C5a-Mediated Leukotriene B₄-Amplified Neutrophil Chemotaxis Is Essential in Tumor Immunotherapy Facilitated by Anti-Tumor Monoclonal Antibody and β-Glucan

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Intravenous and orally administered β-glucans promote tumor regression and survival by priming granulocyte and macrophage CR receptor 3 (CR3, iC3bR and CD11b/CD18) to trigger the cytotoxicity of tumor cells opsonized with iC3b via anti-tumor Abs. Despite evidence for priming of macrophage CR3 by oral β-glucan in vivo, the current study in C57BL/6 and BALB/c mice showed that granulocytes were the essential killer cells in mAb- and oral β-glucan-mediated tumor regression, because responses were absent in granulocyte-depleted mice. Among granulocytes, neutrophils were the major effector cells, because tumor regression did not occur when C5a-dependent chemotaxis was blocked with a C5aR antagonist, whereas tumor regression was normal in granulocyte-depleted mice. The effectiveness of tumor immunotherapy with mAbs can be greatly enhanced by the combined administration of β-glucan (1–3). Without β-glucan, anti-tumor mAbs, such as Herceptin (Genetech) or Rituxan (Genetech), have limited effector mechanisms, including inhibition of growth factor receptor function (Herceptin) (4) or Ab-mediated cellular cytotoxicity, and there is little, if any, contribution from the C system (5). C-dependent cytotoxicity via the membrane attack complex is limited by C regulatory proteins, such as CD46, CD55, and CD59, that are frequently overexpressed on tumor cells (6–8). Moreover, C receptor (CR)6-dependent phagocytosis or cytotoxicity is reserved for microbial pathogens and is not elicited by C3b/iC3b-opsonized tumor cells (9, 10). In particular, the iC3bR CR3 is normally only triggered by iC3b deposited onto yeast or fungal cell walls (11).

This is because the activation of CR3 for phagocytosis or cytotoxic degradation requires its dual ligation to both iC3b and cell wall β-glucan (12). Tumor cells opsonized with iC3b bind to the iC3b-binding site located within the I domain of CD11b (13), but not to the lectin-like domain for β-glucan located within the C terminus of CD11b (14, 15). However, soluble β-glucan isolated from yeast cell walls overcomes the lack of endogenous tumor cell β-glucan by binding to and priming the lectin site of CR3 to mediate cytotoxic degradation in response to tumor cells opsonized with iC3b (9, 15). By using β-glucan in combination with anti-tumor mAbs that activate C, the resulting coat of iC3b on tumor cells can trigger CR3-dependent cytotoxicity by granulocytes (neutrophils and eosinophils), monocytes, macrophages, and NK cells (5).

In vitro experiments have shown that soluble yeast β-glucan can prime CR3 of granulocytes, macrophages, and NK cells to kill iC3b-opsonized tumor cells. However, murine tumor therapy models incorporating i.v. anti-tumor mAbs and soluble yeast β-glucan indicated that the major CR3 effector cell was a granulocyte (2). Because i.v. administered β-glucans are taken up rapidly by CR3 blood granulocytes or liver Kupffer cells (16), little is likely to reach tissue macrophages that could migrate into tumors. Conversely, orally administered yeast β-glucan particles or large soluble molecules of barley β-glucan are taken up by gastrointestinal macrophages; shuttled to bone marrow, spleen, and lymph nodes; and then slowly degraded into smaller soluble β-glucan fragments that prime the CR3 of the margined pool of granulocytes within the bone marrow (3). Thioglycollate-elicited peritoneal neutrophils upon CR3 priming were able to kill iC3b-opsonized tumor cells in a CR3-dependent manner (3). Although bone marrow NK cells are also likely to be primed for killing, experiments with NK cell-depleted mice indicated no important role for NK cells in oral β-glucan tumor therapy (17). In addition to granulocytes, splenic macrophages isolated from mice receiving oral β-glucan were able to kill iC3b-opsonized tumor cells via CR3 in vitro (3). Thus, it was unclear whether granulocytes, macrophages, or both leukocyte types might be involved in killing iC3b-opsonized tumor cells during oral β-glucan-mediated tumor immunotherapy.
Intratumoral release of the chemotactic factors C3a and C5a was hypothesized to be responsible for recruiting granulocytes and/or macrophages into tumors, thus expanding the importance of C activation beyond IC3b opsonization of tumors. Although receptors for C3a and C5a are present on all granulocytes and monocyte/macrophages (18), C5a primarily functions to recruit neutrophils and macrophages (19, 20), whereas C3a recruits eosinophils, basophils, and mast cells (18, 21). The lack of C3aR and C5aR (CD88) on NK cells probably also explains the absent requirement for NK cell function in mAb- and β-glucan-mediated tumor immunotherapy (22, 23).

