B cell-mediated down-regulation of IFN-γ and IL-12 production induced during anti-tumor immune responses in the tumor-bearing state

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Abstract

Unfractionated spleen cells taken from tumor-bearing mice contained tumor-primed T cells which produced lymphokines such as IFN-γ and IL-2 through collaboration with antigen-presenting cells (APC) binding tumor antigens when cultured in vitro. Here, we investigated the regulatory mechanisms underlying IFN-γ production by T–APC interactions. Elimination of B cells from a splenic population of tumor-bearing mice resulted in enhanced IFN-γ production. Adding B cells back into cultures down-regulated IFN-γ production to almost the same levels as those induced by unfractionated spleen cells. IL-2 production was not enhanced by B cell depletion, but rather was significantly suppressed. IFN-γ-selective up-regulation was due to an enhancement of IL-12 production because IL-12 was detected in B cell-depleted cultures and enhanced IFN-γ production was prevented by addition of anti-IL-12 mAb or anti-CD40 ligand (CD40L) mAb capable of inhibiting CD40L-induced IL-12 production. These results indicate that B cells interfere with IFN-γ production induced through interactions between anti-tumor T cells and APC, and this suppressive effect is based on the capacity of CD40⁺ B cells to down-regulate the CD40L-induced IL-12 production by APC.

Introduction

Cytokines play an important role in host defense and inflammatory responses (1–4). Among inflammatory cytokines, IFN-γ, which is produced mainly by Th1-type lymphocytes, exhibits many immunoregulatory effects including the capacity to induce the differentiation and activation of cytotoxic T lymphocytes (CTL) (5,6) and macrophages (7). Recently, IL-12 has been shown to exert a potent anti-tumor effect (8–11). The anti-tumor effects of IL-12 are closely associated with its capacity to stimulate IFN-γ production by T cells and NK cells because neutralization of IFN-γ by administration of anti-IFN-γ mAb results in the abrogation of the anti-tumor efficacy of IL-12 (9,10). Thus, it is becoming increasingly evident that IFN-γ has a critical role in the manifestation of T cell-mediated inflammatory responses including the anti-tumor immune response.

Tumor-bearing hosts develop an anti-tumor immune response. Spleen cells from early stages of tumor-bearing mice produced IFN-γ and IL-2 upon in vitro cultures without addition of exogenous tumor antigens (12). This was demonstrated to be a result of the collaboration between tumor-primed CD4⁺ T cells and antigen-presenting cells (APC) binding and presenting tumor antigens (12). However, it has been shown that these anti-tumor T cell responses are not potent enough to induce tumor regression but rather decrease with the progress of tumor-bearing stages (12,13). Such low/reduced responsiveness of tumor-primed T cells would be explained by several mechanisms, including the interference with T–APC interactions. Recent studies have shown that when T cells are stimulated with antigen plus APC, IL-12 is produced by APC (14,15). Moreover, we found that B cells...
interfere with the interaction between CD40 on APC and CD40 ligand (CD40L) on TCR-triggered T cells to inhibit IL-12 production by APC (16). Because IL-12 stimulates IFN-γ production by T cells (17,18), it is possible that such a B cell-mediated interference may be involved in the regulation of lymphokine production by tumor-primed T cells.

In the present study, we investigated the effect of B cells on lymphokine production by tumor-primed T cells during interactions with APC presenting tumor antigens. The results show that elimination of B cells from spleen cells of tumor-bearing mice, when cultured in vitro, resulted in an enhancement of IFN-γ but not of IL-2 production. Enhanced IFN-γ secretion was associated with IL-12 production induced following B cell elimination and was prevented by the addition of anti-IL-12 or anti-CD40L mAb. Thus, these results suggest that B cells regulate IFN-γ production by tumor-primed T cells through suppressing CD40L-induced IL-12 production during T–APC interactions, providing a new mechanism of immune-suppression in the tumor-bearing state.

Methods

Mice

Male BALB/c mice and female (C57BL/6×C3H/He)F1 (B6C3F1) mice were obtained from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan) and used at 6–9 weeks of age.

