Antibodies to CD40 Prevent Epstein-Barr Virus–Mediated Human B-Cell Lymphomagenesis in Severe Combined Immune Deficient Mice Given Human Peripheral Blood Lymphocytes

By William J. Murphy, Satoshi Funakoshi, Margaret Beckwith, Susan E. Rushing, Denise K. Conley, Richard J. Armitage, William C. Fanslow, Helen C. Rager, Dennis T. Taub, Francis W. Ruscetti, and Dan L. Longo

CD40 is expressed on both normal and neoplastic B lymphocytes. Signal transduction through CD40 in vitro has been shown to exert stimulatory effects on normal B cells and inhibitory effects on Epstein-Barr virus (EBV)-induced B-cell lymphoma lines and some other cell lines derived from patients with aggressive histology lymphoma. The transfer of normal human peripheral blood lymphocytes (huPBL) from EBV-seropositive donors into severe combined immune deficient (SCID) mice has been previously shown to result in the generation of human B-cell lymphomas. These tumors are similar to the highly aggressive EBV-induced lymphomas that can arise clinically after transplantation or in the setting of immunodeficiency. Treatment of huPBL-SCID chimeric mice with anti-CD40 or anti-CD20 monoclonal antibodies (MoAb) significantly delayed the development of EBV-induced B-cell lymphoma. However, the effects of the two MoAb were mechanistically distinct. Anti-CD40 treatment prevented lymphoma generation, while still allowing for functional human B-cell engraftment in the huPBL-SCID mice compared with mice receiving no treatment, all of which succumbed to lymphoma. By contrast, treatment with anti-CD20 significantly inhibited total human B-cell engraftment in the SCID recipients, which accounted for the absence of lymphomas. In vitro assays examining the transformation of human B cells by EBV also indicated that anti-CD40 could directly inhibit EBV-transformation, whereas anti-CD20 antibodies had no effect. Thus, anti-CD40 exerts selective effects to allow for the engraftment of normal human B cells and prevent the emergence of EBV lymphomas. Stimulation of CD40 by antibodies or its physiologic ligand may, therefore, be of significant clinical use in the prevention of EBV-induced B lymphomas that may arise when EBV-seropositive individuals receive immunosuppressive regimens after transplantation or in immune deficiency states, such as acquired immune deficiency syndrome.

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We also examined the effects of anti-CD40 on human B-cell engraftment and Ig production in these mice.

**MATERIALS AND METHODS**

**Flow cytometric analysis.** The protocol for flow cytometric analysis has been described previously. Briefly, lymph node (LN) cells (consisting of pooled brachial, inguinal, and mesenteric nodes) were washed and counted using a Coulter counter (Coulter Electronics, Hialeah, FL). The cells were then adjusted to the appropriate cell concentration and were blocked with 2% human AB serum to prevent nonspecific binding of Ig. The cells were then incubated with the appropriate directly labeled antibodies. The following antihuman monoclonal antibodies (MoAb) were used: CD3 (Leu4) and HLA-DR from Becton Dickinson (Mountain View, CA), HLA-ABC from Olypus (Lake Success, NY), and CD19 (B4 from Coulter). After incubation, the cells were washed and fixed in 1% paraformaldehyde. The cells were then analyzed using an EPICS flow cytometer (Coulter). A Wilcoxon rank sum test was used to determine if values were significantly ($P < 0.01$) different.

**In vivo experiments.** C.B-17 scid/scid (SCID) mice were obtained from the Animal Production Facility, NCI-FCRDC, Frederick, MD. Mice were kept under specific pathogen-free conditions throughout the study. All mice received autoclaved food, water, and bedding. When human cells were to be transplanted into SCID recipients, huPBL were obtained from healthy donors in leukopacks. All huPBL donors were screened for human immunodeficiency virus (HIV-1) and hepatitis B and provided informed consent before donation. The huPBL were then purified by counter-current excentration and the lymphocyte fraction, containing greater than 90% lymphocytes, were obtained. The huPBL (1 x 10^6) were injected intraperitoneally (IP) into recipient SCID mice. On the day of injection of huPBL, all mice received 20 μL of anti-asialo GM1 (anti-ASGM1) (Wako Chemicais, Dallas, TX) intravenously, a procedure that we have found to improve engraftment of huPBL in SCID mice. Some SCID recipients also received 10 μg recombinant human growth hormone (rhGH) (provided by Genentech, South San Francisco, CA) in 0.2 mL Hank's Balanced Salt Solution (HBSS) IP every other day until time of assay 4 to 8 weeks later, a protocol that we have determined to accelerate EBV-induced B-cell lymphomagenesis in this model. All huPBL-SCID mice treated with rhGH developed lymphoma within 3 months compared with 20% to 45% of mice not treated with rhGH. Antihuman CD40 (M3 hybridoma, mouse IgG) was then used to examine the stimulatory effects on B-cell proliferation in vitro (data not shown). Anti-CD20 (clone 1F5, a mouse IgG2a, MoAb) (a kind gift from Dr Kevin Conlon, NCI-FCRDC, Frederick, MD), or mouse IgG, myeloma protein (Cappel, West Chester, PA) were then given. Mice were either treated with mouse IgG, mouse IgG2a, myeloma protein (MlsG2a, anti-CD40, or anti-CD20 (2 μg/0.2 mL phosphate-buffered saline [PBS]) IP every other day for 20 days for a total of 10 injections (Fig 1). In the experiments in which anti-CD40 was given continuously, the mice received 2 μg of either anti-CD40 or MlsG2a IP every other day until termination of the experiment. Some mice that did not succumb to tumor were then assayed 7 to 8 weeks after huPBL transfer for effects on human cell engraftment as assessed by flow cytometry. All experiments had 5 to 10 mice per group. Each experiment used huPBL from a different donor. A Student's $t$-test was used to determine if there were significant ($P < .001$) differences in survival.

