Generation of CD8^+ T cell–mediated immunity against idiotypic-negative lymphoma escapees

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We investigated the ability of CpG-oligodeoxynucleotide to generate an anti-tumor CD8^+ T-cell immune response and to synergize with passive antibody therapy. For these studies, we generated an antibody against the idiotypic on the A20 B-cell lymphoma line. This antibody caused the regression of established tumors, but ultimately the tumors relapsed. The escaping surface IgG-negative tumor cells were resistant to both antibody-dependent cellular cytotoxicity and signaling-induced cell death. Addition of intratumoral CpG to antibody therapy cured large established tumors and prevented the occurrence of tumor escapees. The failure of the combination therapy in mice deficient for CD8^+ T cells demonstrates the critical role of CD8^+ T cells in tumor eradication. When mice were inoculated with 2 tumors and treated systemically with antibody followed by intratumoral CpG in just one tumor, both tumors regressed, indicating that a systemic immune response was generated. Although antibody therapy can eliminate tumor cells bearing the target antigen, it frequently selects for antigen loss variants. However, when a poly-specific T-cell response was generated against the tumor by intratumoral CpG, even large established tumors were cured. Such an immune response can prevent the emergence of antibody selected tumor escapees and provide long-lasting tumor protection. (Blood. 2009;114:4477-4485)

Introduction

In 1982, we reported the successful treatment of a patient with B-cell lymphoma using a custom-made, anti-idiotypic (Id) monoclonal antibody (mAb). This success was followed by a study of 11 patients with B-cell malignancy each receiving an anti-Id mAb. Nearly half of these patients experienced objective remissions of their tumors, although several patients recurred with tumor cell populations that no longer expressed the target of the therapeutic antibody because of down-regulation or mutation of their surface Id.

One way to maximize antibody therapy and potentially prevent tumor escape is to combine it with adjuvant immunotherapy. Immunostimulatory oligonucleotides containing the unmethylated CpG motif (CpG-oligodeoxynucleotide [ODN]) are potent inducers of both innate and adaptive immunity and can serve as vaccine adjuvants. The immunostimulatory effects of CpG oligonucleotides are broad and include induction of B-cell proliferation and immunoglobulin production, up-regulation of costimulatory molecules (including CD80, CD86, and CD40) by B cells, macrophages, and dendritic cells, and secretion of interferon-γ induced by interleukin-12 and interferon-γ from natural killer (NK) cells. This cytokine milieu can induce the differentiation of naive T cells into Th1 cells on encountering specific antigens. We have recently shown that intratumoral injection of CpG-ODN can generate a CD8^+ T-cell immune response against B-cell lymphoma. Here, we investigated whether this in situ vaccination maneuver could prevent the outgrowth of Id-negative variant tumor cells under the selective pressure of passive anti-Id antibodies.

Methods

Reagents

CpG 1826 with sequence 5’-TCCATGACGTTCCTGACGTGTT (the bold nucleotides represent the immunostimulatory CpG sequences) was provided by Coley Pharmaceutical Group. The following mAbs were used for flow cytometry: goat anti-mouse k phycocerythrin (PE), goat IgG PE isotype control, rat anti-mouse IgG2a PE, rat IgG2a PE isotype control, mouse anti–mouse A20 Id IgG2a AlexaFluor 647, and mouse IgG2a AlexaFluor 647 isotype control. With the exception of mouse anti-A20 Id AlexaFluor 647, these antibodies were purchased from BD Biosciences PharMingen. Mouse anti-A20 Id was conjugated to AlexaFluor 647 using an antibody labeling kit from Thermo Scientific. Mouse anti-A20 Id and mouse anti–38C13 Id (both IgG2a) were mAbs generated in our laboratory. The GK1.5 hybridoma-producing rat anti–mouse CD4 mAb was purchased from ATCC.