C5a is a potent chemotactic agent in vitro, with a short in vivo t1/2 due to serum carboperoxidase N and intracellular degradation of C5a by C5aR-bearing cells (24). In addition, C5aR is widely distributed on leukocytes and nonleukocyte types, including epithelial and endothelial cells (25–28). It was hypothesized that these cells, if stimulated by C5a, could play a role in amplifying the chemotactic responses of C5a by releasing leukotriene B4 (LTB4), a potent chemotaxogen for neutrophils and macrophages (29, 30). LTB4 activates the G protein-coupled LTB4R type 1 (BLT-1) to mediate a diverse array of cellular responses in leukocytes, including chemotaxis, calcium mobilization, degranulation, and gene expression (31–33). LTB4 also activates respiratory burst and granule release from neutrophils (34). The current investigation sought to identify the major effector cell in i.v. and oral β-glucan-mediated tumor therapy and to identify the chemotactic factors responsible for recruiting these effector leukocytes into C-opsonized tumors with i.v. or orally administered β-glucan.

Materials and Methods

Abs and other reagents

The following hybridomas were provided: 11C1 IgG2a anti-murine mammary tumor virus (anti-MMTV) (35), Dr. H. Fugi (Roswell Park Cancer Institute, Buffalo, NY); 14G2a IgG2a anti-GD2 mAb (36), Dr. R. A. Reisfeld (The Scripps Research Institute, La Jolla, CA); and rat anti-mouse granulocyte mAb RB6-8C5 (Ly-6G; anti-Gr-1) (37), Dr. E. Unanue (Wadswortih University School of Medicine, St. Louis, MO). Each hybridoma was grown in bioreactor flasks from which the mAb was purified, as determined by hemocytometer. Cells from 50 ml of medium containing 2% FBS and 2 mM EDTA. Total cell counts were number to obtain the number of neutrophils that had migrated into the peritoneal cavity.

Mice and tumor models

The murine tumor therapy protocols were performed in compliance with all relevant laws and guidelines and were approved by the institutional animal care and use committee of the University of Louisville. Normal C57BL/6 and BALB/c mice were purchased from National Cancer Institute-Frederick. A breeding colony of C3aR−/− mice on a C57BL/6 background (41) was provided by Dr. R. A. Wetsel (University of Texas, Houston, TX). Previously described BLT-1−/− mice (30) on a mixed background were backcrossed for eight generations onto the C57BL/6 background for the current studies.

RMA-S is a C57BL/6 lymphoma that expresses G122 ganglioside. For use in an s.c. tumor model, 1 × 106 RMA-S lymphoma cells were implanted s.c. in C57BL/6 wild-type and BLT-1−/− mice in or near a mammary fat pad. After 5–10 days, when tumors of 4–6 mm in diameter were apparent, therapy was initiated with 14G2a anti-GD2 ganglioside with or without β-glucan. The BALB/c mammary adenocarcinoma known as Ptas64 was obtained from Dr. W.-Z. Wei (Karmanos Cancer Center and Wayne State University, Detroit, MI). This tumor cell line expresses MMTV membrane Ags reactive with the 11C1 anti-MMTV mAb, and its use in tumor models treated with β-glucan was previously described (2, 3). In brief, wild-type BALB/c mice and C3aR−/− mice received s.c. injections of 0.5–1.0 × 106 cells in a mammary fat pad, and a tumor was allowed to form over 7–10 days. When the tumor diameter reached 3–4 mm, therapy was initiated with 11C1 anti-MMTV mAb with or without β-glucan.

Tumor therapy with granulocyte-depleted mice

Granulocytes were depleted from C57BL/6 mice by treatment with the rat anti-mouse granulocyte mAb RB6-8C5, i.e., anti-Gr-1, immediately before and during tumor therapy (2, 42). This treatment was previously shown to deplete only peripheral granulocytes and not monocytes, macrophages, or dendritic cells; it was shown that this dosing schedule allowed time for the reconstitution of serum C levels (2).

