A Critical Role for Mast Cells and Mast Cell-Derived IL-6 in TLR2-Mediated Inhibition of Tumor Growth

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A Critical Role for Mast Cells and Mast Cell-Derived IL-6 in TLR2-Mediated Inhibition of Tumor Growth


Several TLR agonists are effective in tumor immunotherapy, but their early innate mechanisms of action, particularly those of TLR2 agonists, are unclear. Mast cells are abundant surrounding solid tumors where they are often protumorigenic and enhance tumor angiogenesis. However, antitumor roles for mast cells have also been documented. The impact of mast cells may be dependent on their activation status and mediator release in different tumors. Using an orthotopic melanoma model in wild-type C57BL/6 and mast cell-deficient KitW-sh/W-sh mice and a complementary Matrigel–tumor model in C57BL/6 mice, mast cells were shown to be crucial for TLR2 agonist (Pam3CSK4)-induced tumor inhibition. Activation of TLR2 on mast cells reversed their well-documented protumorigenic role. Tumor growth inhibition after peritumoral administration of Pam3CSK4 was restored in KitW-sh/W-sh mice by local reconstitution with wild-type, but not TLR2-deficient, mast cells. Mast cells secrete multiple mediators after Pam3CSK4 stimulation, such as IL-6, but not TNF. Mast cell-mediated anticancer properties were multifaceted. Direct antitumor effects in vitro and decreased angiogenesis and recruitment of NK and T cells in vivo were observed. TLR2-activated mast cells also inhibited the growth of lung cancer cells in vivo. Unlike other immune cells, mast cells are relatively radioresistant making them attractive candidates for combined treatment modalities. This study has important implications for the design of immunotherapeutic strategies and reveals, to our knowledge, a novel mechanism of action for TLR2 agonists in vivo. The Journal of Immunology, 2010, 185: 000–000.

The use of TLR agonists as immunological adjuvants in the treatment of cancer has been intensively studied. TLR-based immunotherapies have shown promise clinically for the treatment of multiple tumor types. Specifically, TLR2 agonists have been successfully used as potential therapeutic in animal models of pancreatic and brain carcinoma (1, 2) as well as in clinical trials (3). In general, the successful mechanism of action of TLR agonists is considered to involve dendritic cell maturation with enhanced tumor Ag presentation and generation of Th1 immunity and CD8 antitumor effector cells (4). TLR2 agonists, such as Pam3CSK4, often do not share the strong Th1-polarizing effects of other TLR agonists (5, 6). However, they retain effectiveness in tumor immunotherapy models, opening up the possibility that they have a distinct mechanism of action.

Mast cells are localized at body sites that interface with the environment, such as the skin and mucous membranes, where they play a role in allergic disease and function as sentinel cells involved in the induction and development of effective immunity (7–9). Mast cells also play a role in tumorigenesis. Abundant mast cells are closely associated with blood vessels surrounding solid tumors and often correlate with enhanced tumor angiogenesis and poor prognosis (10–12). Studies using mast cell-deficient KitW-sh/KitW-sh or KitW-sh/KiW-sh mice showed that tumor-associated mast cells are potent tumor angiogenesis promoters, enhancing tumor growth and metastasis (13–15). Although extensive studies demonstrate a protumorigenic role for mast cells (reviewed in Ref. 16), antitumor actions of mast cells have also been reported. In vitro studies have demonstrated mast cells can mediate direct TNF-mediated tumor cell cytotoxicity (17, 18). Several studies have also correlated high mast cell density with improved survival (19–21). The conflicting reports of associations of mast cells with prognosis are potentially largely dependent on effects of the local tumor microenvironment on mast cell activation status and mediator release.

During infection, activation of mast cell receptors including Fcγ receptors, complement receptors, and TLRs results in the release of preformed mediators including histamine and proteases, the synthesis of lipid mediators, and the selective production of multiple cytokines and chemokines. These mediators are important in eliminating pathogens, recruiting other leukocytes such as neutrophils, NK cells, and T cells, increasing vascular permeability, and in mediating proteolytic degradation at the site of infection (9). Although the role of mast cells in protecting against infection is well documented, the effects of activating mast cells in the context of tumorigenesis have not been substantially explored.

Mast cells are prevalent in the skin and are abundant at the periphery of melanomas (12). They respond to TLR2 agonists by producing leukotrienes, cytokines, and chemokines, which could modify the local microenvironment and recruit leukocytes potentially to limit tumor growth (22, 23). We therefore investigated the role of the mast cell in the tumor growth-inhibitory effects of the
TLR2/1 agonist Pam3CSK4 using an orthotopic model of B16.F10 melanoma, a well used, highly aggressive, poorly immunogenic tumor (24). B16.F10 growth was compared in wild-type and mast cell-deficient KitW/ΔΔW-sh mice. Although KitW/ΔΔW-sh mice are mainly white, they are suitable for melanoma growth studies as the KitW/ΔΔW-sh mutation impairs long-term endogenouse melanocyte survival but does not affect melanoblast development or differentiation or the survival of transplanted c-Kit–expressing melanocytes. Homozygote adult animals consistently retain melanocytes at selected sites including the ear pinna (25, 26). Comparative growth studies in KitW/ΔΔW-sh and control C57BL/6 mice identified that Pam3CSK4 inhibits tumor growth in a mast cell and mast cell-derived IL-6-dependent manner. This study demonstrates that selective activation of mast cells via TLR2 can reverse their tumor-promoting potential and induce them to inhibit tumor growth.

