LEUKOCYTE SUBPOPULATIONS ELICITED BY
A NONTUMORIGENIC VARIANT OF B16 MELANOMA:
THEIR ROLE IN DIRECT REJECTION
OF THE MELANOMA AND IN PREVENTION
OF TUMORIGENESIS IN WINN ASSAYS

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A variant of the B16 melanoma, clone C3471, which is nontumorigenic in immunocompetent mice, is capable of immunizing such mice against the parental, tumorigenic clone B559 (1, 2). Clone B559 is not immunogenic and does not induce concomitant tumor immunity (3). The immunogenic C3471 variant was derived from B559 cells by continuous growth in the thymidine analog, 5-bromodeoxyuridine (BrdUrd). C3471 cells have undergone numerous phenotypic changes, including loss of tumorigenicity, pigmentation and plasminogen activator activity, as well as induction of endogenous retrovirus (1, 4–6). Other investigators have also produced immunogenic alterations of tumor cells by xenogenization with viruses (7–9), haptens (10, 11), mutagens (12–15), and with antineoplastic drugs (16–19).

The inability of C3471 cells to form tumors reflects alteration(s) in interactions of these cells with the host's immune system, rather than an intrinsic loss of tumorigenic potential of C3471 cells (20). Immunocompetent mice mount an inflammatory response to C3471 cells within 24 h after inoculation, and inhibit tumor formation by these cells (2, 21). Mice immunologically compromised by treatment with anti-mouse thymocyte serum (ATS) or X irradiation support tumor formation by C3471 cells.

In the experiments reported here, we have mixed B559 cells with peritoneal cells from C3471 cell-immunized mice and inoculated the mixtures into normal and immunosuppressed mice (Winn-type assay) to analyze leukocyte populations responsible for immunity to B559 melanomas. Our results show that normal mice do not develop tumors when inoculated with a mixture of B559 cells and peritoneal cells.
from C471 cell-immunized mice, and that a high percentage of these mice retain long-lasting immunity to B59 cells. Thy-1.2+ peritoneal lymphocytes, or peritoneal macrophages from C471 cell-immunized mice each protect normal mice against melanoma formation when coinoculated with B59 cells. Thy-1.2+ lymphocytes are required for both the generation and maintenance of tumoricidal macrophages in this system. Significantly, tumoricidal macrophages from C471-immune mice do not protect antithymocyte serum treated recipient mice against B59 melanomas, indicating that recipient thymus-dependent leukocytes are required to effect tumor eradication.

Materials and Methods

Cells. The derivation and maintenance of the melanotic tumorigenic B59 clone and the amelanotic, nontumorigenic C471 subclone of the B16 melanoma have been described previously (6, 22). Briefly, cells were maintained in monolayer culture in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, MD), 50 μg/ml gentamycin (Schering Corp., Kenilworth, NJ), 50 μg/ml aureomycin (Lederle Laboratories, Wayne, NJ), and 2.5 μg/ml fungizone (E. R. Squibb & Sons, Inc., Princeton, NJ). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells of the C471 subclone were grown continuously in the presence of 1 μg/ml BrdUrd added to the maintenance medium. Semiconfluent monolayers were dispensed using 0.0125% trypsin (Worthington Biochemical Corp., Freehold, NJ) with 2.6 mM EDTA. Total cell counts were determined with a Coulter counter and cell viability measured by trypan blue dye exclusion using a hemocytometer. Cells were checked periodically for mycoplasma using the Hoechst stain method (23); no mycoplasma were detected.

Mice. Female C57BL/6 mice, 5-7 wk of age, were obtained from the Mammalian Genetics Animal Production Section, Division of Cancer Treatment, National Cancer Institute at the Charles River Breeding Laboratories, Inc., Wilmington, MA.

Immunization. C471 cells used for immunizing mice were dispersed with trypsin, washed with serum-free MEM, and resuspended at 5 × 10⁶ viable cells/ml MEM for inoculation. All mice used as donors of immune leukocytes for Winn assays were immunized by three intraperitoneal inoculations of 10⁶ viable cells at weekly intervals.

