Erythropoietin enhances immune responses in mice

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Erythropoietin (Epo) is the main erythropoietic hormone. Recombinant human Epo
(rHuEpo) is thus used in clinical practice for the treatment of anemia. Accumulating
data reveals that Epo exerts pleiotropic activities. We have previously shown an anti-
neoplastic activity of Epo in murine multiple myeloma (MM) models, and in MM
patients. Our findings that this anti-neoplastic effect operates *via* CD8$^+$ T lymphocytes
led us to hypothesize that Epo possesses a wider range of immunomodulatory functions.
Here we demonstrate the effect of Epo on B lymphocyte responses, focusing on three
experimental models: (i) tumor-bearing mice, (5T2 MM mouse); (ii) antigen-injected
healthy mice; and (iii) antigen-injected transgenic mice (tg6), overexpressing human
Epo. In the MM model, despite bone marrow dysfunction, Epo-treated mice retained
higher levels of endogenous polyclonal immunoglobulins, compared to their untreated
controls. In both Epo-treated wild type and tg6 mice, Epo effect was manifested in the
higher levels of splenocyte proliferative response induced *in vitro* by lipopolysaccharide.
Furthermore, these mice had increased *in vivo* production of anti-dinitrophenyl (DNP)
antibodies following immunization with DNP-keyhole limpet hemocyanin. Epo-treated
mice showed an enhanced immune response also to the clinically relevant hepatitis B
surface antigen. These findings suggest a potential novel use of rHuEpo as an
immunomodulator.

See accompanying commentary: http://dx.doi.org/10.1002/eji.200737401

Introduction

Erythropoietin (Epo), produced mainly in the adult kidney, is the major growth regulator of the erythroid cell lineage. Cloning of the Epo gene has led to the introduction of recombinant human Epo (rHuEpo) into clinical practice as a treatment for various anemias, including anemia related to chronic kidney disease and certain forms of cancer [1, 2]. Detection of the target receptor for Epo (EpoR) in cells other than erythroid progenitors, such as polymorphonuclear leukocytes, megakaryocytes, endothelial, myocardial and neural cells [3–7], suggests that Epo has other biological functions beyond erythropoiesis, and may have further potential therapeutic applications. These effects include improvement in congestive heart failure [8, 9] and neuroprotection [10–17].

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Abbreviations: Epo: erythropoietin · **HBsAg:** hepatitis B surface antigen · **KLH:** keyhole limpet hemocyanin · **MM:** multiple myeloma · **rHuEpo:** Recombinant human Epo · **tg6:** transgenic mice overexpressing HuEpo

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The idea that rHuEpo may have an important effect on the immune system, including both cellular and humoral type responses, derives from several lines of data, as recently reviewed [18]. For instance, clinical observations revealed that treatment with rHuEpo was associated with enhanced antibody production in hemodialysis patients, demonstrated for T cell-dependent antigens, such as tetanus toxoid and hepatitis B [19–22] as well as for the T cell-independent pneumococcal polysaccharide antigen [20]. Others have demonstrated enhancement of basal and mitogen-stimulated immunoglobulin production by cultured peripheral mononuclear cells (MNC) of dialysis patients treated with rHuEpo. Similarly, MNC from healthy subjects secreted higher levels of IgG, IgA and IgM following *in vitro* incubation with Epo [23]. Noteworthy, studies on the effect of Epo on immunological functions in dialysis patients alluded, in some cases, to an increased antibody response to an administered antigen [19, 24], whereas others claimed that there was no statistically significant difference in the level of antibodies detected [25]. For critical elucidation of Epo effects on the various arms of the immune system, it thus appeared important to examine the immune response following *in vivo* arbitrary vaccination in the absence of clinical problems. Our earlier observations in both clinical cases and murine experimental models served as guidelines in the present study.

Our clinical observation on multiple myeloma (MM) patients [26, 27], along with our studies on experimental murine models [28, 29] have implicated Epo as an anti-neoplastic and immunomodulatory agent. The present study was thus designed to delineate the effect of Epo treatment on the humoral immune response, using three different types of experimental models: (i) the 5T2 MM mouse model [30] as a link to our previous observations [28, 29]; (ii) normal BALB/c mice immunized with dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) or hepatitis B surface antigen (HBsAg); and (iii) transgenic mice overexpressing HuEpo (tg6, [31]), immunized with DNP-KLH. Our results demonstrate that rHuEpo treatment significantly augmented B lymphocyte responses in all of these experimental models.

