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Topical Imiquimod has Therapeutic and Immunomodulatory Effects Against Intracranial Tumors

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Abstract

Topical imiquimod cream (trade name, Aldara) is a toll-like receptor (TLR) 7 agonist that is approved for the treatment of cutaneous tumors. Aldara is also employed as a vaccine adjuvant in clinical trials in patients with glioma and other tumors. The main mechanism of action ascribed to Aldara has been the local activation of TLR7⁺ cells near the application site. Here we report the unexpected finding that Aldara has therapeutic and immunomodulatory activity as a single agent in mice bearing intracranial tumors. Repeated administration of Aldara onto the skin significantly increased the survival of mice bearing intracranial GL261 glioma and EMT6 breast carcinoma. Aldara treatment was associated with a reduction in the number CD4⁺Foxp3⁺ regulatory T cells (Treg) in the blood and brain tumor site. Mice treated with Aldara exhibited a generalized lymphopenia in the blood amidst an increase in brain tumor infiltrating CD4⁺ and CD8⁺ T cells and dendritic cells. Brain infiltrating CD8⁺ T cells were tumor-reactive as demonstrated by degranulation and interferon gamma secretion in a GL261-dependent manner. Additionally, soluble imiquimod directly inhibited the proliferation of GL261 cells in a TLR7-independent manner. This is the first report demonstrating that topical application of imiquimod can enhance T cell responses to intracranial tumors as a single agent. The results must be interpreted with caution considering anatomical and biological differences between mice and humans. Nevertheless, Aldara that is being used as a vaccine adjuvant in clinical trials may have direct anti-tumor effects that are independent of exogenous antigen. Further studies in humans are warranted.

Keywords

Imiquimod; Toll-like receptor; Vaccine; Glioma; Immunotherapy

Introduction

Imiquimod (IMQ) is a synthetic small molecule that activates toll-like receptor (TLR) 7 (1). TLR 7 activation induces inflammatory cytokine expression and maturation of antigen presenting cells. For instance, IMQ causes plasmacytoid dendritic cells (DCs) to produce interferon alpha (IFN α) that directly activates lymphocytes and skews the differentiation of

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monocytes into TLR7⁺ DCs that can respond to subsequent IMQ exposure (2,3). In addition to immune stimulation, IMQ has antiangiogenic and proapoptotic activity (4,5), making it an attractive local immunotherapy for cancer. Accordingly, 5% IMQ topical cream (Aldara) was approved for the treatment of basal cell carcinoma and has been used “off label” to treat a plethora of other cutaneous tumors (1). The majority of clinical uses of Aldara involve application at the skin tumor site where both antigen (dying tumor cells) and adjuvant (IMQ diffusing into the tumor) are present (6). Direct intratumoral injection of soluble IMQ induced tumor regression of murine mesothelioma in a CD8 T cell-dependent manner, but had no effect on survival if distal tumors were not treated (7). Whether these results obtained using intratumoral injection of soluble IMQ predict the efficacy of Aldara is unknown.

There are numerous clinical trials ongoing that employ Aldara as a vaccine adjuvant whereby peptide antigen or antigen-loaded DCs are administered at the skin application site (8). Application of Aldara to the injection site enhanced the trafficking of DCs to the draining lymph nodes in a murine model of glioma (9), and several clinical trials have been initiated in glioma patients where Aldara is used in this manner (8). A reasonable assumption of this approach is that IMQ will diffuse into the skin to activate injected DCs or endogenous DCs, thereby enhancing systemic CD8 T cell responses. However, very little is known about how Aldara may affect systemic immune responses as a single agent. The purpose of this study was to better characterize the effects of Aldara on systemic anti-tumor immune responses. Our results reveal that Aldara by itself can profoundly affect systemic immune responses including T cell responses in the brain.

