Abstract. Background/Aim: Tumor-associated macrophages (TAMs), together with splenic CD11b+ cells, help maintain the tumor microenvironment. The immunomodulatory compound imiquimod (IQM) stimulates innate immune cells, including macrophages, to induce antitumor effects. In order to elucidate the effects of IQM on the tumor microenvironment, we investigated the immunomodulatory effect of IQM during melanoma growth by using the B16F10 melanoma model.

Materials and Methods: To elucidate the immunomodulatory effects of IQM on the tumor microenvironment, we isolated CD11b+ TAMs and splenic CD11b+ cells and evaluated the immunomodulatory effects of IQM, using the B16F10 melanoma model. Results: IQM suppressed B16F10 melanoma growth in parallel with reduction of Foxp3+ regulatory T cells (Tregs) at the tumor site, caused by the down-regulation of CCL22 production by tumor-derived and splenic CD11b+ cells. Subsequently, we investigated the antitumor or tumor-loading effects of splenic CD11b+ cells on B16F10 melanoma growth in vivo. B16F10 melanoma growth was accelerated by splenic CD11b+ cells from untreated mice, but was inhibited by splenic CD11b+ cells from IQM-treated mice. Consistent with these results, Foxp3+ Tregs were significantly decreased in tumors of mice implanted with both melanoma and splenic CD11b+ cells from topical IQM-treated mice. Furthermore, intratumoral administration of anti-CCL22 antibody inhibited B16F10 melanoma growth by decreasing Treg recruitment at the tumor site. Conclusion: Our results suggest a possible mechanism for the antitumor immune response induced by IQM through tumor-associated macrophages.

Imiquimod (IQM) is an immunomodulatory, small-molecule compound in the imidazoquinoline family that induces antitumor effects through Toll-like receptor 7 (TLR7). As a TLR7 agonist, IQM stimulates innate immunity, including tumor-associated macrophages (TAMs) (1-3). In an experimental murine model, the addition of IQM to cryosurgery increased the cellular immune response against tumor antigens, leading to complete rejection of B16OVA melanoma (4). Drobits et al. reported that the anti-melanoma effect of IQM is dependent on the recruitment of plasmacytoid dendritic cells (DCs) to the skin through chemokine (C-C motif) ligand 2 (CCL2) produced by mast cells (2). Notably, CCL2 also recruits immature myeloid cells to the tumor microenvironment (5, 6). Singh et al. reported the therapeutic effects of a TLR7/8 dual agonist (3M-052) on B16F10 melanoma in vivo. They concluded that intratumoral administration of 3M-052 significantly suppressed B16F10 melanoma growth by increasing CCL2 production from the tumor microenvironment, which might result in induction of M1 macrophages, and even enhanced the therapeutic effect of immune checkpoint inhibitors, such as anti-CTLA4 antibody (Ab) and anti-PD-L1 Ab (7).

In humans, IQM is clinically effective for the treatment of superficial melanomas such as lentigo maligna (8, 9). Several reports have suggested that IQM may be an optimal reagent for invasive melanoma when used in combination with other therapies (3, 4, 7, 10, 11). Turza et al. reported that IQM was effective in 10 cases of superficial dermal and subcutaneous metastasis of melanoma when used in combination with intrallesional interleukin (IL)-2 (9). They concluded that IQM could be used to control dermal metastatic melanoma, but that intrallesional IL-2 is indispensable for controlling subcutaneous melanoma, suggesting that the abrogation of regulatory T cell (Treg) function is necessary for induction of an antitumor immune response by IQM (9).
CCL22 attracts chemokine (C-C motif) ligand 4 (CCR4)\(^+\) T cells, including Tregs, in the lesional skin of melanoma (12). As Klarquist et al. reported, CCL22-related Tregs drive B16F10 melanoma growth in vivo, and the diversion of Tregs to normal skin by CCL22 vaccination could limit tumor-infiltrating Tregs, subsequently suppressing melanoma growth (12). In addition, as we previously reported, immunomodulatory drugs such as interferons (IFNs) could modulate CCL22 production from TAMs to decrease Treg recruitment to the tumor (13). Moreover, another CCR4 ligand, CCL2, is highly produced in the advanced stage of ret mouse melanoma (14), suggesting the significance of Treg-related chemokines in melanoma development. These findings suggested that reduction of tumor-derived chemokines could be a therapeutic target for melanoma. In the present study, we examined the therapeutic and immunomodulatory effects of topical IQM on established B16F10 melanomas in vivo, focusing on its effects on TAMs, splenic CD11b\(^+\) cells and Tregs.

