Adoptive Transfer of Tumor Reactive B Cells Confers Host T-Cell Immunity and Tumor Regression

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Abstract

Purpose: We investigated the antitumor reactivity of adoptively transferred effector B cells and the mechanisms by which they may mediate tumor regression in a spontaneous metastases model.

Experimental Design: 4T1 breast cancer cells were inoculated into the flanks of syngeneic Balb/C mice to prime draining lymph nodes. Tumor-draining lymph nodes (TDLN) were harvested and B cells activated ex vivo with lipopolysaccharide and anti-CD40 monoclonal antibody. These activated B cells were adoptively transferred into mice inoculated with 4T1 tumor in the mammary fat pad. The induction of host T-cell immunity was evaluated.

Results: Activated 4T1 TDLN B cells secreted immunoglobulin G (IgG) in response to tumor cells which was immunologically specific. These activated B cells were capable of mediating specific lysis of tumor cells in vitro. Transfer of these activated B cells alone mediated the inhibition of spontaneous metastases to the lung. Examination of the host revealed that the transfer of these B cells resulted in the induction of tumorspecific T-cell immunity as measured by cytotoxicity and cytokine (IFNγ and granulocyte-macrophage colony-stimulating factor) production. The combined transfer of activated T and B cells from TDLN resulted in tumor regression, which was greater than either cell population alone, with host B cells capable of producing IgG that mediated lysis of tumor in the presence of complement.

Conclusions: We have found that appropriately primed B cells can mediate tumor regression by itself and confers host T-cell antitumor immunity. Furthermore, effector B cells can serve as a useful adjunct in adoptive T-cell therapy. Clin Cancer Res; 17(15); 4987–95. ©2011 AACR.
CD3 on TDLN T cells and CD40 on TDLN B cells, which comprise more than 30% of the lymph node cells, augments the antitumor reactivity of tumor-primed LN cells (23). These studies established a role for engaging CD40 on TDLN B cells as APCs in the generation of effector T cells. We have also found that interleukin (IL)-21 augments the efficacy of T-cell therapy by eliciting concurrent cellular and humoral responses (24). This study confirmed an interactive role between tumor-specific humoral responses related to IL-21 administration and adoptively transferred effector T cells that resulted in more effective tumor eradication. More recently, we reported that TDLN B cells can be therapeutic effector cells in cancer adoptive immunotherapy of either pulmonary metastases or s.c. tumors by using 2 experimentally induced histologically distinct tumor models (B16-D5 and MCA 205) in B6 hosts (25). However, whether or not the adoptively transferred therapeutic B cells could induce host T-cell immunity was not examined.

Metastasis represents one of the characteristics of cancer and has remained a major reason for the ineffectiveness of therapy. In this article, we investigated the ability of inhibiting cancer spontaneous metastases with B effector cells alone and in combination with T cells. We used the murine 4T1 breast cancer model. Inoculating 4T1 cells into the mammary fat pad results in the development of spontaneous pulmonary metastases. By using this model, we have documented the therapeutic efficacy of in vivo tumor-primed and in vitro activated B cells, as well as B cell therapy–conferred systemic T-cell antitumor immunity in this study.

Translational Relevance

This article describes preclinical evidence about the utility of adoptively transferred tumor-primed B cells in modulating host antitumor T-cell immunity. This is the first article indicating that tumor-primed B cells can inhibit the growth of spontaneous metastases. The administration of effector B cells in combination with effector T cells was therapeutically more efficacious than either cell population alone. The use of activated B cells may be a useful adjunct in the cellular therapy of malignancy.

Inoculating 4T1 cells into the mammary fat pad results in the development of spontaneous pulmonary metastases. MT-901 (a subline of dimethylbenzanthracene-induced mammary carcinoma), Renca (a kidney cancer cell line), and TSA (a highly aggressive mammary adenocarcinoma) are all syngeneic to Balb/C mice and used as specificity controls. Tumor cells were maintained in vitro in complete medium (CM).