Material and Methods

Tumor Cell Culture

Luciferase stable GL261 cells (GL261-Luc) (10) were grown in DMEM media supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin at 37°C in a humidified incubator maintained at 5% CO₂ and 5% O₂. C1498 cells, a syngeneic (H-2^b) myelogenous leukemia, were maintained identically. EMT6 cells (syngeneic to BALB/c mice) were grown in 10% fetal bovine serum in RPMI medium at the same concentration of CO₂ and O₂. Cultures were screened regularly for mycoplasma with the MycoAlert Detection kit (Lonza Group Ltd, Basel, Switzerland).

Tumor Models

All mice were housed in specific pathogen free conditions at the University of Minnesota facility. All experiments were performed in compliance with Institutional Animal Care and Use Committee protocols. Eight to ten week old female C57BL/6J mice (Jackson Laboratories) were used for orthotopic glioma experiments. To establish syngeneic gliomas, 15,000 GL261-Luc cells were stereotactically injected in a 1 µl volume into the right striatum over 5 minutes into the following coordinates: +0.5 mm anterior, 2.5 mm medial from bregma and 3 mm deep from the cortical surface. Tumor burden was monitored by bioluminescent imaging on the indicated days as described (11). The intracranial EMT6 model of brain metastatic breast carcinoma was performed as previously described in BALB/c mice (12).

Imiquimod Treatment using Aldara

Aldara cream (5% IMQ) was purchased from the Boynton pharmacy at the University of Minnesota. Before Aldara was applied, the hair was shaved over four application sites (a 1×1 cm² area over each shoulder and flank); the skin was sterilized with 10% Triadine and 70% ethanol prep pads under mild anesthesia with ketamine (30 mg/kg) and xylazine (3.75 mg/kg). A volume of Aldara corresponding to 2 mg/kg of IMQ per mouse was applied on

the skin by hand using latex gloves. Controls were treated by application of 0.9 % of saline. Therapy started three days after tumor inoculation and was repeated every week or until death for a maximum of six applications.

Flow Cytometric Analyses

All the antibodies were purchased from eBioscience (San Diego, CA). The following antibodies were used: anti-CD4 (clone RM4-5)-eFlu405, anti-CD8 (clone 53-6.7)-perCP-cy5.5, anti-CD45 (clone 30-F11)-PE-cy7, and anti-CD3 (clone 17A2)-Alexa 700, anti-Foxp3 (clone FJK-16s)-PE, anti-CD11c (clone N418)-eFluo 450, anti-CD19 (clone eBio 1D3)-PE-Cy7, anti-CD49b (clone DX5)-APC, anti-mouse MHC-II (clone M5/114.15.2)-PE, and anti-CD107a (clone eBio1D4B)-FITC according to the manufacturer's protocol. On day 22 after tumor challenge, four mice from each group were selected randomly for analysis of blood, cervical lymph nodes (CLNs), spleen, and brain. Blood (150 μ l) was collected from the facial vein and mixed immediately with 500 μ l of 5 mM EDTA. Red blood cells were lysed with ACK buffer, and peripheral blood leukocytes were washed twice with PBS prior to staining. Single cell suspensions from pooled CLNs and spleen were obtained using standard methods. Brain infiltrating lymphocytes (BIL) were harvested from the tumor bearing hemisphere and stained as described (12). All data shown in blood, CLN, spleen, and BIL reflects cells harvested on day 22 as described above. The absolute number of each cell type was calculated by multiplying the percentage of cells by the total cell number counted using a hemocytometer.

To evaluate the effect of IMQ on T cell proliferation, splenocytes from a naïve B6 mouse were labeled with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA). Next, 5×10^6 splenocytes were seeded per well in a 24-well plate in media containing 20 ng/ml PMA, 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO) supplemented with 0, 0.1, 0.5, 1 or 5 μ g/ml of IMQ. Ninety-six hours later, cells were stained and proliferation was determined by CFSE dilution using flow cytometry.