Tumor cell lines

The following three tumor cell lines were used: Rous sarcoma virus-induced CSA1M fibrosarcoma (BALB/c origin) (19), radiation-induced OV-HM ovarian carcinoma (B6C3F1 origin) (20) and methylcholancerene-induced BAMC-1 fibrosarcoma (BALB/c origin) (21). These tumor lines were kindly provided by Dr Takato O. Yoshida (Hamamatsu University School of Medicine, Hamamatsu, Japan), Dr Ohtsura Niwa (Kyoto University, Kyoto, Japan) and Dr Yoichiro Moriya (Chugai Pharmaceutical, Tokyo, Japan) respectively. They were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified incubator (5% CO2) for 24 h, culture supernatant was harvested by centrifugation and stored at −20°C until use.

Preparation of CSA1M tumor-bearing mice

Tumor-bearing mice were prepared by inoculating s.c. 3×105 CSA1M cells, 5×105 OV-HM cells or 1×106 BAMC-1 cells. Mice 2–3 and 6–8 weeks after tumor cell implantation were used as early and late tumor-bearing hosts respectively.

Recombinant lymphokines and cytokines

The following lymphokines/cytokines were obtained: mouse rIL-2 and mouse rIFN-γ were provided by Shionogi Pharmaceutical (Osaka, Japan); mouse rIL-12 was provided by Genetics Institute (Cambridge, MA).

mAb and polyclonal antibodies

The following mAb were used. Anti-CD3 (145-2C11) (22) was purified from the culture supernatant of its hybridoma cells. Anti-mouse IL-12 (C17.8) (23) and anti-CD40L (HM40L-1) (24) mAb were purified from the ascitic fluids of the relevant hybridomas which were kindly provided by Drs G. Trinchieri (Wistar Institute, Philadelphia, PA) and H. Yagita (Juntendoh University School of Medicine, Tokyo). FITC-conjugated anti-mouse IL-12 (C17.8, rat anti-mouse Mac-1 and rat anti-mouse μ chain mAb were purchased from Seikagaku Kogyo (Tokyo, Japan), Pharmingen (San Diego, CA) and Cappel-Coope Biomedical (Malvern, PA) respectively. FITC-conjugated goat anti-rat IgG (Cappel-Coope Biomedical), normal hamster IgG (Cappel, Durham, NC) and normal rat IgG (BioMeda, Foster City, CA) were also purchased.

Preparation of lymphokine/cytokine samples (whole spleen cell culture system)

Unfractionated spleen cells, obtained from a pool of four to five tumor-bearing mice, were cultured without addition of exogenous tumor antigens in 24-well culture plates (Corning 25820; Corning Glass Works, Corning, NY) at a concentration of 5×106 cells/well, unless otherwise indicated in a volume of 1 ml RPMI 1640 supplemented with 10% FCS (12,13). After incubation at 37°C in a humidified incubator (5% CO2) for 24 h, culture supernatant was harvested by centrifugation and stored as −20°C until use.

Assay systems for cytokine activity/concentration

IL-2 activity. Supernatants were assayed for IL-2 activity according to their ability to support the proliferation of the IL-2-dependent T cell line, CTLL-2, as described (12,13). Briefly, CTLL-2 cells (105/well) were cultured with supernatants in a volume of 0.2 ml in 96-well flat-bottomed microplates (Corning 25860) for 24 h at 37°C. Proliferation was assessed by the uptake of [3H]thymidine during 4 h pulsing with 20 kBq/well.

IL-12 activity. The assay system (antibody capture assay) was previously described (25). Briefly, each well of 96-well microculture plates was coated with 10 μg of purified anti-IL-12 mAb (C15.1) (23). Various dilutions of culture supernatants or standard murine rIL-12 solution were incubated in wells of mAb-coated plates. After washing, cells (1.5×105/well) of the IL-12-responsive cell line 2D6 (25) were cultured on anti-IL-12-coated plates for 48 h and pulse-labeled with 20 kBq/well of [3H]thymidine for the final 6 h. All samples for IL-2 and IL-12 were cultured in triplicates. Absolute concentrations of IL-2 or IL-12 were determined by extrapolation from a standard curve generated using known amounts of rIL-2 or rIL-12. Results were expressed as the mean concentration ± SE of triplicate cultures.

