BAT mAb induces lymphopoiesis in nude mice

Britta Hardy, Yaron Niv, Ludmila Fadaeev and Annat Raiter

Felsenstein Medical Research Center, Tel Aviv University School of Medicine and Department of Gastroenterology (Y.N.), Rabin Medical Center, Beilinson Campus, Petah Tikva, 49100, Israel

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Abstract

The athymic nude mouse provides a powerful tool in the study of human tumors, as it enables growth of human tumors due to deficiencies in T cell functions. However, deficiencies in T cell functions might limit research on efficacy of immune modulators in cancer immunotherapy. BAT mAb mediates its anti-cancer activity through modulation of the immune system that involves both NK and T cells. We analyzed lymphocyte populations in blood 5 and 14 days following the injection of BAT antibody alone or following engraftment of human colon carcinoma cells. Our results demonstrate that BAT injection induced lymphopoiesis in the nude mouse. Percentage of CD3 cells increased up to 24%, CD4 cells up to 20% but no increase was found in CD8 T cells in BAT-injected nude mice. Injection of BAT 12 days post-tumor engraftment propagated CD3, CD4 and CD8 cells seen in the blood 5 days later but not seen in the blood 14 days post-BAT injection. It is possible that this decrease is associated with migration of the lymphocytes from the blood to the tumor sites in the livers. The percentage of CD56-positive NK cells increased (up to 18%) by BAT administration alone or post-tumor injection. The presence of tumors alone did not induce lymphopoiesis in the nude mice. Propagation and lymphopoiesis by BAT mAb might have future clinical implications.

Introduction

The athymic nude mouse provides a powerful tool in the study of human tumors, as it enables the growth of human tumors due to deficiencies in the nude mouse T cell functions. Human tumors are also successfully engrafted into SCID mice in which both T and B cell immunity are deficient. Among studies on cancer therapy of human tumors in athymic and SCID animal models, the research on the efficacy of immune modulators in cancers is limited due to the impaired immune system of these mice. Engraftment of lymphocytes or distinct lymphocyte populations into a SCID mouse was shown to overcome the recipient immune deficiencies, thus enabling studies on the effect of immune modulators in cancer therapy (1, 2). The attempts to apply new therapeutics in cancer by immune modulators aim to modulate the immune system response in order to kill tumor cells. Among the immune modulators are mAbs (3) that selectively bind to a specific determinant on T cells (4), thereby either initiating an activation pathway or inducing an inhibitory effect. Such antibodies as antibodies against CD3/TCR (T cell receptor) receptors trigger the proliferation of T cells and increase their cytolytic activity (5, 6). Antibodies against cytotoxic T lymphocyte associated protein 4 (CTLA4) block its inhibitory effect on T cells (7, 8). The mAbs against CD40 are effective substitutes for Tn in conditioning antigen-presenting cells.

The main tumor cell-killing effector cells are CTL, but accumulating evidence suggests that Th are also essential for priming the immune system against tumors (9). Th activate non-specific immune effector mechanisms in tumor destruction by secreting appropriate cytokines (such as IFN-gamma) (10). We have previously described the BAT mAb which mediates its anti-cancer activity through modulation of the immune system to induce tumor regression (11). BAT anti-tumor activity involves the stimulation of both NK cells and CD4 T cells (12). We have previously studied BAT anti-tumor activity both in SCID and nude mice. Therapeutic effects were obtained by BAT treatment in SCID mice only if mice were engrafted with either murine or human lymphocytes. An anti-tumor effect of BAT in athymic nude mice injected with murine B16 melanoma that developed tumors in the lungs was less effective than that in wild-type mice (1). Recently, we evaluated the therapeutic activity of the BAT mAb in a human colon carcinoma model in nude mice. Our data indicated a remarkable anti-tumor activity of BAT in preventing hepatic metastases in the tumor-engrafted nude mice (B. Hardy, S. Morgenstern, A. Raiter, G. Rodinov, L. Fadaeev and Y. Niv. submitted for publication).

