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Requirement for NK Cells in CD40 Ligand-Mediated Rejection of Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia Cells

Tanja A. Gruber,*† Dianne C. Skelton,* and Donald B. Kohn†*†

We have previously developed a murine model of Philadelphia chromosome-positive acute lymphoblastic leukemia by i.v. injection of a pre-B ALL cell line (BM185) derived from Bcr-Abl-transformed BALB/c bone marrow. We are studying the potential to elicit autologous antileukemic immune responses by introducing genes encoding immunomodulators (CD40 ligand (CD40L), CD80, and GM-CSF) into leukemia cells. BM185 cells expressing CD40L or CD80 alone, when injected into BALB/c mice, were rejected in approximately 25% of mice, whereas cohorts receiving BM185 cells expressing two or more immunomodulator genes rejected challenge 50–76% of the time. The greatest protection was conferred in mice receiving BM185 cells expressing all three immunomodulators. Addition of murine rIL-12 treatments in conjunction with BM185/CD80/CD40L/GM-CSF vaccination allowed rejection of preestablished leukemia. BM185 cell lines expressing CD40L were rejected in BALB/c *nu/nu* (nude) mice, in contrast to cell lines expressing CD80 and/or GM-CSF. Nude mice depleted of NK cells were no longer protected when challenged with BM185/CD40L, demonstrating a requirement for NK cells. Similarly, NK cell depletion in immunocompetent BALB/c mice resulted in a loss of protection when challenged with BM185/CD40L, confirming the data seen in nude mice. The ability of CD40L to act in a T cell-independent manner may be important for clinical applications in patients with depressed cellular immunity following chemotherapy.  *The Journal of Immunology*, 2002, 168: 73–80.

A acute lymphoblastic leukemia (ALL)² is the most frequent type of leukemia in children, and represents roughly 30% of all childhood malignancies (1). With current intensive therapy, including CNS prophylaxis, event-free survival in children with ALL has reached 75%, a great improvement from that seen in the 1950s when single agent chemotherapy was employed, resulting in 0% event-free survival (1). The 25% of ALL patients who will subsequently relapse can often be predicted, based on certain prognostic factors, including infants less than 1 year of age and/or the presence of the Philadelphia chromosome (Ph) (2).

Ph results from a translocation between chromosomes 9 and 22, which causes fusion between the cellular genes BCR and ABL, creating a chimeric 190-kDa protein not found in normal cells. The Bcr-Abl oncogene protein has abnormal protein kinase activity with enhanced autokinase and transkinase activity (3). Children with Ph-positive ALL represent a subgroup at very high risk for treatment failure; the Children’s Cancer Group has found that 20.1% of patients (5–16) when compared with Ph-negative patients (75.8%) with current intensive chemotherapy programs (4). Clearly, alternative treatment strategies for this subgroup of ALL patients are needed.

Bcr-Abl-derived peptides are hypothesized to be true tumor-specific Ags because 1) they contain a novel amino acid sequence at the junction of Bcr and Abl (4), and 2) they are exclusively expressed in the leukemia clone. The immunogenicity of the Bcr-Abl oncogene has been extensively studied in leukemia cells of chronic myeloid leukemia patients, which contain a 210-kDa form of the Bcr-Abl oncogene, demonstrating the feasibility of stimulating tumor-specific immune responses as an adjuvant therapy for Ph-positive patients (5–16).

One strategy currently under study is to induce tumor-specific immune responses with tumor cell vaccines. While irradiated tumor cells may not be sufficient to initiate an antitumor response, tumor immunogenicity can often be enhanced by transduction of tumor cells with genes encoding immunomodulator proteins. Genes used to modify tumor cells include MHC class I and II genes, costimulatory molecules such as CD80 and CD86, and various cytokines such as IL-2, IL-7, IL-12, IFN-γ, TNF-α, Flt3 ligand, and GM-CSF. The efficacy of the genes in stimulating tumor-specific immune responses depends on the type of malignancy studied.

Murine tumor models have allowed these types of immune gene therapy strategies to be evaluated with the three main objectives being: 1) the abrogation of tumor establishment, 2) the immunization of naive animals against wild-type tumor, and 3) the treatment of animals with established tumors. Our lab has developed a murine model of Ph-positive ALL to test the efficacy of gene-modified leukemia cells as a potential antileukemic therapy (17). Bone marrow from a male BALB/c mouse was transduced with a retroviral vector containing the p190 Bcr-Abl oncogene. A transformed cell line, BM185, with pre-B cell characteristics was isolated from the transduced bone marrow. When injected into syngeneic male BALB/c mice, as few as 1 × 10³ BM185 cells result in uniform mortality within 3 wk. Upon sacrifice, mice have high...
white blood cell counts, acute infiltration of the spleen and bone marrow by lymphoblasts, as well as the presence of blasts in the peripheral blood.