Cell lines and mice

All studies were approved by the Stanford Administrative Panel on Laboratory Animal Care. A20, a Balb/c B-cell lymphoma line, and CT26, a Balb/c colon carcinoma line, were obtained from ATCC. The A20 cell line was sorted and subcloned for the CD19^+ population. Tumor cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin (both from Invitrogen), and 50 μM 2-mercaptoethanol (Sigma-Aldrich), as complete medium. Cells were grown in suspension culture at 37°C in 5% CO₂. Six- to 8-week-old female Balb/c mice were purchased from The Jackson Laboratory. CD8 knockout (KO) mice on the Balb/c background were provided by Dr C. G. Fathman.


buffered saline (PBS) was added to the whole cell lysis buffer: 5 mL of lysis buffer (phosphate-complete mini-protease inhibitor cocktail tablet, Roche Applied Science) cells were washed with ice-cold serum-free RPMI 1640 and then lysed in anti-38C13 Id mAb (isotype control mAb) was added to the cells at a complete media and allowed to rest for 1 hour at 37°C. Anti-A20 Id mAb or Cells were spun down to a concentration of one million cells in 200/H9262 Tumor cell signaling studies IgG2a (clone 1D2) was used. IgG2b and then finally to IgG2a. For all of the following experiments, the specific immunoglobulin produced by the A20 B-cell lymphoma was obtained by rescue hybridization using a TK
coupled to KLH using maleimide as described. After 2 thrice-weekly injections of Id-KLH conjugate (50 µg of Id), mice were killed and spleens and lymph nodes were harvested for fusion with K6H6B5 cells. Hybridomas, selected in hypoxanthine/aminopterin/thymidine medium, were screened by enzyme-linked immunosorbent assay for secretion of antibodies binding to A20 Id protein. The hybridoma (1G6) was subcloned by limiting dilution together with OP9 spleen cells used as feeder layers. The resulting antibody was a mouse IgG1 κ. Using the protocol for the isolation and cloning of subclass switch variants, the antibody was switched to IgG2b and then finally to IgG2a. For all of the following experiments, the IgG2a (clone 1D2) was used.

**Generation of the A20 anti-Id mAbs**

The specific immunoglobulin produced by the A20 B-cell lymphoma was obtained by rescue hybridization using a TK- variant of the tumor fused to the SP2/0 HPRT- cell line. Id-secreting hybrids were grown in hypoxanthine/aminopterin/thymidine medium to select against both the input A20 cells and the SP2/0 parental cells, as previously described. To generate anti-Id antibodies, Balb/c mice were immunized with A20 Id protein coupled to KLH using maleimide as described. After 2 thrice-weekly injections of Id-KLH conjugate (50 µg of Id), mice were killed and spleens and lymph nodes were harvested for fusion with K6H6B5 cells. Hybridomas, selected in hypoxanthine/aminopterin/thymidine medium, were screened by enzyme-linked immunosorbent assay for secretion of antibodies binding to A20 Id protein. The hybridoma (1G6) was subcloned by limiting dilution together with OP9 spleen cells used as feeder layers. The resulting antibody was a mouse IgG1 κ. Using the protocol for the isolation and cloning of subclass switch variants, the antibody was switched to IgG2b and then finally to IgG2a. For all of the following experiments, the IgG2a (clone 1D2) was used.

**Tumor cell signaling studies**

Cells were spun down to a concentration of one million cells in 200 µL of complete media and allowed to rest for 1 hour at 37°C. Anti-A20 Id mAb or anti-38C13 Id mAb (isotype control mAb) was added to the cells at a concentration of 0.5 µg/mL and further incubated for 1 hour at 37°C. The cells were washed with ice-cold serum-free RPMI 1640 and then lysed in lysis buffer (50 µL of lysis buffer (phosphate-buffered saline [PBS] + 1% NP40 + 0.5% sodium deoxycholate + 0.1% sodium dodecyl sulfate) for 30 minutes on ice. Each cell pellet was lysed with 150 µL of lysis buffer for 30 minutes on ice. The lysate was cleared by centrifugation and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and Western blotting. Immunoblots were probed for total tyrosine phosphorylation using p-Tyr horseradish peroxidase (Santa Cruz Biotechnology).

**Detection of tumor-reactive T cells**

Blood was collected from the tail vein, anticoagulated with 2mM ethylenediaminetetraacetic acid in PBS, then diluted 1:1 with Dextran T500 (Pharmacosmos) in 2% in PBS, and incubated at 37°C for 45 minutes to precipitate red cells. Leukocyte-containing supernatant was removed and centrifuged, and the remaining red cells were lysed with ammonium chloride potassium buffer (Quality Biological). Peripheral blood mononuclear cells (PBMCs) were then cocultured with 5 × 10^5 irradiated A20 cells for 24 hours with 0.5 µg of anti–mouse CD28 mAb (BD Biosciences PharMingen) and in the presence of monensin (Golgistop; BD Biosciences) for the last 5 hours at 37°C and 5% CO2. Tumor specificity of the response was assessed by parallel experiments coculturing PBMCs with 5 × 10^5 irradiated CT26 cells, a BALB/c colon cancer cell line (ATCC). Cells were then washed and stained with anti-CD8 fluorescein isothiocyanate and anti-CD4 APC (BD Biosciences). Intracellular interferon-γ (IFN-γ), tumor necrosis factor, and perforin expression was assessed using BD Cytofix/Cytoperm Plus Kit per instructions and PE-conjugated antibodies (BD Biosciences).