In the current study we analyzed lymphocyte populations in the blood of nude athymic mice following injection of BAT alone.
or following tumor engraftment in order to elucidate BAT activity in the nude athymic mouse.

**Methods**

**BAT antibody**

BAT is a murine mAb developed against a membrane preparation of a Burkitt lymphoma cell line (Daudi). The mAbs were selected by binding to Daudi cells and by their ability to induce human PBMC proliferation (11, 13). The BAT mAb is produced from either in vitro hybridoma cultures grown in RPMI-1640 medium supplemented with 10% FCS or from mice ascites, followed by purification on a protein G Sepharose column (Pharmacia, Uppsala, Sweden).

**Human tumor cell line**

HM7 is a sub-clone of the human colorectal carcinoma (CRC) cell line LS174T, selected for its high mucin synthesis and metastatic potential (14). The cells were obtained as a generous gift from Robert S. Breselier (MD Andersen, USA). The cells were grown in RPMI-1640 supplemented with 10% FCS, L-glutamine (2 mM), Na-pyruvate (1 nmol), penicillin (100 U ml⁻¹), streptomycin sulfate (0.1 mg ml⁻¹) and nystatin (12.5 U ml⁻¹). Cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

**Human colon carcinoma tumor model in nude mice**

We used a liver metastases human colon carcinoma tumor model in nude mice (14). We performed three experiments and each group of mice in each experiment was composed of 10 female mice 9- to 10-weeks old. BALB/c nude mice were anesthetized and their spleens were exposed. HM7 (2 × 10⁶) cells in 0.25 ml PBS were injected into the exposed spleen; after 1 min, the spleens were removed and the excisions were closed. HM7 cells colonize the liver as bulky nodules. Mice were divided into four groups. (i) Nude mice injected with BAT only (10 µg per mouse in PBS). (ii) Nude mice injected with the isotype control mouse IgG3. (iii) Nude mice injected with tumor cells only. (iv) Nude mice inoculated with tumor cells and 12 days post-tumor inoculation injected intravenously with BAT antibodies at 10 µg per mouse in PBS.

**Collecting blood samples of nude mice**

Two blood sampling periods, ‘first’ and ‘second’ sampling, were defined equivalently across all four groups, based on corresponding times after BAT (or BAT control) injection. Thus, blood pooled from five mice from groups 1 and 2 of nude mice was collected on day 5 (first sampling) and day 14 (second sampling) post-antibody and control IgG injections. Experiments with groups 1 and 2 were repeated five times. Blood from groups 3 and 4 was collected 17 days (first sampling) and 26 days (second sampling) post-tumor inoculation, corresponding to 5 and 14 days post-BAT injection (BAT administration on day 12 post-tumor inoculation, plus either 5 or 14 days).

**Fluorocytometric cell analysis of lymphocytes in blood**

Mononuclear cells were isolated from the blood by Ficoll–Hypaque centrifugation. Thereafter, 3 × 10⁶ freshly isolated leukocytes samples were suspended in 50 µl PBS containing 5% FCS and 0.1% Na-azide.

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage of cells</th>
</tr>
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<tbody>
<tr>
<td>CD3</td>
<td>0.8</td>
</tr>
<tr>
<td>CD4</td>
<td>0.1</td>
</tr>
<tr>
<td>CD3/CD4</td>
<td>0.1</td>
</tr>
<tr>
<td>CD8</td>
<td>0.6</td>
</tr>
<tr>
<td>CD3/CD8</td>
<td>0.3</td>
</tr>
<tr>
<td>Thy1.2</td>
<td>29.6</td>
</tr>
<tr>
<td>CD3/Thy1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>CD4/Thy1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>NK 1.1</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Samples containing 0.5 × 10⁶ cells were incubated in PBS + 5% FCS or 0.1% Na-azide with rat anti-mouse CD3 PE labeled (clone number KT3) and anti-mouse CD4 (clone number 191.1) and rat anti-mouse CD8 (clone number KT15) fluorescein labeled (Serotec, Oxford, UK) and anti-mouse Thy1.2 (CD90.2) labeled with biotin (clone number 30-H12, BioLegend, San Diego, CA, USA) followed by streptavidin Cy5 coupled to R-phycocerytin (Dako Cytomation), for 45 min on ice. Triple-staining dot blot analysis was performed using a FACScan (Beckton Dickinson, CA, USA).

