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Lymphoma Immunotherapy with CpG Oligodeoxynucleotides Requires TLR9 Either in the Host or in the Tumor Itself

Jiali Li,* Wenru Song,* Debra K. Czerwinska,* Bindu Varghese,* Satoshi Uematsu,†
Shizuo Akira,‡ Arthur M. Krieg,§ and Ronald Levy2*

Established widely metastatic tumor was cured in a transplanted mouse B cell lymphoma model, by the combination of chemotherapy plus intratumoral injection of oligodeoxynucleotides containing unmethylated C-G motifs (CpG). This therapeutic effect required that the CpG be injected directly into the tumor and was dependent on CD8 T cells. Although the efficacy of CpG oligodeoxynucleotides has been thought to depend on the expression of TLR9, we unexpectedly found that tumor rejection did not require host expression of TLR9. By using a TLR9-deficient tumor and a TLR9KO host, we demonstrate that TLR9 expression either by the host or the tumor is required. These results indicate that activation of Ag presentation by cells within the tumor via TLR9 stimulation can be an effective form of immunotherapy. This study forms the basis of an ongoing clinical trial in patients with lymphoma. The Journal of Immunology, 2007, 179: 2493–2500.

lymphoma, a malignancy of the immune system, is responsive both to passive immunotherapy, such as mAbs (1), and to active immunotherapy (2, 3) We have previously shown that injection of dendritic cells (DC)3 into a single tumor site, in combination with systemic chemotherapy, can completely eradicate established and metastatic lymphoma in an animal model (4). In this model, the injected DC were able to process tumor Ags released from the dying tumor cells and to induce a systemic immune response against the tumor that was mediated by CD8 T cells. However, this approach requires the preparation of DC, which must be MHC matched to the host. For therapy in humans, the DC would need to be derived from and produced on a customized basis for each patient. It would be more convenient to activate the DC directly in situ and to avoid the need for ex vivo cell production. The activation of DC in cancer patients has been challenging because DC at the tumor site are often dysfunctional and unable to prime T cells efficiently (5). Several biological agents, such as CD40L, 4-1BBL (6), TNF (7), and oligodeoxynucleotides (ODN) containing certain unmethylated C-G motifs (CpG) activate DC and B cells by inducing their expression of costimulatory molecules and by triggering cytokine production (8–10). CpG ODNs are ssDNA fragments containing immune stimulatory sequence motifs similar to those found in microbes. DC and B cells have a receptor for CpG, TLR9, which initiates an activating signal for these cells (11, 12). In the current study, we substituted CpG for intratumoral injection of DC in the therapy model for murine B cell lymphomas. The systemic therapeutic effects previously obtained by DC injection were replicated by CpG injection. The therapeutic effect of CpG depended upon its injection directly into a site of dying tumor cells. This course resulted in a systemic CD8-dependent T cell immune response against the tumor. As expected, this immunizing effect of CpG required the presence of the TLR9, but unexpectedly this receptor could be present exclusively either in the host or in the tumor cells.

Materials and Methods

Reagents

CpG 1826 with primer sequence 5′-TCCATGACGTTCCTGACGTT (the bold nucleotides represent the immunostimulatory CpG sequences) and control ODN 2138 with primer sequence 5′-TCCATGACGTTCCTGACGTT were provided by Coley Pharmaceutical Group (Ottawa, Ontario, Canada).

Cell lines and animal models

A20, a BALB/c B cell lymphoma line expressing MHC class I and class II H-2d molecules, was obtained from American Type Culture Collection. Tumor cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Invitrogen Life Technologies), and 50 µM 2-ME (Sigma-Aldrich), as complete medium. Cells were grown in suspension culture at 37°C in 5% CO2. Four to 6-wk-old female BALB/c mice were purchased from Harlan Sprague Dawley Laboratories and were housed at the Laboratory Animal Facility at Stanford University Medical Center (Stanford, CA). CD8 knockout (KO) mice and B cell-deficient mice (JHBD) on the BALB/c background were provided by Dr. C. G. Fathman and Dr. P. Utz (Stanford University School of Medicine, Stanford, CA), respectively. TLR9KO mice (12) backcrossed five generations to BALB/c were obtained from Dr. S. Akira (Coley Pharmaceutical Group, Wellesley, MA). The BALB/c syngeneic A20 lymphoma tumor grows with a similar efficiency in the TLR9−/− BALB/c strain as in wild-type BALB/c mice. All experiments were conducted in accordance with Stanford University Animal Facility and National Institutes of Health guidelines.