Initial studies compared the leukemogenicity of the BM185 cell line transduced with one of three immunomodulators: GM-CSF, IL-2, and CD80 (17). When expressed in BM185 cells, IL-2 and GM-CSF modestly delayed the development of leukemia, but did not induce rejection of the leukemia. CD80 expression, however, induced 37.2% of mice to reject the leukemia when challenged with a dose of $5 \times 10^5$ cells. CD80 protection was lost in BALB/c nu/nu (nuide) mice, and in BALB/c mice debuffed of either CD4 or CD8 T lymphocytes, demonstrating the requirement for T lymphocytes in the antileukemic response. Mice given irradiated CD80-expressing BM185 cells were protected against subsequent challenge with low doses of wild-type BM185 (17). Subsequently, combinations of these three cytokines were evaluated (18). CD80/ GM-CSF proved to be the most efficacious; mice injected with live cells expressing CD80/GM-CSF demonstrated enhanced survival, and 50% of mice survived when vaccinated with irradiated BM185 cells expressing CD80/GM-CSF and subsequently challenged with a lethal dose of wild-type BM185 cells. Vaccination with CD80/ GM-CSF after challenge with wild-type BM185 cells, however, was not protective (18).

CD40 ligand (CD40L) is expressed on T cells, and interacts with its receptor, CD40, expressed on APCs, induces activation of the APC (19). Activation of APCs leads to up-regulation of co-stimulatory molecules as well as cytokine expression and release. The interaction in turn allows subsequent activation of naive T cells, resulting in initiation of the cellular immune response. CD40L expression on solid tumors such as mastocytomas and neuroblastomas has been shown to elicit antitumor responses in murine models (20, 21). This study focuses on the effects of CD40L expression alone, and in conjunction with CD80 and/or GM-CSF in BM185 cells. We found that CD40L expression in BM185 cells protected mice from an otherwise lethal challenge as efficaciously as CD80 expression. This protection, however, did not involve CD4 T lymphocytes as has been shown for CD80, but rather was NK cell and CD8 T lymphocyte dependent. Vaccination with BM185 cells expressing CD40L, CD80, and GM-CSF allowed a percentage of mice to reject subsequent challenge with a lethal dose of BM185wt cells, and, in conjunction with systemic IL-12 treatments, was able to protect mice who had preestablished leukemia. This is the first demonstration of eradication of preestablished leukemia in our murine model of ALL.

Materials and Methods

**Mice**

Six- to 8-week-old male BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal care facility under standard conditions. BALB/c nu/nu mice were purchased from Harlan Bioproducts for Science (Indianapolis, IN). Experiments involving mice were all reviewed and approved by the Animal Care Committee (Children’s Hospital Los Angeles, Los Angeles, CA).

**Cloning of MFGmCD40L retroviral vector**

The pcDNA3.1 mCD40L expression plasmid was kindly provided by M. Brenner (Baylor College of Medicine, Houston, TX). Ncol and Xhol restriction sites were added to the 5’ and 3’ ends, respectively, of the CD40L-encoding sequences by PCR amplification of the gene with the following primers: 5’-GCTCCTGCCCACGGTTAGAAACATACTGCCACCC-3’ and 5’-CCCAAGATGGTTAGACTGACCTCGCGATG-3’. The amplified product was gel purified and directly ligated into the pGEM-T vector (Promega, Madison, WI). mCD40L was isolated with Ncol and Xhol restriction enzymes, gel purified, and ligated into the MFG retroviral vector (22), which had been cut with Ncol and Xhol, and dephosphorylated with calf intestinal alkaline phosphatase. The ligated construct was transfected into competent DH5α bacterial cells (Life Technologies, Grand Island, NY), which were selected on ampicillin, and colonies were analyzed by restriction enzyme analysis. A single clone with the desired construct was sequenced to verify the intactness of the gene.

**Generation of GP + E86 (GPEmCD40L)**

MFmCD40L was cotransfected into the packaging cell line PA317 with pSv2neo using DOTAP, according to manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). Cells were selected in G418 (500 μg/ml) for 2 wk. The pool of G418-resistant cells was seeded at $2 \times 10^5$ cells/10-cm plate in D10 and kept at 32°C overnight. Supernatant was collected on 3 subsequent days, filtered with a 0.45-μm filter, and stored at -80°C. Supernatant from the MFmCD40L-transfected PA317 cells was used to transduce the ectropic packaging cell line GP+E86. Briefly, $10^5$ GPE cells were plated on a 10-cm plate and, on the following day, vector supernatant and protamine sulfate (6 μg/ml) were added. Cells were then incubated at 37°C overnight. The transduction was repeated on 2 subsequent days for a total of three hits. GP+E86 cells were stained for mCD40L using a PE-conjugated hamster anti-mouse CD154 mAb clone MR1 (BD Pharmingen, San Diego, CA). A CD40L-positive pool of cells was obtained with FACS (BD Biosciences, San Diego, CA). This pool was used as a source for MFmCD40L vector supernatant, harvested as described above.