PE-conjugated anti-NK1.1 (clone number PK136) antibodies were used in double staining with anti-mouse CD3 labeled with FITC. Anti-mouse-kappa for detection of kappa-positive B cells was labeled with biotin (Southern Biotechnology Associates, Birmingham, AL, USA) and detected with streptavidin–FITC (Jackson Immuno-Research, West Grove, Palo Alto, CA, USA).

Side scatter and forward scatter of dot plots were used to determine the gates of lymphocytes; PE- or FITC-labeled IgGs (Pharmingen, San Diego, CA, USA) served as isotope controls for PE- or FITC-labeled antibodies. FACS analysis was done using FACSCalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium). Data were analyzed using CELLQuest (Becton Dickinson).

**Results**

**Effect of BAT injection of naive nude mice on lymphocyte populations in the blood**

The proportion of lymphocyte sub-populations in blood of nude mice is demonstrated in Table 1. FACS analysis determined the percentages of CD3, CD4, CD8 T cells and NK cells in the blood of nude mice. Lymphocytes in groups of control nude mice injected with 10 µg of control IgG3 were compared with lymphocytes of nude mice after a single injection with 10 µg per mouse of BAT mAb.

Lymphocytes in blood of control nude mice maintained a low proportion of CD3- and CD4-positive T cells, which varied between 0.6 and 0.8% at 5 and 14 days post-control IgG3 administration. However, a significant increase of CD4 lymphocytes was found in nude mice 5 days after BAT administration. However, a significant increase of CD4 lymphocytes was found in nude mice 5 days after BAT
injection. Percentage of CD3-positive cells increased to 24.5 and was maintained on day 14 (19%). Percentage of CD4 cells was increased to 20.8 ($P < 0.05$) and was maintained on day 14 (14.6%). Double staining of CD3/CD4 populations increased to 19.3%. In contrast to CD4 cell propagation, CD8 T cells in the blood of the nude mice did not increase at any tested day following the injection of BAT antibodies and the values were as low as in the controls (0.3–0.6%). Percentage of CD3/CD8-positive cells confirmed the low number of CD8 cells (Table 1). CD4 cells lymphopoiesis induced by BAT mAb in nude mice is also demonstrated in Fig. 1 that represents FACS analysis of double-staining CD3/CD4 and CD3/CD8 dot plots compared with control. The number of NK cells increased from 7.3% in controls to 18.5% after 5 days following BAT administration (Table 1) and increased to 24.2% on day 14.

Triple staining that includes anti-mouse Thy1.2, CD4 and CD3 T cells is represented in Fig. 2 by dot plot FACS analysis. Results showed that 30% of cells in the blood of untreated nude mice are Thy1.2 positive. BAT mAb injection did not induce an increase in the percentage of Thy1.2 cells. However, BAT mAb induced an increase in the percentage of CD3/Thy1.2 from 0.4 to 24% and CD4/Thy1.2 from 0.1 to 21% (Table 1).

**Effect of HM7 human colon carcinoma engraftment of nude mice on lymphocyte populations in blood**

Percentage of lymphocyte sub-populations in the blood of nude mice engrafted with tumor only was followed on the first and second samplings. The effect of tumor only on blood lymphocytes determined by FACS analysis is presented in Table 2. As can be seen, the percentage of CD3, CD4 and CD8 T cells was low and statistically non-significantly changed by tumor administration: 1.2 to 3.5% for CD3, 1.1 and 2.6% for CD4 and 0.3 and 1.1% for CD8 cells. The number of anti-kappa-positive B cells increased with time. NK cells were 7.4% on first sampling and 0.3% on second sampling (Table 2).