Flow cytometry

The following mAbs were used in this study: PE anti-mouse CD80, PE anti-mouse CD86, PE anti-mouse H-2d, FITC anti-mouse I-A4, PE
anti-mouse IgM, PE anti-mouse B220, and PE anti-mouse CD19, which were all purchased from BD Pharmingen. PE anti-mouse IgG1, PE rat isotype control and FITC mouse isotype control were from eBioscience. Cells were stained in standard FACS buffer (PBS, 1% FBS, and 0.01% sodium azide), studied by flow cytometry on a BD FACSCalibur System and analyzed using CellQuest Pro program.

Tumor transplantation and immunotherapy

A20 lymphoma cells were implanted s.c. on the lower back in 4- to 6-wk-old BALB/c mice at a dose of 10^5 cells in 100 μl of PBS. Treatments began when tumors reached ~1.5 cm^2, which typically occurred at day 20 post-inoculation of tumor. The chemotherapy agent cyclophosphamide (CTX) was then i.p. administrated at a dose of 100 mg/kg on two consecutive days. CpG was then given intratumorally at 100 μg/dose in a volume of 100 μl on days 1, 2, 3, 6, and 8 postchemotherapy. The growth of tumor was monitored by a caliper three times per week, and expressed as length by width in square centimeters. Mice were sacrificed when tumor size reached 5 cm^2 or when tumor sites ulcerated. In some experiments, CpG 1826 was given either intratumorally, peri-tumorally, or at a distant s.c. site.

Detection of tumor reactive T cells

Mice were sacrificed nearly 1 wk after CpG vaccine; spleens were harvested and made into single-cell suspensions, and the RBCs were lysed. A total of 5 × 10^5 splenocytes were cocultured with 5% irradiated A20 cells (10^5) for 24 h in the presence of monensin at 37°C and 5% CO2. Afterward, cells were washed and surface stained with anti-CD8 FITC and anti-CD4 allophycocyanin (BD Biosciences). Intracellular IFN-γ expression was assessed using BD CytoFix/Cytoperm Plus kit (catalog no. 554715) per instructions, and BD anti-IFN-γ PE-conjugated Ab. Cells were analyzed using a FACSCalibur and CellQuest Pro software (BD Immunocytometry Systems).

Depletion of CD4 and CD8 T cells

Ascitic fluid was harvested from mice bearing lymphoma GK1.5, 2.43, or SFR8-B6 producing anti-CD4, anti-CD8 Abs, or isotype control, respectively. The ascites were diluted in PBS and filtered. Diluted ascitic fluid containing 1 mg/ml mAbs were injected i.p. at a dose of 0.5 mg in a volume of 500 μl on 3, 2, 1, and 0 days before treatment started, and weekly thereafter for the duration of the experiment. These depletion conditions were validated by flow cytometry of splenocytes using PE-conjugated anti-CD4 (Caltag Laboratories) and anti-CD8 (BD Pharmingen) mAbs that do not compete with the earlier Abs used for in vivo depletion. More than 95% of the relevant cell subset was depleted, whereas all of the other subsets remained at normal levels.

Detection of anti-A20 Ab in the serum

Mice from each treatment group were bled on day 30, and serum was pooled and collected by centrifugation. A total of 5 × 10^5 A20 cells in 100 μl of FACS buffer were incubated with 1 μl of undiluted serum for 30 min at 4°C, cells then washed with FACS buffer twice, stained with PE-conjugated anti-mouse IgG1 for 30 min, and washed to remove unbound Ab. The bound Ab level was detected by flow cytometry.