Immunohistochemistry staining of tumors

Wild-type or BLT-1−/− animals bearing RMA-S tumors were treated, or not, with 14G2a anti-GD2 ganglioside mAb and orally administered WGP or barley β-glucan or i.v. administered NSG as described previously. After 3 wk of therapy, tumor tissues were removed and snap-frozen in tissue freezing medium (OCT; Sakura Finetechnical). The sections were sectioned and fixed with cold acetone. To detect tumor-infiltrating neutrophils, the sections were blocked with Tris-saline/3% BSA buffer and then incubated with an avidin/biotin blocking kit (Vector Laboratories) and stained with anti-Gr-1-biotin for 1 h at room temperature. After three 10-min washings with blocking buffer, the sections were stained with streptavidin-HRP (Southern Biotechnology Associates) for 1 h at room temperature. After an additional three washes, HRP-substrate (Vector Laboratories) was added for 30 min at room temperature. After additional washes, the sections were counterstained with hematoxylin to provide morphological detail. The numbers of infiltrating neutrophils were calculated as the mean of the number of Gr-1+ cells in 10 representative high power fields (>400 total magnification).

Neutrophil chemotaxis upon C5a stimulation in BLT-1−/− and BLT-1+/+ mice

Seven- to 8-wk-old BLT-1−/− (n = 5) and BLT-1+/+ (n = 5) mice were injected i.p. with 1 ml of PBS containing 20 μg of recombinant human C5a (Sigma-Aldrich). BLT-1−/− (n = 5) and BLT-1+/+ (n = 3) control mice received 1 ml of PBS i.p. After 4 h, mice were killed by CO2 asphyxiation, and a peritoneal lavage was performed with 10 ml of ice-cold RPMI 1640 medium containing 2% FBS and 2 mM EDTA. Total cell counts were determined by hemocytometer. Cells from 50 μl of lavage fluid were deposited onto a glass slide using a cytocentrifuge and stained with Wright-Giemsa. The proportions of neutrophils were determined by counting 200 total cells in each smear. These values were multiplied by the total cell number to obtain the number of neutrophils that had migrated into the peritoneal cavity.

Graphing and statistical analysis of data

Data were entered into PRISM 4.0 (GraphPad) to generate graphs of tumor regression or neutrophil chemotaxis, and Student’s t test was used from within the program to determine the significance of differences between two datasets. The Mann-Whitney rank-sum test was used to compare C5a-induced chemotaxis between BLT-1−/− and BLT-1+/+ mice. Survival curves were created using the Kaplan-Meier method, and statistical analyses of survival curves used a log-rank test.

Results

Granulocytes are required as killer cells for mAb- and oral β-glucan-mediated immunotherapy

Previous research has shown that orally administered yeast WGP or barley β-glucans are taken up by gastrointestinal macrophages that partially degraded the large β-glucan molecules and released small soluble fragments of β-glucan that function to prime the CR3 of margined granulocytes and tissue macrophages in such a way that they were capable of mediating the cytotoxicity of iC3b-opsonized tumor cells in vitro (3). Although macrophages are essential for taking up the orally administered β-glucan and processing it into a form that could prime granulocytes and macrophage CR3, it appears possible that the more numerous granulocytes might play the major role in killing iC3b-opsonized tumor
cells. To determine the requirement for granulocytes in oral β-glucan tumor therapy, mice were injected i.p. with anti-Gr-1 rat anti-mouse granulocyte mAb before and during therapy to deplete peripheral granulocytes, but not monocytes, macrophages, or dendritic cells (2). Therapy of RMA-S-MUC1 tumors consisted of a combination of i.v. 14.G2a anti-GD2 ganglioside mAb and oral yeast WGP β-glucan (Fig. 1). As observed previously with this model (3), combined mAb and oral yeast WGP β-glucan induced significantly more tumor regression than did treatment with mAb alone (p < 0.01). However, granulocyte-depleted mice exhibited no tumor regression compared with untreated controls (Fig. 1). Moreover, depletion of granulocytes, despite combined therapy with oral WGP and anti-tumor mAb, resulted in significantly diminished survival compared with similarly treated animals that were not granulocyte depleted. Thus, tumor regression mediated by mAb and oral β-glucan is dependent upon granulocytes.