IFN-γ concentrations. IFN-γ concentration was measured by ELISA: mouse IFN-γ ELISA kits were purchased from Genzyme (Cambridge, MA), and our own ELISA system was prepared using two types of anti-mouse IFN-γ mAb [XMG1.2 (Endogen, Cambridge, MA) and biotinylated R4-6A2 (R4-6A2 was purified from R4-6A2 hybridoma and biotinylated in our laboratory)] as well as mouse rIFN-γ. In our ELISA system, 1 U/ml corresponded to ~100 pg/ml in Genzyme ELISA kits.

Preparation of various lymphoid populations

Spleen cells were depleted of B cells by immunomagnetic negative selection as described (26). Briefly, spleen cells were incubated with magnetic particles bound to goat anti-
Down-regulation of IFN-γ and IL-12 production by B cells

**Table 1. Enhanced IFN-γ production induced by depleting of B cells from spleen cells of CSA1M tumor-bearing mice**

<table>
<thead>
<tr>
<th>Spleen cells from micea</th>
<th>B cell depletab</th>
<th>IFN-γ production (U/ml)c</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>–</td>
<td>6.5 ± 0.5</td>
<td>4.0 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Early tumor bearing</td>
<td>–</td>
<td>40.0 ± 0.7</td>
<td>57.0 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>Late tumor bearing</td>
<td>–</td>
<td>6.0 ± 0.9</td>
<td>3.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>5.0 ± 0.3</td>
<td>4.0 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Early tumor bearing</td>
<td>+</td>
<td>190.0 ± 1.2</td>
<td>272.0 ± 13.9</td>
<td></td>
</tr>
<tr>
<td>Late tumor bearing</td>
<td>+</td>
<td>38.0 ± 0.7</td>
<td>30.0 ± 5.4</td>
<td></td>
</tr>
</tbody>
</table>

*aSpleen cells were obtained from normal or tumor-bearing mice 2-3 (early) or 6-8 weeks (late) after tumor cell implantation.
>bSpleen cells were depleted of B cells as described in Methods.
>cUnfractionated spleen cells or B cell-depleted fractions (5 x 10⁶/well) were cultured in 24-well culture plates for 48 h.

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mouse Ig (Advanced Magnetic, Cambridge, MA). Surface Ig-negative cells were obtained by removing cell-bound magnetic particles with a rare earth magnet (Advanced Magnetic). A B cell-enriched population was prepared by positive selection. Briefly, spleen cells were labeled with superparamagnetic microbeads conjugated to rat anti-mouse IgM mAb (Miltenyi Biotec, Sunnyvale, CA). Labeled cells were separated from unlabeled cells by magnetic cell sorting using the MiniMACS (Miltenyi Biotec) according to the technology described in detail (27). The magnetic cells were retained in a MiniMACS column inserted into the MiniMACS magnet while the non-magnetic cells passed through. Labeled cells were eluted after the column was removed from the magnet.

**Flow cytometry analysis**

Cells were first incubated with anti-CD16 mAb (28) to prevent the binding of staining mAb with Fc receptors and then stained directly with FITC-conjugated mAb or with a primary (rat anti-mouse) mAb or control rat IgG followed by FITC-conjugated goat anti-rat IgG. Stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA).

**Results**

**B cell-depleted splenocytes from tumor-bearing mice induce enhanced levels of IFN-γ production**

Our earlier study demonstrated that unfractionated spleen cells from tumor-bearing mice produce IL-2 and IFN-γ when cultured in vitro without addition of exogenous tumor antigens (12). This was shown to be based on the collaboration between tumor-primed T cells and APC binding tumor antigens (12). We have confirmed this (Table 1). Unfractionated spleen cells from BALB/c mice bearing syngeneic CSA1M fibrosarcoma 2–3 weeks after CSA1M cell inoculation (early stages of tumor growth) produced significant levels of IL-2 and IFN-γ. The results also show that this initial lymphokine-producing capacity decreases at late stages of tumor growth. We investigated the effect of B cell-depletion on the lymphokine production induced through T–APC collaboration. Splenocytes from normal or CSA1M tumor-bearing mice were depleted of B cells by immunomagnetic negative selection as described in Methods. The efficacy of B cell depletion from splenic populations is shown in Fig. 1. The results of Table 1 demonstrate that elimination of B cells from early and late stages of CSA1M tumor-bearing mice results in an enhancement of IFN-γ production. Similar patterns of enhanced IFN-γ production were found to be induced following B cell depletion in two other tumor models (Table 2). Enhanced levels were much higher in B cell-depleted fractions from early than late tumor-bearing mice (Table 1 for CSA1M and data not shown for OV-HM and BAMC-1). Therefore, the mechanisms underlying the enhanced IFN-γ production following B cell depletion were examined using spleen cells from early stages of CSA1M tumor-bearing mice in most of the following experiments.