To determine the effect of immunization route on the capacity of the animal to reject a tumor challenge, a similar immunization schedule was followed using subcutaneous injection of C471 cells into the flank, followed by inoculation of 2 × 10⁶ viable Bs59 cells (the minimal number required to yield tumors in 100% of mice injected) 7 d after the last immunization, either intraperitoneally or subcutaneously, into the opposite flank. Mice receiving subcutaneous challenge with Bs59 cells were monitored for tumor development by examination of the inoculation site for palpable melanoma nodules. Tumor development in the peritoneal cavity was monitored initially by swelling of the abdomen, and confirmed by dissection 40 d after challenge. Intraperitoneal Bs59 tumors are easily visualized macroscopically as pigmented masses or extensive “peppering” of the mesentery.

Irradiation. Mice were exposed to 650 rad from a 2,400 Ci caesium source gamma cell (located at Memorial Sloan-Kettering Cancer Center, New York), and were maintained in sterile bedding and provided with sterile drinking water acidified to pH 2.0 with 1 N HCl.

In Vivo Depletion of T Lymphocytes. Naive or C471 cell-immunized mice were inoculated intraperitoneally with 0.2 ml rabbit ATS (M. A. Bioproducts) on days 6, 5, 3, 2, and 1 before use in an experiment. On day 0, mice were either inoculated with 2 × 10⁶ Bs59 tumor cells, inoculated with mixtures of peritoneal cells and Bs59 cells for Winn assays, or killed for harvest of peritoneal exudate cells (PEC). Wright-Giemsa staining of cytocentrifuged PEC from ATS-treated C471-immunized mice revealed 65% macrophages, 2% lymphocytes, 16% neutrophils, 17% eosinophils, and 0.5% mast cells.

PEC. PEC were harvested by lavage of the peritoneal cavities of naive or C471 cell-immunized mice with balanced salt solution containing 1 mM EDTA and 1 mM Hepes.
according to a modification of the procedures of Cohn and Benson (24) as described by Michl et al. (25). Immune PEC were collected 7 d after the last inoculation of C3471 cells. Lysis of contaminating erythrocytes was done routinely using hypotonic salt solutions. The leukocytes were then centrifuged (600 g/12 min/4°C) and resuspended in MEM without serum. Cells were enumerated using a hemocytometer and viability determined by trypan blue dye exclusion. Wright-Giemsa staining of cytocentrifuged PEC from C3471 cell-immunized mice revealed 82% mononuclear leukocytes (macrophages and lymphocytes), 6% eosinophils, 3% neutrophils, and 3% mast cells.

**Fractionation of PEC Subpopulations**

**GLASS ADHERENT AND NONADHERENT.** Subpopulations of C3471-immune PEC were separated on the basis of adherence to glass. Glass-adherent leukocytes were separated from nonadherent cells by incubation of 3–4 × 10^6 PEC in 100-mm sterile glass petri dishes with MEM supplemented with 15–20% FBS for 2–2.5 h at 37°C in a humidified atmosphere containing 5% CO₂. Approximately 95% of PEC that adhered to glass in a 6-h incubation adhered in the first 2.5 h after plating. An average of 47% ± 5% C3471-immune PEC are glass adherent within 2.5 h. Nonadherent PEC were transferred to tubes on ice. The adherent PEC monolayers were washed three times each with 5 ml of MEM at 25°C. Adherent PEC were washed twice more with ice-cold PBS containing 1 mM EDTA and 1 mM Hepes (Sigma Chemical Co., St. Louis, MO). After discarding these fluids, adherent cell monolayers were incubated with cold PBS containing EDTA-Hepes for 5–10 min at 4°C. Adherent PEC were then gently scraped using a sterile rubber policeman and transferred to tubes on ice. Both adherent and nonadherent PEC were pelleted (600 g/12 min/4°C) and resuspended in MEM without serum for counting and viability determinations as above. Wright-Giemsa staining of cytocentrifuged preparations of glass-adherent immune PEC revealed 97% mononuclear leukocytes, 1% mast cells, and 1% neutrophils. Over 95% of these mononuclear leukocytes were macrophages as measured by their capacity to bind and/or ingest glutaraldehyde-fixed erythrocytes coated with IgG (Table I). Similar staining of leukocytes in the fraction not adherent to glass revealed 90% mononuclear leukocytes, 3% mast cells, and 5% eosinophils. Less than 7% of these leukocytes ingested any IgG-coated erythrocytes.