**Granulocyte chemotaxis into tumors is mediated by C5a, but not by C5aR**

C activation by naturally occurring tumor-reactive Abs or exogenous anti-tumor mAbs not only targets tumor cells with covalently bound iC3b, but also releases the chemotactic factors C3a and C5a (1–3, 38). Receptors for C3a and C5a are expressed on all myeloid-derived leukocytes, such as neutrophils, eosinophils, monocytes, and macrophages. Neutrophils, however, express far more C5aR (CD88) than C5aR, whereas eosinophils express more C3aR than C5aR (18). To examine whether C5a or C5aR plays a critical role in the recruitment of neutrophils, which are required for combined anti-tumor mAbs and β-glucan tumor immunotherapy, protocols were conducted using a C5aR antagonist peptide to block C5aR in wild-type mice or mice deficient in C3aR (Figs. 2 and 3). Blockade of C5aR completely inhibited (p < 0.05) the tumor regression mediated by either i.v. (Fig. 2A) or orally administered (Fig. 2B) β-glucan. In contrast, in the tumor model that compared wild-type to C3aR/−/− BALB/c mice, there was no significant difference in tumor regression mediated by i.v. yeast β-glucan (Fig. 3A) or oral barley or yeast β-glucan (Fig. 3B). In addition, tumor histology displayed similar levels of tumor-infiltrating granulocytes in these animals (not shown). These findings in combination with the observation that tumors did not contain significantly increased numbers of eosinophils relative to neutrophils indicate that neutrophils recruited via C5a are probably responsible for the tumor regression mediated by anti-tumor mAb and β-glucan therapy.

**FIGURE 1.** A, Granulocytes are required for tumor regression mediated by anti-tumor mAb and oral yeast β-glucan. Groups of six C57BL/6 mice were implanted s.c. with 1 ¥ 10^6 RMA-S-MUC1 cells. Tumor therapy consisting of i.v. 14.G2a anti-GD2 mAb (100 μg every 3 days) with or without oral yeast WGP β-glucan (400 μg/day) was started when a palpable tumor formed (day 8). Two groups of mice received i.p. injections of 500 μg of anti-Gr-1 rat anti-mouse granulocyte mAb 3 days before beginning immunotherapy and subsequent i.v. injections of 250 μg of anti-Gr-1 at 3-day intervals. Mice were killed if tumor diameter exceeded 12 mm. Each data point represents the mean ± SEM for the six mice in each group. B, Long term survival was monitored in mice, extending to 100 days beyond the initiation of therapy. Granulocyte-depleted mice, despite treatment with oral WGP and anti-tumor mAb, were observed to have decreased survival with respect to their treated counterparts, who were not granulocyte depleted (**, p < 0.005 vs neutrophil-depleted counterparts).

**FIGURE 2.** Chemotaxis of neutrophils via C5aR is required for tumor regression mediated by anti-tumor mAb and either i.v. or oral yeast β-glucan. Groups of six BALB/c mice were implanted in a mammary fat pad with 1 ¥ 10^6 Ptas64 mammary carcinoma cells. Tumor therapy consisting of i.v. 11Cl anti-MMTV mAb (200 μg every third day) or oral yeast WGP β-glucan (400 μg/day) was started when a palpable tumor formed (day 8). Two days after implanting tumor cells and then at 3-day intervals during therapy, some groups of mice were injected i.v. with 25 μg of either a C5aR antagonist peptide (C5aR-Ant.) or a nonsense peptide (N.S. peptide) of the same size. Mice were killed if the tumor diameter exceeded 12 mm. Each data point represents the mean ± SEM for the six mice in each group.
LTB₄ and LTB₄ receptors (BLT-1) are required to amplify C5a-mediated neutrophil chemotaxis

Although C5a is a potent chemoattractant, it has a short half-life in vivo due to both its rapid inactivation by serum carboxypeptidase N and its intracellular degradation by C5aR-bearing cells (24). It was hypothesized that C5a stimulation of nearby C5aR-intratumoral vascular endothelial cells and granulocytes could produce a burst of LTB₄, thus amplifying the short-lived chemotactic signal mediated by C5a. To this end, tumor therapy was conducted in BLT-1/-/- mice on a C57BL/6 background vs wild-type C57BL/6 mice (Fig. 4), and these data indicated a complete loss of tumor regression in BLT-1/-/- mice given either i.v. yeast (Fig. 4A) or oral yeast (or barley) β-glucan therapy (Fig. 4B). Furthermore, immunohistochemistry staining of tumors extracted from treated and nontreated BLT-1/-/- mice given either i.v. yeast (Fig. 4A) or oral yeast (or barley) β-glucan therapy (Fig. 4B). These data suggest that the amplification of C5a-mediated neutrophil chemotaxis requires the involvement of LTB₄.