**Materials and Methods**

**Ethics statement for animal experiments.** The protocol for the animal study was approved by the ethics committee of Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (Permit number: 2014-153). The research practices comply with the Tohoku University Graduate School of Medicine’s Animal Experimentation Ethics guidelines and policies. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

**Animals and the melanoma cell line.** C57BL/6 mice and BALB/c mice (5 to 8 weeks old) were purchased from Japan Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in the animal facility at the Tohoku University Graduate School of Medicine. The murine melanoma cell line, B16F10, was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s minimal essential medium (Sigma, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Tokyo, Japan). All mice were bred under specific pathogen-free conditions at the Tohoku University Graduate School of Medicine.

**Reagents.** Imiquimod cream (5%) was a kind gift from Mochida Pharmaceutical Co., Ltd (Tokyo, Japan). Blocking monoclonal Ab against mouse CCL22 (158132) was purchased from R&D Systems (Minneapolis, MN, USA), and rat IgG was purchased from Syn Pharmaceutical Co., Ltd (Tokyo, Japan). DNase1 and lipopolysaccharide (LPS) were purchased from Sigma (Tokyo, Japan). DNase1 and lipopolysaccharide (LPS) were purchased from Sigma (Tokyo, Japan).

**Tumor inoculation and treatment.** B16F10 melanoma cells (100 μl of 2x10\(^6\) cells/ml) were subcutaneously injected into female C57BL/6 mice as described previously (13). For quantitative real-time PCR (qRT-PCR) and FACS analysis, 5\% imiquimod cream was topically administered on day 7, and the tumor was harvested on day 9. For qRT-PCR, whole tumors were frozen with liquid nitrogen and then crushed with a Cryo-Press (Microtec, Chiba, Japan). Total RNA was extracted by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. For FACS analysis, whole tumors were incubated with collagenase 4 and DNase I for 20 min at 37°C, followed by mechanical disruption, as previously described (13). After single-cell suspensions were obtained, FACS analysis was performed. A total of 1x10\(^6\) cells were re-suspended in PBS/1% FCS and incubated with a combination of Abs for 30 min at 4°C. For the detection of Foxp3 expression, cells were fixed and permeabilized by using Cytofix/Cytoperm solution (BD Bioscience) according to the manufacturer’s protocol. After washing, the cells were kept in PBS/1% FCS. For all assays, cells were analyzed by using a C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA).

For the therapeutic experiments, the size of established tumors was measured with a caliper (Mitutoyo, Utsunomiya, Japan), and tumor volume was estimated by using the formula: \( \frac{1}{2} \times \text{length} \times \text{width} \times \text{depth} \). Starting on day 6, imiquimod cream (5%) was topically administered to the tumors three times a week. Tumor-bearing animals were sacrificed when the tumors displayed severe ulceration or reached a size of 1,000 mm\(^3\).

**RNA extraction and quantitative real-time PCR experiments.** Total RNA was extracted by using an RNAeasy Micro kit (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions. RNA was eluted with 14 μl of RNase-free water. Contaminating genomic DNA was removed with DNase I treatment (RNase-Free DNase Set; Qiagen). Reverse transcription was performed with the SuperScript VILO cDNA synthesis kit (Invitrogen). Amplification reactions were performed by using an MX 3000P real-time quantitative PCR system (Stratagene, Tokyo, Japan). Relative mRNA expression levels were calculated for each gene and each time point after normalization against GAPDH by using the ΔΔCt method. The experiments were repeated at least five times under identical conditions. The results are shown as the mean±standard deviation (SD) of all experiments.

**Purification of CD11b\(^+\) cells.** CD11b\(^+\) cells were isolated from tumors and spleens by using MACS beads (Miltenyi, Bergisch-Gladbach, Germany), as previously reported (15). Briefly, tumors were incubated with collagenase 4 and DNase I for 20 min at 37°C, followed by mechanical disruption. After single-cell suspensions were obtained, dead cells were depleted by using a dead cell removal kit from Miltenyi, according to the manufacturer’s protocol. Thereafter, aliquots of 10\(^7\) cells were incubated for 15 min with 10 μl of anti-CD11b beads (clone M170/15.11.5) (Miltenyi) in 100 μl MACS buffer (PBS, 0.5 mmol EDTA, 0.5% BSA) at 4°C, washed twice with MACS buffer and subjected to two consecutive rounds of separation via magnetic MS columns (Miltenyi). This procedure yielded predominantly CD11b\(^+\) cells with purity greater than 80% from tumors and greater than 95% from spleens, as assessed by FACS analysis.
IgG2a (0.1 mg) was administered intratumorally (0.1 mg) on days 7, 10, in tumors from IQM-treated and untreated mice by using qRT-PCR. Compared to untreated mice, topical IQM significantly decreased the mRNA expression of Th2/Treg-related chemokines (CCL17 and CCL22) in the tumor microenvironment (Figure 2A). The mRNA expression of Th1 chemokines (CXCL9, CXCL10 and CXCL11) or CCL2 was not significantly augmented (Figure 2A).