Results

Prolonged survival and increased immunoglobulin levels in rHuEpo-treated 5T2 MM mice

We have previously shown Epo effects on tumor regression in several murine experimental models. As such, treatment of MOPC-315 MM-bearing mice with rHuEpo culminated in regression of the tumors in 30–60% of the mice [28] and in reduction of tumor load even in those mice that eventually died from the disease [29]. Similarly, we found that rHuEpo treatment significantly prolonged the survival of mice bearing another tumor, the 5T33 MM [28]. As we have recently demonstrated [29], rHuEpo-treated MM patients displayed improved cellular immune functions. Here we focused on the effects of Epo on the humoral immune system, using a murine MM model. Since the goal of the present study was to explore the effect of Epo on B cell immune responses, we sought a model whereby production of the pathological monoclonal Ig can be kinetically uncoupled from the production of endogenous polyclonal Ig. We therefore chose the 5T2 MM model, which is less aggressive than the 5T33 model, displaying pathological Ig at a late stage of the disease, and also more closely resembling the natural history of the disease in humans [32]. The slow progression of the disease thus enabled us to specifically monitor the levels of endogenous Ig, at a time when the pathological protein is not detected. First, we raised the basic question as to whether treatment with rHuEpo would prolong the survival of 5T2 MM-bearing mice, as it did in the MM models that we studied previously. At 27 days following injection of 5T2 MM tumor cells (3×10^5, i.v.) the mice were treated with ten daily injections of rHuEpo (s.c.; 30 U/injection), or an equivalent volume of the diluent; followed by three injections per week for an additional period of 3 weeks. The results, summarized in Fig. 1A, show that the median survival in the control group was 75 days, whereas in the rHuEpo-treated group it was 97 days (Kaplan-Meier analysis, $p=0.029$). Thus, rHuEpo treatment significantly prolonged the survival of the tumor-bearing mice.

We next examined the effect of Epo on endogenous Ig production in mice bearing this 5T2 MM tumor. We have previously shown that rHuEpo treatment reduced the level of the pathological monoclonal M protein in the MOPC-315 murine MM model [28]. The fact that the pathological protein in the 5T2 MM model is detected at a very advanced stage of the disease, close to the time of death [32], enabled a study determining whether Epo treatment has any effect on endogenous Ig, at a time before the pathological Igκ becomes detectable [33]. The results show that 6 weeks after tumor challenge, sera from mice treated with rHuEpo for the previous 2 weeks displayed higher levels of endogenous κ light chains and γ2a heavy chains, as determined by Western blot analysis (Fig. 1B). Hence, we consider it possible that the lower levels of endogenous normal Ig in the diluent-injected mice reflected the expansion of MM cells in the bone marrow, which may have been attenuated in the rHuEpo-injected mice.
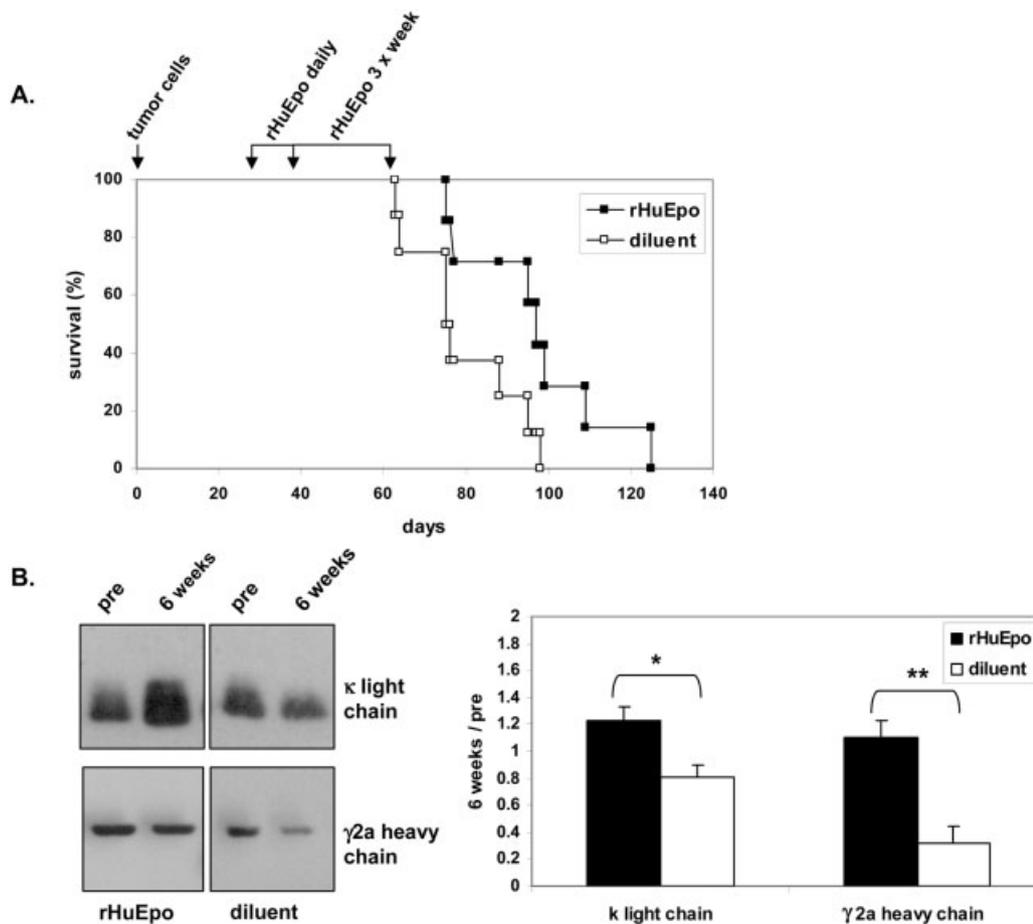
Figure 1. rHuEpo treatment prolongs survival and elevates endogenous immunoglobulins in 5T2 MM mice. Fifteen mice were injected i.v. with 3×10^5 bone marrow cells from a 5T2 MM mouse. At 27 days following 5T2 challenge, seven mice were injected s.c. with 30 U rHuEpo for 10 consecutive days, followed by three injections per week, for 3 additional weeks. The rest of the mice, serving as control, were injected with the diluent using the same schedule of injections. (A). Survival over 130 days is depicted. Median survival was 75 and 97 days for control and rHuEpo-treated mice, respectively; significance of the Kaplan-Meier was 0.029. (B). A representative Western blot analysis of sera from rHuEpo- and diluent-injected control mice, 0 ("pre"), and 6 weeks following 5T2 cell injection; immunoblotted with anti-κ light chain or anti-γ2a heavy chain antibodies. Quantification of Western blots of sera from the seven rHuEpo-treated and eight diluent-treated mice is depicted on the right; *p=0.019, **p=0.002.