Materials and Methods

Mice

C57BL/6, B6.129S6-TgrmGf1/J, B6.129S2-B6ESkol/J, B6.129P2-Ccrl1−/−, and B6.Cg-KitW/+/H2Mexacm1 (KitW/ΔΔW-sh) were purchased from The Jackson Laboratory (Bar Harbor, ME). KiPSA mice were bred in-house from this stock. B6.129-Tlr2pm1Ker/J mice were provided by Dr. S. Akira (Osaka, Japan) and bred in-house or purchased from The Jackson Laboratory. Matched groups of male or female mice were used by Dr. S. Akira (Osaka, Japan) and bred in-house or purchased from The Jackson Laboratory. Matched groups of male or female mice were used 8–12 wk of age and housed under pathogen-free conditions with food and water provided ad libitum. All experiments were performed according to protocols approved by the animal research ethics board of Dalhousie University (Halifax, Nova Scotia, Canada).

Generation of bone marrow-derived mast cells and local reconstitution of mast cell-deficient mice

Bone marrow-derived mast cells (BMMCs) were generated from wild-type C57BL/6 and gene-deficient TLR2−/−, TNF−/−, IL-6−/−, or CCL3−/− mice as previously described (27) and were used at >98% purity. BMMCs derived from selected gene-deficient animals were functionally similar to control C57BL/6 BMMCs (Supplemental Fig. 1). BMMCs were cultured for 16–24 h in the absence of PGE2 prior to in vitro experiments. Eight- to ten-week-old KitW/ΔΔW-sh mice were reconstituted on the dorsal surface over the right and left flanks in 2 × 2 cm2 areas via a series of injections with BMMCs (1 × 106 intradermal and 1 × 106 s.c.), which resulted in mast cell levels comparable with those of wild-type C57BL/6 mice (Supplemental Fig. 2).

Established tumor growth experiments

B16.F10 cells (1 × 105) (American Type Culture Collection, Manassas, VA) in 100 μl PBS were injected s.c. above the right and left flanks. At days 4 and 9 (and day 14 as indicated), mice were injected s.c. at the tumor site with 3 μg Pam3CSK4 or FSL-1 (EMC Microcollections, Tübingen, Germany) in 50 μl PBS or diluent control. Tumor volume (V) was determined by caliper measurements (V = L × W × 2). After 14 to 19 d, mice were euthanized, and tumors and removed and weighed. Percentage tumor reduction = (tumor + BMMC treated tumor average – PBS treated tumor average)/PBS treated tumor average × 100.

In vivo Matrigel–tumor growth experiments

Growth factor–reduced Matrigel (Becton Dickinson, San Jose, CA) suspensions containing 1.5 × 105 B16.F10 or LLC1 cells (American Type Culture Collection), with or without 10 μg/ml Pam3CSK4 (EMC Microcollections) and/or 3 × 105 BMMCs, were prepared. All Matrigel suspensions contained 3 ng/ml murine rIL-3 (PeproTech, Rocky Hill, NJ). C57BL/6 mice (8–12 wk) were injected s.c. in four sites with 0.3 or 0.15 ml suspensions containing 3 ng/ml murine rIL-3 (PeproTech, Rocky Hill, NJ). C57BL/6 or IL-6−/− BMMCs incubated for 24 h in BM GMC culture medium with or without 10 μg/ml Pam3CSK4, tumor cell apoptosis in B16.F10 (0.5 × 105/ml)–BMMC (1 × 105/ml) cocultures was assessed using an annexin V–FITC–propidium iodide apoptosis detection kit (BD Biosciences, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. BMMCs were excluded from analysis via biotinylated anti-CD45 (eBioscience, San Diego, CA) and streptavidin (SA)–allophycocyanin (BD Biosciences) staining.

Cell count and flow cytometry

Established tumors were collagenase digested as above. Matrigel tumors were digested in 2.24 U/ml Dispase (Life Technologies, Carlsbad, CA) in PBS. Overall tumor cellularity was assessed by flow cytometry of digested tumors with reference to a known number of 15 μm Polystyrene microspheres (Polysciences, Warrington, PA). Total leukocytes were stained with biotinylated anti-CD45 (eBioscience) and SA–PerCP (BD Biosciences), anti-TCR–PERCP (eBioscience) or anti-CD3–FITC (BD Biosciences), anti-CD8–PE (eBioscience), and anti-NK1.1–allophycocyanin (BD Biosciences). Biotinylated rat IgG2a isotype (eBioscience) with SA–PerCP staining did not exceed 0.1% of total. Samples were gated on CD45+ cells and leukocyte subsets quantified within this gate. Staining using relevant isotype control Abs (eBioscience) did not exceed 0.2% of CD45+ cells.

Chemotaxis assays

C57BL/6 spleens were dispersed by pushing through a 100-μm nylon mesh. Erythrocytes were lysed using 0.14 M ammonium chloride plus 20 mM Tris (pH 7.4). Adherent cells were removed via incubation for 20 min at 37°C in 0.1% BSA in RPMI 1640. Chemotaxis of nonadherent splenocytes was performed using 24-well Transwells with a 5-μm pore size (Corning, Lowell, MA). Test supernatant (600 ng/ml) was added to the bottom chamber, and 1 × 106 splenocytes in 100 μl RPMI 1640 containing 1% BSA was added to the top. Chemotaxis was carried out for 2 h at 37°C. To enumerate total migrating cells, each well received 15-μm polybead polystyrene microspheres (Polysciences) as an internal standard. NK1.1+, CD11b+, and CD8+ cells were assessed by flow cytometry, as described earlier. Total migrated subsets were assessed as a percentage of the starting cell subset population and were calculated based on absolute numbers of each cell subset.

