Overcoming Immunoescape Mechanisms of BCL1 Leukemia and Induction of CD8+ T-Cell–Mediated BCL1-Specific Resistance in Mice Cured by Targeted Polymer-Bound Doxorubicin

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Abstract
BALB/c mice bearing syngeneic BCL1 leukemia, a mouse model of human chronic lymphocytic leukemia, were treated with polymer-bound doxorubicin conjugate targeted with BCL1-specific monoclonal antibody. Such treatment can cure up to 100% of mice and the cured mice show long-lasting resistance to BCL1 leukemia. We show that both CD4+ and CD8+ T cells are required for establishment of the resistance, but only CD8+ T cells are necessary for its maintenance. BCL1 cells express MHC class I and II and also costimulatory molecules CD80 and CD86, which can aid eliciting of antitumor response. On the other hand, BCL1 cells also use several immunoescape mechanisms, such as expression of PD-L1, PD-L2, and interleukin-10. BCL1 cells thus can be recognized by BCL1-specific T cells, but instead of effective priming, such T cells are anergized or deleted by apoptosis. Moreover, BCL1 leukemia progression is accompanied by robust expansion of CD4+CD25+Foxp3+ regulatory T (Treg) cells. Although it has been shown that depletion of Treg cells in tumor-bearing mice can retard tumor growth, direct evidence that expansion of Treg cells can promote tumor growth was lacking. In this study, we provide first direct evidence that expanded Treg cells can indeed promote tumor progression by using mice with selectively expanded Treg cells before inoculation of BCL1 leukemia. Finally, we have also shown that elimination of some immunoescape mechanism (e.g., deletion of Treg) can significantly improve the therapeutic outcome of chemotherapy.

Introduction
In this study, we used a conjugate of a synthetic, water-soluble, and biocompatible copolymer based on N-(2-hydroxypropyl)methacrylamide (HPMA) with doxorubicin bound via a Gly-Phe(D,L)-Leu-Gly spacer (1, 2) and containing either human polyclonal or monoclonal antibody (mAb). B1 mAb specifically binds with high affinity to an idiotype of surface IgM on BCL1 leukemia (3). Thus, we used a conjugate containing potent cytostatic drug specifically targeted to BCL1 cells (2), which enabled us to effectively treat mice bearing the leukemia while causing only minimal damage to the immune system (1, 4).

Because genetic instability is a hallmark of all malignant cells (5), tumors accumulate enormous number of mutations, and although that only small proportion of these occur in open reading frames of genes expressed by tumor cells, it is inevitable that any given cancer cell will express at least few new antigenic determinants that could be recognized by the immune system (6). Numerous innate and adaptive immune effector cells participate in the recognition and destruction of cancer cells, a process that is known as cancer immunosurveillance (5–7). Cancer cells can escape innate and adaptive immune either by immunosubversion or by immunoselection (8–12). Immunosubversion is a process of active suppression of the immune response by tumor cells. Tumors use numerous different mechanisms (further referenced as immunoescape mechanisms) of immunosubversion (8, 13–16), including down-regulation of MHC class I expression (9, 10) and up-regulation of expression of CD95L (16), indoleamine-2,3-dioxygenase (14, 15), and arginase-1 or transforming growth factor-β and interleukin (IL)-10 (17). Moreover, a significant expansion of regulatory T (Treg) cells, which are capable of inhibiting both CD4+ and CD8+ T-cell responses, was observed during progression of many types of tumors both in mice and humans (18).

It was reported previously that conjugates based on poly(HPMA) containing doxorubicin are capable to completely cure tumor-bearing mice and that some of these mice show tumor-specific resistance (1, 4). The goal of our study was to investigate this resistance; specifically, we aimed to determine which subset of immunocompetent cells is necessary for the establishment and which for the maintenance of the resistance. Another aim was to determine which immunoescape mechanisms can be used by BCL1 cells to avoid rejection by immune system. We identified several mechanisms used by BCL1 leukemia and we also showed that their abrogation can significantly improve the therapeutic outcome of chemotherapy. Moreover, we provided the first direct evidence that mice with expanded Treg cells show faster tumor progression than mice with normal counts of Treg cells.