BLT-1/-/- mice have impaired neutrophil chemotaxis in response to C5a

Both C5a and LTB₄ are powerful chemoattractants that recruit granulocytes into the inflammatory site (31, 43). However, neutrophils from BLT-1/-/- mice display normal responses to C5a (30), suggesting that neutrophils from BLT-1/-/- mice have a functional C5aR. To confirm that C5a-mediated chemotaxis can be amplified by LTB₄, BLT-1/-/- C57BL/6 mice vs wild-type C57BL/6 mice were injected i.p. with rC5a. C5a-mediated neutrophil recruitment to the peritoneal cavity was evaluated by counting neutrophil numbers. As shown in Fig. 6, 71% fewer neutrophils were recruited in response to C5a in BLT-1/-/- mice compared with wild-type mice (p = 0.0079). These data show a very important link between C5a and LTB₄ in neutrophil chemotactic responses and explain why tumor reduction responses require both C5aR and BLT-1.

Discussion

These studies demonstrated the essential role of C-mediated chemotaxis of neutrophils in tumor regression mediated by mAb and β-glucan. Among granulocytes, the chemotactants C5a and C3a preferentially recruit neutrophils and eosinophils, respectively. Because tumor regression mediated by mAb and β-glucan depended on C5aR, but was independent of C3aR, a role for neutrophils, rather than other granulocytes, was suggested. The loss of tumor regression after selective depletion of granulocytes indicated that macrophages, although sensitive to C5a, could not mediate tumor regression independently from neutrophils. Lastly, the functional activity of C5a in vivo was shown to require amplification by
LTB₄, because rC5a-mediated neutrophil recruitment to the peritoneal cavity and tumor regression were suppressed in mice deficient in BLT-1. Indeed, many fewer infiltrating neutrophils were observed in the tumors of BLT-1⁻/⁻ mice compared with wild-type animals (Fig. 5). In a zymosan-induced peritonitis model, in which the alternative pathway of C is activated, LTB₄ was shown to be important in neutrophil recruitment (30, 44). Although a second C5aR as well as a low affinity LTB₄R (BLT-2) were recently identified (45, 46), they did not seem to influence neutrophil recruitment in these experiments. These data support the hypothesis that C5a initiates a chemokine cascade in which C5a induces LTB₄, making LTB₄ the effective chemoattractant mediating inflammatory events initiated by C activation. Moreover, these studies provide additional molecular mechanisms for the effectiveness of combined β-glucan and anti-tumor mAb immunotherapy and show a dual role for C activation in the opsonization of tumors with iC3b and the C5a-mediated recruitment of neutrophils to the tumor.

The tumor microenvironment suppresses effective immune responses and may promote host immune tolerance. Altering the tumor milieu to promote inflammation enhances the recruitment of immune cells, particularly APCs and T cells, to tumors, leading to clinical benefit (47). In contrast, chronic inflammation can increase mutagenesis and promote tumor development (48, 49). Indeed, DNA damage resulting from chronic inflammation is believed to be the mechanism by which infection can cause cancer (50). Therefore, manipulation of inflammation toward therapeutic benefit would significantly improve the efficacy of tumor immunotherapy. Our data expand those observations to include the significant contribution of neutrophil chemotaxis in response to C5a and LTB₄ to mediate tumor regression by combined oral β-glucan and anti-tumor mAb therapy. Notably, an abundant neutrophil infiltrate in the regressing tumors of treated animals implies that the recruited β-glucan-primed neutrophils were efficient killers. Thus, it is hypothesized that strategies to improve the quantity of infiltrating primed neutrophils should improve therapeutic efficacy.

Tumor immunotherapy mediated by anti-tumor mAbs and β-glucan is particularly novel, in that it recruits neutrophils as effector cells and can use humanized mAbs already in clinical use.
(e.g., Herceptin, Erbitux, Campath, (Im)Clone Systems) and Rituxan (Berlex)). The critical feature required of such therapeutic mAbs is that they activate C, thus requiring a human IgG1 or IgG3 framework, allowing iC3b opsonization of tumors and C5a-mediated recruitment of neutrophils (17). Immunotherapy with mAb and β-glucan can be coupled easily to vaccine therapies that elicit immune CTL, exploiting the concurrent production of anti-tumor Abs. C5a-rejected neutrophils and immune CTL would probably kill tumors more effectively than would CTL alone. Tumor escape from CTL through down-regulation of MHC class I would also be prevented, because neutrophils are targeted to the iC3b on tumors and not to MHC class I.

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Disclosures
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References


