injected with DNP-KLH showed increased levels of IgG1, reflecting a Th2 response [8]. We thus propose that the role of EPO in the preferential induction of either a Th1 or a Th2 response may depend not only on the type of antigen involved, but also on the regimen of EPO treatment.

The mechanisms of EPO affecting immune response are still largely unknown, particularly since lymphocytes

do not seem to express EPO-R. In that respect, our search for possible mechanisms led us to the novel discovery that dendritic cells express EPO-R, and that stimulation with EPO enhances their survival and function [11, 12].

In conclusion, we demonstrate that in the context of CP treatment, EPO can enhance humoral immunity in addition to its erythropoietic activities. Our findings emphasize a role for EPO as an immune modulator, particularly when given as combination treatment. Moreover, the specific pattern of the immune response to be elicited depends on several factors, including the target antigen, the type of treatment, its schedule and EPO doses. Our results thus point to the possibility of applying EPO, ei-

ther alone or combined with other agents, in the development of cancer vaccines, as well as in the vaccination of immunocompromised patients.

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