Tumor-draining lymph nodes

To induce TDLN, 1 × 10^6 4T1 tumor cells in 0.1 mL PBS were injected s.c. into the lower flanks of normal Balb/C mice. The draining inguinal lymph nodes were collected 9 days later and processed by using mechanical dissociation. Multiple inguinal TDLNs were pooled from groups of mice for lymphoid cell suspension preparation. CD3+ T cells and CD19+ B cells were purified from TDLN cells or splenocytes by using antibody-coupled Microbeads and MACS separator (Miltenyi Biotec. Inc.).

T- and B-cell activation and expansion

TDLN T cells and/or B cells were activated with immobilized anti-CD3 plus anti-CD28 mAbs in CM containing hrIL-2 and/or lipopolysaccharide (LPS; Sigma-Aldrich) plus anti-CD40 (FGK45) mAb ascites as previously described (25). Activated and expanded TDLN T and/or B cells were used for adoptive immunotherapy, phenotype, and immune function analyses. To test systemic immune responses post-TDLN T- and/or B-cell adoptive transfer, spleens were harvested at the end of therapy. Purified splenocyte T and/or B cells were activated as described for TDLN cells.

Cytokine and antibody production assessment

Supernatants at the end of TDLN cell or splenocyte activation were collected and analyzed for IFNγ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and immunoglobulin M (IgM), IgG production by using ELISA (BD PharMingen). In addition, we tested IgG and IFNγ production in response to tumor antigen. After activation and expansion, 1 × 10^6 TDLN or splenocyte effector T and/or B cells were cocultured with 2.5 × 10^5 irradiated (6,000 cGy) tumor stimulator cells in 24-well tissue culture plates for 24 hours. The supernatant was then collected and analyzed for the production of IgG and IFNγ in response to tumor.

Adoptive T- and/or B-cell therapy of spontaneous pulmonary metastasis

Healthy Balb/C mice were inoculated with 5 × 10^6 4T1 cells into the mammary fat pad to induce spontaneous pulmonary metastases. Two weeks after tumor inoculation, tumor-bearing mice were treated with tail vein i.v. injection of activated 4T1 TDLN T and/or B cells. Commencing on the day of the effector cell transfer, intraperitoneal (i.p.) injections of IL-2 (40,000 IU) were administered in 0.5 mL of PBS and continued twice daily for 8 doses. Approximately 2 weeks after T- and/or B-cell...
transferred, all mice were sacrificed and lungs were harvested for enumeration of spontaneous pulmonary metastatic nodules.

**Flow cytometry analysis**

Cell surface expression of CD3, CD19 was analyzed by immunofluorescence assay. All fluorescein isothiocyanate (FITC)- or PE-conjugated antibodies were from BD Pharmingen. Binding of IgG produced by splenocyte T+B or B cells to tumor cells was detected by using FITC-anti-mouse IgG (BD Biosciences) following the incubation of tumor cells with splenocyte T+B or B-cell culture supernatants.

**Antibody and complement-mediated cytotoxicity**

Tumor cell lysis mediated by antibodies produced by splenocytes obtained from the treated host was assessed by incubating tumor cells with splenocyte T+B or B-cell culture supernatants in test tubes on ice for 1 hour, followed by cell culture in the presence of rabbit complement (CalBiochem) in a 37°C water bath for another hour. Viable cells were then counted after trypan blue staining to calculate cell lysis.

**CTL cytotoxicity**

CTL cytotoxicity against 4T1 tumor cells was tested by using the lactate dehydrogenase (LDH)-release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega) with the LDH-release assay (Cytotox 96; Promega). Viable cells were then counted after trypan blue staining to calculate cell lysis.

Cytotoxicity was calculated by using the following formula:

\[
\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector spontaneous} - \text{Target spontaneous}}{\text{Target maximum} - \text{Target spontaneous}} \times 100
\]

**Statistical analysis**

The significance of differences in numbers of metastatic nodules, the concentration of cytokine and immunoglobulins, and cell lysis was determined by using 1-way ANOVA (Newman–Keuls post hoc test). Values of \( P < 0.05 \) were considered statistically significant between the experiment groups.