B cell depletion increases the proportions of T cells and APC in a spleen cell suspension. Analysis of IFN-γ production by different numbers of unfractionated or B cell-depleted cells revealed that IFN-γ production by the unfractionated population even at a cell density as high as 8 x 10⁶ cells/well was lower than that induced by the B cell-depleted population even at a cell density as low as 1–2 x 10⁵ cells/well (Table 3). Moreover, addition of B cells to a given number of the [T + APC] fraction resulted in down-regulation of IFN-γ production to almost the same levels as those observed for unfractionated spleen cells (Fig. 2). Thus, enhanced IFN-γ production induced after B cell depletion is not explained simply by an increase in the numbers of T cells and APC in a given cell density of cultures. The results also show that inhibition of IFN-γ production induced by addition of B cells was observed irrespective of whether B cells were prepared from normal spleen cells or from cells of early tumor-bearing mice.

IFN-γ-selective enhanced production following B cell depletion

Because IL-2 is also produced through interactions between anti-tumor T cells and APC from tumor-bearing mice (12), we examined whether B cell depletion induces enhanced production of IL-2 as well. Figure 3 shows that IFN-γ production was again enhanced following depletion of B cells from spleen cells of tumor-bearing mice, whereas IL-2 production in the same culture was not elevated but was rather significantly reduced. Because the peak of IL-2 production was observed 24 h but not 48 h after cultures (12,13), data for IL-2 production 24 h after culture are shown in Fig. 3. IL-2 production was not enhanced in B cell-depleted cultures even after 48 h (data not shown). These results indicate that up-regulation of lymphokine production induced by B cell depletion is observed selectively for IFN-γ. This may be consistent with the hypothesis that there exists a mechanism capable of enhancing IFN-γ production specifically rather than the possibility that enhanced IFN-γ production is due to an increase in the number of T cells and APC after B cell depletion.

IL-12 production in cultures of splenocytes from tumor-bearing mice following B cell depletion

We have recently found that whereas stimulation of unfractionated normal spleen cells with anti-CD3 mAb failed to generate IL-12 production, the same cell preparation depleted of B cells produced ~100 pg/ml IL-12 (16). In considering that IL-12 is produced by APC interacting with TCR-stimulated T cells in the absence of B cells, we investigated whether IL-12
Down-regulation of IFN-γ and IL-12 production by B cells

Fig. 1. Flow cytometry analysis of splenocytes before and after B cell depletion. Unfractionated spleen cells and B cell-depleted fractions from normal mice or early or late stages of tumor-bearing mice were stained for Thy-1.2, surface Ig (µ) and Mac-1 as described in Methods.

Table 2. Enhanced IFN-γ production induced by depleting B cells from spleen cells of tumor-bearing mice in two other models

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Spleen cells from mice</th>
<th>B cell depletion</th>
<th>IFN-γ production (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>OV-HM</td>
<td>normal</td>
<td>−</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>tumor bearing</td>
<td>−</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>tumor bearing</td>
<td>+</td>
<td>28.7 ± 0.9</td>
</tr>
<tr>
<td>BAMC-1</td>
<td>normal</td>
<td>−</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>tumor bearing</td>
<td>−</td>
<td>36.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>tumor bearing</td>
<td>+</td>
<td>139.4 ± 26.5</td>
</tr>
</tbody>
</table>