Materials and Methods
Mice. Female BALB/c mice were obtained from the breeding colony at the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i. Mice were used at 9 to 15 wk of age. All experiments were approved by the Animal Welfare Committee at the Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

Cell lines. The murine B-cell leukemia BCL1, B16F10 melanoma, and RAW264.7 cell lines were purchased from the American Type Culture Collection.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Marek Kovař, Department of Immunology and Gnotobiology, Institute of Microbiology and Gnotobiology, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, Prague 4-Krc 14220, Prague, Czech Republic. Phone: 420-241-062-366; Fax: 420-241-721-143; E-mail: makovar@biomed.cas.cz.

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Collection. DC2.4 dendritic cell line and J774.A1 were kindly provided by Professor Jonathan Sprent (Garvan Institute, Sydney, Australia).

Monoclonal antibodies. The following anti-mouse mAbs have been used for cell surface staining: CD80-APC (eBioscience), CD86-APC (CalTag-Innvetiro), CD4-PE, CD4-PerCP, H2Kd-PE, 1-A/E-PE (BD Pharmingen), CD8-PB (Serotec), CD80-biotin, CD86-biotin (Southern Biotechnology Associates), and CD25-APC. Unconjugated anti-mIg-2 mAb JES6-1A12, anti-mouse Foxp3-PE, and IFN-γ-PE mAb were purchased from eBioscience. B1 mAb was prepared in our laboratory as described earlier (2).

Staining for surface antigens. Single-cell suspension was prepared from spleens. After RBC lysis, cells were resuspended in flow cytometry buffer (PBS/2% FCS/0.05% azide), blocked by 10% mouse serum for 30 min on ice, and stained with mAbs for 30 min on ice in the dark. When biotinylated mAbs were used, cells were additionally incubated for 10 min on ice with fluorochrome-conjugated streptavidin. Cells were washed twice after each step in flow cytometry buffer and fixed in 4% paraformaldehyde before analysis.

Intracellular staining. Foxp3 staining buffer set (eBioscience) was used for Foxp3 staining and IC Fixation buffer and Permeabilization buffer (eBioscience) were used for IFN-γ staining. Surface antigens were stained as described above. Cells were then resuspended in 100 μL of Fixation/Permeabilization working solution and incubated for 30 to 60 min on ice followed by washing twice with Permeabilization solution. Afterwards, cells were blocked by 2% mouse serum for 10 min on ice. mAb-PE conjugate was added and cells were incubated for 30 min on ice in the dark. Finally, cells were washed twice in Permeabilization buffer and fixed in 4% paraformaldehyde.

Flow cytometry. Flow cytometric analysis was performed on LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Depletion of CD4+, CD8+, and CD25+ populations. CD4+, CD8+, and CD25+ subpopulations were depleted by i.p. injection of the following mAbs: 0.034 mAb (clone GK 1.5), 0.028 mAb (clone 53-6.72), and 0.025 mAb (clone PC 61.5). Two hundred fifty micrograms per mice of all mAbs were injected.

Reverse transcription-PCR. Total RNA was isolated from 5 × 10^6 cells by using 1 mL of Trizol reagent (Life Technologies-Invitrogen) according to the manufacturer’s protocol. RNA (1 μg) was reverse transcribed using oligo(dT)12-18 primer and SuperScript II RNase H−Reverse Transcriptase (Invitrogen). Resulting cDNAs were used for PCR with specific primers for human polyclonal IgG instead of B1 mAb were not able to cure any mice, despite the fact that the treatment significantly prolonged survival (Fig. 1A, right). Neither free doxorubicin nor free B1 mAb was able to cure any BCL1 bearing mice even when doxorubicin was given at maximal tolerated dose (Supplementary Fig. S2). Interestingly, when the cured mice were rechallenged with a lethal dose of BCL1 cells, the mice were resistant to BCL1 leukemia (Fig. 1B). This resistance was observed even if the mice were reinoculated very long time after the treatment (4 months); thus, the phenomenon can be characterized as a long-lasting immune memory. The resistance is BCL1 leukemia specific because mice are not resistant to other BALB/c-derived tumors (RAW264.7 and J774.A1; data not shown). In the next step, we investigated whether the time when treatment is administered affects the strength of the induced resistance. Indeed, when the treatment is provided very early after BCL1 cell inoculation, the cured mice are not resistant to BCL1 leukemia at all (Fig. 1C). Similarly, treatment provided relatively late during the progression of BCL1 leukemia did not leave any mice able to survive rechallenge with BCL1 cells. The optimal time frame of treatment to establish effective resistance against BCL1 leukemia was between day 7 and 11 after BCL1 cell inoculation.