**Results**

**Tumor-primed and ex vivo activated 4T1 TDLN cells produce antibody and cytokine spontaneously and in response to 4T1 tumor**

We obtained the 4T1 mammary carcinoma which when inoculated into the mammary fat pad of the Balb/C host will spontaneously metastasize to the lungs. We evaluated the ability to generate B as well as T effector cells by using this model. 4T1 tumor cells were inoculated into the flanks of mice to generate TDLN. These LNs were harvested 9 days later and T and B cells purified by using immunomagnetic beads. Flow cytometry analysis indicated that in addition to the 60% to 70% of CD3+ T cells in the TDLN (Fig. 1, day 0), the remainder of the approximately 30% to 40% TDLN cells are CD19+ B cells. Enrichment by using the antibody-coupled magnetic beads resulted in highly purified B cells and T cells (94%–98%). Purified TDLN B cells and T cells were activated with LPS plus anti-CD40 for B cells and

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Figure 1. Purification of T, B cells from 4T1 TDLN, and the phenotype of these cells post in vitro activation and expansion. Unfractionated TDLN cells prepared from freshly harvested 4T1 TDLNs and the purified TDLN B cells and T cells were double-stained with FITC-anti-CD3 and PE-anti-CD19 (day 0). Activated and expanded cells were double stained similarly (day 4). Data are representative of 3 experiments conducted.
anti-CD3/anti-CD28 plus IL-2 for T cells, respectively. Post in vitro activation and expansion (Fig. 1, day 4), B cells remained CD19^+ (97%), whereas T cells remained CD3^+ (97%). After exposure of unfractionated TDLN cells to LPS, the CD19^+ B-cell percentage increased to approximately 70%, suggesting that LPS stimulates B-cell proliferation (data not shown). Activation with anti-CD40 or LPS/anti-CD40 resulted in significant decreases in CD38 and CD23 B-cell markers, indicating that anti-CD40 promotes B-cell differentiation (data not shown).

The culture supernatants were collected and assessed for cytokines and Ig production. Activated T cells secreted large amounts of IFNγ and GM-CSF as opposed to B cells which did not (data not shown). By contrast, large amounts of IgM and IgG were produced by activated B cells and not by T cells as expected. To determine whether the activated 4T1 TDLN cells were reactive to tumor antigen, we carried out coculture experiments of the activated TDLN cells with irradiated tumor cells and assessed the release of IgG and IFNγ (Fig. 2). Activated B cells secreted significantly large amounts of IgG in response to 4T1 tumor cells compared with their culture in CM. Importantly, this elicited IgG production was tumor antigen specific. This was revealed by the use of irrelevant Renca tumor cells in the same experiment. On the other hand, activated T cells when cultured with irradiated 4T1 tumor cells produced increased IFNγ compared with the absence of tumor cells (CM) or to the irrelevant Renca tumor cells. These experiments indicated that tumor-primed and ex vivo activated 4T1 TDLN B cells and T cells produce antibody and cytokine in response to the 4T1 tumor cells specifically.

Adoptively transferred TDLN B effector cells inhibit 4T1 spontaneous pulmonary metastases by conferring host T-cell anti-4T1 immunity

We proceeded to assess the in vivo antitumor reactivity of these B effector cells in adoptive immunotherapy experiments. Balb/C mice were inoculated with 4T1 cells in the mammary fat pad and, 14 days later, were treated by the adoptive transfer of activated 4T1 TDLN T or B cells i.v. Approximately 2 weeks later, mice were euthanized and the