OV-HM or BAMC-1 tumor-bearing mice were prepared by inoculating s.c. OV-HM (5×10^5/mouse) or BAMC-1 (1×10^6/mouse) to syngeneic B6C3F1 or BALB/c mice, respectively. Spleen cells (5×10^6 cells/well) from early stages (~2 weeks after tumor cell implantation) of tumor-bearing mice with or without B cell depletion were cultured for 48 h.

production is detected in cultures of B cell-depleted spleen cells containing tumor-primed T cells and APC (Fig. 4). The results show that IL-12 was only marginally detected in cultures of unfractionated spleen cells, whereas B cell depletion from splenocytes of tumor-bearing mice resulted in significant levels of IL-12 production. An elevation of IL-12 production was also observed in the B cell-depleted fraction from late stages of tumor-bearing mice.
Table 3. IFN-γ production by different numbers of splenocytes from tumor-bearing mice before versus after B cell depletion

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>B cell depletion</th>
<th>No.</th>
<th>IFN-γ production (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>–</td>
<td>8x10^6</td>
<td>15 ± 0.8</td>
</tr>
<tr>
<td>Normal</td>
<td>–</td>
<td>4x10^6</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>Normal</td>
<td>–</td>
<td>2x10^6</td>
<td>5 ± 0.2</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>–</td>
<td>8x10^6</td>
<td>72 ± 16.2</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>–</td>
<td>4x10^6</td>
<td>55 ± 14.1</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>–</td>
<td>2x10^6</td>
<td>30 ± 3.2</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>4x10^6</td>
<td>15 ± 0.8</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>2x10^6</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>1x10^6</td>
<td>8 ± 0.4</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>+</td>
<td>4x10^6</td>
<td>276 ± 14.2</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>+</td>
<td>2x10^6</td>
<td>204 ± 12.5</td>
</tr>
<tr>
<td>Tumor bearing</td>
<td>+</td>
<td>1x10^6</td>
<td>96 ± 1.8</td>
</tr>
</tbody>
</table>

aVarious numbers of spleen cells that were not or were depleted of B cells were cultured in 24-well culture plates for 48 h.

In our previous study (16), IL-12 production was shown to be mediated by CD40+ APC and dependent on the stimulation with CD40 ligand (CD40L) induced on T cells activated with anti-CD3 stimulation (16). This was demonstrated by inhibiting IL-12 production using anti-CD40L mAb. In the present system, we examined whether anti-CD40L mAb reduces IL-12 production by APC interacting with anti-tumor T cells. Table 4 demonstrates that addition of anti-CD40L mAb to B cell-depleted splenocyte cultures reduces IL-12 production. Thus, these results are consistent with the notion that B cells regulate CD40L-induced IL-12 production in APC during interactions between T cells and APC from tumor-bearing mice.

The induction of IL-12 is responsible for enhanced IFN-γ production

We determined whether IL-12 induction is responsible for enhanced IFN-γ production following B cell depletion from splenocytes of tumor-bearing mice (Fig. 5). The B cell-depleted fraction of spleen cells from early stages of tumor-bearing mice again generated enhanced levels of IFN-γ production (Fig. 5, right panel). Addition of anti-IL-12 or anti-
Down-regulation of IFN-γ and IL-12 production by B cells

Fig. 4. IL-12 is produced in cultures of splenocytes from tumor-bearing mice following B cell depletion. Unfractionated or B cell-depleted spleen cells from normal (N), early (E) or late (L) stages of tumor-bearing mice were cultured for 18 h and culture supernatants were assayed for IL-12 production according to the procedure described in Methods.

Table 4. Inhibition of IL-12 production in cultures of B cell-depleted fraction by addition of anti-CD40L mAb

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Addition of antibody</th>
<th>IL-12 production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>–</td>
<td>0.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>control IgG</td>
<td>4.6 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>anti-CD40L</td>
<td>3.4 ± 4.6</td>
</tr>
<tr>
<td>B cell depleted</td>
<td>–</td>
<td>28.7 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>control IgG</td>
<td>29.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>anti-CD40L</td>
<td>8.3 ± 0.8</td>
</tr>
</tbody>
</table>

Unfractionated spleen cells from early stages of tumor-bearing mice or its B cell-depleted fraction were cultured in the presence of 20 μg/ml anti-CD40L mAb or control IgG for 18 h. Culture supernatants were assessed for IL-12 concentrations as described in Methods.