**In vitro transformation of human B cells.** Fresh cell-free 5 days postestablishment supernatants from the EBV-producing cell line B95-8 were used as a source of transforming virus. Anti-CD40 and anti-CD20 were coated on the wells at a concentration of 10 μg/mL. HuPBL were elutriated to remove monocytes, and the lymphocyte fraction was then run over a nylon wool column to enrich for B cells. The cells remaining in the column were removed by smashing the columns and found to be enriched for B cells. The purified B cells were incubated for 2 hours in 3 mL of undiluted viral preparation at a concentration of 5 x 10^6 cells/mL at 37°C in T25 flasks. A total of 17 mL of RPMI-1640-10% fetal calf serum (FCS) was added, then the cell suspension was transferred into 24-well plates previously coated with anti-CD40, anti-CD20, or antitransforming factor-β (anti-Tgβ) antibodies, as described above. Transformation was assessed by examining surviving cells after 28 days. Any transformed cell arising under these conditions was analyzed by flow cytometry for expression of CD3 and CD19. An anti-Tgβ antibody (ID-11) was obtained from Celltrix (Santa Clara, CA) and was used as an isotype-matched (IgG1) control antibody for anti-CD40.

**Serum Ig and diaphragm and tetanus toxoid (DT)-specific Ig determination.** For determination of human Ig in the serum, samples were assayed by enzyme-linked immunosorbent assay (ELISA) as follows: flat bottom 96-well microtiter plates (Corning Glass Works, Corning, NY) were coated with goat antihuman Ig (Kirkegaard & Perry Laboratory, Gaithersburg, MD) at 1 μg/mL in PBS. After washing two times, a blocking step was carried out using 5% goat serum. This was followed by the addition of titration of human IgM + IgG standard (DAKO Corp, Santa Barbara, CA). After washing four times, alkaline phosphatase-conjugated goat antihuman Ig (Kirkegaard & Perry Laboratory, Gaithersburg, MD) was added. After washing, the reaction was developed using substrate and optical density (OD) measured at 402 nm. A Wilcoxon rank sum test was used to determine if the values differed significantly ($P < .01$). For the immunization studies, huPBL-SCID mice were immunized IP with various doses of a DT (Connaught Laboratories, Inc, Swiftwater, PA) 1 day after huPBL transfer. The huPBL-SCID mice then received 2 μg injection of rhGH with or without 2 μg of anti-CD40 IP given every other day for 20 days. The mice were bled 2 weeks after immunization and the amount of DT-specific antibodies was determined by ELISA using plates coated with DT and using the human capture assay described previously. The plates were then read at 450 nm and the OD values were plotted. A Wilcoxon rank sum test was used to determine if the values obtained from immunized mice were significantly ($P < .01$) greater than values obtained from unimmunized mice.
RESULTS