Figure 3. A, adoptively transferred 4T1 TDLN B cells mediated effective inhibition of the spontaneous pulmonary metastasis of 4T1 breast cancer cells injected into the fat pad. Balb/C mice were inoculated into the fat pad with 4T1 tumor cells. Two weeks later, the tumor-bearing mice were treated with tail vein injection of 4T1-primed and in vitro activated 4T1 TDLN T cells or B cells. Approximately 14 days after TDLN cell transfer, all mice were randomized and sacrificed, and lungs were harvested for enumeration of pulmonary metastatic nodules. B, adoptively transferred 4T1 TDLN T+B cells mediated inhibition of the spontaneous pulmonary metastasis of 4T1 in a dose-dependent manner. Spontaneous 4T1 pulmonary metastases were treated with 4T1-primed and in vitro activated 4T1 TDLN B cells or T+B cells.
pulmonary metastases enumerated. The results of this experiment are summarized in Figure 3A. Infusion of $3 \times 10^6$ activated T or B cells alone each resulted in significant ($P < 0.05$) reduction of the spontaneous 4T1 metastases. To test potential host T-cell–mediated 4T1 cytotoxicity after TDLN B-cell–adoptive immunotherapy, we isolated PBMCs from the 4T1 tumor–bearing host subjected to TD LN B-cell treatment, IL-2 treatment, or no treatment. PBMCs were cultured in low concentrations of IL-2 (60 IU/mL) in the presence of anti-CD3/CD28. The resultant cells were approximately 90% T cells (data not shown), as we have achieved in our previous studies (18, 25). Such activated cells were then cultured with 4T1 tumor target cells. The lysis of the target cells was analyzed by the LDH release assay as described in Materials and Methods. As shown in Figure 4, CTLs generated from the PBMCs harvested from hosts subjected to 4T1 TD LN B-cell treatment killed 4T1 tumor cells significantly ($P < 0.05$) more than CTLs prepared from hosts subjected to IL-2 treatment or no treatment. This CTL activity was tumor antigen specific as revealed by the decrease lysis of 2 irrelevant tumor targets.

In a similar fashion, we assessed the ability of host T cells to produce IFNγ and GM-CSF in response to tumor antigen after the adoptive transfer of effector B cells. Splenocytes were harvested and activated with anti-CD3/anti-CD28 plus IL-2 as described above. After activation, the T cells were cocultured with irradiated tumor cells and supernatants harvested 24 hours later. As illustrated in Figure 5, activated splenocytes from hosts that received effector B cells secreted significantly ($P < 0.05$) higher levels of IFNγ and GM-CSF in response to 4T1 tumor compared with splenocytes from mice that received IL-2 or no treatment. This cytokine release was immunologically specific. Together, these data indicate that adoptively transferred effector B cells alone can inhibit 4T1 pulmonary metastases and induce systemic host T-cell–mediated antitumor immunity as measured by tumor lysis as well as cytokine release.

Adoptive transfer of 4T1 TD LN T + B cells confers systemic host cellular and humoral antitumor immunity

We proceeded by examining the antitumor reactivity of B cells in combination with T cells to treat 4T1 spontaneous metastases (Fig. 3B). Mixed T and B cells purified from the 4T1 TD LN were activated with anti-CD3/anti-CD28/IL-2 + LPS/anti-CD40. B-cell transfer alone resulted in significantly ($P < 0.05$) diminished lung tumors as observed in the previous experiment (Fig. 3A), whereas the combination of T + B cells resulted in a significantly greater (approximately 90%) reduction of lung tumors that was dose dependent on the number of TD LN T and B effector cells given.

We evaluated the antitumor reactivity of host splenocytes after the adoptive transfer of T + B effector cells compared with no treatment. Purified T and B splenocytes were activated with anti-CD3/CD28 plus IL-2 or with LPS and anti-CD40, respectively. As illustrated in Figure 6A, supernatants from the activation culture showed large quantities of IFNγ or IgG released by the activation of splenic T cells or B cells, respectively. The activated T cells or B cells were then cocultured with irradiated tumor cells and supernatants collected 24 hours later. As shown in Figure 6B and C, the

![Figure 4. CTL-mediated 4T1 cytotoxicity.](#)