CD40L mAb to these cultures resulted in almost complete inhibition of IFN-γ production. These results demonstrate that IL-12 produced in B cell-depleted cultures plays a critical role in the induction of enhanced IFN-γ production. Although only marginal levels of IL-12 were detected in cultures of unfractionated spleen cells (Fig. 4), IFN-γ production by unfractionated splenocytes was inhibited by anti-IL-12 or anti-CD40L mAb (Fig. 5, left panel). Thus, the results suggest that the undetectable levels of IL-12 are produced in unfractionated splenocyte cultures and contribute to inducing significant levels of IFN-γ production. Taken together, these observations indicate that B cells regulate IL-12 production induced via the CD40L–CD40 interaction between T cells and APC, and consequently inhibit the production of IFN-γ by anti-tumor T cells.

Discussion

A large number of studies have reported that the tumor-bearing state induces suppressive effects on the host’s general (29,30) or anti-tumor (31–33) T cell responses. Apart from these previous reports, recent studies have implied the operation of new mechanisms underlying tumor-induced immunosuppression. For example, a structural abnormality was reported to be induced in the TCR–CD3 complex (34-37): the loss of the ζ chain within the CD3 complex was detected in T cells from peripheral blood or lymphoid organs (34,37) or tumor-infiltrating T cells (35,36), providing a new explanation for reduced T cell responsiveness. Although this possibility may have aspects that need further elucidation (38), these observations accentuated the necessity of reconsidering the mechanisms of tumor-induced immunosuppression.

Cell-mediated immunosuppression may be achieved when a particular lymphoid population interferes with a T cell response as has been proposed previously for the classical suppressor T cell induction (31,32,39) or recently for the Th1–Th2 cross-regulation (40,41). The results obtained here provide a novel model for this line of immunosuppression. Namely, the production of a representative anti-tumor effector cytokine, IFN-γ, by tumor-primed T cells contained in spleen
cells is down-regulated by co-existing B cells, but elimination of these B cells from the splenic population induces an enhancement of IFN-γ production. This enhancement was not due simply to an increase in the numbers of responding T cells and stimulating APC because enhanced lymphokine production was selective to IFN-γ but not to IL-2 and addition of normal B cells to a given number of [T cell + APC] population resulted in down-regulation of IFN-γ production. More directly, enhanced IFN-γ production was demonstrated to be mediated by IL-12 produced at detectable levels only in B cell-depleted cultures. CD40L-induced IL-12 production by APC, which was prevented in the presence of CD40+ B cells, could be restored by elimination of B cells capable of interfering with T cell (CD40L+)–APC (CD40+) interactions. Thus, the present results provided evidence for a new mechanism of immunosuppression that would operate in the tumor-bearing state.

The role of IFN-γ as an anti-tumor effector cytokine has been manifested through investigating the anti-tumor efficacy of IL-12. Administration of rIL-12 into tumor-bearing mice resulted in complete tumor regression or tumor growth inhibition (8–11). The majority of work has indicated that the involvement of T cells is critical: in T cell-deficient mice the anti-tumor activity of IL-12 was marginal (8), and elimination of both CD8+ and CD4+ T cell subsets abrogated IL-12-induced efficacy (9,10). Moreover, studies have shown that the critical biologic property for the IL-12 effects is the ability of this cytokine to induce IFN-γ production in T cells and/or NK cells (9,10). High levels of IFN-γ are detected in tumor-bearing mice following administration of IL-12 (42). Injection of a neutralizing anti-IFN-γ mAb before IL-12 treatment led to almost complete inhibition of the IL-12-induced anti-tumor effect in various mouse tumor models (9,10). Thus, both T cells (regardless of the specific T cell subset) and IFN-γ appear to be critical for the implementation of anti-tumor effects by IL-12.