Epo-associated shift in splenic lymphocyte levels

We next questioned whether higher levels of Epo are associated with a shift in the lymphoid cell populations of the spleen, under conditions not involving tumors. We therefore determined the expression of the CD4, CD8 and CD19 cell surface markers, reflecting the abundance of T helper, T cytotoxic/suppressor and B cell population of lymphocytes, respectively. As shown in Fig. 2A and B, the levels of CD4$^+$ cells were significantly reduced in association with rHuEpo administration (Fig. 2A), or overexpression of the hormone, in the Epo transgenic mice (tg6), which possess constitutively increased (10–12-fold) Epo plasma levels [31] (Fig. 2B). A slight, yet significant reduction in the levels of CD8$^+$ cells was observed in the rHuEpo-injected mice, and not in the tg6

mice. A similar reduction in CD4$^+$ and CD8$^+$ was also observed in the peripheral blood (data not shown).

rHuEpo–mediated effects on mitogen-induced splenocyte proliferation

The above data raised the question of whether rHuEpo treatment also impacts the *in vitro* mitogen-driven proliferative response of splenic lymphocytes. Anti-CD3-driven proliferation was lower in splenocytes derived from Epo transgenic and from rHuEpo-injected mice (Fig. 2C), as compared to their wild-type (wt) or diluent-injected counterparts. In contrast to the findings on decreased levels of CD4$^+$ and CD8$^+$ T cells, in both rHuEpo-injected and the tg6 mice, the values of CD19$^+$ cells were not affected by the high Epo levels *in vivo*
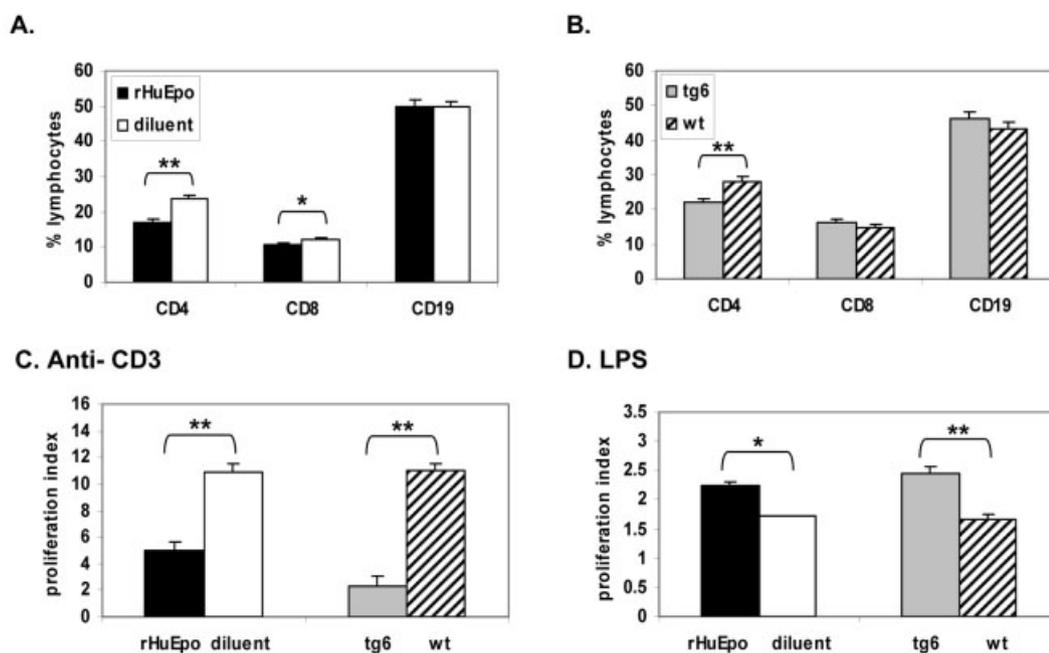
Figure 2. Epo-associated splenocyte phenotype and response to mitogens. (A, B) Flow cytometry analysis of splenocytes for expression of CD4, CD8 and CD19 markers. (A). Splenocytes from rHuEpo-injected BALB/c mice; three to five injections, 30 U rHuEpo (black bars) and diluent-injected controls (white bars), (n=9/group), and (B) from tg6 (gray bars) and their wt C57BL/6 littermates (striped bars) (n=12/group); **p=0.0001 and 0.002 for CD4 decrease in rHuEpo-injected mice and tg6, respectively, *versus* their controls; *p=0.03 for CD8 decrease in rHuEpo-injected mice *versus* their controls. (C, D) Proliferation assay (MTT method) of splenocytes from BALB/c mice injected thrice weekly with 180 U rHuEpo, or with diluent (control), and from tg6 and C57BL/6 wt mice. The proliferation values presented are ratios of mean ± SEM of stimulated *versus* non-stimulated cells, from at least five separate experiments. (C) Cells incubated on anti CD3-coated plates for 4 days. **p<0.005. (D) Stimulation with 2 μg/mL LPS for 4 days *in vitro*. *p< 0.05, **p< 0.001. Basal proliferation values of unstimulated splenocytes were in the range of 0.1 OD.