![Figure 5. Tumor-specific cytokine production by anti-CD3/anti-CD28–activated and IL-2–expanded splenocytes.](#)
activated T and B cells isolated from the spleens of the animals subjected to TDLN T + B-cell therapy produced significantly ($P < 0.05$) more IFN$\gamma$ and IgG in response to 4T1 in a tumor antigen–specific manner than T or B cells from the spleens of animals that received no treatment. Noteworthy is the observation that LPS/anti-CD40 activation of splenic B cells from nontreated 4T1 tumor–bearing mice (Fig. 6C) did not result in specific antitumor reactive B cells, indicating that the in vivo activation process does not result in promiscuous B effector cells. It is only in the context of in vivo B-cell priming in TDLN (Fig. 2) or with the adoptive transfer of tumor reactive B cells that you can secondarily activate B lymphoid cells to differentiate into B effector cells.

**Immune host splenic B-cell–produced IgG binds to 4T1 tumor cells and results in tumor cell lysis**

To define the immunologic significance of host splenic B-cell–produced IgG after adoptive transfer of TDLN effector cells, we tested the interaction of these antibodies with 4T1 tumor cells. Cell staining and flow cytometry revealed that IgG secreted by the B splenocytes obtained from the animals subjected to TDLN cell therapy were able to bind to 4T1 tumor cells (10.2%, Fig. 7A). The MHC I staining served as a positive control, and the supernatants produced by splenic T cells served as a negative control. We then tested the consequence of the binding of IgG to tumor cells by testing complement-mediated cytotoxicity. In Figure 7B, antibody produced by splenic B cells obtained from hosts given TDLN T + B cells significantly ($P < 0.05$) lysed 4T1 tumor cells in the presence of complement, more efficiently than antibody produced by B cells obtained from nontreated host ($P = 0.14$ compared with complement alone).

**Activated TDLN B cells directly mediate tumor-specific cytotoxicity**

In addition to producing antibody that can kill tumor cells via complement-mediated cytotoxicity, we examined whether 4T1-activated B cells can mediate tumor lysis directly. B cells from 4T1 TDLN were isolated and activated ex vivo with LPS/anti-CD40. The activated B cells were then assessed for tumor cytotoxicity by using the LDH release assay. As shown in Figure 8, activated 4T1 TDLN B cells were able to mediate lysis of 4T1 tumor cells in vitro in 2 independent experiments. The cytotoxicity was specific when compared with the irrelevant Renca and TSA tumor targets respectively.

**Discussion**

This article documents the effectiveness of tumor-specific B effector cells after adoptive transfer in suppressing the development of spontaneous metastatic carcinoma. More importantly, we provide experimental evidence in this article that adoptively transferred B effector cells induce host T-cell antitumor immunity. Antigen-specific systemic T-cell immunity was documented in the recipient hosts after adoptive transfer of activated TDLN B cells. This was evident by the presence of host CIL activity and tumor-specific T-cell production of cytokines against 4T1 tumor.

The modulation of host T-cell antitumor immunity by adoptively transferred effector B cells in this setting...
represents a new finding and was shown to significantly augment the efficacy of adoptive T-cell therapy.

In contrast to resting B cells, several reports have described the application of activated B cells in the immunotherapy of cancer. The majority of these reports have indicated how activated B cells can be used as effective APCs for in vitro T-cell sensitization (5–8, 12). In another setting, activated B cells have been reported to enhance the ability to generate tumor-infiltrating lymphocytes ex vivo in a culture system involving anti-CD3 activation and IL-2 expansion (26). By our knowledge, there is only one report that has shown the utility of activated B cells to mediate regression of solid tumor after adoptive transfer. Harada and colleagues reported that LPS-activated B cells bound to anti-CD3 resulted in a modest increase in survival in mice bearing B16 lung metastases after adoptive transfer (27). The authors postulated that the activated B cells provided additional costimulatory signals to a T-cell response of the host. Besides antigen presentation or costimulation, activated B cells may mediate direct tumor cell death. Kemp and colleagues reported that CpG-containing oligodeoxynucleotide stimulation of B cells results in the induction of TRAIL/Apo-2 ligand expression that can mediate tumor cell killing (28). In our study, we have shown that LPS/anti-CD40–activated B cells can directly mediate lysis of tumor cells in standard in vitro cytotoxicity assays. This may represent one mechanism by which adoptively transferred effector B cells mediate tumor regression.