The CD107a mobilization assay was conducted as reported (13) with several modifications. Briefly, 0.1×10^6 BIL were restimulated by co-culture with irradiated GL261-Luc (12000 Rad) at a 1:2 ratio of tumor cells to BIL for six hours. The following reagents were added per well at the beginning of the co-culture: 0.5 μ g anti-CD107a-FITC, 0.14 μ l of golgi Stop (2 μ M menosine, BD Biosciences, San Jose, CA,) and 0.1 μ l of brefeldin A solution (1 μ g/ml, eBioscience). The cells were then harvested and stained, followed by fixation. Non-stimulated and cells stimulated with irrelevant antigen (C1498 cells) were used negative controls.

For determination of IFN γ secretion, 0.2×10^6 BIL were stimulated with 0.1×10^6 splenocytes that had been pulsed with 100 μ g of GL261-Luc lysate or C1498 lysate then irradiated (3000 Rad). The stimulated cells were cultured for four days. Fifty microliters of tissue culture supernatant was collected from each sample and run triplicate. IFN γ was quantified using a flow cytometric bead array according to the manufacturer's instructions (BD Biosciences, San Jose, CA). All samples were acquired on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Between 50,000–100,000 events were collected for cellular staining, 500 events were collected for bead array. All Data were analyzed with Flowjo software (Tree Star, Inc, Ashland, OR.)

Cytotoxicity Assays

The cytotoxicity of IMQ was determined by plating 5,000 GL261-Luc cells per well in a 96-well plate in complete media supplemented with soluble Imiquimod (R837, Invivogen, San Diego, CA) for 72 hours. Each concentration of IMQ was tested in triplicate. The number of

viable cells per well was determined using a standard curve obtained by plating 0 to 15,000 GL261-Luc cells per well. Thirty microliters of D-Luciferin (0.7 mg/ml) solution was added to each well and luminescence was measured with a plate reader (Synergy2, BioTek Instruments, Inc, Winooski, Vermont) after a 7 min delay according to the manufacture's instructions. The number of viable cells in each well was determined assuming 100% viability when no IMQ was added (control) according to the following formula: [viability (%) = $[1 - (\text{cell number in IMQ-treated sample} / \text{cell number in control})] \times 100$]. A standard MTT assay was run in parallel yielding similar results (Supplementary Figure 1A), although the luciferase assay was more sensitive. The effect of IMQ on EMT6 cell growth was measured by the MTT assay.

TLR 7 RT-PCR

Total RNA was extracted from GL261-Luc and splenocytes from a naïve C57BL/6J mouse using the RNeasy plus Mini Kit (Qiagen, Valencia, CA). A one-Step RT-PCR kit was used according to the manufacturer's instructions (Qiagen). Ten nanograms of total RNA was used as template for each reaction. The following PCR parameters were applied: 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 45 seconds, for a total 35 cycles. The following primers were used to amplify a 96 bp segment from exon 2: 5'-GCTGAACCATCTGGAAGAAATG-3'; 5'-TGCAG CCTCT TGGTACACACAT-3'. PCR products were visualized on a 2% agarose gel with ethidium bromide and photographed with an Alpha Imager EC Gel Documentation and Fluorescent Imaging System (Cell Biosciences, Inc. Santa Clara, CA).

Statistical Analysis

All data were graphed and analyzed statistically using Prism 4 software (GraphPad Software, Inc. La Jolla, CA). Unless explicitly stated, data were analyzed by ANOVA using post-hoc comparisons by t-test. The Log rank test was used to analyze mouse survival. Differences between groups were considered significant if P was less than 0.05.

Results

Aldara Inhibited the Growth of Intracranial Glioma and Breast Carcinoma

The therapeutic activity of Aldara against intracranial gliomas was initially revealed in a vaccination experiment whereby Aldara was used as a vaccine adjuvant. B6 mice bearing established GL261-Luc glioma were vaccinated with GL261-Luc cell lysates alone, or lysates injected intradermally into an Aldara application site. Mice receiving saline or Aldara alone were used as controls. Mice treated by lysate alone did not survive significantly longer than saline controls (Supplementary Fig 2A). Although 20% of mice treated by lysate plus Aldara survived long-term, this difference was not statistically significant from the Aldara alone group. Mice treated by Aldara reproducibly exhibited a 50% increase in median survival relative to saline treated mice (28 days increased to 42; Fig 1A). Bioluminescent imaging demonstrated that Aldara reduced tumor burden by 3-fold on day 21 after tumor implantation (Fig 1B–C). To further evaluate the effect of Aldara on brain tumor growth, mice bearing intracranial EMT6 breast carcinoma were treated with Aldara identically to the regimen used against GL261 glioma. Aldara increased the median survival of EMT6-bearing mice by 22% ($p=0.007$; Supplementary Fig 2B). Although the therapeutic efficacy in both models was modest, these unexpected and intriguing results using Aldara as a single agent warranted further studies to delineate possible mechanisms.