(Fig. 2A, B). We thus compared *in vitro* LPS-driven proliferation of splenocytes from the rHuEpo-treated mice *versus* the control, diluent-treated mice. Our data demonstrate that splenocytes from rHuEpo-treated mice exhibited increased levels of proliferation induced by LPS (Fig. 2D). Further support for the role of Epo in augmenting B cell functionality was provided by our experiments using the tg6 mice. We correspondingly found that LPS-induced proliferation of splenocytes from the tg6 mice was significantly greater than that of their wt counterparts (Fig. 2D).

rHuEpo increases specific anti-DNP antibody response

To assess whether Epo also augments the immune response to an injected antigen, we chose the model of antibody response to the well-characterized hapten determinant, DNP. To ascertain whether rHuEpo conferred an increase in specific anti-DNP antibody levels, BALB/c mice were injected with 10 μg DNP-KLH. The choice of this antigen dose was based upon our initial calibration, showing that the resulting immune response was in the linear range 2 weeks following injection of DNP-KLH at doses between 1 and 50 μg

(data not shown). The immunized mice were injected with 180 U rHuEpo, three times weekly for 2 weeks, or with the diluent serving as the control. Blood samples were drawn before and 2 weeks after rHuEpo treatment. Measurement of anti-DNP antibodies was based on ELISA. Fig. 3 shows a significant Epo-associated increase in anti-DNP IgG1 antibody levels, which characterize most of the anti-DNP immune response [34]. This increase, compared to that of the diluent-injected control mice, was evident following 2 weeks of rHuEpo injections. No increase was evident after 4 weeks of rHuEpo treatment (data not shown), suggesting that, in the case of this antigen, Epo enhanced the initial stage of antibody response. Immunization of the tg6 mice with DNP-KLH resulted in similar high levels of anti-DNP IgG1 antibodies (Fig. 3). We did not detect any effect of rHuEpo on the IgG2a response to DNP in either murine model (tg6 or Epo-treated BALB/c; data not shown).