**Effect of BAT treatment of human colon carcinoma of nude mice on peripheral blood leukocytes**

Treatment of the tumor-bearing mice with a single administration of BAT (10 µg per mouse) 12 days post-tumor administration induced a remarkable anti-tumor activity, manifested as prevention of liver metastases (B. Hardy, S. Morgenstern, A. Raiter, G. Rodinov, L. Fadaeev and Y. Niv. submitted for publication). Blood analysis tested on the first sampling (5 days post-BAT injection) resulted (Table 3) in a significant 33.6% increase in CD3 cells ($P < 0.05$) and 20.8% in CD4-positive cells ($P < 0.05$). A similar effect of BAT treatment was also seen.

**Fig. 1.** FACS analysis of T cell sub-populations in blood of nude mice injected with mouse IgG3 as control or BAT mAb. Double-staining dot plots of CD3/CD4 and CD3/CD8 lymphocyte populations 5 days after BAT mAb administration. Lymphocytes were stained with PE-labeled anti-mouse CD3 and FITC-labeled anti-mouse CD4 or CD8. Acquisition of 10 000 gated cells was confirmed by CD3-labeled cells.

**Fig. 2.** FACS analysis of CD3/Thy1.2 and CD4/Thy1.2 T cell sub-population in blood of nude mice injected with mouse IgG3 as control or BAT mAb. Dot plots of CD3/Thy1.2 and CD4/Thy1.2 lymphocyte populations 5 days after BAT mAb administration. Lymphocytes were stained with anti-mouse CD3 or CD4 and anti-mouse and biotin-labeled Thy1.2 followed by streptavidin–RPE-Cy5. Isotype controls were biotinylated IgG1 and FITC- or PE-labeled.

**Table 2.** FACS analysis of lymphocytes in blood of nude mice injected with human colon carcinoma HM7 cells

<table>
<thead>
<tr>
<th>Days post-tumor administration</th>
<th>Percentage of cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
</tr>
<tr>
<td>CD3</td>
<td>1.2</td>
</tr>
<tr>
<td>CD4</td>
<td>1.1</td>
</tr>
<tr>
<td>CD8</td>
<td>0.3</td>
</tr>
<tr>
<td>B cells</td>
<td>52.7</td>
</tr>
<tr>
<td>NK</td>
<td>7.4</td>
</tr>
</tbody>
</table>

First reading at day 17 post-tumor administration and second reading at day 26.
in increased number of NK cells on the first sampling (22.1%) and CD8 cells (9.7%). B cells bearing kappa light chain were present in all groups of mice and were not significantly affected by BAT treatment (52.8%). On second sampling (14 days after BAT treatment, 26 days after tumor inoculation), CD3, CD4 and CD8 and NK cell sub-population sizes decreased to the control nude mice values in the blood as seen in Table 3.

**Table 3. FACS analysis of lymphocytes in blood of nude mice injected with human colon carcinoma HM7 cells and treated with BAT mAb**

<table>
<thead>
<tr>
<th>Reading</th>
<th>Percentage of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>CD3</td>
<td>33.6</td>
</tr>
<tr>
<td>CD4</td>
<td>20.8</td>
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<tr>
<td>CD8</td>
<td>9.7</td>
</tr>
<tr>
<td>Anti-kappa</td>
<td>52.6</td>
</tr>
<tr>
<td>CD66</td>
<td>22.1</td>
</tr>
</tbody>
</table>

First reading at 17 days post-tumor administration and 5 days post-BAT injection and second reading at 26 days post-tumor administration and 14 days post-BAT injection.