**Flow cytometry**

Cells were surface-stained in fluorescence-activated cell sorter buffer (PBS, saline; 1% fetal bovine serum (FBS), and 0.01% sodium azide), subjected to flow cytometry on a BD FACSCalibur System, and the data were analyzed using Cytobank (http://cytobank.stanford.edu/public). Intracellular IFN-γ expression was assessed using BD Cytofix/Cytoperm Plus kit per the manufacturer’s instructions.

**Cell proliferation studies**

Tumor cells were plated in 96-well plates at a concentration of 1000 cells in 200 µL of complete media. Cells were incubated with media alone, anti-A20 Id mAb, or anti-38C13 Id mAb for 4 days. Antibodies were prepared in complete medium and added at a dose ranging between 0 and

(Stanford University School of Medicine). Fcer1g (FcRKO) mice were purchased from Taconic Farms. All mice were housed at the Laboratory Animal Facility at Stanford University Medical Center.

**Figure 1. Anti-A20 Id mAb binds A20 tumor cells and inhibits A20 tumor growth in vitro.** (A) A20 tumor cells were washed and stained with an Alexa-Fluor 647-labeled anti-A20 Id mAb or with an Alexa-Fluor 647-labeled isotype control mAb. (B) A20 tumor cells were stimulated with anti-A20 Id mAb (S) or an isotype control mAb (I) for 1 hour at 37°C. The proteins were lysed, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blotted, and probed for total tyrosine phosphorylation expression. (C) A20 cells or 38C13 cells were incubated in the presence of anti-A20 Id mAb. Cells were then pulsed with [3H]thymidine and harvested. Data are represented as mean ± SD of triplicate values.
Saline in CD8-/- Mice

More than 95% of the relevant cell subset was depleted, whereas all of the anti-CD4. This mAb was found not to compete with the mAb used for in vivo depletion. Anti-A20 Id mAb or mAb against the Id on 38C13 cells (anti-38C13 Id mAb) was administered intraperitoneally (anti-A20 Id mAb or anti-38C13 Id mAb) was administered intraperitoneally with saline, anti-A20 Id mAb, or an isotype control mAb (anti-38C13 Id) 3 hours later. Numbers in parentheses indicate animals cured by therapy. Depletion of CD4

Tumor cells were implanted subcutaneously on the lower back of Balb/c female mice at a dose of 10⁷ cells in 100 μL of saline. Antibody therapy (anti-A20 Id mAb or anti-38C13 Id mAb) was administered intraperitoneally once at a dose of 100 μg/mouse on day 0. CpG therapy was then given intratumorally at a concentration of 0.1 mg/dose in 100 μL on days 2, 3, 4, 6, and 8. Tumor growth was monitored by a caliper and expressed as the product of length and width. Mice were killed when tumor size reached 4 cm² or when tumor sites ulcerated. All studies were performed using 10 mice per group and repeated a minimum of 3 times to confirm results.

Depletion of CD4+ T cells

Ascites fluid was harvested from SCID mice bearing the GK.1.5 hybridoma (ATCC) producing rat anti–mouse CD4 antibody. The ascites was diluted in saline and filtered. Diluted ascitic fluid was administered intraperitoneally at a dose of 0.5 mg antibody in a volume of 500 μL on days –3, –2, –1, and 0 and weekly thereafter for the duration of the experiment. These depletion conditions were validated by flow cytometric analysis of PBMCs using PE-conjugated anti-CD4. This mAb was found not to compete with the mAb 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.

Cytotoxicity assay

Splenocytes were isolated from either naive mice or tumor-bearing mice that were cured with anti-A20 Id + CpG therapy. The splenocytes were counted and incubated with ⁵¹Cr-labeled A20 at the indicated effector-to-target cell ratios for 4 hours in triplicate wells. ⁵¹Cr release was determined by analyzing the supernatants in a γ counter (Wallac Microbeta 1450 Liquid Scintillation and Luminescence Counter, PerkinElmer Life and Analytical Sciences). All groups were studied in triplicate.