**FRACTIONATION ON NYLON WOOL.** Nonadherent PEC were further purified for enrichment of T lymphocytes by fractionation on nylon wool columns based on the method of Julius et al. (26). Nylon wool from Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Deerfield, IL) was soaked in 0.2 N HCl, boiled in distilled water, and autoclaved. Before addition of leukocytes, the columns were washed and preincubated with PBS containing 5% heat-inactivated FBS prewarmed at 37°C. After addition of 0.5–1.5 × 10^8 viable glass-nonadherent PEC suspended in prewarmed PBS + 5% FBS, columns were incubated sealed for 1 h at 37°C. PEC not adhering to the nylon wool were eluted dropwise with at least two column volumes of warm PBS + 5% FBS into centrifuge tubes, and cell number and viabilities were determined. Wright-Giemsa staining of cytocentrifuged nylon wool eluate cells revealed a virtually pure (98%) population of lymphocytes.

**Activation of Adherent PEC In Vitro.** PEC harvested from naive C57BL/6 mice or from ATS-treated C3471-immunized mice were plated on glass petri dishes to separate glass adherent and nonadherent fractions as above. Adherent cell monolayers were washed to remove nonadherent leukocytes, and PEC in the glass-nonadherent or nylon wool-purified fractions from either unimmunized normal C57BL/6 mice or from C3471-immunized C57BL/6 mice were incubated

| Ingested or bound particle | Phagocytic index* | Percent control | Binding index* | Percent control |
|---------------------------|-------------------|-----------------|---------------|----------------|
| Resident                  | IgG-GRBC          | 442.5 (92)†‡    | 100           | 937.2 (96)†‡   | 100            |
| C3471-immune             | IgG-GRBC          | 738.7 (95)      | 165           | 1324.8 (96)‡   | 141            |

* Phagocytic and binding indices were determined as described previously (3).
†‡ Percent macrophages binding or ingesting IgG-GRBC.
with the glass-adherent cells for ~18 h in MEM containing 10% FBS. At the end of incubation, nonadherent PEC were removed and adherent cells were washed with MEM, PBS, and then PBS containing 1 mM EDTA and 1 mM Hepes, to minimize contamination with nonadherent leukocytes. PEC still adherent to the glass petri dishes were then harvested by scraping, as above, counted and coinjected with B559 cells in Winn assays.

Antiserum. Immune lysis of T lymphocytes from unfractionated and fractionated PEC was carried out using either rabbit ATS having a cytotoxic titer of 1:3,200, or monoclonal mouse anti-Thy-1.2 (gift of Dr. U. Hammerling, Memorial Sloan-Kettering Cancer Center) having a titer of 1:10,000. 4-6-wk-old rabbit complement obtained from Pel-Freez Biological, Inc. (Rogers, AR) was used at a 1:4 dilution to effect lysis. PEC pellets containing 0.25–2.5 × 10^7 viable cells were resuspended in a total volume of no more than 200 μl of either rabbit ATS or ant-Thy-1.2 in PBS (with Ca++ and Mg++), incubated on ice for 30–40 min, washed in PBS, resuspended in 200 μl PBS containing rabbit complement at 1:4 dilution, and incubated for 20 min at 37°C in a CO_2 incubator. Cell viability was estimated by trypan blue dye exclusion.

Tumor Neutralization Assay. A modification of the adoptive tumor neutralization assay described by Winn (27) was used to evaluate the effectiveness of immune peritoneal cell populations in preventing tumor formation by B559 melanoma cells. Unfractionated PEC, harvested from naive or immune mice as described above, were washed in PBS and resuspended in MEM without serum. Subpopulations of immune PEC were similarly prepared after fractionation. PEC were mixed with freshly harvested B559 cells to give the appropriate PC/B559 cell ratio in a final volume of 0.2 ml. This cell mixture was inoculated subcutaneously into the flank of C57BL/6 mice and animals were checked two to three times weekly for appearance of palpable tumor nodules. Tumor formation is reported for all animals used in each experiment.