Effects of anti-CD40 and anti-CD20 on the prevention of human lymphomagenesis in vivo. Because we have previously shown that anti-CD40 could directly inhibit the growth of EBV-induced lymphomas in vitro,12 we wanted to determine if anti-CD40 could also prevent or delay the occurrence of EBV-lymphomas in huPBL-SCID mice when EBV-seropositive donors were used as a source of normal huPBL (Fig 1). The effects of anti-CD40 on the prevention of lymphoma development was assessed by effects on survival, histological evaluation for evidence of tumor, and the ability to culture B-cell lymphomas from various lymphoid organs in the mice. The results showed that treatment of huPBL-SCID chimeric mice with anti-CD40 at the time of huPBL transfer significantly prevented the development of and death from human B-cell lymphoma (Table 1). While treatment of the huPBL-SCID mice with rhGH was used to accelerate the EBV-induced B-cell lymphoma development, it was not required for the antitumor effects of anti-CD40, as anti-CD40 treatment also delayed lymphoma development in mice not receiving rhGH (Fig 2). As the data show, the absence of rhGH in this experiment resulted in a longer mean day of death from lymphoma in the control mice. However, survival was also significantly (P < .01) prolonged with the administration of anti-CD40. These long-term studies did, however, indicate that with certain donors, most of the huPBL-SCID mice eventually developed lymphoma after cessation of anti-CD40 treatment when only 10 injections of CD40 antibody were administered. We then examined whether continuous administration of anti-CD40 would allow huPBL-SCID mice to remain permanently disease-free. HuPBL-SCID mice then received anti-CD40 (2 μg) given every other day for the duration of the experiment. We also administered rhGH to promote lymphoma development and assure the occurrence of aggressive lymphoma. The results show that when continuous administration of anti-CD40 was performed, 9 of 11 treated huPBL-SCID mice remained disease-free for over

Table 1. Effect of Anti-CD40 Versus Anti-CD20 Administration in EBV-Induced B-Cell Lymphoma Development in huPBL-SCID Chimeras

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment*</th>
<th>No. of Mice</th>
<th>Mean Day of Death</th>
<th>Incidence of Lymphoma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>mlgG</td>
<td>6</td>
<td>31.4 ± 3.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anti-CD40</td>
<td>6</td>
<td>&gt;50†</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>mlgG</td>
<td>8</td>
<td>&gt;26.3 ± 2.1†</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>anti-CD40</td>
<td>8</td>
<td>&gt;55‡</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>mlgG</td>
<td>5</td>
<td>33.2 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anti-CD40</td>
<td>5</td>
<td>&gt;53‡</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>anti-CD20</td>
<td>5</td>
<td>&gt;53†</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hu-PBL chimeric mice were generated as described in Materials and Methods. The mice then received IP injections of either anti-CD40, anti-CD20 or mlgG control antibodies at 2 μg administered every other day for 20 days and total of 10 injections. Mice were then monitored for tumor development and progression. Moribund mice were killed. All mice were necropsied for evidence of tumor.
† Mice were moribund with evidence of extensive tumor nodules in the peritoneal cavity and evidence of lymphoma by histologic assessment.
‡ Treatment with anti-CD40 or anti-CD20 resulted in no deaths due to lymphoma and no evidence of lymphoma when assayed 2 to 6 weeks after cessation of treatment. Anti-CD40 or anti-CD20 treatment significantly (P < .001) increased survival compared with control recipients.
§ One of 8 mice surviving and showing no evidence of tumor.

Fig 2. Effect of anti-CD40 administration on prevention of EBV-induced B-cell lymphoma in huPBL-SCID mice. HuPBL-SCID mice did not receive rhGH in this experiment. Mice received either anti-CD40 (--; or mlgG (---) (2 μg) IP every other day for 20 days and a total of 10 injections. Moribund mice were killed and showed evidence of extensive lymphoma. Treatment with anti-CD40 significantly (P < .01) increased survival of mice.

Fig 3. Effect of long-term administration of anti-CD40 on lymphoma development in huPBL-SCID mice. In these experiments, mice received rhGH to promote lymphoma development. Mice then received either 2 μg of anti-CD40 (--; or mlgG; (---) IP every other day until termination of the experiment. Treatment with anti-CD40 significantly (P < .001) increased survival of mice; 9 of 11 were disease-free 130 days after huPBL transfer, whereas 18 of 18 control mice had died of lymphoma by day 76.
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150 days, whereas 18 of 18 of the control mice were dead by day 76 (Fig 3). Thus, long-term administration of anti-CD40 was effective in preventing the development of B-cell lymphomas in the majority of huPBL-SCID mice.

We then compared the in vivo efficacy of anti-CD40 with a MoAb directed toward anti-CD20 as a binding control antibody. To our initial surprise, treatment of the huPBL-SCID mice with anti-CD20 (clone 1F5) also significantly delayed lymphoma development (Table 1). This occurred despite the fact that we had found that the 1F5 MoAb had no effect on the in vitro proliferation of various EBV-induced B-cell lymphomas (Funakoshi et al, manuscript in preparation). Signalling through CD20 has been shown to exert stimulatory effects normal human B cells and it is possible that while inhibitory effects were not detected on B lymphoma lines in vitro that direct antitumor effects may occur from this MoAb in vivo. However, the 1F5 antibody (a mouse IgG2a isotype, whereas the M3 anti-CD40 clone is a mouse IgG1) has been previously shown to result in the in vivo clearance of both normal and neoplastic B cells, which could account for the absence of human lymphoma development. It was then important to determine the effects of both anti-CD40 and anti-CD20 treatment on human B-cell engraftment in the surviving huPBL-SCID mice.