Generation of TLR9KO lymphoma cell line

Primary bone marrow cells were isolated from 4-wk-old TLR9KO donor mice under sterile conditions, and cultured on a monolayer of irradiated stromal cell line AC6.21. The retrovirus vector MSCV-neo/p190Bcr-Abl, which carries the oncogene Bcr-Abl, was a gift from Dr. M. Cleary and Dr. K. Smith (Stanford University School of Medicine, Stanford, CA) (13). Virus was produced by transfecting the Phoenix-E packaging cell line. Primary bone marrow cells were infected by the recombinant retrovirus and the transduced cells were selected and cultured in complete RPMI 1640 medium. The emerging transformed cell line was designated TLR9KO B/A. The phenotype of this cell line was determined by flow cytometry, which showed that it expressed the B lineage markers B220 and CD19, as well as CD80 and CD86 at levels similar to A20 cells, but was negative for MHC II and IgM. As expected, unlike wild type B cells and unlike the A20 tumor, it does not respond to CpG, and does not increase its expression of CD80 and CD86 after exposure to CpG. To obtain a stable clone growing in vivo, TLR9KO B/A cells were implanted at a dose of 5 × 10^6 into 10 wild-type BALB/c mice. On day 10 postinoculation, the mouse that had the largest tumor (1.2 cm in diameter) was sacrificed. The tumor was removed and dissected under sterile conditions, and a single-cell suspension was cultured in complete RPMI 1640 medium. The cells were then expanded and frozen as a working cell bank.

CpG IMMUNOTHERAPY REQUIRES TLR9 IN HOST OR TUMOR

FIGURE 1. CpG induces the expression of costimulatory molecules on A20 cells in vitro. A20 cells (10^5 cells/ml) were incubated for 72 h with either CpG 1826 or control ODN 2138 at a final concentration of 3 μg/ml, or with medium alone. Cells were analyzed for the expression of CD80 (a) and CD86 (b) by direct immunofluorescence and flow cytometry.

Results

CpG 1826 induces the expression of costimulatory molecules on A20 cells

The expression of TLR9 by A20 B cell lymphoma cells was confirmed by Western blot analysis and real-time PCR (data not shown). We next tested whether the A20 cells could respond directly to CpG ODN. A20 cells were incubated with CpG (3 μg/ml) for 72 h, and the expression of the costimulatory molecules CD80 and CD86 was measured by flow cytometry. As shown in Fig. 1, CpG 1826 significantly induced the expression of both CD80 and CD86 on these A20 cells, an effect that was not seen with the non-CpG control ODN 2138 (14).

CpG inhibits the proliferation of A20 cells in vitro

We then tested for a direct effect of CpG on the proliferation of A20 lymphoma in vitro. Tumor cells were incubated for 3 days with CpG at concentrations from 0.0003 to 30 μg/ml. Proliferation of A20 cells was measured by [3H]thymidine incorporation. CpG 1826 inhibited A20 proliferation in a dose-dependent manner, whereas the control ODN had no effect (Fig. 2). This inhibitory effect of CpG 1826 on the growth of the A20 lymphoma cells was
striking and in contrast to its potent proliferative and antiapoptotic effects on normal B cells (15).

CpG in combination with chemotherapy can cure large established tumors

A20 lymphoma cells implanted s.c. grow progressively and reach \( \sim 1.5 \text{ cm}^2 \) by day 20. At this time the malignancy is also widely metastatic in the animals, intratumoral injection of CpG caused only transient tumor regression (Fig. 3a). Treatment with systemic CTX likewise caused a transient regression of the tumor (Fig. 3b). In contrast, the combination of intratumoral CpG with systemic CTX resulted in complete and permanent regression of the local s.c. tumors and cure of the animals (Fig. 3c). This permanent therapeutic effect was dependent on the injection of the CpG directly into the tumor nodule. When the CpG was injected into a distant s.c. site or even into a peritumoral site, the therapeutic effects were no greater than that seen with CTX alone (Fig. 3, d and e). This result implied that a direct encounter between CpG and a cell within the tumor nodule, either the malignant B cells or with an infiltrating APC, was required to induce a systemic therapeutic effect.