Results

B1 mAb-targeted conjugates are able to cure BALB/c mice bearing BCL1 leukemia and induce BCL1-specific resistance. B1 mAb recognizes idotype of surface IgM on BCL1 cells and thus B1 mAb-targeted conjugates specifically kill BCL1 cells. Two structures of antibody-containing conjugates, called classic and star structure (20), were used (Supplementary Fig. S1). Cytostatic activity of B1 mAb-targeted conjugates against BCL1 cells in vitro was determined (Supplementary Table S1). To examine therapeutic efficacy of B1 mAb-targeted conjugates in vivo, BALB/c mice were i.p. inoculated with BCL1 cells and treated by i.v. injected conjugates. Two doses of star structure conjugate cured all experimental mice, whereas conjugate of classic structure cured 75% of BCL1 leukemia-bearing mice (Fig. 1A, left). Conjugates containing human polyclonal IgG instead of B1 mAb were not able to cure any mice, despite the fact that the treatment significantly prolonged survival (Fig. 1A, right).
Role of different subsets of immune cells in conferring resistance to BCL1 leukemia following treatment with targeted polymeric drugs. We hypothesized that immune system may contribute to efficient treatment of BCL1 leukemia by B1 mAb-targeted conjugates. Therefore, we compared the efficacy of the treatment in immunocompetent BALB/c mice versus immunocompromised nu/nu mice. Nu/nu mice do not have thymus and thus they lack T cells, but they have B cells as well as natural killer cells. Treatment with B1 mAb-targeted conjugate at a dose that cured 50% of BALB/c mice did not cure any nu/nu mice (Fig. 2A), which implies that T cells augment the therapeutic effect. To elucidate which subset of T cells plays a key role in the resistance to BCL1 leukemia, we performed experiments on BALB/c mice with depleted CD4+, CD8+, or both subsets of T cells. Surprisingly, treatment with B1 mAb-targeted conjugate was more effective in CD4+-depleted mice than in normal BALB/c mice (Fig. 2B, left). which implies that T cells augment the therapeutic effect. To elucidate which subset of T cells plays a key role in the resistance to BCL1 leukemia, we performed experiments on BALB/c mice with depleted CD4+, CD8+, or both subsets of T cells. Surprisingly, treatment with B1 mAb-targeted conjugate was more effective in CD4+-depleted mice than in normal BALB/c mice (Fig. 2B, left). However, all mice that were CD4+ depleted during treatment died on rechallenge with BCL1 cells, although significantly later than control mice (Fig. 2B, right). When CD8+ or both CD4+ and CD8+ cells were depleted, establishment of BCL1-specific resistance was completely abrogated (Fig. 2C). To confirm that CD8+ cells are essential for the resistance to BCL1 leukemia, we depleted CD8+ or CD4+ cells in mice that survived treatment of BCL1 leukemia with B1-targeted conjugate as well as subsequent rechallenge with BCL1 cells. Depletion of CD8+ cells but not CD4+ cells before second rechallenge with BCL1 cells caused abrogation of the resistance (Fig. 3A). CD4+ cells are thus required for establishment of the resistance but CD8+ cells are responsible for maintaining long-lasting BCL1-specific resistance. Next, we tried to elucidate whether BCL1-specific memory T cells can be found only in CD8+ compartment or also in CD4+ T-cell subset. As shown in Fig. 3B, both CD8+ and CD4+ T-cell subsets in spleen of BCL1-resistant mice contain ~1% of cells, which express IFN-γ on coculture with BCL1 cells. Almost all of these BCL1-specific CD8+ and CD4+ T cells are CD44high, showing that they have phenotype of memory T cells. When splenocytes from BCL1-resistant but not from naive BALB/c mice were cocultured with BCL1 cells, very high concentrations of IFN-γ and also TNF-α were found in the supernatants (Fig. 3C). IFN-γ and TNF-α production was BCL1 specific, as these cytokines were not detected after coculture of splenocytes from BCL1-resistant mice with other BALB/c-derived tumor cells (J774A.1; data not shown). Once we realized that BCL1-specific T cells produce IFN-γ and TNF-α on contact with BCL1 cells, we decided to test the effect of these cytokines on proliferation of BCL1 cells. Figure 3D shows that proliferation of BCL1 cells is quite sensitive to IFN-γ (50% inhibition of BCL1 cell proliferation at ~1 ng/mL), whereas TNF-α has no effect on proliferation of BCL1 cells within the tested concentration range (10 pg/mL to 10 ng/mL).