**Cell lines**

The BM185 cell line has been previously described (2). All BM185 cell lines were maintained in RPMI 1640 medium supplemented with 5% FCS, 2 mM l-glutamine, 10^{-5} M 2-ME, and 100 μM penicillin/streptomycin. The A20 murine B cell lymphoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 with 10% FCS, 5 \times 10^{-5} M 2-ME, and 100 μM penicillin/streptomycin. The apoptotic packaging cell line PA317 (23), ectropic packaging cell line GP+E86 (GPE) (24), and NIH3T3 cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM l-glutamine, and 100 μM penicillin/streptomycin (D10). PA317 and NIH3T3 were obtained from the ATCC. GP+E86 was kindly provided by A. Bank (Columbia University, New York, NY).

NIH3T3 cells expressing murine CD40 ligand (mCD40L) were generated by transfecting the pcDNA3.1 mCD40L expression plasmid using the transfection reagent DOTAP. A transfected pool was obtained by selection in D10 supplemented with G418 (500 μg/ml) for 2 wk. NIH3T3 cells expressing human CD40L were kindly provided by A. Cardossa (Dana-Farber Cancer Institute, New York, NY). NIH3T3 cells expressing mCD40L from the retroviral vector, MFmGmCD40L (NIH3T3 mCD40L), were generated by transduction of NIH3T3 cells with supernatant from the GPEmCD40L-packaging cell line, and selecting a mCD40L-positive pool by FACS (BD Biosciences).

Hybridoma cell lines producing Abs to murine CD4 and murine CD8 (OKI.5 and 53-6.72, respectively) were both obtained from the ATCC. OK1.5 was maintained in IMDM with 1.5 g/l sodium bicarbonate, supplemented with 20% FCS and 100 μM penicillin/streptomycin. The 53-6.72 line was maintained in RPMI 1640 supplemented with 10% FCS and 100 μM penicillin/streptomycin. All cell lines were incubated at 37°C in 5% CO₂.

**Generation of BM185 cell lines expressing CD40L**

BM185 cells were seeded at $3 \times 10^3$ cells/well in a six-well Costar tissue culture plate (Corning, Corning, NY) and incubated overnight at 37°C in 5% CO₂. The following day, supernatant obtained from the MFmCD40L cell line was added to the cells with 6 μg/ml proteamine sulfate. Cells were spinoculated at 32°C for 2 h, $1000 \times g$. After spinoculation, the cells were incubated at 37°C overnight. The spinoculation was repeated with fresh supernatant on 2 subsequent days. Transduced cells were expanded, and a mCD40L-positive pool was selected by FACS. Clones were obtained from the mCD40L-positive pool by an automated cell distributor unit on the FACSVantage sorter (BD Biosciences). The mCD40L-positive pool was selected by FACS. Clones were obtained from the mCD40L-positive pool by an automated cell distributor unit on the FACSVantage sorter (BD Biosciences).

**Leukemia challenges and vaccinations**

BM185 cell lines were harvested, washed twice in HBSS, and resuspended in HBSS with 50 μM heparin. Leukemia challenges were administered by injecting 100 μl vol containing live cells into the tail vein of mice. Cells
used for vaccinations were washed twice in HBSS, resuspended in HBSS supplemented with 50 µM heparin, and irradiated at 3000 rad in a 137Cs gamma irradiator (JL Shepherd & Associates, San Fernando, CA). Vaccinations of 100 µl vol were delivered by s.c. injection in the inguinal region of mice.

**Murine rIL-12 administration**

Mice were administered 2.5 µg murine rIL-12 (rmlL-12; R&D Systems) s.c. in the inguinal region daily for 5 serial days on days 0–4 and 14–18 after i.v. challenge with BM185 cells.

**In vivo depletions**

BALB/c and nude BALB/c mice were depleted of CD4+ T cells (hybridoma GK1.5; ATCC) or CD8+ T cells (hybridoma 53-6.72; ATCC) by i.p. injections of 0.5 mg Ab. Mice were depleted of NK cells by i.v. injection of 50 µl antiasialo GM1 (Wako Pure Chemical Industries, Osaka, Japan). Mice in the control group were given 0.5 mg polyclonal rat IgG (Rockland, Gilbertsville, PA). Injections were administered on days −6, −3, +1, and +4, and twice weekly thereafter to maintain depletion. One mouse per cohort was sacrificed on days −1, +15, +30, +45, and +60 to verify in vivo depletion. A portion of the splenocytes taken from sacrificed mice was analyzed for CD4+, CD8+, and B220 subpopulations by flow cytometry, and the remaining splenocytes were used in a NK assay against YAC-1 targets to test for NK function. Abs used to verify depletions by flow cytometry included PE-conjugated rat anti-mouse CD8α clone 53-6.72, PE-conjugated rat anti-mouse CD4 clone H129.19, and PE-conjugated rat anti-mouse CD45R/B220 clone RA3-6B2 (BD Pharmingen).