In vitro cell stimulation, protein array analysis, and ELISA

B16.F10 cells (0.5 × 105/ml) and/or BMMCs (1 × 105/ml) were incubated with or without 10 μg/ml Pam3CSK4, FSL-1, LPS (Sigma-Aldrich), PGN (Invogen), and polyinosinic-polycytidylic acid (Calbiochem, San Diego, CA) or A23187 ionophore (Sigma-Aldrich) positive control at 37°C in RPMI 1640 containing 10% FBS, 3 ng/ml murine rIL-3 (PeproTech), and 100 μg/ml soybean trypsin inhibitor (Sigma-Aldrich). Cell-free supernatants were assessed using the murine CytoCline III Ab array (Raybiotech, Norcross, GA) and ELISA kits to quantify levels of TNF and IL-13 (both eBioscience), IL-6 (PeproTech), and CCL1 and CCL3 (both R&D Systems, Minneapolis, MN).

Statistics

Samples were analyzed using unpaired t test or non-parametric Mann-Whitney U test as indicated based on D’Agostino and Pearson omnibus normality test. Matrigel–tumor experiments were assessed with repeated measures Friedman’s test and between-group comparisons assessed by Wilcoxon matched pairs test (10 d) or repeated measures ANOVA with Bonferroni’s post hoc test as indicated based on D’Agostino and Pearson omnibus normality test.
Results

TLR2 agonist treatment inhibits B16.F10 tumor growth via mast cell activation

The tumor-inhibitory effects of the TLR2/1 agonist Pam3CSK4 were investigated using an orthotopic model of B16.F10 melanoma. When treated with Pam3CSK4, tumors showed a significant reduction in volume (73%; Fig. 1A) and cellularity (74%; Fig. 1B). Mast cells were detected in close proximity to blood vessels at the periphery of both Pam3CSK4-treated and control tumors (Fig. 1C). As the ability of mast cells to exert a protective role in tumorigenesis may be dependent on an intratumoral distribution (28, 29), we assessed the distribution of mast cells in control and Pam3CSK4-treated tumors. Mast cells were predominantly found in the stroma as opposed to intratumorally for both PBS-treated (2.3 ± 0.5/mm² versus 0.5 ± 0.1/mm², n = 6) and Pam3CSK4-treated (3.3 ± 0.7/mm² versus 1.7 ± 0.9/mm², n = 9) tumors. The percentage of mast cells that were found intratumorally did not significantly differ between PBS-treated and Pam3CSK4-treated tumors (21 ± 5% versus 28 ± 11%). The protumorigenic role of mast cells has been well characterized in several models (14, 15). Consistent with these findings, baseline tumor growth rate, in the absence of TLR2 agonist treatment, was significant and all pooled data are presented as mean ± SEM.

To investigate a possible role for mast cells in Pam3CSK4-mediated tumor growth inhibition, B16.F10 tumors were treated in mast cell-containing C57BL/6 mice or mast cell-deficient KitW-sh/W-sh mice. In KitW-sh/W-sh mice, Pam3CSK4 did not inhibit tumor growth (Fig. 2A), whereas tumor growth was decreased in parallel groups of mast cell-containing mice (59%; Fig. 2B), suggesting a mast cell-dependent mechanism of Pam3CSK4 action. Decreased end-point tumor weight (65%; Fig. 2C) and tumor blood volume (69%; Fig. 2D) were also mast cell dependent. Enhanced growth of diluent-treated control tumors was observed in C57BL/6 compared with KitW-sh/W-sh, further confirming the protumorigenic role of mast cells in the absence of TLR2 activation.

Whereas KitW-sh/W-sh mice have been documented to be similar to their wild-type littermates with respect to myeloid and lymphoid cell subsets in multiple anatomical sites (30), others have demonstrated myeloid differences compared with wild-type C57BL/6 mice (31). To overcome any inherent defects of the mast cell-deficient mouse model and assess the physiological role of mast cells in Pam3CSK4-mediated effects, KitW-sh/W-sh mice were locally reconstituted with BMMCs, which were allowed to mature in situ prior to performing tumor growth experiments. We also extended the time course of the tumor growth experiment to ensure the lack of effect of Pam3CSK4 in KitW-sh/W-sh mice was not attributable to delayed tumor growth kinetics (Fig. 2A). Even with tumor size reaching levels comparable with those of C57BL/6 mice, the antitumor activity of Pam3CSK4 was absent in mast cell-deficient mice (Fig. 3A). Local reconstitution with C57BL/6, but not TLR2−/− BMMCs, restored the Pam3CSK4-mediated decrease in tumor volume (61%, Fig. 3A) and tumor weight (63%, Fig. 3B).

TLR1 and TLR2 are expressed on both murine BMMCs (32) and B16 melanoma (33) (see also Supplemental Fig. 3). To confirm the specific involvement of mast cell-associated TLR2 in the antitumor effects of Pam3CSK4, the ability of wild-type and TLR2−/− BMMCs to inhibit tumor growth were compared using a Matrigel–tumor model. Even within the context of the tumor growth-promoting Matrigel environment (34), TLR2-expressing BMMCs significantly inhibited melanoma growth in the presence of Pam3CSK4 (49% inhibition), whereas Pam3CSK4-activated TLR2-deficient BMMCs did not (Fig. 3C). Pam3CSK4 or BMMCs alone had no inhibitory effect on tumor growth as weight of Matrigels containing B16.F10 plus BMMCs (0.295 ± 0.046 g) did not differ from that of B16.F10 alone (0.244 ± 0.028 g) or B16.F10 plus Pam3CSK4 (0.250 ± 0.016 g).