Results

Anti-A20 Id mAb specifically binds tumor cells and inhibits tumor growth in vitro

An antibody against the Id region of the B-cell receptor (BCR) on the A20 B lymphoma-cell line was generated as described in “Methods.” The directly conjugated antibody bound the A20 cell surface Id as demonstrated by flow cytometry (Figure 1A). The antibody was further analyzed for its ability to induce signal transduction. Tumor cells were incubated with either anti-A20 Id mAb or mAb against the Id on 38C13 cells (anti-38C13 Id mAb)
Anti-A20 Id mAb can inhibit tumor cell growth in vivo, but ultimately Id-negative tumor cells escape.

(A) Escapee tumor cells from anti-A20 Id mAb-treated mice or A20 tumors derived from nontreated mice were stained for surface or intracellular IgG2a expression and analyzed by flow cytometry. Histograms are gated on live lymphocytes and are representative of 5 separate tumors. (B) Escapee tumor cells from anti-A20 Id mAb-treated mice or A20 tumors derived from nontreated mice were surface-stained with anti-κ light chain antibody or anti-A20 Id mAb. Graphs are gated on live lymphocytes and are representative of 5 separate tumors. (C) Escapee cells were plated for 4 days in the presence of either anti-A20 Id mAb or anti-38C13 Id mAb. Cells were then pulsed with [3H]thymidine for 12 hours and harvested. (D) Escapee tumor cells were cultured in vitro for 24 hours in complete media. Cells were then stimulated with anti-A20 Id mAb (S) or an isotype control mAb (I) for 1 hour at 37°C. The proteins were lysed, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blotted, and probed for total tyrosine phosphorylation expression. (E) Wild-type mice were inoculated with escapee tumor cells subcutaneously and then treated intraperitoneally with saline, anti-A20 Id mAb, or an isotype control mAb (anti-38C13 Id) 3 hours later. Each line represents a group consisting of 10 mice.

Figure 3. Anti-A20 Id mAb can inhibit A20 tumor cell growth in vivo, but ultimately Id-negative tumor cells escape.
Anti-A20 Id mAb can inhibit tumor cell growth in vivo, but ultimately Id-negative tumor cells escape.

To characterize the relapsing tumors that emerged after antibody therapy, escapee tumors were assessed for BCR expression. A20 BCRs have been previously characterized as having IgG2a heavy chain and κ light chains. Escapee cells were stained for both surface and intracellular expression of BCR. Interestingly, escapee cells no longer expressed surface IgG2a but retained expression of intracellular IgG2a (Figure 3A). Similarly, escapee cells no longer expressed surface Id (stained by AlexaFluor 647-labeled anti-A20 Id) and no longer expressed surface κ light chain (Figure 3B). Positive staining for these molecules was observed intracellularly, however (data not shown).

Escapee cells (from 5 mice with escaping tumors) were plated in vitro and incubated with anti-A20 Id for 4 days. Unlike wild-type A20 cells, the growth of escapee cells could no longer be inhibited by anti-A20 Id antibody (Figure 3C). Further, the escapee cells were no longer able to transmit an anti-Id-induced activation signal as measured by intracellular tyrosine phosphorylated proteins (Figure 3D).

We next investigated the ability of these escapee cells to respond to anti-A20 Id therapy in vivo. Despite retaining intracellular expression of Id, the escapee cells were no longer inhibited by antibody therapy (Figure 3E).

A20 lymphoma cells were implanted subcutaneously into Balb/c mice and allowed to grow to a size of approximately 1.0 cm². By this time, the tumors are widely metastatic (B.V., J. Li, and M. Goldstein, unpublished data, February 2007). Treatment of these large tumors with intratumoral injection of CpG resulted in only temporary tumor growth delay (Figure 4). Treatment with systemic anti-A20 Id in these large tumors was also incapable of inducing tumor regression (Figure 4). In contrast, the combination of intratumoral CpG with systemic anti-A20 Id resulted in complete and permanent tumor regression of the local subcutaneously tumors in 8 of 10 mice (Figure 4). This result implies that Id-negative tumor cells could be effectively treated by inducing a poly-specific immune response.