**CTL assays**

Spleens were harvested from mice, and single cell suspensions of splenocytes were made and cocultured in vitro with irradiated (2500 rad) BM185/CD80 cells for 5 days. Cultures were supplemented with 4 U/ml rmIL-12 (R&D Systems). Stimulated splenocytes were incubated with BM185 cells labeled with 51Cr (sodium chromate; New England Nuclear, Boston, MA) at various E:T ratios and incubated for 4 h to determine their ability to lyse with cytotoxic target and release 51Cr into the supernatant. Percent specific lysis was calculated with the following equation: Percent specific lysis = (experimental lysis – spontaneous lysis)/(total lysis – spontaneous lysis) × 100.

**NK cell assays**

Spleens were harvested from mice, and single cell suspensions of splenocytes were made and cocultured in vitro with irradiated (2500 rad) BM185/CD80 cells for 5 days. Cultures were supplemented with 4 U/ml rmIL-12 (R&D Systems). Stimulated splenocytes were incubated with BM185 cells labeled with 125I (sodium iodide; Amersham Life Science, Arlington Heights, IL) at various E:T ratios and incubated for 4 h to determine their ability to lyse with cytotoxic target and release 125I into the supernatant. Percent specific lysis was calculated with the following equation: Percent specific lysis = (experimental lysis – spontaneous lysis)/(total lysis – spontaneous lysis) × 100.

**Results**

**Construction of BM185 cell lines expressing mCD40L**

To conduct studies on the immunogenicity of CD40L-expressing leukemia cells, mCD40L was cloned from the expression plasmid pcDNA3.1 mCD40L into the MFG retroviral vector, which was packaged into the GPE ecotropic packaging cell line (Fig. 1A). To ensure biologically active mCD40L was transferred to cells transduced with MFGmCD40L vector, NIH3T3 cells were transduced with MFGmCD40L vector, NIH3T3 cells were transduced and cocultivated with the A20 murine lymphoma cell line. CD80 has been previously shown to be up-regulated in A20 cells induced and cocultivated with the A20 murine lymphoma cell line (17, 18). To determine whether CD40L expression alone, or in combination with CD80 and/or GM-CSF expression, allows a delay in the development of leukemia, and, in a percentage of mice, allows rejection of the leukemia (17, 18). To determine whether CD40L expression alone, or in combination with CD80 and/or GM-CSF confers greater protection, cohorts of mice were challenged i.v. with BM185 cells expressing CD40L, alone, or in combination with CD80 and GM-CSF. Mice challenged with BM185 cells expressing CD80 alone, or CD80 in combination with GM-CSF have been shown to develop tumor-specific T lymphocyte responses, allowing a delay in the development of leukemia, and, in a percentage of mice, allowing rejection of the leukemia (17, 18). To determine whether CD40L expression alone, or in combination with CD80 and/or GM-CSF confers greater protection, cohorts of mice were challenged i.v. with 5 × 10^3 live cells and followed for survival (Fig. 2). All transduced cell lines conferred protection compared with mice challenged with wild-type cells, as determined by the log rank test (p = 0.000000). BM185/CD80/CD40L/GM-CSF-challenged mice demonstrated the greatest protection, with 76.1% of mice surviving challenge. BM185/CD40L, BM185/CD80, and BM185/CD80/CD40L offered the least protection, with 22.9, 28.6, and 21.7% of mice surviving, respectively. Interestingly, the combination of CD80 and CD40L did not provide protection above those cell lines expressing only one of these genes, while CD40L expression alone with GM-CSF and CD40 expression with GM-CSF did provide greater protection, 70.8 and 50% of mice surviving, respectively, than cell lines expressing only one of the genes (p = 0.000151, p = 0.001792, respectively).