rHuEpo increases anti-HBsAg antibody response

To evaluate the clinical significance of these Epo effects on the antibody response, we focused on the response to HBsAg. Twenty BALB/c mice were injected with 200 ng
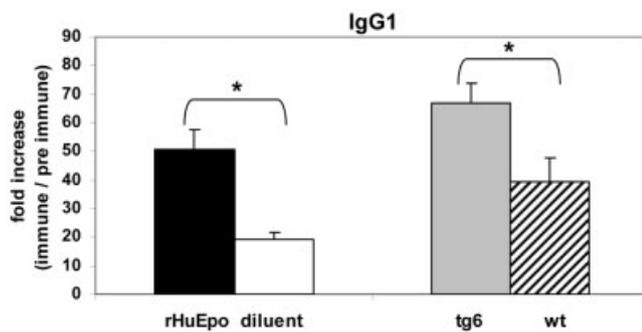
Figure 3. Epo increases the levels of anti-DNP IgG1 antibodies in DNP-KLH injected mice. BALB/c and C57BL/6 mice (tg6 and wt) were immunized s.c. with 10 µg DNP-KLH. The BALB/c mice were injected three times per week with 180 U rHuEpo or with diluent as a control, for 2 weeks, starting on the day of antigen injection. Blood samples were drawn before and 2 weeks following immunization. Results are depicted as the fold increase of IgG1 antibody levels measured using ELISA in sera samples of immunized mice and as related to those measured in the respective pre-immune sera. Sera dilution (1:5000) was chosen to ensure OD values for the immune sera in the range of 0.2–1 OD at 450 nm. The OD values obtained for pre-immune sera of the rHuEpo and diluent-injected mice were the same. Values represent the mean ± SEM of samples from four mice in each experimental group. IgG1 levels in sera of rHuEpo (black bars) and diluent (white bars) injected BALB/c mice, tg6 mice (gray bars) and C57BL/6 wt mice (striped bars). The figure represents results of one experiment out of four, all of which demonstrated similar results. *$p=0.002$ for rHuEpo-treated BALB/c mice *versus* control, *$p=0.047$ for IgG1 of tg6 *versus* wt C57BL/6 mice.

HBsAg, and divided into two groups. Ten mice received thrice weekly rHuEpo injections throughout the experiment, and the other ten served as the diluent-injected control. Blood samples were drawn before rHuEpo treatment, and at 2, 4 and 8 weeks of treatment. As shown in Fig. 4, a significant increase in the amount of anti-HBsAg Ig was observed after 4 and 8 weeks in the rHuEpo-treated mice, by both ELISA and by dot blot analysis (Fig. 4A and D, respectively). The increase observed in anti-HBsAg IgG after 8 weeks of rHuEpo treatment was significant in IgG2a, and not in IgG1 levels (Fig. 4B and C, respectively). These findings thus reflect an activation of the Th1 response, which has been shown to contribute to the successful treatment of hepatitis B [35].

Discussion

The study based on experimental murine models shows that Epo affects both T and B lymphocyte populations. Furthermore, while lending additional support to our previous finding on the anti-MM effects of Epo [26, 27], we now demonstrate that Epo also augments B cell

responses, manifested in endogenous normal polyclonal immunoglobulin production, LPS-induced proliferation of splenocytes, as well as specific antibody response to antigen stimulation (DNP and HBsAg).

The study was driven by our previous demonstration of the anti-neoplastic effects of Epo treatment in human MM patients [26] and in various murine experimental models [28, 29], attributing this activity to Epo-mediated immunomodulation. Moreover, our recent study showing Epo-associated improvement of immune functions in MM patients lends further support to the supposition that Epo acts on the immune system. The working hypothesis underlying the current study was that Epo acts as an immunomodulating hormone, affecting both the cellular and humoral immunological arms. To examine the effects of Epo on B cell responses, we sought an experimental model whereby the effect of Epo on endogenous Ig could be scrutinized without interference from the production of the pathological MM paraprotein. We chose to study the murine 5T2 MM [30], since the pathological Ig appears at a very late stage of the disease in these mice. Our initial important finding was that Epo exerted an anti-tumor effect in this model, as it did in the other models that we have tested previously, thereby further substantiating our original observations pointing to an anti-tumor effect of Epo [28, 29]. The next important finding was that Epo has an effect on endogenous Ig levels in these tumor-bearing mice. We demonstrated that higher levels of endogenous Ig κ light chains in the Epo-treated mice, prior to the time of appearance of the pathological paraprotein [32], was associated with improved survival. This Epo-mediated enhancement of the normal Ig is of particular importance in a disease characterized by bone marrow dysfunction, caused by excessive plasma cell proliferation and secretion of the pathological protein.