The Matrigel model was also used to assess the frequency of mature blood vessels at the tumor periphery because the decreased tumor blood volume of Pam3CSK4-treated tumors (Fig. 2D) suggested mast cells could inhibit tumor angiogenesis. Matrigel plugs in which B16.F10 was grown with Pam3CSK4-activated BMMCs had 60% fewer vessels than those that contained melanoma cells together with BMMCs alone (Fig. 3D).

FIGURE 1. Pam3CSK4 treatment decreases B16.F10 tumor growth. C57BL/6 mice were injected s.c. with B16.F10 melanoma cells. Tumors were treated s.c. on days 4 and 9 post-tumor injection (arrows) with Pam3CSK4, or PBS control (n = 10 tumors per group). A, Tumor volume was monitored daily. **p = 0.004. B, After 14-d growth, overall cellularity of collagenase-disrupted tumors was assessed. Right panel depicts representative diluent PBS- or Pam3CSK4-injected tumors. *p = 0.038. C, Tumor-associated mast cells were examined in tissue sections stained with Alcian blue–safranin O (original magnification ×400). Adjacent tumor cell areas are indicated by black arrows. D, Mast cell-deficient KitW-sh/W-sh and C57BL/6 mice were injected s.c. with B16.F10 melanoma cells (n = 10 per group), and tumor volume was followed for 16 d. Pooled data from two independent experiments: *p = 0.026.

Dunnett’s multiple comparison post test, using B16.F10 alone as control (4 d), based on normality tests. The p values < 0.05 were considered significant, and all pooled data are presented as mean ± SEM.

**p = 0.004. After 14-d growth, overall cellularity of collagenase-disrupted tumors was assessed. Right panel depicts representative diluent PBS- or Pam3CSK4-injected tumors. *p = 0.038. C, Tumor-associated mast cells were examined in tissue sections stained with Alcian blue–safranin O (original magnification ×400). Adjacent tumor cell areas are indicated by black arrows. D, Mast cell-deficient KitW-sh/W-sh and C57BL/6 mice were injected s.c. with B16.F10 melanoma cells (n = 10 per group), and tumor volume was followed for 16 d. Pooled data from two independent experiments: *p = 0.026.
Proliferation was unaffected by Pam3CSK4 treatment but was decreased by TNF from mast cells was not critical for tumor inhibition. We further assessed direct antitumor effects in vitro. B16.F10 demonstrated TNF from mast cells was not critical for tumor inhibition, at day 10, in the presence of Pam3CSK4 and BMMCs (Supplemental Fig. 5). To determine the involvement of TLR2-activated mast cells in early NK and T cell recruitment, we performed flow cytometry of B16.F10 tumors in Matrigel, on day 4, in the presence or absence of Pam3CSK4 and/or BMMCs. Tumor growth was significantly decreased in the presence of BMMCs and Pam3CSK4 and was associated with significant local increases in both NK and T cells (Fig. 5B).

CCL3 induces recruitment of both mature NK cells (35) and activated CD8+ T cells (36, 37). To determine whether TLR2-activated mast cells directly induce the migration of NK and CD8+ T cells and specifically to assess the role of mast cell-derived CCL3, we performed in vitro chemotaxis assays of C57BL/6 splenocytes using supernatants generated from wild-type and CCL3−/− BMMCs (Fig. 5C). Pam3CSK4-activated BMMC supernatants induced significant chemotaxis of NK1.1+ NK cells and both CD3+CD8+ and CD3−CD8+ T cell subsets (all p < 0.05). In contrast, supernatants from mast cells that could not produce CCL3 did not induce migration of NK or T cell subsets, demonstrating a role for CCL3 in mediating mast cell-dependent effector cell recruitment.

**TLR2-activated mast cell inhibition of B16.10 tumor growth is TNF independent**

Mast cells have been shown to induce tumor cytotoxicity in a TNF-dependent manner (17, 18). In vitro Pam3CSK4-stimulated BMMCs have elevated secretion of TNF (Fig. 4A). However, coculture of BMMCs and B16.F10 in the presence of Pam3CSK4 did not induce tumor cell apoptosis (Fig. 4B). Local reconstitution of KitW-sh/KitW cells with BMMCs derived from TNF-deficient mice restored the antitumor effect of Pam3CSK4 as indicated by significant reductions in tumor volume (57%; Fig. 4C), end-point tumor weight (122 ± 30 mg versus 78 ± 22 mg, p = 0.030), and tumor blood volume (72.0 ± 16.1 versus 42.8 ± 22.1 RU, p = 0.035), demonstrating TNF from mast cells was not critical for tumor inhibition. We further assessed direct antitumor effects in vitro. B16.F10 proliferation was unaffected by Pam3CSK4 treatment but was decreased by 18% in the presence of Pam3CSK4-activated C57BL/6 BMMC supernatants (Fig. 4D).

**TLR2-activated mast cells recruit NK and T cell subsets**

To identify potentially important mast cell mediators, supernatants of BMMCs cultured with or without Pam3CSK4 and/or B16.F10 cells were examined by cytokine protein array. In BMMC–B16.F10 cocultures, Pam3CSK4 induced IL-6, IL-13, CCL1, CCL2, CCL3, CCL5, CXCL1/2, CXCL3, and CXCL5 production (Supplemental Fig. 4). Such mediator production was independent of mast cell degranulation assessed via β-hexosaminidase release (Supplemental Fig. 1). The presence of melanoma cells further enhanced mast cell mediator production, as confirmed by ELISA (Fig. 5A).