IQM decreased CCL22 production from CD11b+ TAMs in B16F10 melanoma. To validate the immunomodulatory effects of topical IQM on cancer stroma cells, we examined the effects of IQM on chemokine production from CD11b+ TAMs, which are the main population of tumor-infiltrating lymphocytes (TILs) in B16F10 melanoma. CD11b+ cells isolated from IQM-treated and untreated tumors were cultured for 48 h with LPS. The chemokines in the culture supernatant were then quantified by ELISA. Consistent with the IQM-induced decrease in CCL22 mRNA expression within the tumor, CCL22 production by these isolated CD11b+ cells was also significantly decreased by IQM treatment as compared to that of CD11b+ cells from untreated tumors (p<0.05) (Figure 2B). There was no significant difference in the production of CXCL10 from CD11b+ cells of IQM-treated and untreated tumors (Figure 2C). Unexpectedly, CCL17, CXCL9 and CXCL11 were not detected in the culture supernatants (data not shown).

IQM increased the expression of perforin and granzyme B and CD8+ T cell recruitment in B16F10 melanoma. To investigate the immunomodulatory mechanisms responsible for the decrease of Tregs, we evaluated the immunomodulatory effects of topical IQM. We determined the mRNA expression of type 1 and 2 T helper cell (Th1/Th2)-related chemokines and CCL2, which is a key chemokine for the induction of antitumor effects in B16F10 melanoma (3, 4, 7, 10), in tumors from IQM-treated and untreated mice by using qRT-PCR. Compared to untreated mice, topical IQM significantly decreased the mRNA expression of perforin and granzyme B in tumors from IQM-treated and untreated mice by using qRT-PCR. Compared to untreated mice, topical IQM significantly increased the mRNA expression of perforin and granzyme B in the tumor microenvironment (p<0.05) (Figure 3A).

Next, we evaluated the immunomodulatory effects of topical IQM on the profiles of TILs in the tumors by flow cytometry. Whole tumor cells were analyzed 48 h after stimulus with or without topical IQM. As shown in Figure 3B, the main population of TILs was CD11b+ TAMs. The percentage of CD8+ T cells within the CD45+ cell population was significantly increased by topical administration of IQM as compared to untreated mice (p<0.05). In contrast, there was no significant difference in the number of CD4+ T cells responsible for the decrease of Tregs, we evaluated the immunomodulatory effects of topical IQM. We determined the mRNA expression of type 1 and 2 T helper cell (Th1/Th2)-related chemokines and CCL2, which is a key chemokine for the induction of antitumor effects in B16F10 melanoma (3, 4, 7, 10), in tumors from IQM-treated and untreated mice by using qRT-PCR. Compared to untreated mice, topical IQM significantly decreased the mRNA expression of perforin and granzyme B (Figure 2B). There was no significant difference in the production of CXCL10 from CD11b+ cells of IQM-treated and untreated tumors (Figure 2C). Unexpectedly, CCL17, CXCL9 and CXCL11 were not detected in the culture supernatants (data not shown).
or CD11b+ cells within the CD45+ cell population in the presence or absence of IQM (Figure 3B). In addition, there was no significant difference in the percentage of PD-L1+ cells within the CD11b+ cell population in IQM-treated and untreated tumors (Figure 3C). There was no significant difference in the percentage of CD69+ cells and PD-1+ cells within the CD4+ and CD8+ T cell populations in IQM-treated and untreated tumors (Figure 3D).

**Immunomodulatory effect of IQM on splenic CD11b+ cells in vivo.** Since CD11b+ cells are the main population of CD45+ leukocytes in B16F10 melanoma, and since the number of CD11b+ cells isolated from B16F10 melanoma is extremely low, we next examined the immunomodulatory effects of topical IQM on splenic CD11b+ cells instead of TAMs. First, we evaluated the chemokine production of splenic CD11b+ cells from tumor-bearing mice treated with or without IQM.