Systemic Effects of Aldara on Lymphocytes and Dendritic Cells

Aldara was administered to glioma-bearing mice every seven days starting three days after tumor implantation. Immune monitoring assays were conducted on day 22 after tumor implantation because tumor burden was significantly different between saline and Aldara treated mice at this time point. The percentage and absolute number T cells, B cells, NK cells, and DCs was quantified in the cervical lymph nodes (CLN), spleen, and blood. The effect of Aldara on each cell type was significantly different depending on the tissue analyzed. In the blood, Aldara significantly depleted T and B lymphocytes (Fig 2B). The effect of Aldara on B cells (over 20-fold reduction) and Tregs (3-fold reduction) measured in blood was particularly dramatic (Fig 2B). In contrast, mice treated with Aldara exhibited a noticeable but statistically insignificant increase in circulating NK cells and DCs compared to mice in the saline group (Fig 2B). Thus, in the blood Aldara selectively depleted cells of the adaptive immune system. In contrast to the blood, treatment with Aldara had negligible effects on measured leukocytes in the spleen; with the exception of B cells, which were significantly depleted (Fig 2C).

Aldara affected the CLNs, which are the draining lymph nodes of the brain and therefore drain the tumor site. Contrary to the blood, Aldara significantly increased the percentage and absolute number of CD4 T cells, CD8 T cells, and DCs in the CLNs (Fig 2D). A similar increase was documented for NK cells, although it failed to reach statistical significance. The percentage and absolute number of B cells and Tregs in the CLNs was not significantly different relative to saline-treated controls. Collectively, these data reveal that Aldara has systemic effects on the adaptive and innate immune system that varies greatly depending on the tissue analyzed.

Aldara Modulates T Cell Responses at the Brain Tumor Site

The effect of Aldara on leukocyte infiltration and function was determined at the brain tumor site. Brain infiltrating leukocytes (BIL) were dissociated from glioma-bearing mice that had been treated by repeated application of Aldara or saline. Mice treated with Aldara exhibited no appreciable changes in brain infiltrating B cells or NK cells (Fig 2E). Aldara nearly doubled the number of DCs and CD4 T cells and more than tripled the number of CD8 T cells in the brain relative to saline controls. However, mice treated by Aldara exhibited a significant decrease in the frequency and absolute number of Tregs in the brain (Fig 2E).

The number of BIL recovered was very limiting, preventing us from performing CTL assays or ELISpot assays. Therefore the frequency of tumor-reactive CD8 T cells was tested by a degranulation assay measuring CD107a cell surface mobilization. Treatment with Aldara was associated with a 4-fold increase in the number of brain infiltrating CD8 T cells that degranulated when challenged with GL261 antigen relative to saline controls (Fig 3A). In order to establish tumor-specificity, BIL were co-cultured with B6 mouse splenocytes that had been pulsed with cell lysates from GL261 cells, or C1498 cells as an irrelevant antigen control. Soluble IFN γ was then measured in the tissue culture supernatant. No appreciable IFN γ was measured using BIL from saline treated mice, suggesting that these T cells had impaired effector function or were not tumor-reactive. In marked contrast, BIL from Aldara treated mice elaborated IFN γ in a GL261 antigen-dependent manner (Fig 3B). Collectively, these data demonstrated that topical administration of Aldara significantly increased the number of DCs and tumor-reactive T cells that reach the brain tumor site.