In Vitro Cytotoxicity Assay. A colony assay (V. H. Freedman, G. Kaplan, C. S. Copeland, and S. C. Silverstein, manuscript in preparation) was used to measure cytotoxicity. Briefly, tumor cells were plated in 16-mm Costar wells (Costar, Data Packaging, Cambridge, MA), and overlaid with PEC from immunized mice. After 24 h incubation, the tumor cells were trypsinized, diluted, and plated in 60-mm petri dishes. Colonies were counted after 7 d. The percent B559 cells killed was calculated as number of colonies in experimental sample divided by the number of colonies in control sample times 100.

Results

Effect of Immunization Route on Rejection of B559 Melanoma. We compared the efficacy of subcutaneous vs. intraperitoneal immunization with C3471 cells in protecting C57BL/6 mice against challenge with various numbers of malignant B559 melanoma cells. Fig. 1 shows that at all tumor cell doses tested, mice immunized intraperitoneally were more resistant to intraperitoneal challenge with B559 cells than were mice immunized subcutaneously. Subcutaneous immunization provided good protection against subcutaneous or intraperitoneal challenge only at the lowest tumorigenic dose (2 × 10^6 B559 cells). B559 tumors developed in 100% of unimmunized animals. As will become evident, the peritoneal cavities of mice immunized with C3471 cells contain tumoricidal leukocytes. Fig. 1 shows that repeated intraperitoneal immunization with C3471 cells was the most efficient method of eliciting these leukocytes. Therefore, all subsequent studies were performed using peritoneal leukocytes elicited by intraperitoneal immunization.

Effects of Immunosuppression on Rejection of B559 Melanoma by C3471 Cell-immunized Mice. C57BL/6 mice treated with ATS before inoculation with C3471 cells develop progressive amelanotic C3471 tumors (20, 21, 28). To determine whether ATS alters established immunity to the malignant parental melanoma (clone B559), mice immunized with C3471 cells were treated with ATS before subcutaneous challenge with B559 cells. All of these mice developed melanotic tumors. In contrast, all of the immunized control mice that received normal rabbit serum (NRS) were protected
Fig. 1. Effect of immunization route on rejection of subcutaneous vs. intraperitoneal challenge with B59 melanoma cells. C57BL/6 mice were immunized by either three subcutaneous (s.c.) or intraperitoneal (i.p.) inoculations of 10⁶ C471 cells, each 7 d apart. Immunized mice were challenged by either subcutaneous or intraperitoneal injection of the indicated numbers of B59 melanoma cells, 7 d after the last C471 cell inoculation. □, Unimmunized C57BL/6 control mice challenged either i.p. or s.c.; □, s.c. immunized mice challenged i.p.; ■, i.p. immunized mice challenged i.p.; □, s.c. immunized mice challenged s.c.; □, i.p. immunized mice challenge s.c. Mice were monitored for tumor formation as described in Materials and Methods. Data represent averages from four experiments.

**TABLE II**

Lack of Protection Against B59 Melanoma in ATS-treated* C471-immunized Mice

| Mice injected subcutaneously with 2 × 10⁵ B59 cells | Mice with tumors/mice inoculated | MLP ± SD |
|-------------------------------------------------|---------------------------------|---------|
| C471-immunized                                  | 0/8                             | —       |
| ATS-treated, C471-immunized                     | 12/12                           | 9.9 ± 2.1 |
| NRS-treated, C471-immunized                     | 0/4                             | —       |
| Unimmunized controls                            | 12/12                           | 12.9 ± 7.6 |

* Rabbit ATS or normal rabbit serum injected intraperitoneally into C57BL/6 mice immunized with C471 cells (Materials and Methods).

against tumor formation (Table II). These results indicate that established immunity can be abrogated by ATS treatment; they suggest that ATS-sensitive leukocytes are involved in both the afferent and efferent pathways of C471 cell-elicited host defense.