Elevated chemokine production by Pam3CSK4-activated mast cells suggested mast cell-mediated tumor-inhibitory effects might involve effector cell recruitment. Flow cytometry of disrupted tumors demonstrated that Pam3CSK4 treatment was associated with infiltrating CD45+ leukocytes, including CD8+ TCRβ+ and NK1.1+ cell subsets. B16.F10 tumors grown in Matrigel also had a marked mononuclear cell infiltration, at day 10, in the presence of Pam3CSK4 and BMMCs (Supplemental Fig. 5). To determine the involvement of TLR2-activated mast cells in early NK and T cell recruitment, we performed flow cytometry of B16.F10 tumors in Matrigel, on day 4, in the presence or absence of Pam3CSK4 and/or BMMCs. Tumor growth was significantly decreased in the presence of BMMCs and Pam3CSK4 and was associated with significant local increases in both NK and T cells (Fig. 5B).

**TLR2-activated mast cells inhibit B16.10 tumor growth via an IL-6-dependent mechanism**

Pam3CSK4-activated BMMCs secreted substantial amounts of IL-6 (Fig. 5A). The requirement of mast cell IL-6 in tumor growth inhibition by Pam3CSK4 was confirmed via lack of Pam3CSK4 effect after local reconstitution of KitW-sh/KitW sh with BMMCs derived from IL-6−/− mice. In contrast, tumor growth was decreased in a parallel group of wild-type C57BL/6 mice (Fig. 6A). We further confirmed the requirement for mast cell-secreted IL-6 using the Matrigel–B16.F10 model. Tumor growth was not inhibited by Pam3CSK4 treatment in the presence of IL-6−/− mast cells. Furthermore, tumor blood volume and the number of mature erythrocyte-containing blood vessels were not decreased in the presence of Pam3CSK4-activated IL-6−/− BMMCs (Fig. 6B). We further confirmed the requirement for mast cell-secreted IL-6 using the Matrigel–B16.F10 model. Tumor growth was not inhibited by Pam3CSK4-activated IL-6−/− BMMCs, in contrast with results of similar studies with wild-type BMMCs. As in vitro and in vivo experiments have demonstrated IL-6 can induce melanoma growth inhibition (38–41), we assessed the effect of Pam3CSK4-activated IL-6−/− BMMC supernatants on B16.F10 proliferation. In vitro, supernatants from Pam3CSK4-activated IL-6−/− BMMCs did not significantly decrease B16.F10 cell proliferation (Fig. 6C).

**TLR2-activated mast cells inhibit lung carcinoma growth**

To ascertain whether the inhibitory effects of TLR2-activated mast cells are unique to melanoma or can be extended to other tumor types, we investigated the ability of Pam3CSK4-activated mast cells to inhibit the growth of the lung cancer model LLC1, at day 10, using the Matrigel–tumor model system in C57BL/6 mice. TLR2 expressing BMMCs significantly inhibited LLC1 growth in the presence of Pam3CSK4 (58%), whereas Pam3CSK4-activated TLR2-deficient BMMCs did not (Fig. 7A). In contrast, Pam3CSK4 or BMMCs alone had no effect on tumor growth (Fig. 7B) as weight...
of Matrigels containing LLC1 plus BMMCs (0.236 ± 0.028 g) did not differ from that of LLC1 alone (0.257 ± 0.036 g) or LLC1 plus Pam3CSK4 (0.241 ± 0.019 g). In a manner comparable with the B16.F10 melanoma, decreased LLC1 tumor growth in the presence of Pam3CSK4-activated mast cells was also associated with mononuclear cell infiltration (Fig. 7C) and decreased angiogenesis (Fig. 7D).

**A TLR2/6 agonist can mediate mast cell-dependent tumor growth inhibition**

To assess the broader applicability of mast cell-dependent antitumor responses to TLR-based immunotherapies, we evaluated the ability of mast cells to produce mediators in response to a panel of TLR agonists. These studies demonstrate that at doses similar to that used for Pam3CSK4 in this study (10 μg/ml), the synthetic diacylated lipopeptide FSL-1 (a TLR2/6 agonist) and LPS (a TLR4 agonist) induced mediator production at least 2-fold over media control, similar to that seen with Pam3CSK4. The synthetic dsRNA analogue polyinosinic-polycytidylic acid (a TLR3 agonist) and bacterial peptidoglycan (activates TLR2/6 and other receptors) failed to induce mediator profiles from BMMCs similar to Pam3CSK4 upon 24-h stimulation (Supplemental Table I). To investigate whether additional TLR2 agonists could mediate mast cell-dependent antitumor effects in vivo, we performed similar studies using the TLR2/6 agonist FSL-1. FSL-1 treatment significantly inhibited B16.F10 tumor growth in C57BL/6 mice but not in mast cell-deficient KitW-sh/W-sh mice (Fig. 7E). Mast cell dependence in the antitumor effect of FSL-1 was confirmed via restoration of the antitumor effects of FSL-1 in KitW-sh/W-sh mice locally reconstituted with C57BL/6 BMMCs (day 15 tumor volume: 476 ± 153 mm³ [PBS] versus 55 ± 27 mm³ [Pam3CSK4], n = 4).