Soluble Imiquimod Directly Inhibits the Growth of TLR7⁻ GL261 Glioma Cells

Topical application of Aldara results in measurable concentrations of IMQ in the serum of humans (14). Although the brain penetration of IMQ was not assessed, the dysfunctional

blood-brain barrier in the central core of intracranial tumors raises the possibility of IMQ exposure directly to tumor cells. Therefore, we tested the ability of soluble IMQ to directly inhibit the growth of GL261-Luc cells to determine if this could be an additional mechanism by which Aldara inhibited glioma growth. IMQ inhibited GL261-Luc cell growth in a dose dependent manner (Figure 4A, Supplementary Fig 1). In order to test if this effect required TLR7 expression, RT-PCR was conducted for TLR 7 on RNA isolated from GL261-Luc cells. We were not able to detect TLR7 RNA in GL261-Luc cells despite TLR7 expression in B6 mouse splenocytes as a positive control (Figure 4B). Therefore the growth inhibitory effects of IMQ on glioma cells do not require TLR7 expression.

Discussion

Aldara is being used in multiple clinical trials as a vaccine adjuvant for cancer and other diseases (8). Aldara is attractive for this purpose because of extensive preclinical and clinical data demonstrating local activation of antigen presenting cells that can subsequently enhance T cell priming (1,6). The design of such clinical studies does not include patients treated by Aldara alone, and, consequently, it will not be possible to distinguish the effects of the adjuvant and antigen. We initially discovered the anti-tumor efficacy of Aldara against intracranial tumors by including an Aldara alone group in a vaccine experiment as a “confirmatory” control. To our knowledge this study is the first demonstration that topical Aldara can modulate systemic immune responses including T cell responses in the immunologically specialized central nervous system. However, the impact of our findings on human immunology remain entirely unclear. The thickness of the skin and subsets of cells that express TLR7 are different between rodents and humans. Moreover, the surface area of skin to which Aldara was applied in our study was large relative to standard human applications. Thus, in mice, the serum levels of IMQ and responding cell types are expected to differ relative to humans. Our data should therefore be interpreted with caution. Nevertheless, topical application of Aldara results in appreciable serum concentrations of IMQ in humans ranging from 0.1–1.6 ng/ml, and systemic side effects including fever have occurred (14). These observations suggest that Aldara could impact anti-tumor immune responses systemically in patients and warrants further study. Additionally, our data suggest that perhaps systemic administration of IMQ or IMQ-like drugs should be revisited using conservative, metronomic dosing to mimic the systemic exposure that likely occurred in our murine studies. It is tempting to speculate that systemic exposure to TLR7 ligands could facilitate the depletion of Tregs and B cells, which could be exploited to enhance anti-tumor T cell responses.

We identified at least four plausible mechanisms by which Aldara could inhibit glioma growth in the GL261 animal model. First, topical application markedly increased the number of CD8 T cells that reached the brain tumor site. Not only did more CD8 T cells reach the brain, but they had significantly enhanced effector function as assessed by degranulation and IFN γ elaboration relative to saline-treated mice in which CD8 T cells failed to perform either function. We do not believe CD8 T cell reactivity was specific to luciferase because we tested this extensively in prior studies and found no evidence for luciferase-reactive T cells even following vaccination with GL261-Luc lysates and strong adjuvants (15). Second, Aldara reduced the frequency and absolute number of Tregs at the tumor site, which may have enhanced the tumoricidal function of CD8 T cells. Unfortunately, due to the limited cell numbers recovered from the BIL, we were not able to directly measure tumoricidal function in CTL assays. Third, Aldara increased the number of CD4 T cells and DCs in the CLNs and tumor site, which could enhance CD8 T cell priming and restimulate CTLs at the tumor site. Fourth, soluble IMQ directly inhibited the growth of GL261-Luc cells in culture. Since a fraction of Aldara gets into the serum, it is possible that the repeated serum exposure of IMQ achieved brain penetration where it could act on tumor

cells directly. This effect did not require TLR7 because GL261 cells did not express TLR7. However, soluble IMQ did not inhibit the growth of EMT6 breast carcinoma cells, illustrating that the direct effect of IMQ on tumor cells may not be applicable to all tumors (Supplementary Fig 1B).