Previous reports, mostly regarding dialysis patients, have implied that Epo treatment is associated with improved immunological functions [19, 20, 36, 37]. rHuEpo therapy in these patients was associated with repair of T cell functions [36, 38], probably *via* indirect mechanisms [39], as well as normalization of pro-inflammatory and anti-inflammatory cytokine levels [40, 41]. The discrepancies in data on dialysis patients, showing in some cases increased antibody response to an administered antigen [19, 24], and in others no statistically significant difference in the level of antibodies detected [25] deserve proper attention. This may be explained in light of our current study by the fact that different antigens were used in the various studies, or that the timing of samples analysis varied. In support of the latter, are our current data showing that the augmenting effect of Epo was preferential at the earlier stages of antibody (IgG) generation. However, it should be noted that we did not detect any significant effect of
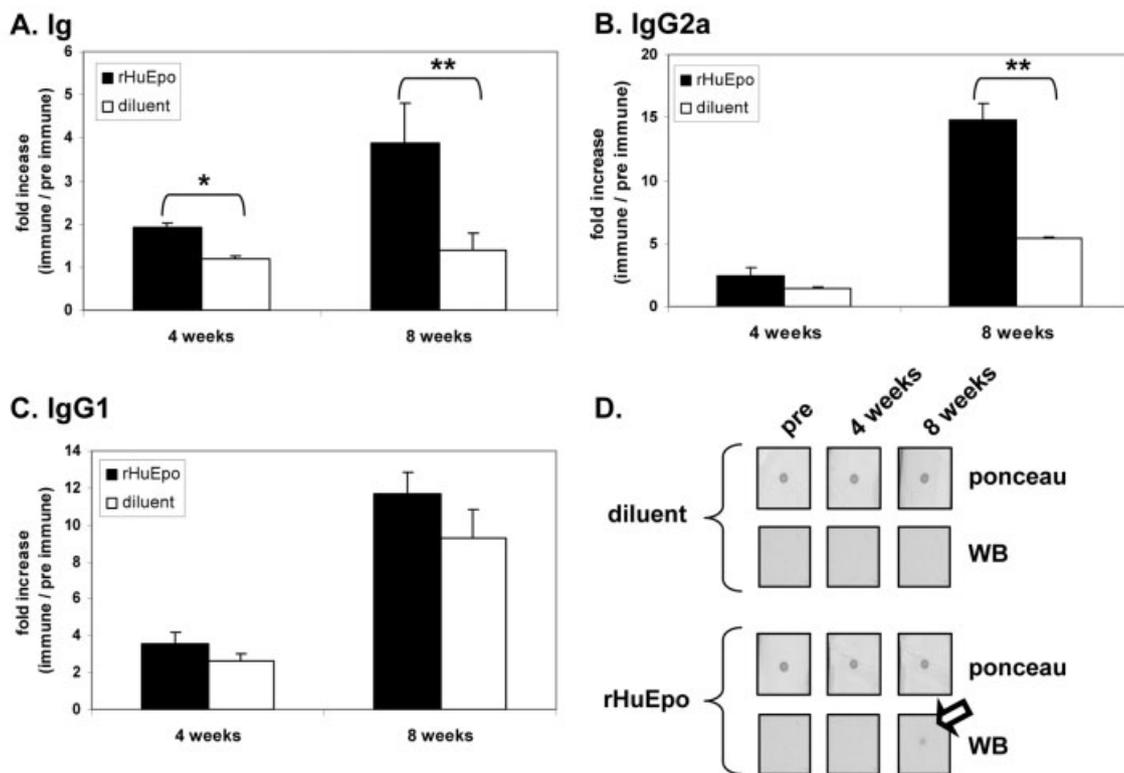
Figure 4. Epo augments anti-hepatitis B antibodies in mice injected with HBsAg. HBsAg (200 ng) was administered s.c. to BALB/c mice, injected three times per week with 180 U rHuEpo (black bars) or with diluent (white bars) as control, until the end of the experiment. Results represent the mean ± SEM of fold increase of ELISA measured anti-HBsAg antibody levels after immunization, with respect to the pre-immune levels (ten mice/group). OD values for the immune sera were in the range of 0.2–1 OD at 450 nm, serum dilution 1:10. Similar OD values were obtained for pre-immune sera of the rHuEpo and control mice. (A) Anti-HBsAg Ig, (B) anti-HBsAg IgG2a, (C) anti-HBsAg IgG1; *$p < 0.05$, **$p < 0.001$. (D) Serum samples from the rHuEpo-treated and control mice were pooled separately. Western blots (WB) of HBsAg (20 ng) spotted onto a nitrocellulose membrane filter were overlaid with the indicated pools of anti-HBsAg sera (diluted 1:200) from the rHuEpo or control mice (samples from ten mice in each pool). Antibody binding to HBsAg was detected by incubation of the blots with HRP-labeled goat anti-mouse antibodies, followed by the ECL reaction. Equal loading of HBsAg was ensured by Ponceau staining of the antigen blot. Preimmune sera and serum samples obtained 4 and 8 weeks following HBsAg injection, were tested for binding to HBsAg, as depicted. The arrow marks HBsAg detected by antisera from rHuEpo-injected mice, collected 8 weeks following immunization.

rHuEpo treatment on IgM antibody levels (data not shown).

Although studies on hemodialysis patients point to Epo effects on immune responses, the mechanisms underlying this effect cannot be deduced under these conditions. It should be emphasized that the aforementioned studies were carried out in patients who exhibited a range of immune system abnormalities, such as impaired T cell-associated activities [42–44], decreased antibody response [45], increased susceptibility to infections [46] and high levels of proinflammatory cytokines [47]. Accordingly, the effects of Epo on immunological functions in hemodialysis patients are likely to be influenced by other physiological parameters, in addition to Epo administration.

The use of *in vivo* murine models in our present study enabled a critical approach to determine whether Epo affects the B cell arm of the immune response, without the confounding factor of hemodialysis and the associated therapy. Furthermore, studying the murine model permitted the analysis of shifts in T and B lymphocytes in the spleen, in addition to the assessment of Ig and specific antibodies in the peripheral blood as performed in the clinical studies.