Mice lacking CD8+ T cells were not cured by the combination therapy of anti-Id antibody and intratumoral CpG (Figure 5A). Interestingly, CD8-/- mice treated with the combination therapy survived significantly longer than CD8-/- mice treated with either anti-A20 Id or CpG alone (Figure 5B), suggesting the enhanced therapeutic effect may be a result of improved ADCC.
incubated for 4 hours in the presence of 51Cr-labeled A20 cells (target cells). Cytotoxic T cells from vaccinated mice, but not naive mice, had survived for 100 days. Splenocytes were used as effector cells against 51Cr-labeled A20 target cells at the indicated ratio for 4 hours, and specific release of 51Cr was measured. Each line is representative of 4 mice.

Figure 5. Anti-A20 Id mAb + CpG combination therapy cures large established A20 tumors using a CD8-dependent, CD4-independent mechanism. (A-B) CD8−/− mice were inoculated with 10⁶ A20 tumor cells. When tumors reached 1 cm² (day 0), mice were treated once intraperitoneally with anti-A20 Id mAb. CpG was given intratumorally on days 2, 3, 4, 6, and 8. Numbers in parentheses indicate animals cured by therapy. Each line in panel B represents a group consisting of 10 mice. (C-D) Balb/c mice were inoculated with 10⁶ A20 tumor cells. When tumors reached 1 cm² (day 0), mice were treated once intraperitoneally with anti-A20 Id mAb. CpG was given intratumorally on days 2, 3, 4, 6, and 8. Numbers in parentheses indicate animals cured by therapy. Each line in panel D represents a group consisting of 10 mice. (E) Splenocytes were isolated from naive mice or from tumor-bearing mice that were cured with anti-A20 Id mAb and had survived for 100 days. Splenocytes were used as effecter cells against 51Cr-labeled A20 target cells at the indicated ratio for 4 hours, and specific release of 51Cr was measured. Each line is representative of 4 mice.

To study the role of CD4+ T cells in the anti-tumor response mediated by combination therapy, mice were depleted of CD4+ T cells using anti-CD4 antibodies. Depletion of CD4+ T cells had no effect on the therapeutic outcome (Figure 5C-D).

We then compared splenocytes (effecter cells) isolated from cured mice to those of naive mice. These effector cells were incubated for 4 hours in the presence of 51Cr-labeled A20 cells (target cells). Cytotoxic T cells from vaccinated mice, but not naive mice, recognized and killed the A20 tumor cells (Figure 5E).

Fifteen days, 3 days, and 1 day after the last day of therapy, T cells from PBMCs and splenocytes were analyzed for intracellular IFN-γ, tumor necrosis factor, and perforin expression in response to A20 tumor cells. No significantly increased expression was detected in mice treated with combination therapy compared with controls (data not shown). These data indicate that either these cytokines do not play a key effector role in A20 killing or that analysis was performed at a time after tumor challenge and therapy as to escape assay detection.

**Anti-A20 Id + CpG combination therapy can mediate tumor destruction at local and distant tumor sites**

To examine whether anti-A20 Id + CpG therapy had a systemic anti-tumor effect, mice were inoculated with A20 cells at 2 different sites (left and right sides) of the abdomen. Anti-A20 Id was injected intraperitoneally as in all other experiments (Figure 6). CpG was injected intratumorally into only the left tumor site. CpG alone had no effect on the growth of the untreated tumor located on the right (Figure 6), and there was no significant difference in survival between CpG and control groups (data not shown). Furthermore, the combined anti-A20 Id + CpG treatment significantly inhibited the growth of tumor at both the treated and
untreated sites and inhibited tumor recurrence. The combination of anti-A20 Id with CpG significantly prolonged the survival of mice compared with either anti-A20 Id or CpG alone (Figure 6). Nine of 10 mice receiving the combination therapy achieved complete cures, and none of the 9 surviving mice experienced tumor relapses. The response of the untreated tumor implied the generation of a systemic immune response.

**Anti-A20 Id mAb + CpG combination therapy prevents the emergence of Id-negative tumor escapees**

A20 tumor cells escaping passive anti-Id antibody therapy lost expression of surface Id (Figure 2). To assess the ability of mice cured of A20 tumor with anti-A20 Id + CpG to recognize tumor antigens other than the Id, we challenged the cured mice with Id-negative escapee tumor cells isolated from anti-Id–treated mice. Each cured mouse was also inoculated at 2 other sites with wild-type A20 tumor cells and CT26 colon carcinoma cells. Naive mice that had neither seen tumor nor been treated with any therapy showed progressive growth of all 3 tumor cell lines. Mice cured by the combination therapy with anti-Id antibody and intratumoral CpG were capable of rejecting a challenge with both wild-type A20 tumor and with Id-negative escapee tumor, whereas CT26 cells were unimpeded. These results demonstrate that a specific immune response had been generated in the cured mice against targets shared by wild-type A20 lymphoma cells and their variants (Figure 7).