How might IMQ trigger such dramatic effects that appear selective to certain tissues, cell types, and in the case of GL261, TLR7 independent? The mechanism by which IMQ inhibited GL261 cell growth could be due to adenosine receptor antagonism which has been recently demonstrated by Schon and colleagues (16). Adenosine receptors regulate many important processes including the concentrations of intracellular cyclic AMP and numerous signal transduction pathways (reviewed in (17)). Aldara induced profound lymphopenia in the blood in T and B lymphocytes selectively (NK cells and DCs were conversely enriched), but only depleted B cells in the spleen (Fig 2). We also documented that soluble IMQ inhibited the proliferation of CD4 and CD8 T cells in tissue culture (Supplementary Fig 3). We speculate that higher concentrations of IMQ were obtained in the blood relative to the spleen following topical Aldara treatment, triggering more pronounced lymphopenia in the blood. Both TLR7 and adenosine receptors could be involved in immune modulation via direct and indirect mechanisms including alterations in cytokine expression and concentrations of free adenosine. Further studies will be required including IMQ pharmacokinetics and flow cytometry in mice deficient for TLR7 and adenosine receptors to better define exactly how Aldara modulates leukocyte populations *in vivo*.

In summary, our study demonstrates that topical application of Aldara can affect systemic anti-tumor immune responses in murine models. Aldara markedly enhanced the number of tumor-reactive T cells that entered the brain and significantly reduced brain tumor growth in two animal models. As Aldara and other TLR agonists are increasingly used in clinical trials it will be important to consider their effects as single agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Aldara inhibits glioma growth

(A) B6 mice bearing GL261-Luc were treated by Aldara or saline weekly starting three days after tumor implantation. Kaplan Meier survival plot is shown (** $p=0.0021$; $n=6$ /group). (B) Representative bioluminescent imaging of individual mice longitudinally over time illustrates tumor burden in each group. (C) Aggregate bioluminescent imaging data from mice in A–B. (** $p<0.01$; error bars represent standard deviation). These experiments were repeated three times with similar results.

Figure 2. Aldara selectively modulates the percentage and absolute number of leukocytes

On day 22 after GL261-Luc challenge, four mice from each group were sacrificed and leukocytes from the indicated tissues were analyzed by flow cytometry. (A) Representative gating scheme used to define leukocyte subsets in B-E. The percentage and absolute numbers were calculated in the blood (B), spleen (C), pooled cervical lymph nodes (D), and brain infiltrating leukocytes (E). All graphs represent the mean $\pm$ SEM (* p <0.05, ** p <0.01, *** p <0.001). The experiments were repeated at least twice with similar results.

A **B**

Figure 3. Aldara increased the number of brain infiltrating tumor-reactive T cells

On day 22 after tumor implantation, brain-infiltrating lymphocytes (BIL) were dissociated from four mice in each group and analyzed by flow cytometry. (A) BIL were co-cultured with irradiated GL261-Luc target cells and the number of degranulating CD8 T cells was measured by flow cytometry as described in the methods. (B) BILs were co-cultured with B6 mouse splenocytes that had been pulsed with cell lysates from GL261-Luc or C1498 cells as a negative control. Four days later, soluble IFN γ was measured in the supernatant. Averages are graphed $\pm$ SEM (* p <0.05, *** p <0.001). The experiments were repeated at least twice with similar results.

Figure 4. Soluble imiquimod inhibits GL261 proliferation despite lack of TLR7 expression

(A) A bioluminescent cell viability assay was conducted whereby GL261-Luc cells were cultured in the indicated concentrations of IMQ for 72 hours. Data are normalized to cells treated with no IMQ as 100% viable. (B) RT-PCR was performed for TLR7 using 10 ng of total RNA from GL261-Luc cells or B6 mouse splenocytes. Tubes run without the RT step were used as a “No RT control”.