**BALB/c nu/nu mice reject BM185 cells expressing CD40L alone, or in combination with CD80 and GM-CSF**

To determine the mechanism of protection seen in immunocompetent BALB/c mice challenged with BM185 cell lines, cohorts
of BALB/c nu/nu (nude) mice that lack a thymus and therefore functional T lymphocytes, alongside cohorts of BALB/c mice, were given i.v. challenges with $5 \times 10^3$ live cells. A large portion of BALB/c mice receiving BM185/CD80/GM-CSF rejected the leukemia, whereas this protection was absent in nude mice ($p = 0.000126$) (Table I), in accordance with previous observation (17, 18). BALB/c and nude mice receiving BM185/CD40L, however, had similar survival, indicating protection mediated by a non-T lymphocyte population, such as NK cells. In light of these data, we expected survival of nude mice receiving any BM185 cell line expressing CD40L. This was the case with BM185/CD80/CD40L and BM185/CD80/CD40L/GM-CSF; nude mice receiving BM185/CD80/CD40L had enhanced survival compared with BALB/c mice ($p = 0.015255$) (Table I). This was not the case, however, for nude mice receiving BM185/CD80/GM-CSF, as the protection seen in BALB/c mice was absent in nude mice ($p = 0.0002292$). Conversely, no significant enhanced survival in nude mice was seen from any cell line not expressing CD40L.

The greater survival of nude mice receiving BM185/CD80/CD40L compared with BALB/c mice suggested that an immune effector, such as NK cells, was more abundant in nude mice than BALB/c. We found absolute NK cell numbers, however, to be similar in both mice (data not shown).

To test the hypothesis that protection from BM185/CD40L challenge in nude mice was mediated by NK cells, nude mice were depleted of NK cells and challenged i.v. with BM185/CD40L. As expected, nude mice depleted of NK cells no longer demonstrated protection from BM185/CD40L challenge (Fig. 3). Mice receiving control Ab had 50% long-term survivors, a figure similar to our previous experiments with BM185/CD40L challenge (75%, Table I). In comparison, untreated mice had only 20% long-term survivors. This discrepancy is most likely due to experimental variation, as each cohort consisted of 10 mice (5 mice per cohort per each experiment).

Depletion of NK cells in BALB/c mice abrogates survival following challenge with BM185/CD40L

Nude mice were protected from BM185/CD40L challenge by NK cells, as NK depletion in these mice led to a loss of protection. To determine whether the protection seen in BALB/c mice was also

Table 1. Survival of BALB/c and BALB/c nu/nu mice challenged with BM185 cell linesa

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>BALB/c</th>
<th>nu/nu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number surviving</td>
<td>% LTSb</td>
</tr>
<tr>
<td>BM185wt</td>
<td>1/20</td>
<td>5</td>
</tr>
<tr>
<td>BM185/CD40L</td>
<td>6/10</td>
<td>60</td>
</tr>
<tr>
<td>BM185/CD80</td>
<td>4/10</td>
<td>40</td>
</tr>
<tr>
<td>BM185/CD80/CD40L</td>
<td>2/8</td>
<td>25</td>
</tr>
<tr>
<td>BM185/CD40L/GM-CSF</td>
<td>5/9</td>
<td>56</td>
</tr>
<tr>
<td>BM185/CD80/GM-CSF1</td>
<td>8/10</td>
<td>80</td>
</tr>
<tr>
<td>BM185/CD80/GM-CSF</td>
<td>8/10</td>
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a Data are compiled from four separate experiments with similar results. Challenge dose, $5 \times 10^3$ cells.
b LTS, long term survivors.
c MST, mean survival time ± SD.
d Values of $p$ represent survival of BM185 cell lines within mice cohorts compared to BM185wt as determined by the log rank test.

d Cohorts with statistically significant difference in survival of BALB/c mice compared to nu/nu mice. Values of $p$ are as follows: BM185/CD80/CD40L = 0.015255, BM185/CD80/GM-CSF = 0.002292, BM185/CD80/GM-CSF = 0.000126.
mediated by NK cells, cohorts of mice were depleted of either CD4 T lymphocytes, CD8 T lymphocytes, or NK cells, and followed for survival following BM185wt/CD40L challenge (Fig. 4). Mice depleted of CD4 lymphocytes demonstrated protection similar to mice treated with control Ab and untreated mice, indicating a lack of requirement for the CD4 subset of cells. In contrast, mice depleted of CD8 lymphocytes had decreased survival when challenged with BM185wt/CD40L. In addition to CD8 lymphocytes, there was also a requirement for NK cells, as all mice depleted of NK cells did not survive BM185wt/CD40L challenge, supporting the results seen in nude mice.

Immunologic memory is induced following primary challenge

To determine whether mice develop immunologic memory against wild-type BM185 cells following challenge with transduced BM185 cells, we challenged mice with 5 × 10^3 BM185wt cells (Table II). A total of 70% of mice who had survived a primary challenge with BM185wt/GM-CSF rejected subsequent challenge with BM185wt, while 50% of mice who had survived a primary challenge with BM185wt/CD80/CD40L/GM-CSF rejected subsequent challenge with BM185wt cells. This difference was not found to be statistically significant, demonstrating that both cell lines were equally effective in initiating an antileukemic memory response.