**Discussion**

This study demonstrates a previously unrecognized mechanism for TLR2-based immunotherapy with a critical role for the mast cell. TLR2 expression on mast cells was crucial for mediating the inhibitory effects of Pam3CSK4 on melanoma growth, even though multiple other effector cells, and the melanoma cells themselves, are known to express TLR2. The KitW-sh/W-sh model provides a useful tool to study the role of mast cells as KitW-sh/W-sh mice are profoundly mast cell deficient in multiple anatomical sites, with only rare mast cells detected in the skin of adult animals (30, 42). Restoration of the tumor-inhibitory effect of Pam3CSK4 in KitW-sh/W-sh mice via local reconstitution of mast cells indicates that other potential differences between mast cell-deficient mice and controls (31) were not responsible for the lack of tumor growth inhibition by Pam3CSK4 in KitW-sh/W-sh mice. TLR2-mediated activation completely reversed the normal protumorigenic role of mast cells in this model and instead harnessed them to initiate complex tumor-inhibitory effects that include recruitment of immune effector cells and angiogenesis inhibition.
Although mast cells have been demonstrated to display antitumor activity via TNF-mediated cytotoxicity (17, 18), the direct effects of activated mast cells on tumor growth occurred independently of mast cell-mediated TNF cytotoxicity and apoptosis induction. Pam3CSK4 did not exhibit direct antitumor effects in vitro. However, decreased tumor cell proliferation via Pam3CSK4-activated C57BL/6 but not IL-6−/− mast cell supernatants demonstrates the tumor-inhibitory effects of TLR2-activated mast cells are likely to be, in part, mediated via the direct effects of mast cell-derived IL-6. The role of IL-6 in tumorigenesis is controversial and appears to be dependent on the stage of disease and tumor type (39). However, in vitro studies have demonstrated IL-6 is directly growth inhibitory.

FIGURE 4. Pam3CSK4-activated mast cells decrease B16.F10 tumor growth in a TNF-independent manner. A, TNF secretion was measured by ELISA of cell-free supernatants. A23187 ionophore induction of TNF was included as a positive control at the 6-h time point (n = three experiments/group). *p < 0.05; ***p < 0.001 (compared with media control). B, Annexin V–propidium iodide (PI) staining of B16.F10 cells cocultured with Pam3CSK4 and/or C57BL/6 BMCCs (n = 3 experiments/group). CPT was included as a positive control with DMSO vehicle control. ***p < 0.001. C, Mast cell-deficient KitW-sh/W-sh mice were locally reconstituted with TNF-deficient BMCCs 2 wk prior to tumor injection then treated on days 4 and 9 (arrows) with Pam3CSK4 (n = 12 mice from two independent experiments per group). *p = 0.012. D, B16.F10 proliferation was assessed in the presence of increasing doses of Pam3CSK4 (pooled data from three independent experiments) and cell-free supernatants from C57BL/6 BMCCs (pooled data from 12 independent experiments) treated for 24 h with or without Pam3CSK4. CPT was included as a positive control. Data are expressed as fold change over media control for BMCC supernatants and fold change over DMSO vehicle control for CPT. ***p < 0.0001. ns, not significant; UD, undetectable.

FIGURE 5. Pam3CSK4-activated mast cells recruit immune effector cells in a CCL3-dependent manner. A, IL-6, IL-13, CCL1, and CCL3 secretion was measured by ELISA of cell-free BMCC supernatants (n = 3 experiments). *p < 0.05; **p < 0.01. B, C57BL/6 mice were injected with Matrigel (0.15 ml) containing B16.F10 cells in the presence or absence of Pam3CSK4 and/or BMCCs derived from C57BL/6 mice (n = 6). After 4 d, Matrigels were removed and weighed by flow cytometry. B16+F10 alone. C, C57BL/6 splenocyte chemotaxis was assessed in response to supernatants from C57BL/6 or CCL3−/− BMCCs (n = 3 independent experiments for C57BL/6 BMCC and n = 2 independent experiments for CCL3−/− BMCCs). *p < 0.05.
for several tumor cell types, including melanoma (38, 43–45). The melanoma growth-inhibitory effects of IL-6 have also been confirmed in vivo (40, 41). Studies in melanoma cell lines, including B16.F10.9 cells, have demonstrated IL-6–induced tumor cell growth inhibition is enhanced in the presence of soluble IL-6R and occurs via a STAT-dependent mechanism that involves upregulation of cyclooxygenase-2 gene expression and induction of cell cycle arrest (46–48). IL-6–transfected B16.F10 tumors exhibit a significant decrease in angiogenesis via an in vivo intradermal angiogenesis assay (40). Other mast cell-derived mediators may also suppress tumor angiogenesis. For example, Pam3CSK4–activated mast cells also secrete large amounts of IL-13 (Fig. 5A), a potent antiangiogenic cytokine (54).

The crucial role of mast cells for the mobilization of immune effector cells in response to infection is well documented (7, 8). However, their potential to enhance the recruitment of effector cells to tumor sites has not been previously explored. Decreased tumor growth in the presence of Pam3CSK4–activated mast cells is associated with the early recruitment of significant numbers of both NK and T cell subsets. We have demonstrated that TLR2-activated mast cells secrete large amounts of CCL3, which has been demonstrated to be chemotactic for mature NK cells (35) and activated CD8+ T cell subsets in this model. We have also identified multiple other cytokines and chemokines produced from Pam3CSK4–activated mast cells that may enhance effector cell recruitment and activation in vivo. For example, TLR2 ligand-activated BMMCs secrete substantial amounts of CCL1, which can induce migration of NK cells via CCR8 in other systems (55).