CpG in combination with CTX results in a systemic antitumor effect

To test whether CTX-CpG therapy had a systemic antitumor effect, mice were inoculated with lymphoma cells at two different sites (upper back and lower back (Fig. 4a)), and then divided into four groups: 1) control (no treatment), 2) CTX alone, 3) CpG alone, and 4) CTX combined with CpG. CTX was injected i.p. as in all other experiments. CpG was injected only into the tumor site on the upper back. CpG alone had no effect on the growth of tumor at the nontreated sites on the lower back, and there was no significant difference in survival between CpG and control groups (data not shown). The combination of CpG with CTX (group 4) significantly prolonged the survival of mice compared with the group 2 (control group receiving CTX alone; \( p < 0.001 \)) as shown in Fig. 4b. In group 4 (CTX plus CpG), six of nine mice had tumor remission during 60 days observation, and the recurrence of tumor growth was significantly delayed as compared with group 2 (CTX alone). Fig. 4c shows the comparison of average tumor growth at sites on the upper and lower back in each group. The data indicate that the combined CTX plus CpG treatment significantly inhibited the growth of tumor at both the treated and untreated sites and delayed the tumor recurrence (\( p < 0.001 \)).
Host B cells are not required for the antitumor effects of CpG plus CTX

TLR9 is expressed both in the A20 tumor and in the host immune cells, including normal B cells and DC (data not shown). To address whether host B cells were required for the therapeutic effect of CpG plus CTX, experiments were performed in B cell-deficient (JHD) mice. A20 cells were inoculated into wild-type BALB/c mice or into JHD mice. Mice were treated with the CTX combined with CpG therapy as described. The treatment cured the tumor-bearing JHD mice with results identical with those observed in wild-type hosts (Fig. 5). Most of the wild-type mice, but not the JHD mice, made Abs against the A20 tumor, but there was no relationship between Ab titers and tumor growth or survival in individual mice (data not shown). From these results we conclude that neither host B cells nor Abs are involved in the therapeutic effect.

FIGURE 5. The therapeutic effect of combination of chemotherapy plus CpG is not dependent on B cells. Wild-type BALB/c mice (a) and B cell-deficient (JHD) mice (b) were inoculated with A20 cells and mice were treated with CTX followed by intratumoral injection of CpG as described. Each curve represents serial measurements of tumor size of an individual mouse.

FIGURE 6. T cell immune response. In two separate experiments, splenocytes alone from mice inoculated with A20 tumor and then treated with chemotherapy alone vs CTX plus CpG (CTX + CpG) (a) or CpG alone vs chemotherapy plus CpG (CTX + CpG) (b) were cocultured with irradiated A20 cells. CD8 and CD4 T cells were assayed for intracellular IFN-γ using a PE-conjugated Ab. The percentages of CD8 and CD4 T cells expressing IFN-γ in the CTX mouse (A) and the CpG mouse (B) were similar at 0.5% and 0.6% vs 0.2% and 0.2%, respectively. This expression is in comparison to the CTX with CpG mouse in which the IFN-γ expression was 3.7% and 9.1% of the CD8 T cells vs 1.0% and 1.1% of the CD4 T cells, respectively.

FIGURE 7. The therapeutic effect of chemotherapy plus CpG is dependent on CD8 T cells, but not on CD4 T cells. CD4 or CD8 T cells were depleted by i.p. injection of ascitic fluid containing 0.5 mg of anti-CD4 T cells (GK1.5 hybridoma) or anti-CD8 T cells (2.43 hybridoma), respectively. Complete depletion of the CD8 T cell or the CD4 T cell populations was confirmed by flow cytometry of peripheral blood. Abs were given days 3, 2, 1, and 0 before chemotherapy and the depletion was maintained by weekly injections. Alternatively, BALB/c CD8 KO mice were used. Tumor-bearing mice were treated with the combination of chemotherapy plus CpG and tumor growth was measured. Groups included immune-compotent mice (a), CD4 T cell-depleted mice (b), CD8 T cell-depleted mice (c), and CD8 KO mice (d). Each line represents the serial measurement of tumor size of an individual mouse. In the CD8-depleted and CD8 KO groups, tumor size represents the sum of local injected site plus the measurable metastatic sites. Dorsal (e) and ventral (f) sides of the same three CD8-depleted mice are shown. The primary tumor site and sites of metastatic tumors are indicated.
A T cell immune response is induced against the tumor