Anti-CD40 treatment allows for human B-cell engraftment in vivo whereas anti-CD20 treatment resulted in the clearance of human B cells. We then determined the effects of anti-CD40 and anti-CD20 treatment on human cell engraftment to gain insight to the mechanism(s) by which EBV-lymphoma was prevented in the huPBL-SCID mice. When the surviving mice (treated with anti-CD40 or anti-CD20) were later examined for the presence of human cells in the peripheral lymphoid tissues (6 to 8 weeks after huPBL transfer), markedly contrasting effects on B-cell engraftment were seen depending on the MoAb used. Anti-CD40 treatment of the huPBL-SCID mice resulted in significantly greater human cell engraftment, including the engraftment of human B cells (HLA-ABC, CD19, CD3 -) as determined by flow cytometric assessment of LN cells (Fig 4, Table 2) and determination of serum levels of human Ig (Fig 5). Interestingly, with some donors, the predominant cells to engraft after anti-CD40 treatment were human B cells (Table 2, experiment A), whereas we and others have previously found that the usual predominant cell-type to engraft in huPBL-SCID mice is T cells. Regardless of whether human B cells or T cells were the predominant cell-type to engraft, anti-CD40 treatment resulted in significant human B-cell engraftment in these mice without the appearance of B-cell lymphoma. In contrast, the surviving huPBL-SCID chimeras that received anti-CD20 showed little evidence of...
B-cell engraftment and the predominant human cells to engraft were always HLA-ABC⁺, CD3⁺ T cells (Fig 3, Table 2). Human Ig levels were also significantly lower in the sera of mice treated with anti-CD20 (Fig 5) with most mice negative for human Ig supporting the idea that the 1F5 MoAb was clearing all human B cells in these mice. The nonspecific depletion of all human B cells (both normal and neoplastic) by anti-CD20 would also account for the prevention of B-cell lymphoma and subsequent increases in survival of the huPBL-SCID mice while at the same time allowing for engraftment of functional human B cells.

**Anti-CD40 inhibits EBV transformation of B cells in vitro.** We then examined the effect of anti-CD40 on the transformation of human B cells by EBV in vitro. Purified human B cells were placed in culture with supernatants from the B95-8 cell line as a source of EBV. This technique has been used extensively for the generation of lymphoblastoid B-cell lines and the only cells to survive after several weeks in culture are transformed. Some wells either received an isotype-matched control AB to TGFP, anti-CD20, or anti-CD40. After 28 days, the extent of EBV-induced B-cell transforma-

![Graph](image_url)
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Fig 6. Effect of anti-CD40 treatment on the function of human B cells in huPBL-SCID mice. HuPBL-SCID mice were treated as described in Materials and Methods. The mice were given anti-CD40 (2 μg) every other day for 20 days and a total of 10 injections to prevent lymphoma induction. Two weeks after huPBL transfer, mice were immunized with various concentrations of a DT vaccine preparation. One week later the mice were bled and the amount of DT-specific antibodies were determined by using a human Ig capture DT-specific ELISA. Values are presented as OD values. Mice treated with anti-CD40 displayed significant (P < .01) increases in DT-specific antibodies as a result of DT-immunization.