CTL induction correlates with BM185wt survival postvaccination

BM185 cells expressing one or more immunomodulators demonstrate increased immunogenicity. To more closely mimic a clinical setting in which patients would receive irradiated tumor cells expressing immune stimulating genes as a vaccine, cohorts of BALB/c mice were vaccinated twice with 5 × 10^6 irradiated cells (3000 cGy) 1 wk apart, and subsequently challenged 1 wk later with 5 × 10^3 wild-type BM185 cells (Fig. 5A). BM185wt/CD40L/GM-CSF, BM185wt/CD80/CD40L/GM-CSF, BM185wt/CD80/GM-CSF, and BM185wt/CD80 cohorts each had a higher percentage of mice able to reject i.v. challenge compared with unvaccinated mice, whereas BM185wt, BM185wt/CD40L, and BM185wt/CD80/CD40L cohorts did not have higher levels of survival compared with unvaccinated mice. When mice were analyzed for CTLs specific for BM185wt cells, cohorts of mice that demonstrated protection from vaccinations had statistically higher levels of CTL responses compared with mice that did not have significant protection conferred from vaccinations (Fig. 5B). BM185wt/CD40L/GM-CSF, BM185wt/CD80/CD40L/GM-CSF, BM185wt/CD80/GM-CSF, and BM185wt/CD80 cohorts had CTL levels that were not statistically different from each other, but differed statistically to BM185wt, BM185wt/CD40L, BM185wt/CD80/CD40L, and naive cohorts with p values ranging from 0.000098 to 0.054158 (Fig. 5B). Survival of mice vaccinated and subsequently challenged with wild-type BM185 cells was significantly correlated with the level of CTL induction in the different cohorts (R = 0.66) (Fig. 5C).

Systemic rmIL-12 treatment in conjunction with BM185/GM-CSF vaccinations protects against preestablished leukemia

Previous studies have shown that mice that were first i.v. challenged with a low dose of nonirradiated BM185 cells and then vaccinated with irradiated BM185wt/CD80/GM-CSF were not protected compared with unvaccinated mice, despite the development of CTLs (18). If challenged s.c., however, three of five mice subsequently vaccinated with BM185wt/CD80/GM-CSF were able to reject the preestablished leukemia. Thus, although systemic immune responses were generated, mice were unable to eradicate preestablished systemic leukemia. This may be due to the high malignancy of our model. Because CD40L mediates protection in part by NK cells in our model, we hypothesized that this immediate innate immunity may be able to keep the tumor burden low until CD80 and GM-CSF expression is able to initiate tumor-specific systemic T lymphocyte immunity. Thus, we challenged mice with 1 × 10^3 BM185 cells i.v. and subsequently vaccinated mice with BM185wt/CD80/CD40L/GM-CSF on days +1, +5, and +12. Unfortunately, BM185wt/CD80/CD40L/GM-CSF-vaccinated mice were not protected, and all succumbed to the leukemia at the same rate as mice vaccinated with BM185wt/CD80/GM-CSF or as mice receiving no vaccination (Fig. 6A).

A recent report demonstrated a delay in the development of leukemia with systemic rmIL-12 treatments in a murine model of acute myeloid leukemia (26). To determine whether this was the case in our murine model of ALL, we gave cohorts of mice s.c. rmIL-12 injections following i.v. challenge with 1 × 10^7 BM185 cells (Fig. 6B, primary challenge). Surprisingly, 80% of mice...
treated with rmIL-12 alone following BM185wt challenge survived. Upon rechallenge, however, all the mice succumbed to the leukemia, indicating a lack of immunologic memory (Fig. 6B, secondary challenge). When IL-12 treatments were combined with BM185/CD80/CD40L/GM-CSF vaccination following BM185 challenge, 100% of mice survived primary challenge. In addition, all of these mice rejected subsequent rechallenge with BM185wt cells, suggesting long-term memory (Fig. 6B, secondary challenge). This is the first demonstration of eradication of established systemic leukemia in our murine model of ALL.

**Discussion**

We have examined the ability of immunomodulatory gene products to induce antileukemic immune responses in a murine model of Ph-positive ALL. The Ph-positive murine BM185 cell line was used in a hierarchy of challenge models, including: 1) injection of live cell lines expressing one or more immunomodulators, 2) pre-vaccination with irradiated cells expressing immunomodulator genes, followed by challenge with live leukemia cells, and 3) challenge with live leukemia cells, followed by vaccination with irradiated cells expressing immunomodulator genes.