To search for a T cell immune response against the tumor we examined the spleens of tumor-bearing mice 2 wk after being treated with CTX alone (group 2), CPG alone (group 3), or with CTX plus intratumoral Cpg (group 4), at a time when tumors had regressed in response to treatment. After stimulation in vitro with irradiated A20 tumor cells, the CD8 and CD4 T cells were separately analyzed for the production of IFN-γ by intracellular flow cytometry. We found CD8 T cells responding to A20 tumor stimulation in mice treated with CTX plus CpG but not in the mice treated with CTX alone, CPG alone (Fig. 6), or from normal mice (data not shown). These results are representative of two independent experiments.

CD8 T cells, but not CD4 T cells, are required for the antitumor effects of CpG with CTX

To study the role of individual T cell subsets in the therapeutic regimen, CD4 or CD8 T cells were depleted from the host by treatment with the respective mAbs. The therapeutic maneuver was also assessed in CD8-deficient mice. The body tumor burden (including both local tumor and distant lymph nodes) was compared among different treatment groups as shown in Fig. 7. Depletion of CD4 T cells had no effect on the therapeutic outcome either at the local site or at the distant sites (Fig. 7b). By contrast, depletion of CD8 T cells abolished the therapeutic effect, as shown by recurrence of tumors by day 60 in 100% of these mice (Fig. 7c). Results in CD8 T cell-deficient animals (Fig. 7d) confirmed the dependence on CD8 T cells. Interestingly, in CD8-depleted animals, the treatment was effective in controlling tumor growth at the primary injected sites (Fig. 7e). However, mice in this group had progressive distant tumor metastasis, as shown in Fig. 7f. This result suggested that CD8 T cells are required to control systemic tumor cells, but that control of the local injected site may be due either to a local innate immunity triggered by CpG or a direct antiproliferative effect of CpG.

FIGURE 8. Combined chemotherapy plus CpG remains effective in A20-bearing cells. A20 cells (107 cells) were inoculated into wild-type BALB/c mice and into TLR9KO mice. Treatment began when tumors reached 1.5 cm². Mice were treated with CTX, Cpg, or CTX with Cpg as described in Fig. 4. The growth curves of tumors in wild-type BALB/c (WT) mice (a) and TLR9KO mice (b) are shown. Each line represents serial measurement of the tumor size of an individual mouse.

TLR9 expression by host cells is dispensable for the antitumor effect of CpG plus CTX

To test the requirement for interactions between CpG and host immune cells, A20 tumor cells were inoculated into wild-type BALB/c mice or into TLR9KO. In the latter, only the tumor cells express TLR9. The A20 tumor grew equally well in both these mouse strains (data not shown). As before, monotherapy with CTX alone or with Cpg alone transiently delayed the growth of A20 lymphoma, and this delay was the case in wild-type as well as TLR9KO hosts (Fig. 8a and b, left and middle panels). Surprisingly, the combination of CTX with intratumoral injection of Cpg was effective both in wild-type hosts (Fig. 8a, right) and in TLR9KO hosts (Fig. 8b, right). It is possible that tumor regression was more rapid in the wild-type host than in the TLR9KO host, suggesting an additive effect when TLR9 was present both in the tumor and in the host. This result was confirmed in two independent experiments as well as in sixth generation backcross TLR9KO mice. Just as in wild-type mice, the antitumor effect of the treatment in the TLR9KO hosts was dependent on CD8 T cells (data not shown). Taken together, these results suggest that the TLR9 expressed in the host APC is not required to initiate the therapeutic T cell immune response.

The therapeutic effects of CpG plus CTX require the TLR9 in the host if TLR9 is absent from the tumor

The experiments described suggested that the TLR9 present in the lymphoma cells might be sufficient to mediate the antitumor effects of CpG plus CTX. This hypothesis predicts that if TLR9 were missing from the tumor then the TLR9 would be required in the
host. To test this hypothesis, we generated a new TLR9KO lymphoma line from a TLR9KO mouse. This TLR9KO B/A pre-B cell lymphoma grew equally well in wild-type and in TLR9KO mice, but with a much more rapid growth rate compared with the A20 lymphoma cell line. As expected, it showed no phenotypic or in vitro growth effects in response to CpG in vitro (data not shown).