![Figure 1](image-url)
After isolation, these CD11b+ cells were cultured for 48 h with LPS, and then the chemokines in the culture supernatant were quantified by ELISA. In parallel with the IQM-induced decrease in CCL22 production from tumor-derived CD11b+ cells, CCL22 production from splenic CD11b+ cells was also significantly decreased by topical IQM as compared to that in cells from untreated tumors (p<0.05) (Figure 4). In addition, in vitro TLR7 agonist stimulation significantly decreased CCL22 production (p<0.05), which suggests that IQM directly suppresses CCL22 production from splenic CD11b+ cells. CXCL10 production was also decreased, although the decrease was limited (Figure 4).

IQM did not affect the suppressive function of splenic CD11b+ cells in B16F10 melanoma-bearing hosts. To evaluate the suppressive function of splenic CD11b+ cells in vivo, CD11b+ cells were isolated from the spleen to test their capacity to suppress T cell proliferation. Isolated CD11b+ cells were cocultured with syngeneic CD4+ T cells and allogeneic BMDCs as stimulators. There was no significant difference in suppressive function of splenic CD11b+ cells in each group (Figure 4C).

**Splenic CD11b+ cells modulated B16F10 melanoma growth in vivo.** To determine if the topical IQM-induced decrease in CCL22 production from splenic CD11b+ cells (Figure 4A) and suppression of Treg recruitment to the tumor (Figure 1B) influence tumor cell growth, we investigated the effects of splenic CD11b+ cells on B16F10 melanoma growth in vivo. We isolated CD11b+ cells from the spleens of IQM-treated or untreated mice and implanted them together with B16F10 melanoma cells. B16F10 melanoma growth was accelerated by splenic CD11b+ cells from untreated mice but was inhibited by splenic CD11b+ cells from IQM-treated mice (Figure 5A). To confirm the effects of splenic CD11b+ cells on Treg recruitment, we evaluated the number of Tregs in the tumors by flow cytometry. The percentage of Foxp3+ Tregs within the CD4+ T cell population was significantly decreased by splenic CD11b+ cells from IQM-treated mice (p<0.05) (Figure 5B). Representative FACS data are shown in Figure 5C.

**CCL22 Ab significantly suppressed splenic CD11b+ cells and modulated B16F10 melanoma growth in vivo.** To determine if the decrease in Tregs is due to decreased CCL22 production from splenic CD11b+ cells (Figure 4A), we investigated the effects of anti-CCL22 Ab on B16F10 melanoma growth in the presence of splenic CD11b+ cells in vivo. We isolated splenic CD11b+ cells from untreated mice and implanted them together with B16F10 melanoma. We then treated B16F10 melanomas (5 mm in diameter) on the backs of mice by intratumoral injection of anti-CCL22 Ab (0.1 mg/mouse) or control IgG (0.1 mg/mouse). B16F10 melanoma growth was inhibited by intratumoral injection of anti-CCL22 Ab (Figure 5D). To confirm the effects of intratumoral injection of anti-CCL22 Ab on Treg recruitment, we evaluated the number of Tregs in the tumors by flow cytometry. The percentage of Foxp3+ Tregs within the CD4+ T cell population was significantly decreased by intratumoral injection of anti-CCL22 Ab (p<0.05) (Figure 5E). Representative FACS data are shown in Figure 5F.
Discussion

TAMs and circulating immature myeloid cells, so-called myeloid-derived suppressor cells (MDSCs), play crucial roles in maintaining the tumor microenvironment together with Tregs (16-20). In the differentiation cascade of myeloid cells in the tumor-bearing host (21), TAMs are derived from Ly6C⁺ monocytes (monocytic MDSCs) in the blood (21). The majority of TAMs are alternatively activated M2 macrophages, which represent the main population of inflammatory cells in solid tumors (21-24). Since some populations of TAMs are derived from MDSCs, the mechanisms of suppressive activities of TAMs are similar to those of MDSCs (16, 17, 21, 22, 25). For instance, the...
suppressive function of TAMs and MDSCs is partly mediated by the metabolism of L-arginine, which is determined by the expression of high levels of arginase 1 and inducible nitric oxide synthase (iNOS), both of which inhibit T cell proliferation (16, 17, 21, 22, 25). In addition, both TAMs and MDSCs produce Treg-related chemokines to recruit Tregs to the tumor microenvironment (16, 17, 21, 22, 25-27). Moreover, TAMs and MDSCs express immune checkpoint molecules, such as PD-L1 and B7-1, to suppress the activities of effector memory T cells in the tumor microenvironment.
Figure 5. Continued
microenvironment (15, 25). Notably, TAMs are a heterogeneous population of cells that could be polarized to M1-like macrophages in the tumor microenvironment (21). Therefore, TAMs could be a promising immunotherapy target in the treatment of melanoma (13, 28).