Figure 1. B1 mAb-targeted conjugates are able to cure BALB/c mice bearing BCL1 leukemia and establish BCL1 resistance. A, BALB/c mice bearing BCL1 leukemia were treated either with B1 mAb-targeted conjugates (left) or conjugates containing irrelevant human polyclonal IgG (right) of either star or classic structure. Treatment was provided i.v. on days 11 and 14 after tumor cell inoculation (5 × 10⁵ i.p.) and one dose contained 5 mg/kg doxorubicin. Control mice were injected with the same volume of saline (300 µL). B, completely cured mice from A were reinoculated i.p. with lethal dose of BCL1 cells (5 × 10⁴) on day 110 and left without any treatment. C, BALB/c mice bearing BCL1 leukemia were treated with single dose of star structure of HPMA copolymer-bound doxorubicin conjugate targeted with B1 mAb. One dose containing 5 mg/kg doxorubicin was injected i.v. at selected intervals after i.p. inoculation of 5 × 10⁵ BCL1 cells (day 3, 7, 11, or 15). The table on the right shows portion (%) of LTS (>90 d) after treatment as well as portion (%) of LTS after i.p. inoculation of 5 × 10⁴ BCL1 cells (the numbers in brackets show mean survival time). All experiments were done at least twice with similar results.
BCL1 cells use several immunoevasive mechanisms to abrogate immune response. Because many types of cancer evade immune response by down-regulation of MHC I expression, we looked on the expression of this key molecule on BCL1 cells. Surprisingly, we found that BCL1 cells express MHC I at level comparable with normal B cells (data not shown). This applies also for MHC II and two costimulatory molecules, CD80 and CD86 (Fig. 4). CD80 expression was relatively high, whereas the expression of CD86 was intermediate. Strikingly, these data imply that BCL1 cells could work as antigen-presenting cells (APC) and thus could prime BCL1-specific CD4+ and CD8+ T-cells. On the contrary, BCL1 cells also express PD-L1, PD-L2, and IL-10 (Fig. 4B). IL-10 was also detected in supernatant of ex vivo–cultured BCL1 cells but not in supernatant of ex vivo–cultured splenocytes from normal BALB/c mice (Fig. 4B). IL-10 was also detected in supernatant of ex vivo–cultured BCL1 cells but not in supernatant of ex vivo–cultured splenocytes from normal BALB/c mice. Furthermore, we found that level of IL-2 was significantly decreased in serum of mice bearing BCL1 leukemia. This implies that the serum contained p40 dimers, which are potent IL-12 antagonists. Next, we decided to examine whether BCL1 leukemia has any effect on population of Treg cells, which are known to hamper antitumor immunity by suppressing both CD4+ and CD8+ T-cell responses. As shown in Fig. 5A, relative number of Treg cells sharply increased during progression of BCL1 leukemia. Determination of Treg cells was based on CD25+Foxp3+ double positivity in CD4+-gated cells. We showed that the increase of Treg cells is not only relative (Fig. 5B, left and middle) and that also absolute number of Treg cells increased (Fig. 5B, right). No significant increase of CD4+CD25+Foxp3+ (i.e., activation of naive CD4+CD25+ cells) was observed. Then, we performed experiment when we either deliberately expanded or depleted population of Treg cells in BALB/c mice before inoculation of BCL1 leukemia. Mice with expanded Treg cell population (40% of CD4+ cells) died significantly earlier than control mice, whereas mice depleted from Treg cells died significantly later (Fig. 5C and D). This finding strongly suggests that BCL1 leukemia uses expansion of Treg cell population to protect itself from immune response.
Elimination of T<sub>reg</sub> cells improves treatment with suboptimal dose of B1 mAb-targeted conjugate. We examined whether abrogation of some immunoescape mechanisms of BCL1 leukemia (i.e., depletion of T<sub>reg</sub> cells) could increase therapeutic activity of B1 mAb-targeted conjugate. BCL1-bearing mice depleted from T<sub>reg</sub> cells were treated with suboptimal dose of B1 mAb-targeted conjugate. Indeed, treatment was more effective in T<sub>reg</sub> cell–depleted mice than in control mice containing normal numbers of T<sub>reg</sub> cells (Fig. 6A). This effect is rather modest than strong (three versus one cured mouse), but on the other hand, the experiment was done twice using relatively large experimental groups (eight mice), and thus we believe that elimination of T<sub>reg</sub> cells indeed improves treatment with suboptimal dose of B1 mAb-targeted conjugate. All mice cured in these two experiments were resistant to BCL1 leukemia, which was proved by rechallenge of these mice with lethal dose of BCL1 cells (data not shown).