Mice of the two strains were inoculated with the TLR9KO B/A tumor and then treated as described. Once again, CpG monotherapy induced transient tumor regression in wild-type mice but, as expected, it had no such transient effect on the TLR9KO mice (Fig. 9a, left and middle panels vs b, left and middle panels). Nevertheless, the combined CpG with CTX therapy was completely effective on this TLR9KO tumor in the wild-type BALB/c hosts (Fig. 9a, right). This suggests that TLR9 expression by host cells is sufficient to mediate the antitumor effect of CpG plus CTX. As expected, in the TLR9KO hosts inoculated with the TLR9KO B/A tumor (Fig. 9b, right), the combined CTX-CpG treatment had no therapeutic effect and 100% of mice in this group died of progressive lymphoma.

Discussion
We have shown in this study that the combination of intratumoral injection of CpG together with systemic administration of CTX can cure animals with advanced local and widely metastatic lymphoma. This antitumor effect was dependent on a number of factors including: 1) the presence of TLR9 either in the tumor or in the host; 2) the induction of a CD8 T cell immune response; and 3) the introduction of CpG locally into the region of dying tumor cells. This approach is particularly attractive for human application because CpG has been introduced into the clinic and has a track record of safety and even antitumor efficacy (16).

CpG has been shown to have an antitumor effect in a variety of tumor models (17). This effect has been attributed to activation of host NK cells and DC, resulting in a combination of innate and acquired immunity. In addition, CpG can directly inhibit proliferation of TLR9-positive tumor cell lines, as was the case in our lymphoma model. We observed that CpG inhibited the growth of the A20 lymphoma in vitro and CpG has been shown to induce apoptosis in malignant B cells of human chronic lymphocytic leukemia patients (18). Certain chemotherapeutic drugs, in particular CTX, have been shown to synergize with immunotherapies such as CpG (19). The role of CTX in our model could simply have been to retard the tumor, allowing time for a T cell immune response to occur. Alternatively, it could have contributed in a more active way, inducing danger signals resulting from dying tumor cells or even altering the balance between activated T cells and Treg cells (20–22). We know from prior work with this model that the induction of local tumor cell death is critical and that radiotherapy just to the CpG-injected tumor site can substitute for CTX (our unpublished data). As opposed to other reports (23, 24), it was clear that the systemic antitumor effect depended on the injection of CpG directly into the tumor. These data suggest that an interaction of CpG with tumor cells or with DC at the tumor site was critical for the induction of a systemic CD8 T cell response in our model. Other reports have shown that close physical and temporal association between Ag and activation signal are important for the activation of DC (25).

DC play a central role in the immune system and are likely candidates for the initiation of tumor Ag-specific CTLs. Studies conducted by Levitsky and colleagues and others (26–28) have shown that bone marrow-derived DC, not tumor cells, are the APC.

FIGURE 9. Combined chemotherapy plus CpG is effective against TLR9KO tumors in wild-type mice but ineffective in TLR9KO tumors in TLR9KO mice. TLR9KO B/A cells (5 × 10⁶ cells) were inoculated s.c. in the lower back of wild-type BALB/c mice or TLR9KO mice. Treatment began when tumor reached 1.5 cm² on days 10–12. Mice from each background were treated with CTX, CpG, or CTX with CpG. CTX was given i.p at a dose of 100 mg/kg on two consecutive days. CpG was then given intratumorally on days 1, 2, 3, 6, and 8 postchemotherapy at a dose of 100 μg in a volume of 100 μl. Tumor sizes in wild-type BALB/c (WT) mice (a) and TLR9KO mice (b) are shown. Each curve represents the serial measurement of tumor size of an individual mouse.
that activate tumor-specific CTL, especially in nonhematologic tumor models. To initiate a cross-presentation, bone marrow-derived APC, which do not express tumor Ags themselves, must both capture extracellular Ags and receive specific activation signals, as, for example, through CD40 (29, 30). TLR9 activation in DC enhances their ability to cross-present Ags (31). In our studies, a TLR9KO tumor could be cured by CpG plus CTX in wild-type hosts, suggesting that activation of host APC was sufficient to induce the CD8 T cell immune response against tumor-derived Ags. Blander and Medzhitov (25) have shown recently that CpG can enhance cross-priming of DC. One interesting observation in our study was that CD4 T cells were not required for the therapeutic effect, whereas CD4 T cells have been shown to be essential for generation of antitumor CD8 CTL in other models (32). It is known that CpG has direct effects on DCs that are similar to the effect of CD4 T cells (33, 34). Hence, in our tumor model, CpG effects on DC or other APC may bypass the requirement for CD4 T cells. CpG might also stimulate the migration of DC into the local draining lymph nodes of the injection site where the cross-presentation can occur (35).