**Discussion**

Anti-idiotypic antibodies have shown remarkable success in the clinic. The therapeutic response of patients to anti-Id antibodies was correlated with the ability of their tumor cells to respond to the antibody in vitro by signal transduction through their BCR, as measured by intracellular tyrosine phosphorylation. Thus, a direct anti-tumor effect of the anti-Id antibodies was implicated in the mechanism of tumor destruction. Other evidence implicated ADCC as an additional mechanism.

Many patients whose tumors regressed initially relapsed later with tumor cells no longer reacting with the therapeutic antibody, although immunoglobulin expression remained intact. This phenomenon of tumor escape was explained by the selection of genetic variants in the tumor population that were generated by ongoing somatic point mutation in their immunoglobulin variable genes.

Here we developed an animal model of this escape phenomenon to test for possible therapeutic solutions. We produced an anti-idiotypic mAb against the mouse A20 B lymphoma. The resulting antibody was able to induce signal transduction through the surface BCR on A20 cells, just as was the case with the responding human tumors. We further demonstrated that the anti-A20 Id mAb inhibited tumor cell proliferation in vitro (Figure 1C). The antibody had a strong therapeutic effect in vivo that was at least partially dependent on ADCC (Figure 2C). Just as in patients, therapy of the mouse tumor with the anti-Id antibody led to the emergence of...
tumor escape variants lacking Id expression. Unlike the case with the human somatic mutants, the escape variants in this mouse system lacked surface Ig expression but retained it intracellularly.

Numerous investigators have demonstrated therapeutic synergy between immunostimulatory CpG and antibody therapy.20-23 Woolridge et al have shown that CpG enhances the ADCC of NK cells and macrophages, thereby improving mAb therapy by this mechanism.22 On the other hand, we have previously shown that a T-cell anti-tumor immune response is generated when intratumoral CpG6 is combined with systemic tumor destruction by low-dose chemotherapy. The role of antibody in our current model could have been to retard tumor growth, allowing time for a CpG-mediated T-cell immune response to occur. Alternatively, the antibody could have contributed to therapy in a manner similar to low-dose chemotherapy, by killing tumor cells by ADCC or by direct cytotoxicity, releasing tumor antigens to be cross-presented by host antigen-presenting cells. Many mAbs, including rituximab, have been shown to have similar lytic activity against tumor cells.26-31 suggesting the potential application of an immunostimulatory CpG vaccination maneuver to these mAb therapies.

Another possibility to consider is that the tumor cell in this model is, in itself, an antigen-presenting cell. Because CpG-ODN induces up-regulation of several antigens involved in antigen presentation and communication with T cells (including MHC I and MHC II, CD40, CD80, and CD86) in both primary tumor cells32-33 and in vitro cell lines,6,34,35 the tumor B cell may be able to directly process and present tumor antigens to CD8+ T cells. These models suggest possible mechanisms in which CD4+ T cells would not be necessary and are supported by our results here showing that CD4+ T cells are not required for therapy with anti-Id antibody combined with CpG.

One drawback to mAb therapy has always been the possibility of tumor escape. Selective pressure of an antibody exerted on a single tumor antigen may lead to the selective outgrowth of tumor cells not expressing the particular antigen from a heterogeneous tumor population.3 Moreover, mAbs can lose their effectiveness against highly mutagenic targets, such as immunoglobulin V regions. It is probable that antigenic escape from mAbs is a more general phenomenon and not restricted to Id targets. Therefore, the induction of additional killing mechanisms, such as the generation of a polyclonal CD8+ T-cell response, should be able to address tumor cells that would escape the effects of single mAbs. We demonstrate here that the addition of intratumoral CpG injection can augment the therapeutic effect of mAbs, both by the enhancement of ADCC tumor killing and by the induction of a polyclonal T-cell response against other tumor antigens. The combination of a passive mAb therapy with an active immunotherapy can prevent the outgrowth of antigenic variants that escape the antibody therapy and lead to cure of high burden systemic disease.

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Authorship

Contribution: J.D., B.T., A.W., and J.T. performed experiments; D.K.C. analyzed results; B.V. performed experiments, analyzed results, and made the figures; and B.V., S.L., and R.L. designed the research and wrote the paper.

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