Discussion

The conjugates based on poly(HPMA) containing doxorubicin as cancerostatic drug and their antitumor activity were previously described in several different tumor models (1, 2, 4). We and others have previously highlighted the advantage of use of such conjugates in comparison with free drug, and now we show some interesting features and important immunologic mechanisms induced by the treatment with this novel class of anticaner drugs.

Single-dose treatment of BALB/c mice at various time points after BCL1 cell inoculation showed that the treatment must be given in optimal time frame (day 7–11) to establish BCL1-specific protective immunity in cured mice. As we show in Supplementary Table S2, a small mass of death tumor cells could be a reason explaining ineffective establishment of protective antitumor immunity in mice cured too early after tumor cell inoculation. Although this study was done with EL4 T-cell lymphoma, we believe that the results are also applicable to BCL1 leukemia as we have strong experience with both these cell lines and we know that the pattern of anti-EL4–specific and anti-BCL1–specific protective immunity is very identical. We also checked whether the failure to establish effective anti-BCL1 leukemia when the treatment is given too late could be caused by increased numbers of T<sub>reg</sub> cells. However, this explanation is not the right one, as T<sub>reg</sub> cell numbers remain constant at least until day 15 after i.p. inoculation of 5 × 10<sup>5</sup> BCL1 cells at day 0 (Supplementary Fig. S3).