We report here that anti-CD40 can prevent human EBV-induced B-cell transformation in vivo. However, it is still not determined whether anti-CD40 prevents the transformation by EBV or suppresses the growth of the cells after they are transformed. The in vitro data do indicate that anti-CD40 may be directly capable of inhibiting EBV-induced B-cell lymphoma development in vivo, while at the same time allowing for functional human B-cell engraftment in SCID mice. This is in contrast to the prevention of lymphoma development in the huPBL-SCID mouse by anti-CD20. The nonspecific removal of human B cells in these mice, as a consequence of anti-CD20 treatment, also prevented lymphoma development due to the removal of the potential target cells that would eventually give rise to EBV lymphoma. The IFS MoAb has been previously shown clinically to result in the clearance of normal B cells. Therefore, treatment with either anti-CD40 or anti-CD20 prevents the development of human EBV-induced B-cell lymphomas in huPBL-SCID mice, but by different mechanisms. Anti-CD40 appears to selectively prevent B-cell lymphomagenesis while allowing for functional human B-cell engraftment, whereas treatment with anti-CD20 results in the removal of the total human B-cell population. However, anti-CD20 may also directly inhibit B-cell lymphoma growth in vivo as CD20 stimulation by the IFS clone has been shown to activate human B cells. Interestingly, treatment with anti-CD20 also resulted in lower T-cell engraftment in some experiments, but this was dependent on the huPBL donor (Table 2). Because the amount of huPBL injected appears to play a critical role in subsequent engraftment in the SCID mouse, the removal of the human B cells by anti-CD20 may reduce the amount of human cells present and thus affect human T-cell engraftment in a nonspecific fashion. On the other hand, B- and T-cell engraftment may be codependent with T-cell products facilitating B-cell engraftment and vice versa. The mechanism by which the anti-CD20 MoAb (mouse; IgGk) prevents human B-cell engraftment in vivo appears to depend on AB-dependent cell-mediated cytotoxicity. This has also been reported to occur using the human/mouse model when antibodies to CD23 and CD24 were used to treat established human B-cell lymphomas engrafted into SCID mice. The model we are using is significantly different because we are evaluating the effect of anti-CD40 on lymphomagenesis prevention, not treatment of established tumors and are determining the effects on normal B-cell engraftment and survival. We have found that blocking Fc receptors in vivo could abrogate the efficacy of the anti-CD20 MoAb in mice bearing human B-cell lymphomas, but had no effect on the antitumor effects of anti-CD40 (Funakoshi et al, manuscript in preparation). This, and our

Table 3. Effect of Anti-CD40 on EBV Transformation of Human B Cells In Vitro

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8/8</td>
<td>8/8</td>
<td>18/18</td>
</tr>
<tr>
<td>Anti-CD20</td>
<td>8/8</td>
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<tr>
<td>Anti-CD40</td>
<td>0/8</td>
<td>0/8</td>
<td>1/18</td>
</tr>
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* Anti-CD40 or anti-CD20 were coated (10 μg/mL) to plates overnight as described in Materials and Methods. Partially purified human B cells were then placed in the wells at 1 × 10⁶ per mL and supernatant (50% vol/vol) from the B98 cell line was placed in the wells. The wells were then monitored for the presence of transformed cells after 28 days, which were determined by flow cytometry, to be CD19⁺ and CD3⁺. Wells not scored positive had no viable cells present.
previous data showing that a recombinant soluble human ligand is also capable of inhibiting lymphoma proliferation in vitro and provides antitumor effects in vivo (manuscript in preparation), would suggest that anti-CD40 is capable of directly inhibiting lymphomagenesis in vivo.

We have previously shown that CD40 stimulation can result in the inhibition of a variety of human B-cell lymphomas both in vitro and in vivo. This is presumably through an "activation-induced growth arrest" that has been reported to occur with some B- and T-cell lymphomas. Not all B-cell lymphomas appear susceptible to this phenomenon; for example, we have found that anti-CD40 does not inhibit the growth of human myeloma cell lines even after cross-linking (data not shown). Additionally, there may be significant differences concerning the effects of different anti-CD40 MoAbs, depending on the particular epitope they recognize.

While the mechanism underlying the ability of anti-CD40 to prevent EBV-induced B-cell lymphoma development in the huPBL-SCID model is still under investigation, the in vitro data suggest at least two possible mechanisms: inhibition of transformation by EBV or inhibition of proliferation of the neoplastic cell after EBV transformation. We are currently examining the effect of CD40 stimulation on viral promoters in the EBV-transformed lines. These results do suggest that treatment with anti-CD40 or CD40 ligand may be of considerable use to prevent EBV-lymphoma development after bone marrow transplantation in which anti-CD40 or CD40 ligand would only need to be administered until the patient’s immune system successfully reconstituted. Indeed, because of its reported immunostimulatory effects, CD40 stimulation may also accelerate immune reconstitution, while preventing the occurrence of lymphoma.

The huPBL-SCID mouse should prove useful in evaluating the immunologic effects of CD40 stimulation using human cells in vivo. While we show here that anti-CD40 allows for antigen-specific human secondary antibody responses in SCID mice showing that the human B cells in these mice are functional, it will be of interest to determine if anti-CD40 can actually permit primary antigen responses in huPBL-SCID mice. Anti-CD40 may therefore also be of use to optimize the huPBL-SCID model for examination of human B-cell function and Ig production without the complication of EBV-lymphoma induction. In light of recent data showing that CD40 is expressed on human monocytes and T cells, studies are currently underway evaluating CD40 stimulation in the huPBL-SCID model to promote human primary immune responses.

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