We showed an increase in the proliferative response to LPS and a reduced response to anti-CD3 in both rHuEpo-injected and tg6 mice. These findings thus indicate that Epo augments certain B cells functions. The enhanced LPS-mediated proliferation of splenocytes persisted when the adherent cells were removed from the culture (data not shown), in support of the notion that macrophages did not mediate this response. The Epo-associated increase in humoral immune responses was also shown using two antigen models. HBsAg was chosen in view of its clinical relevance, and the hapten DNP made it possible to focus on the particular response to DNP, separate from the response to the carrier protein conjugate. The DNP hapten elicits mainly a Th2-

mediated response [34], typically reflected in the production of IgG1 antibodies [48], whereas the HBsAg elicits mainly a Th1-mediated response culminating in the generation of specific IgG2a antibodies [49]. Our finding that treatment with Epo significantly increased the levels of anti-HBsAg IgG2a reflects activation of the Th1 response. Of note, activation of Th1 pathways has been shown to contribute to the successful treatment of hepatitis B [35]. It is possible that in these cases Th cells are involved in Epo-associated augmentation of the humoral response. Although the splenic cell populations measured in the absence of antigen challenge were decreased by Epo treatment, this decrease was not observed in the antigen-injected mice (data not shown). The increased antibody response to the DNP haptenic determinant, measured separately from the carrier molecule used for immunization, corroborates the idea that in addition to effects on the T cell arm, Epo affects also the ultimate production of antibodies by B cells.

Direct effects of Epo on B cell functions were demonstrated in cultured cell lines [50, 51] that may possess EpoR. Other studies have suggested that Epo-mediated regulation of antibody production *in vitro* is indirect and may be mediated by T cell- and monocyte-dependent mechanisms [22]. Our results showing Epo effects on both Th1 and Th2 type responses are in line with the possibility that Epo acts on mediator cells, other than T cells. It should be noted that we could not detect EpoR mRNA transcripts on mouse splenic lymphocytes (data not shown) or on human T cells, based on RT-PCR analysis [27]. In addition, we did not detect any direct effect of Epo on T cell activation or proliferation when applied *in vitro* [26, 27] . Other cells to be considered as potential mediators of these Epo immuno-potentiating effects are APC [52], which are pivotal in mediating immune responses. Thus far, EpoR expression on APC has not been demonstrated; however, the premise that Epo may act indirectly *via* these cells warrants further investigation.

Our finding that rHuEpo augmented the antibody response to HBsAg suggests that rHuEpo might have new clinical applications as an immunomodulator. Higher doses of rHuEpo are required to mount a significant increase in antibody titer, compared to those required for erythropoiesis. This may reflect the requirements of target cells other than erythroblasts, although the experimental conditions (*e.g.*, amount of antigen injected, time schedules and duration of treatment) may also contribute to this finding. Effects of Epo on Th cells could have been exerted indirectly *via* other cells at an early stage of B cell responses, before the increase in Ig production was manifested. Yet, identifying the target cell(s) upon which Epo exerts its direct non-erythroid effect remains a major challenge. Finally, although Epo was shown to have immune-mediated

anti-MM effects, there have been reports that in the case of other cancers rHuEpo might even accelerate the progression of disease [53–56]. The different effects of rHuEpo treatment may thus be determined by the expression of EpoR on the malignant cells [57–59].

Altogether, our study is significant in showing that Epo mediates an augmented B cell response, indicating that rHuEpo therapy might be implemented in improving the immune response of immunocompromised patients. These findings point to further potential therapeutic applications for rHuEpo, as an immunomodulator, beyond its current use in promoting erythropoiesis, *i.e.* augmenting various human vaccination programs.

Materials and methods

Mice

Female mice of the inbred strain BALB/c, aged 6–8 weeks, were obtained from the Tel-Aviv University Animal Breeding Center. The transgenic mouse line overexpressing HuEpo (tg6) has been previously described [31]. The resulting mouse line was bred by mating hemizygous males to wt C57BL/6 females. Half of the offspring were hemizygous for the transgene and the other half were wt and served as controls. All experiments were performed on female mice aged 3–5 months. The 5T2 MM cell line [60–62], generously provided by K. Vanderkerken (VUB, Brussels), was propagated in C57BL/KaLwRij mice (Harlan, CPB Zeist, The Netherlands). Bone marrow cells from these MM-carrying mice were transferred by i.v. injection, as described [30], into 8–10-week-old syngeneic recipients. Experiments were conducted under permit numbers M-03–167, M-04–090 and M-04–148, authorized by the Animal Care Committee at the Sackler Faculty of Medicine.