B cells (36), macrophages (37), and some tumor cells can also function as APC (38, 39). Tumor cells are not usually efficient APCs because they lack significant expression of costimulatory molecules (40) and may secrete cytokines that inhibit CD8 T cells (41). In the special case of B cell malignancies, however, it is possible that the tumor can become its own APC, with increased expression of costimulatory molecules, enabling it to present its own Ags directly to T cells (9, 42). A20 cells, an MHC class I- and class II-expressing B cell lymphoma, has long been recognized as a powerful APC (43, 44). Indeed, we demonstrated that CpG enhances the expression of CD80 and CD86 on A20 lymphoma cells and it has been shown by others that CpG increases the costimulatory capacities of the A20 lymphoma cells (45, 46). In the situation where host APC did not express TLR9 the host DC could still have played the key APC role. In the TLR9KO mouse, DC cannot respond directly to CpG (47) but could respond to other activation signals derived from dying tumor cells (48). Dissection of the key player, tumor or host DC, in the APC function in our model will require experiments in hosts that not only lack TLR9 but also lack APC function.

Surprisingly, we found that TLR9 expression on the lymphoma cells was sufficient to obtain the full antitumor effect of the CpG plus CTX. Therefore, B cell lymphoma provides a unique opportunity for CpG therapy through the multiple mechanisms of a direct antitumor effect, a stimulation of host NK cell function, an enhancement of Ag presentation function by the tumor and synergy with cytotoxic agents to induce a T cell immune response against the tumor.

In summary, CpG may have unique effects in B cell lymphoma by triggering immune mechanisms through TLR9 stimulation either of the host or of tumor cells. Human B cell lymphomas express TLR9. The results presented here provide the basis for an ongoing clinical trial of CpG therapy in patients with B cell lymphoma.

Acknowledgments

We thank Risini Weeratna at Coley Pharmaceutical Group (Ottawa, Ontario, Canada) for breeding the TLR9KO mice and for testing the histocompatibility of A20 tumor in these hosts. We thank Alain Vicari for editorial suggestions. We also thank Shoshana Levy for valuable discussions throughout this study.

Disclosures

Arthur M. Krieg is an employee of Coley Pharmaceuticals.

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13. Smith, K. S., J. W. Rhee, and M. L. Cleary. 2002. Transformation of bone marrow B cells (36), macrophages (37), and some tumor cells can also function as APC (38, 39). Tumor cells are not usually efficient APCs because they lack significant expression of costimulatory molecules (40) and may secrete cytokines that inhibit CD8 T cells (41). In the special case of B cell malignancies, however, it is possible that the tumor can become its own APC, with increased expression of costimulatory molecules, enabling it to present its own Ags directly to T cells (9, 42). A20 cells, an MHC class I- and class II-expressing B cell lymphoma, has long been recognized as a powerful APC (43, 44). Indeed, we demonstrated that CpG enhances the expression of CD80 and CD86 on A20 lymphoma cells and it has been shown by others that CpG increases the costimulatory capacities of the A20 lymphoma cells (45, 46). In the situation where host APC did not express TLR9 the host DC could still have played the key APC role. In the TLR9KO mouse, DC cannot respond directly to CpG (47) but could respond to other activation signals derived from dying tumor cells (48). Dissection of the key player, tumor or host DC, in the APC function in our model will require experiments in hosts that not only lack TLR9 but also lack APC function.

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