The antiangiogenic effect of Pam3CSK4–activated mast cells is particularly noteworthy as the presence of mast cells correlates with enhanced angiogenesis in melanomas (12). Further, experimental studies using a transgenic model of skin cancer identified that mast cells are crucial for the induction of angiogenesis in premalignant lesions (14). Studies in mast cell-deficient KitW-sh/W-sh mice, using the B16.1BL6 melanoma model, demonstrated that delayed tumor growth kinetics in mast cell-deficient mice is attributable to a delayed and decreased angiogenic response (13). Mast cell IL-6–dependent angiogenesis suppression is consistent with previous findings that IL-6–mediated melanoma growth inhibition is independent of B and T cell responses (41) and that the antitumor action of IL-6 is due in part to angiogenesis inhibition (53). Further, IL-6–transfected B16.F10 tumors exhibit a significant decrease in angiogenesis via an in vivo intradermal angiogenesis assay (40). Other mast cell-derived mediators may also suppress tumor angiogenesis. For example, Pam3CSK4–activated mast cells also secrete large amounts of IL-13 (Fig. 5A), a potent antiangiogenic cytokine (54).

The crucial role of mast cells for the mobilization of immune effector cells in response to infection is well documented (7, 8). However, their potential to enhance the recruitment of effector cells to tumor sites has not been previously explored. Decreased tumor growth in the presence of Pam3CSK4–activated mast cells is associated with the early recruitment of significant numbers of both NK and T cell subsets. We have demonstrated that TLR2-activated mast cells secrete large amounts of CCL3, which has been demonstrated to be chemotactic for mature NK cells (35) and activated CD8+ T cell subsets (36, 37). Our data from in vitro chemotaxis experiments using CCL3+ BMMCs demonstrates a clear role for mast cell-derived CCL3 in the recruitment of NK and CD8+ and CD8+ T cell subsets in this model. We have also identified multiple other cytokines and chemokines produced from Pam3CSK4–activated mast cells that may enhance effector cell recruitment and activation in vivo. For example, TLR2 ligand-activated BMMCs secrete substantial amounts of CCL1, which can induce migration of NK cells via CCR8 in other systems (55).

The tumor-inhibitory effects of Pam3CSK4 in vivo may also involve activation of other TLR2-expressing immune cells. Indeed, TLR2 engagement via Pam3CSK4 can enhance proliferation, survival, and effector function of CD8+ T cells and lower their threshold of activation (56). However, the lack of Pam3CSK4–mediated antitumor effects in KitW-sh/W-sh mice and their restoration via wild-type, but not TLR2-deficient, mast cells demonstrates a critical role for mast cell TLR2 expression. Furthermore, the use of the Matrigel–tumor model system, containing only mast cells and tumor cells, demonstrates that although Pam3CSK4 may later act on other TLR2-expressing immune cells, the mast cell is both necessary and sufficient for initiating the antitumor effects after TLR2 immunotherapy. This is in keeping with the role of the mast cell as a sentinel cell in initiating immune responses after pathogen recognition (9).

Our studies of the LLC1 tumor model demonstrate that the mast cell-mediated antitumor effects of TLR2 immunotherapy are
Mast cells decrease LLC1 tumor growth in a TLR2-dependent manner and are required for B16.F10 tumor growth inhibition by a TLR2/6 agonist. A, C57BL/6 mice were injected with Matrigel (0.3 ml) containing LLC1 cells in the presence or absence or Pam3CSK4 and/or BMCCs derived from C57BL/6 mice or TLR2−/− mice (pooled data from three independent experiments, n = 9 C57BL/6 BMCC, n = 13 TLR2−/− BMCC). Dashed line indicates weight of Matrigel with BMCCs plus Pam3CSK4 in the absence of tumor (0.092 ± 0.008 g, n = 8). **p = 0.008. B, Representative growth of LLC1 in Matrigels containing Pam3CSK4 and/or C57BL/6 BMCCs. C, H&E-stained sections were examined for mononuclear cell infiltration (black arrows) among tumor cells (arrowheads). Original magnification ×400. D, Mature blood vessels were quantified at tumor periphery of H&E-stained sections. Data are pooled from two separate experiments (n = 7 mice). *p = 0.016. E, C57BL/6 mice or mast cell-deficient KitW−/W− mice were injected s.c. with B16.F10 cells. Tumors were treated s.c. on days 4 and 9 post-tumor injection (arrows) with FSL-1 or PBS diluent control. Data pooled from four independent experiments (n = 26 to 28 for C57BL/6, n = 16 for KitW−W−), ***p < 0.001.
immunotherapy to ensure that other medications that interfere with mast cell function or survival are not being used concurrently.

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Disclosures
The authors have no financial conflicts of interest.