IL-12 is now known to be produced predominantly by macrophages and dendritic cells (DC) (43) when these are exposed to various pathogens or their products (43). IL-12 production is also induced through interactions of macrophages/DC with activated T cells via CD40–CD40L interactions (16,44,45). The most important aspect of the present study concerns the IL-12 production by splenocytes from tumor-bearing mice and cell-mediated regulation of IL-12 production. The results show that tumor-bearing mice develop the capacity to produce IL-12. However, IL-12 production was not detected or only marginally detected in cultures of unfractionated spleen cells from tumor-bearing mice but could be induced only after depletion of B cells from spleen cells. These results may be understood based on our previous study (16) which demonstrated that B cells regulate CD40L-induced IL-12 production in APC during T–APC interactions. Namely, although stimulation of unfractionated normal spleen cells with anti-CD3 mAb failed to generate IL-12 production, the same cell preparations depleted of B cells produced IL-12 and this IL-12 production was inhibited by anti-CD40L mAb. Thus, the results indicated that the CD40L expressed on anti-CD3-activated T cells induces APC to produce IL-12 through the CD40–CD40L interaction, but this pathway is competitively inhibited by CD40+ B cells incapable of producing IL-12 upon stimulation with CD40L (16). In the present system, it is possible that when tumor-primed T cells are reactivated with APC presenting tumor antigens, they express CD40L and stimulate APC to produce IL-12 depending on the elimination of CD40+ B cells as competitors from a responding population. IL-12 production was found to be responsible for enhanced production of the anti-tumor cytokine, IFN-γ. Thus, the present study demonstrated that B cells down-regulate a series of T cell 1 immune responses involving IL-12 and IFN-γ production.

While IL-12 expression induced enhanced IFN-γ production, IL-2 production was rather suppressed in the same B cell-depleted cultures. Previously, we found that when unfractionated spleen cells from tumor-bearing mice were stimulated in vitro with IL-12, they produced enhanced levels of IFN-γ, whereas the same splenic preparations exhibited reduced levels of IL-2 and tumor necrosis factor production (10). This is consistent with our present observations. Thus, it appears that IL-12 functions in the production of T cell-derived lymphokines in a different manner.

In addition to B cells, Th2-derived cytokines have also been described to regulate IL-12 production (43). The representative of these cytokines is IL-10 (46), and IL-10 inhibits APC to express IL-12 both in T-independent and T-dependent pathways (47), resulting in the suppression of IFN-γ production by T cells (46). Because B lineage cells have the potential to produce IL-10 (48), the involvement of IL-10 in the B cell-mediated down-regulation of IL-12/IFN-γ production should be further investigated. IL-4 and IL-6 do not appear to down-regulate T cell-dependent IL-12 production (47). However, we have observed that serum IL-6 levels increase with the progress of tumor-bearing stages (49) and that IL-6 inhibits the secretion of IFN-γ but not IL-2 by anti-tumor T cells upon stimulation with APC presenting tumor antigens (13). High levels of IL-6 in late stages of tumor-bearing mice (49) may account, in part, for the fact that the restoration of IFN-γ production following B cell depletion is apparently weaker in spleen cells from late stages than from early stages of tumor-bearing mice. Thus, the production of the anti-tumor cytokine, IFN-γ, by anti-tumor T cells appears to be regulated at various levels by a number of components participating in humoral immunity.

Our results illustrate that while an anti-tumor immune response (IFN-γ production) is induced once the tumor is initiated, such a response is down-regulated by B cells. The B cell-mediated down-regulatory mechanism is not restricted to immunoregulation in the tumor-bearing state, as observed for IL-12 production during anti-CD3-triggered T cells and APC (26). Thus, the three major immune cell populations (T cells, APC and B cells) form a network in which stimulatory/inhibitory interactions are induced in each combination of these populations. The tumor-bearing state may be regarded as a representative pathophysiological condition in which such a complicated network comes into operation. For example, it is well known that the number of B cells increases in the tumor-bearing state. This facilitates the B cell-mediated down-regulatory mechanism as shown in this study. Further studies will also be required to investigate whether qualitative changes in the capacity to regulate IFN-γ/IL-12 production are generated along with the progress of tumor-bearing
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stages, including an increase in CD40 expression and soluble CD40 production.

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Abbreviations

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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</table>

References

of mice bearing primary tumors induced by Moloney sarcoma virus. J. Exp. Med. 139:1473.