All mice receiving BM185 cells expressing one or more immunomodulators had enhanced survival compared with mice challenged with BM185wt cells (p = 0.000000). The percentage of long-term survivors was dependent on the combination of immunomodulators expressed within the cells. The rank order of the genes from most efficacious to least was as follows: CD80/CD40L/GM-CSF > CD40L/GM-CSF = CD80/GM-CSF > CD80 = CD40L = CD80/CD40L. This hierarchy is most likely to be specific for this leukemia model, as different genes have been shown to confer different levels of protection depending on the tumor model. The reasons for these discrepancies among different tumor models are likely to be reflections of specific aspects of the tumor cells themselves and the Ags available for immune recognition. The Ags presented in our BM185 cells are unknown. Potentially Bcr-Abl, the gene responsible for transforming the BALB/c bone marrow and containing a novel peptide at the fusion site, may be one of the Ags recognized by the immune system in this model.

CD80-mediated protection has been previously determined to require both CD4 and CD8 T lymphocytes in this model (17). Much of the focus of these current studies was to elucidate the mechanism whereby CD40L confers protection. Initial studies in nude mice revealed that protection from leukemia by expression of CD40L was not solely mediated by T cells, and protection was lost in these mice after NK depletion, demonstrating a requirement for NK cells. This was confirmed in BALB/c mice, although CD8 T cells were also shown to be involved in the immune response in these immunocompetent mice. Although CD8 effectors can act alone when killing target cells, their differentiation from naive CD8 lymphocytes often requires help from CD4 lymphocytes. In order for this priming to occur, both the CD8 and CD4 lymphocytes must recognize Ag on the same APC. More recently, however, it has been shown that signaling through the CD40 receptor on the APCs can replace the requirement for the CD4 Th cells, which may explain the lack of requirement for CD4 lymphocytes in the antitumor responses induced by CD40L (27, 28). Based on these data, we propose that APCs within the host take up apoptotic or necrotic BM185 cells and cross-present leukemia-associated Ags to the immune system. Live leukemia cells expressing CD40L then activate these APCs, allowing up-regulation of costimulatory and adhesion molecules, resulting in direct CD8 T cell activation. In addition, activated APCs release cytokines such as IL-12 that can directly stimulate NK cells. Both BM185wt cells and BM185/CD40L/CD80 were found to be susceptible to NK lysis in vitro at levels similar to YAC-1 targets, supporting this hypothesis (data not shown).

Although 23% of mice were able to reject BM185/CD40L challenge, vaccination with these cells provided no protection. This lack of protection in a prevaccination setting most likely reflects suboptimal memory induction by CD40L, with the beneficial effects of CD40L mainly stemming from activation of NK cells, cells involved in the innate arm of the immune system that lacks memory. Thus, in a clinical setting, vaccinations would consist of cells expressing CD40L to eliminate residual leukemia cells and additional molecules such as CD80 and GM-CSF to establish long-lasting memory.

The CD80/CD40L/GM-CSF combination was highly protective in live challenge experiments. This cell line also induced high levels of CTL activation comparable with that of CD80 alone. The enhanced survival of the mice receiving the combination most likely reflects the ability of these immunomodulators to recruit multiple arms of the immune system, including CD4, CD8, and NK cells. In vaccination experiments whereby mice were prevaccinated and subsequently challenged with wild-type leukemia, BM185/CD80/CD40L/GM-CSF protected mice at a similar level as BM185/CD80, BM185/CD80/GM-CSF, and BM185/CD40L/GM-CSF. These four cell lines were also found to induce the greatest CTL responses, indicating that in this setting, in which antileukemic immunity is established before leukemia challenge, CTL memory cells are a critical component in antitumor immunity.

Previously, it has been shown that vaccination with CD80/GM-CSF was insufficient to protect against preestablished leukemia in

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**Table II. Immunologic memory against BM185wt cells in long term survivors**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Primary Challenge</th>
<th>Secondary Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number surviving</td>
<td>% LTS&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naive (no primary challenge)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BM185wt</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>BM185/CD80</td>
<td>25/47</td>
<td>53</td>
</tr>
<tr>
<td>BM185/CD80/CD40L/GM-CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM185/CD80/CD40L/GM-CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26/32</td>
<td>81</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are compiled from two separate experiments with similar results.

<sup>b</sup> Primary challenges consisted of 5 × 10<sup>4</sup> of the indicated cell line; all secondary challenges consisted of 5 × 10<sup>3</sup> BM185wt cells.

<sup>c</sup> LTS, long term survivors; mice surviving past 60 days postchallenge.

<sup>d</sup> Values of p represent survival of mice compared to those treated with BM185wt cells as determined by Fisher’s exact test with 95% confidence. Statistically significant p values are in bold.