Figure 3. CD8<sup>+</sup> T cells are essential for long-lasting resistance to BCL1 leukemia. A, BALB/c mice completely cured from BCL1 leukemia with B1 mAb-targeted conjugate were i.p. reinoculated with 5 × 10<sup>4</sup> BCL1 cells. Left, LTS (>90 d) were either depleted from CD8<sup>+</sup> cells or not and again i.p. reinoculated with 5 × 10<sup>4</sup> BCL1 cells; right, LTS that were depleted either from CD4<sup>+</sup> cells or from both CD8<sup>+</sup> and CD4<sup>+</sup> cells. B, spleen cells from LTS were cocultivated under standard conditions (37°C, 5% CO<sub>2</sub>, humidified atmosphere) for 7 h with BCL1 cells. Brefeldin A was added for the last 5 h of incubation (1 μmol/L). Cells were then stained for extracellular markers, washed, and fixed. Next, cells were permeabilized and stained for mouse IFN-γ. C, splenocytes isolated from LTS or naive BALB/c mice were cultured either alone or with BCL1 cells. After 24 h, supernatants were collected and concentration of selected cytokines was measured by ELISA. D, BCL1 cells were isolated from spleens of BALB/c mice bearing BCL1 leukemia (>94% purity). rmTNF-α and rmIFN-γ were added at desired concentrations and cells were incubated under standard condition for 72 h. [3H]thymidine (18.5 kBq) was added for the last 16 h. Cell proliferation for each experimental condition is expressed as % of [3H]thymidine incorporation into control cells (higher than 20,000 cpm/well in all experiments). Each experiment was done twice with similar results.
The role of CD8+ cells in effective antitumor response is well established, and particularly in BCL1 model, CD8+ cells were shown to be vital for the allogeneic elimination of clonogenic leukemia cells (22). Once we confirmed that CD8+ T cells are indeed the essential subset responsible for long-lasting BCL1-specific immune memory, we focused our interest on possible interactions of BCL1 cells with the immune system and immunoevasive mechanisms of this tumor. First, we found that BCL1 cells do not mask against the immune system by down-regulation of MHC class I expression, which is a common mechanism of many tumors to evade the immune system (9, 10). Moreover, BCL1 cells express not only MHC class I but also MHC class II and two main costimulatory molecules, CD80 and CD86. Because coexpression of these molecules on tumor cells is rather striking, we confirmed this finding by three different methods [i.e., by flow cytometry, reverse transcription-PCR (RT-PCR), and Western blotting; Fig. 4A]. The BCL1 cells could therefore work as APCs and thus should be capable of priming BCL1-specific CD4+ and CD8+ T cells. To figure out how can BCL1 cells escape self-rejection while expressing molecules involved in T-cell priming, we intensively searched for molecules that are known to impair T-cell activation. We found that BCL1 cells express PD-L1, PD-L2, and also IL-10. It is well established that PD-L1 and/or PD-L2 expression on APCs renders those APCs strongly tolerogenic because engagement of PD-L1 or PD-L2 with their receptor PD on T cells causes either anergy or apoptosis of these T cells (23, 24). IL-10 has immunosuppressive activity on T cells, both indirectly via modulation of dendritic cell (25) and also in direct fashion (26, 27), particularly by inhibiting proliferation as well as cytokine synthesis by CD4+ cells. In addition, IL-10 inhibits the monocytic production of IL-12, an essential mediator for the development of effector functions of CD8+ T cells (28–31). Furthermore, the presence of IL-10 during the activation of CD4+ cells results in the development of regulatory phenotype of these cells (32). Increased levels of IL-10 were not found in the serum of BALB/c mice bearing BCL1 leukemia.

Figure 4. Phenotype of BCL1 cells resembles phenotype of APCs with tolerogenic activity. A, BCL1 cells express both MHC class I as well as MHC class II and also two major costimulatory molecules, CD80 and CD86 (gray, isotype control). Expression was detected by either flow cytometry (left) or RT-PCR (middle). Right, detection of CD86 molecule was carried out also by Western blotting of BCL1 cell lysates as well as in other different cell types. Membrane fraction was isolated from each type of cells and protein concentration was measured. The same amount of membrane fraction (in terms of protein content) from each sample was used for analysis. Relative amount of CD86 in analyzed samples was measured by densitometry. DC2.4 is an established cell line of dendritic cells with semimature phenotype, which can be matured by IFN-γ. B, expression of selected molecules detected by RT-PCR in splenocytes from naive BALB/c mice (*) and BCL1 cells (*). RayBio Mouse Cytokine Antibody Array was used to detect various cytokines (see Materials and Methods) either in the supernatant of ex vivo–cultured BCL1 cells (B, right) or in the serum (C) of healthy BALB/c mice versus BALB/c mice bearing BCL1 leukemia. *, significant difference (P < 0.05) determined by Student’s t test. B, far right, production of IL-10 by ex vivo–cultured BCL1 was measured by ELISA using rmIL-10 as a standard. Experiments were done twice with similar results.
leukemia, showing that IL-10 produced by BCL1 cells works only in paracrine fashion. However, we found that serum of BALB/c mice bearing BCL1 leukemia had significantly decreased IL-2 level, which is also an unfavorable condition for T-cell priming. More importantly, serum of BALB/c mice bearing BCL1 leukemia contained p40 dimers, strong IL-12 antagonists (33, 34), which can severely impair the effector functions of CD8+ T cells (30). In conclusion, BCL1 cells show a phenotype of tolerogenic APCs (i.e., use rather unique immunescape strategy while taking advantage of their ability to make cell-cell contact with T cells and anergize or delete those that are BCL1 specific).