References

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Figure S1: Gene-deficient BMMC are functionally similar to wild-type C57BL/6 BMMC. All BMMC release significant levels of β-hexosaminidase in response to A23187 ionophore but not Pam3CSK4. BMMC (1 x 10^6/mL) in modified HEPES-Tyrode’s buffer were treated with Pam3CSK4 (10 μg/mL) or A23187 (0.5 μM) for 20 minutes and the level of degranulation was assessed via β-hexosaminidase release. Data are pooled from three independent BMMC activations, performed in triplicate.
Figure S2: Local reconstitution with BMMC restored skin mast cell numbers to levels comparable to wild-type C57BL/6 mice. 9-10 week old Kit<sup>W-sh/W-sh</sup> mice were reconstituted on the dorsal surface over right and left flanks in 2 x 2 cm<sup>2</sup> areas via a series of injections with BMMC (1 x 10<sup>6</sup> i.d. and 1 x 10<sup>6</sup> s.c.). Following 9 weeks skin samples were removed and fixed in Carnoy’s fixative. Mast cells were visualized via Alcian blue (pH 0.5) – safranin O staining. Mast cells were detected in all skin layers in all samples (N=8-12 skin samples per group).
**Figure S3:** TLR1 and TLR2 are expressed on B16.F10 melanoma cells and BMMC. Agarose gel electrophoresis of PCR products of TLR1 and TLR2 from RT-PCR of cDNA amplified from RNA isolated from B16.F10 melanoma and BMMC using RNeasy® Mini Kit and RNase-Free DNase Set (Qiagen) according to manufacturer’s protocol. Murine HPRT was used as a positive control. The negative control consisted of DNase treated BMMC RNA. Reverse transcription was performed using the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer’s instructions. PCR of cDNA was performed using GoTaq® Green DNA polymerase Master Mix (Promega) and primers synthesized by Sigma Genosys: mHPRT: forward 5’-CACAGGACTAGAACACCTGC-3’, reverse 5’-GCTGGTGAAAAGGACCTCT-3’; TLR1: forward 5’-GTTGTCACTGATGTCTTCAGC-3’, reverse 5’-GCTGTACCTTAGAGAATTCTG-3’; TLR2: forward 5’-GCCTTGACCTGTCTTTCAAC-3’, reverse 5’-GGACTGATAATTCCGGAGAC-3’. Sequence specificity was confirmed by sequencing RT-PCR products (data not shown).
**Figure S4:** Pam$_3$CSK$_4$ activated BMMC secrete multiple mediators. Protein array analysis of supernatants of mast cells (BMMC) (1x10$^6$/mL) co-cultured for 24 hrs at 37°C with B16.F10 melanoma cells (0.5 x 10$^6$/mL) in the absence (A) or presence (B) of Pam$_3$CSK$_4$ (10 μg/mL). Pam$_3$CSK$_4$ treatment induced secretion, at least 2-fold excess over media control, of (1) IL-6, (2) CCL2 (MCP-1), (3) CCL1 (TCA-3), (4) CCL3 (MIP-1α), (5) IL-13, (6) CXCL1/2 (MIP-2), (7) CXCL3 (KC), (8) CXCL5 (LIX) and (9) CCL5 (RANTES), as determined by spot density analysis. 

C-D) Protein array analysis of supernatants of B16.F10 melanoma cells (0.5 x 10$^6$/mL) cultured for 24 hrs at 37°C in the absence (C) or presence (D) of Pam$_3$CSK$_4$ (10 μg/mL). Pam$_3$CSK$_4$ treatment induced secretion, at least 2-fold excess over media control, of (6) CXCL1/2 (MIP-2) and (7) CXCL3 (KC), as determined by spot density analysis.
Figure S5. Pam₃CSK₄ activated mast cells recruit immune effector cells to B16.F10 tumors. A) Male C57BL/6 mice were injected s.c. with B16.F10 melanoma cells. Tumors were treated s.c. on days 4 and 9 post tumor injection with Pam₃CSK₄ or PBS control (n=7 tumors per group). After 14 days growth, tumors were removed, collagenase digested and assessed by flow cytometry. Established tumors treated with Pam₃CSK₄ contain elevated leukocytes (*p=0.027), including CD₈⁺TCRβ⁺ T cells (*p=0.019), and NK1.1⁺ natural killer cells (*p=0.013), compared to controls. B) Hematoxylin and eosin stained formalin-fixed tissue sections reveal a marked mononuclear cell infiltrate (dark arrows) amongst tumor cells (open arrows) in B16.F10 tumors (0.5 x 10⁶/mL) grown for 10 days in Matrigel (0.3 mL) containing Pam₃CSK₄ (10 μg/mL) and BMMC (1 x 10⁶/mL) as compared to tumors alone.
Supplementary Table 1: Cytokine and chemokine production by C57BL/6 BMMC cultured with a panel of TLR agonists

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (pg/mL)</th>
<th>IL-13 (pg/mL)</th>
<th>CCL1 (pg/mL)</th>
<th>CCL3 (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Media</td>
<td>49.18 ± 43.99</td>
<td>237.7 ± 155.1</td>
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<td>29.69 ± 10.68</td>
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<tr>
<td>FSL-1</td>
<td>515.7 ± 125.8</td>
<td>1784 ± 438.0</td>
<td>424.1 ± 130.3</td>
<td>117.4 ± 35.43</td>
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<tr>
<td>PGN</td>
<td>74.02 ± 31.37</td>
<td>355.0 ± 138.7</td>
<td>421.3 ± 110.6</td>
<td>75.70 ± 37.29</td>
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<tr>
<td>LPS</td>
<td>2087 ± 699.5</td>
<td>11557 ± 4148</td>
<td>3885 ± 2557</td>
<td>1857 ± 1442</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>60.23 ± 36.78</td>
<td>241.6 ± 168.7</td>
<td>35.24 ± 28.31</td>
<td>30.51 ± 10.08</td>
</tr>
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</table>

C57BL/6 BMMC (1 x 10^6/mL) were cultured for 24 hours in the presence of Media or 10 µg/mL of TLR agonist (TLR2/6 - FSL-1 and PGN; TLR4 – LPS; TLR3 – Poly(I:C)). Data are presented as mean ± SEM of 3 independent experiments). Bold numbers indicate values ≥2-fold over Media control.