<sup>e</sup> Mean survival times of BM185/CD80/GM-CSF and BM185/CD80/CD40L/GM-CSF are statistically different in the primary challenge (p = 0.015963) but not the secondary challenge (p = 0.231782).
this model (18). Although BM185/CD80/GM-CSF was as protective in a prevaccination setting as BM185/CD80/CD40L/GM-CSF, NK recruitment provided by CD40L expression was postulated to be able to keep the tumor burden low until sufficient CTLs developed to eradicate the leukemia. However, when mice with preestablished leukemia were vaccinated with the CD80/CD40L/GM-CSF combination, there was no delay in the development of the leukemia, and all mice succumbed to the disease. This most likely reflects the high malignancy of the BM185 cell line. As few as 1 \times 10^3 BM185 cells result in 100% mortality within 2–3 wk. Mice dying of this challenge dose demonstrate massive infiltration of lymphoblasts in the spleen, bone marrow, and peripheral blood. The kinetics between tumor cell growth and the generation of tumor-specific immune responses has been shown to be a critical factor in the rejection of tumor cells in murine models (29). The doubling time of BM185 cells in vitro is approximately 12 h, and

**FIGURE 5.** CTL induction correlates with BM185wt survival postvaccination. A, BALB/c mice were vaccinated with 5 \times 10^6 irradiated cells of the indicated cell lines. Naïve mice were not vaccinated. Vaccinations were given on days −14 and −7, and mice were subsequently challenged with 5 \times 10^3 BM185wt cells on day 0. Data are compiled from two separate experiments with similar results. B, BALB/c mice were vaccinated with 5 \times 10^6 irradiated cells of the indicated cell lines. Naïve mice were not vaccinated. Vaccinations were given on days −14 and −7. On day 0, mice were sacrificed and splenocytes were stimulated with irradiated BM185/CD80 cells for 5 days and subsequently tested for their ability to lyse BM185wt cells at an E:T ratio of 100:1. Percent lysis is shown as the mean value of five mice per cohort. Data shown are a representative experiment of two. C, Mean values from graphs A and B above were used as mean values to plot the percentage of long-term survivors as a function of percent specific lysis of BM185wt targets. Survivorship correlated with percent specific lysis, as indicated by the linear regression line (R = 0.66).

**FIGURE 6.** Vaccination in combination with s.c. IL-12 administration protects against preestablished leukemia. A, Mice were challenged with 1 \times 10^3 BM185wt cells on day 0 and subsequently vaccinated with either 1 \times 10^6 irradiated BM185/CD80/GM-CSF or 1 \times 10^6 irradiated BM185/CD80/CD40L/GM-CSF on days 1, 5, and 12. One cohort of mice was left untreated. B, Mice were challenged with 1 \times 10^3 BM185wt cells on day 0 and subsequently vaccinated with either BM185/CD80/CD40L/GM-CSF (Vacc), rmIL-12 (IL-12), or BM185/CD80/CD40L/GM-CSF in combination with rmIL-12 treatments (IL-12 + Vacc), or were given no treatments at all (No Treatment). Cell vaccinations consisted of 1 \times 10^6 irradiated cells on days 1, 5, and 12. IL-12 treatments consisted of s.c. injection of 2.5 μg rmIL-12 on days 0, 1, 2, 3, 4, 14, 15, 16, 17, and 18. Long-term survivors were rechallenged with 1 \times 10^3 BM185wt cells on day 61.
hence, by the time CTLs develop 7 days postvaccination, the tumor burden in the mice can be estimated to be as high as 1.6 × 10⁷ cells if challenged with 1 × 10⁶ cells.

CD40L activates NK cells indirectly by stimulating APCs. This indirect stimulation may be inadequate in mice with these large tumor burdens. The addition of rmIL-12 as an adjuvant with the vaccinations, however, allowed mice with preestablished systemic leukemia to survive and reject subsequent rechallenge with the wild-type strain. Surprisingly, mice given rmIL-12 alone also rejected preestablished leukemia, although these mice lacked immunologic memory against leukemia cells, as determined by subsequent rechallenge. The mechanism of IL-12 protection has not yet been determined in this model, although previous studies have shown CD8, NK, or Vα14 NKT cell-mediated responses to be induced by IL-12, depending on the tumor model used (30–34). Due to the lack of immunologic memory in these experiments, the protection is most likely to be mediated by either NK or Vα14 NKT cells. Further studies focusing on the effect of IL-12 in this model are underway.

The ability of IL-12 in combination with a cellular vaccine is encouraging, as this is the first demonstration of eradication of established leukemia in this highly malignant model of Ph-positive ALL. This combination may be potent enough to provide therapeutic benefit for patients in remission following chemotherapy. The induction of an adequate immune response in patients in remission may allow the immune system to eradicate minimal residual disease, and thereby decrease the rate of relapse in Ph-positive ALL patients. Furthermore, the ability of CD40L to act in a T cell-independent manner may be an important factor in this type of vaccine, as patients have depressed cellular immunity following chemotherapy.

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References