Immunization

Mice were immunized s.c. with 10 μg DNP-KLH (Calbiochem), or 200 ng HBsAg (SmithKline Beecham Biologicals ENG5279A4) in 100 μL PBS, emulsified in 100 μL complete Freund's adjuvant (CFA; DIFCO).

rHuEpo injections

For the 5T2 MM model, 30 U rHuEpo (Epoetin alfa, Eprex®, Janssen-Cilag), or diluent (control) were injected for 10 consecutive days, followed by three injections each week for 3 weeks, as previously described [28, 29].

To establish the optimal injection schedule of rHuEpo on the immune response of DNP-KLH injected mice, we tested the effects of different immunization regimens based on various rHuEpo doses (30, 60, 90 and 180 U/injection) and time schedules (daily treatment for 10 consecutive days with 30 U [28, 29], or three injections per week for the other Epo doses). The maximal immune response was obtained in mice receiving thrice weekly injections (s.c.) of 180 U rHuEpo into the back

flank, for the duration of the experiment. This rHuEpo-treatment schedule was thus used throughout the study, unless otherwise stated.

ELISA

Antibody levels in sera were measured using ELISA. Antibodies to DNP were assayed using DNP-BSA (Calbiochem), thus providing data on the net response to the DNP hapten. Antibodies to HBsAg were assayed using a specific kit (BioELISA anti-HBsAg from Biokit, Gamidor Diagnostics LTD, Petach Tikva, Israel) [63]. Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig and donkey anti-mouse IgG were obtained from Sigma (St. Louis, MO). HRP-conjugated rat anti-mouse IgG2a and IgG1 were obtained from PharMingen (San Diego CA).

Serum samples from immunized mice were used at a dilution yielding OD values >0.2 and <1.0, in the linear range. Typical OD levels of sera from pre-immune mice were under 0.05. The results are presented as the ratio between the antibody levels in serum samples of immunized mice and their respective pre-immune sera. The values obtained for pre-immune sera of the rHuEpo and diluent-injected mice were generally similar. Values represent the mean ± SEM of samples from at least four mice in each experimental group, as specified.

Proliferation of murine splenocytes stimulated with *Escherichia coli* LPS or anti-CD3

Mice were killed, and their spleens were immersed in PBS and forced through 200-μm pore-size wire mesh, using the plunger from a 5-mL syringe to produce a single cell suspension. The cells were pelleted by centrifugation, and erythrocytes were lysed by hypotonic shock (10 s in sterile distilled water), followed by the addition of 0.1 vol of ×10 Hanks' buffered saline solution. Splenocytes were centrifuged and washed once in PBS, prior to dilution in RPMI culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1000 U/mL penicillin/streptomycin and 2 mM glutamine. Isolated splenocytes (200 000/well/200 μL) were seeded into 96-well flat-bottom microtiter plates and incubated with LPS (*E. coli* strain 0127:B8, Sigma, UK) at 2 μg/mL. Following incubation for 96 h at 37°C in 5% CO_2–air, cell proliferation was assessed using the colorimetric methyl thiazol tetrazoliumbromide (MTT) assay for measuring cell proliferation [64]. For anti-CD3 driven proliferation, the 96-well plates were coated with 5 μg/mL mAb anti-CD3 (clone 145–2C11, BioLegend, CA), prior to addition of the splenocytes. Culture medium for anti-CD3-mediated proliferation was supplemented with 50 μM β-mercaptoethanol. For the rHuEpo or diluent-injected BALB/c mice, splenocytes were cultured 1 day after the last rHuEpo or diluent injection.

Lymphocyte surface markers

Spleen cells (1×10^6) were immersed in 100 μL PBS and incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated mAb to CD4, CD8 or CD19 (Southern Biotech, AL). Isotype-matched mAb were used as negative controls. After 30 min on ice, the cells were washed with PBS and analyzed on FACSort flow cytometer (Becton Dickinson, San Jose, CA), using WINMDI software.

Western blot analysis

Western blot analysis was performed essentially as described [65]. Briefly, serum samples (0.5 μL) were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with HRP-conjugated goat anti-mouse IgG2a antibodies (γ2a chain specific) (Southern Biotech) diluted 1:4000, or anti-κ light chain antibodies (ICN Biomedicals, USA) diluted 1:1000, followed by HRP-conjugated donkey anti-rabbit antibodies (Jackson Immuno Research Laboratories, PA) diluted 1:10 000. For dot blot analysis, HBsAg (20 ng/5 μL) was spotted on nitrocellulose membrane filters. The filters were subsequently incubated with pooled sera from the HBsAg-injected mice (diluted 1:200), followed by HRP-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, PA) diluted 1:10 000, followed by the ECL reaction.

Statistical analysis

Kaplan-Meier statistical analysis of the survival curve was performed using the SPSS statistical software package (SPSS 12.0 for Windows, SPSS Inc., Chicago, IL). Student's unpaired two-tailed *t*-test was used to determine a significant difference in the mean values of Epo-mediated fold increase in antibody levels and LPS-stimulated proliferation. Values of $p < 0.05$ were defined as statistically significant.

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