Discussion

T cell development and selection require the fully mature and diverse epithelial microenvironment of the thymus. Although the role of the thymus in the development of a normal repertoire of T cells is undisputed, there are reports that extra-thymic maturation of T cells can occur (15–17). Populations of T cells from athymic mice have been analyzed phenotypically for a variety of surface markers. It was found that CD8 and CD4 cells exist in low detectable numbers (18). Different activation pathways may lead to the development of distinct extra-thymic populations (19).

Proliferative responses of T cells derived from nude mice were studied upon stimulation with Con A, polyvalent anti-CD3 antibodies and phorbol myristate acetate plus ionophore. None of the mitogenic stimuli induced significant proliferation in athymic nude CD4 T cells (20).

However, despite the fact that nude mice are unable to reject allografts and xenografts, CD4, CD8 and CD3 T cell clones reactive to allogeneic stimulation in vitro were cloned from an athymic mouse and maintained in long-term cultures (21). In vivo stimulation with allogeneic cells or Ig resulted in extra-thymic lymphocyte expansion. Lymphocytes were preferentially CD4 cells and could confer a functional immune-competent system to the nude host, with the ability to reject allogeneic skin grafts (19). In our study we found that BAT mAb induced lymphopoiesis in the nude mouse. CD3 T cells were expanded by BAT mAb alone to ~25%, which is half of the normal percentage of CD3-positive cells in the wild-type BALB/c mice. These CD3 T cells are Thy1.2 positive, suggesting that BAT mAb induced differentiation of Thy1.2-positive/CD3-negative to Thy1.2-positive/CD3-positive T cells. Most of the CD3 cells expanded in the BAT-injected nude mice are CD4 positive as demonstrated by double-labeling FACS analysis. CD8 T cells were not expanded and remained in a low percentage throughout this study. These results comply with our previous work in which we demonstrated that BAT mAb injected to wild-type mice or incubated for 5 days with human peripheral blood lymphocytes stimulates CD4 T cells to proliferate and secrete IFN-gamma (12, 22). Tumor regression is related to the immune stimulatory properties of BAT mAb since the anti-tumor activity was found to be transferred by adoptive transfer experiments in which splenocytes from mice treated with BAT induced anti-tumor activity in tumor-bearing recipient mice (1). It is therefore possible that intravenous injection of BAT mAb to nude mice induced proliferation of CD4 T cells with anti-tumor activity. This is supported by our study in nude mice injected with HM7 human colorectal carcinoma and treated with a single intravenous inoculation of BAT mAb. BAT injection induced a significant reduction of the number of colorectal carcinoma liver metastases and liver weights (B. Hardy, S. Morgenstern, A. Raiter, G. Rodinov, L. Fadaeev and Y. Niv. submitted for publication). The presence of tumors alone did not induce lymphopoiesis in the nude mouse while the injection of BAT mAb into tumor-engrafted nude mice did propagate CD4 lymphocytes. However, the proportion of lymphocytes in these tumor-bearing mice was not seen in the blood at 14 days post-BAT injection. We assume that these lymphocytes migrated to the tumor sites. This assumption is supported by histological examinations of colorectal carcinoma metastatic livers of BAT-treated mice in which we have found granulomatous reactions consisting of dense lymphocytic rings by the border of microscopic tumors (B. Hardy, S. Morgenstern, A. Raiter, G. Rodinov, L. Fadaeev and Y. Niv. submitted for publication).

As BAT may also stimulate NK cells, we have also evaluated the percentage of NK cells in BAT-injected nude mice. The percentage of NK cells in the blood of the nude mouse is similar to that of the wild-type mouse (7–12%). We have found a significant 2-fold increase in NK cells in nude mice injected with BAT mAb. These NK cells are CD3 negative as we observed in double-staining FACS analysis (data not shown).

This study demonstrates that BAT mAb can propagate T cells of the T cell-deficient nude athymic mice. The implications of that activity will further be explored.

**Abbreviation**

TCR T cell receptor

**References**