Naturally arising CD25+ CD4+ Treg cells, which constitute 5% to 10% of peripheral CD4+ T cells in normal rodents and humans, are produced in the thymus as a functionally mature and distinct subpopulation of T cells (18, 35). Constitutive high expression of CD25 plus forkhead winged-helix (Foxp3) transcription factor (36) is a typical feature of Treg cells distinguishing them from recently activated T cells, which also express CD25. They play key roles not only in the maintenance of immunologic self-tolerance (i.e., prevention of autoimmunity; refs. 37–39) but also in the control of aberrant or excessive immune responses to various invading infections (40, 41). A significant role for Treg cells has also been implicated in abrogating effective antitumor immunity (42). In this study, we showed that progression of BCL1 leukemia is accompanied by gradual increase of Treg cells (Fig. 5 A and B). The increase in Treg cell numbers is relatively common for many other types of

Figure 5. Robust expansion of Treg cells during progression of BCL1 leukemia: another immunescape mechanism used by BCL1 leukemia. A, BALB/c mice were i.v. inoculated with 5 x 10^6 BCL1 cells. Mice were euthanized at different time points after BCL1 cell inoculation and Treg cells were detected either as CD4+CD25+ or CD4+CD25+Foxp3+ cells (top or bottom row, respectively) by flow cytometry. Number in each dot plot shows their ratio (%) to all CD4+ cells. Each dot plot shows one representative mouse of three experimental animals. B, kinetics of relative increase of CD4+CD25+ (left) and CD4+CD25+Foxp3+ (middle) cell populations in BALB/c mice inoculated as in A (four mice per each experimental group) and absolute numbers of CD4+CD25+ and CD4+CD25+Foxp3+ cells (black columns) in spleen at day 12 (white columns). White columns, control mice. C, three groups of BALB/c mice, each with different size of Treg cell population, were i.p. inoculated with 5 x 10^5 BCL1 cells at day 0. Control group was not manipulated in Treg cell population. Next group was depleted from Treg cells (Treg depleted) and third group had strongly expanded Treg cell population (Treg expanded). Left, size of Treg cell populations in these three experimental groups at days 0 and 7; right, survival of mice in each experimental group. D, mean survival time (left) and an increase in body weight reflecting the progression of the disease (right) among the experimental groups described above in C. *, statistically significant difference (P < 0.05, Student's t test). Experiments were done twice with identical results.
The efficiency of Treg cell depletion was checked by flow cytometry from small samples of peripheral blood taken at day 11. On the same day, one group with normal Treg cell population was i.v. inoculated with 5 \times 10^5 BCL1 cells at day 0 and distributed into four groups, each containing eight mice. Control group was left untreated, whereas mice in two other groups were i.p injected with anti-CD25 mAb (150 μg/mice) on day 8. 

Depletion of Treg cells by anti-CD25 mAb was shown to augment antitumor immunity in several models (44–47). Such depletion can use even very low concentration of IL-2 in tumors and can be seen both in humans and rodents (43, 44). Depletion of Treg cells by anti-CD25 mAb can use even very low concentration of IL-2. Higher number of Treg cells may explain the significantly lower serum concentration of IL-2 in BCL1 leukemia-bearing mice compared with healthy mice (Fig. 4C). Treg cells are IL-2 dependent (49) and can use even very low concentration of IL-2. Higher number of Treg cells using IL-2 thus can cause a substantial decrease of IL-2 in the serum.

About the establishment of BCL1 resistance triggered by the therapy with targeted conjugates, we hypothesize that the treatment causes a massive death of BCL1 cells, whereas a considerable amount of cell fragments and other material is released and available for cross-presentation on dendritic cells. It is of special note that our targeted conjugate has negligible immunosuppressive activity (1, 4), and thus, nonimpaired immune system is ready to elicit BCL1-specific response. As we showed in this study, BCL1 cells are theoretically capable of antigen presentation to both CD4+ and CD8+ T cells but in tolerogenic/proapoptotic fashion. Thus, the cross-presentation of BCL1-specific antigens on host dendritic cells is particularly important to establish BCL1-specific resistance.

In conclusion, our results show that identifying the immunoescape mechanisms used by the particular tumor to evade immune response could be very important for the therapy. Elimination of these mechanisms most likely augments standard treatment procedure and can lead to complete remission with an establishment of long-lasting resistance to the tumor. Here, we showed that it could be possible to turn a tumor into its own cellular vaccine by combination of chemotherapy and immunomodulation.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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