Figure 7. A, immune host B splenocyte–produced IgG binds to 4T1 cells. 4T1 cells were incubated with the culture supernatant of T and B splenocytes harvested from the 4T1 tumor–bearing mice subjected to 4T1 TLN T + B-cell treatment. Bound IgG onto the tumor cells was detected by secondary antibody FITC-anti-mouse IgG. FITC-coupled secondary antibody isotype-matching control was also used. Numbers within each immunofluorescence dot plots indicate the percentage of viable cells stained positive with the FITC-coupled antibodies. Data are representative of 2 independently conducted experiments. B, antibody and complement-mediated 4T1 cytotoxicity. Viable 4T1 cells (0.5 × 10⁶) were cultured with supernatant produced by activated B cells purified from the spleens harvested from the 4T1 tumor–bearing mice subjected to 4T1 TLN T + B-cell treatment or no treatment. CM only and complement alone served as controls. After antibody incubation, rabbit complement was added followed by incubation for another 1 hour. Viable cells were then counted under the microscope after trypan blue staining. Percentage (%) of viable cells was calculated by dividing the remaining viable cells with 0.5 × 10⁶. Data are representative of 2 independent experiments conducted.
We have begun to evaluate methods to enhance the antitumor efficacy of adoptively transferred B effector cells. In a previous report, we found that lymphodepletion prior to B-cell transfer in tumor-bearing mice significantly augmented the antitumor effects mediated by B cells (25). The enhanced antitumor reactivity of activated B cells in the lymphodepleted host may be analogous to what was observed with the adoptive transfer of immune T cells which has been reported to be due to depletion of suppressor cells such as Tregs or the elaboration of homeostatic cytokines that expand adoptively transferred lymphoid cells (29–31). Recently, there have been several reports documenting the presence of regulatory B cells that can suppress antitumor responses. Yanaba and colleagues have reported a subset of B cells that secrete IL-10 that can have adverse effects on immune T cells (32). In preliminary studies, we have documented the presence of such cells in our activated B-cell effector population. Depletion of these cells either in the activation cultures or in the tumor-bearing host may potentially result in enhanced antitumor responses. Another approach in modulating the effectiveness of adoptive B-cell therapy may be the exogenous administration of biological agents that could enhance the persistence or survival of the adoptively transferred B cells. Zhang and colleagues reported that anti-CD137 mAb enhanced the proliferation, survival, and function of activated B cells in vitro (33). It would seem logical that the in vivo administration of anti-CD137 concomitantly with effector B-cell transfer could enhance their antitumor efficacy in a similar fashion to adoptive T-cell therapy which we have reported (34).

The use of LPS plus anti-CD40 as TDLN B-cell stimuli in this article may represent a combined stimulation of innate immunity (by LPS) and adaptive immunity (by anti-CD40 to mimic the CD40L signaling from T helper cells). Evaluation of additional B-cell activation pathways, such as CD137 (33) and the engagement of the B-cell antigen receptor by using anti-Ig in the form of F(ab')2 may lead to further B-cell activation in vitro (35). Furthermore, studies of the trafficking and accumulation of infused B cells in tumor would increase our understanding of the events behind the observed therapeutic efficacy of adoptively transferred effector B cells.

In spite of the findings that activated B cells can mediate tumor regression in adoptive immunotherapy, we do not envision this as a monotherapy. Rather, we view this as an adjunct to T-cell therapy. The collaborative interactions between activated T and B cells are currently unknown as are the interactions with host immune cells. Our laboratory is currently investigating this area to optimize this approach. Collectively, the findings we report herein may provide alternative strategies in developing more effective adoptive cellular therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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