In the present study, we show the immunomodulatory effects of IQM on TAMs and splenic CD11b+ cells, leading to the inhibition of B16F10 melanoma growth in vivo. As Lawrence et al. have reviewed (21), we demonstrated that the production of several chemokines (CCL17, CCL22, CXCL9, CXCL10 and CXCL11) of splenic CD11b+ cells resembled those of TAMs. Subsequently, we investigated the effects of splenic CD11b+ cells on B16F10 melanoma growth in vivo. B16F10 melanoma growth was accelerated by splenic CD11b+ cells from untreated mice but inhibited by splenic CD11b+ cells from IQM-treated mice. Moreover, Foxp3+ Treg recruitment to the tumor was significantly decreased when splenic CD11b+ cells from IQM-treated mice were used. Notably, CCL22 production by both tumor-derived and splenic CD11b+ cells was significantly decreased by IQM treatment compared to that of CD11b+ cells from untreated hosts. As previous reports suggested, CCL22 diverts Tregs and controls B16F10 melanoma growth (13), suggesting the reduction of tumor-derived CCL22 could suppress melanoma growth. Indeed, the blockade of CCL22 by intratumoral injection of anti-CCL22 Ab significantly inhibited Treg recruitment, leading to inhibition of B16F10 melanoma growth in vivo. We also evaluated the mRNA expression of CCL2, which is a known ligand of CCR4 and reported to increase in advanced melanoma in an experimental murine model (14). Unlike CCL22 mRNA expression, IQM treatment did not affect the expression of CCL2 mRNA on B16F10 melanoma. These reports suggested that inhibition of tumor-derived chemokines could be a therapeutic target for melanoma by the reduction of Treg recruitment at the tumor site.

Tregs play a crucial role in maintaining peripheral tolerance that actively suppresses effector T cells (24). Tregs maintain the immunosuppressive microenvironment and promote tumor growth together with TAMs at the tumor site (10, 24, 29). Indeed, both MDSCs, which are known as immunosuppressive immature macrophages, and Tregs determine the prognosis of advanced melanoma patients (30). In addition, Mahnke et al. reported that Treg depletion by denileukin diftitox (Ontak®; Eisai Medical Research, Tokyo, Japan) in melanoma patients resulted in the substantial development of antigen-specific CD8+ T cells in vaccinated individuals (31). Another report also suggested that Treg depletion had a significant clinical effect in unresectable stage IV melanoma patients (32). These reports suggested that Treg depletion could be a supportive therapy for human melanoma.

The therapeutic effect of a TLR7 agonist (including IQM) or TLR7/8 dual agonist on melanoma growth in vivo has been previously reported (2, 4, 7, 33). These reports revealed the immunostimulatory effect of these agonists through
effector cells, such as CD8+ T cells, plasmacytoid DCs or M1 macrophages, and decrease the Tregs and MDSC in the tumor lesion, leading to the inhibition of B16F10 melanoma growth in vivo. In addition, more recently, several clinical trial has proved the antitumor effects of IQM against melanoma in situ (34, 35). In our present study, we further elucidated the immunomodulatory effect of IQM on TAMs and splenic CD11b+ cells during melanoma growth in vivo, especially focusing on the production of Treg-related chemokines and effector T cells-related chemokines. Indeed, we showed that topical IQM reduced the immunosuppressive function of TAMs in B16F10 melanomas and even promoted the subpopulation of splenic CD11b+ cells that inhibited B16F10 melanoma growth. Our present study revealed another possible mechanism of the antitumor effect of IQM.

Acknowledgements

This study was supported, in part, by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (Nos. 23791249 and 25461682). The 5% imiquimod cream was a kind gift from Mochida Pharmaceutical Co., Ltd (Tokyo, Japan).

Conflicts of Interest

The Authors declare no conflicts of interest.

Funding

This study was supported, in part, by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (Nos. 23791249, 25461682 and 16K10143).

References