To evaluate the radiosensitivity of the cells that mediate B59 tumor cell rejection, C57BL/6 mice were immunized with C471 cells and exposed to 650 rad of X irradiation 24 h before inoculation with B59 cells. These mice were unable to reject the B59 cells (data not shown), indicating that radiosensitive effector cell(s) are required for destruction of B59 cells in C471 cell-immune mice.

**Characterization of the Leukocytes Elicited by C471 Cells.** To confirm that leukocytes from C471 cell-immunized mice are responsible for the rejection of B59 cells, we mixed varying numbers of C471-immune peritoneal leukocytes with 2 × 10⁵ B59 cells and inoculated them subcutaneously into naive C57BL/6 mice. C471 cell-immune PEC markedly inhibited tumorigenesis by B59 cells, completely abrogating tumor formation at PEC/melanoma cell ratios of 30:1 or greater, and inhibiting tumor formation in 90% of mice inoculated with a 25:1 ratio. The latter is therefore the ratio of PEC/melanoma cells used for all subsequent Winn assays. In those few
animals that did develop tumors, the mean latent period (MLP) for development of palpable subcutaneous nodules increased progressively as the ratio of PEC/melanoma cells decreased (Fig. 2). As expected, at all ratios tested (10:1 to 40:1), PEC from unimmunized C57BL/6 mice had no inhibitory effect on tumor formation by B59 cells (data not shown).

To identify the kinds of leukocytes responsible for the tumoricidal effects of C471-immune PEC, we carried out tumor neutralization assays using glass-adherent (macrophage-enriched) and glass-nonadherent (lymphocyte-enriched) subpopulations. $5 \times 10^6$ leukocytes from each subpopulation were as effective in protecting mice against B59 melanoma as unfractionated immune PEC (Table III). For those few mice that developed B59 tumors, the MLP was nearly doubled. Similar protection was obtained when mice were inoculated with a mixture of $2.5 \times 10^6$ each of glass-

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**Figure 2.** Inhibition of B59 tumor formation by varying numbers of C471-immune PEC. Peritoneal leukocytes, from mice immunized by three successive weekly intraperitoneal inoculations of $10^6$ viable C471 cells, were co-injected subcutaneously, in varying ratios with $2 \times 10^5$ viable B59 melanoma cells into immunocompetent naive C57BL/6 mice. Mice were monitored for B59 tumor formation by palpation of injection sites two to three times weekly. Data represent averages from seven experiments.

**Table III**

PEC from C471 Cell-immunized Mice Inhibit Tumor Formation by B59 Cells

| C471-immune PEC* | Mice with tumors/ mouse inoculated | MLP ± SD |
|------------------|-----------------------------------|----------|
| Unfractionated   | 5/26                              | 20.6 ± 15.5 |
| Glass adherent   | 4/30                              | 21.0 ± 9.9  |
| Glass nonadherent| 6/33                              | 21.3 ± 6.1  |
| Glass adherent + glass nonadherent‡ | 5/18 | 18.0 ± 9.8  |
| Controls§        |                                   |          |
| Unfractionated normal C57BL/6 | 29/32 | 13.7 ± 8.4  |
| PEC + B59 cells  | 11/12                             | 11.6 ± 4.2  |

* PEC from C471-immunized mice inoculated in a mixture with B59 melanoma cells at a 25:1 ratio using $2 \times 10^6$ viable melanoma cells.
‡ Glass adherent and nonadherent immune PEC mixed together at a 1:1 ratio after separation on glass as described in Materials and Methods.
§ $2 \times 10^6$ B59 cells inoculated either alone or together with $5 \times 10^6$ PEC from naive mice.
adherent and nonadherent fractions together with B59 cells (Table III). These findings differ from those we reported previously using glass-adherent and nonadherent fractions of PEC elicited by immunization with heat-killed bacillus Calmette-Guerin (BCG) (3). In those experiments, only the glass-adherent leukocytes were able to confer protection against B59 tumor formation.

To characterize the cells in the nonadherent PEC population that were responsible for rejection of B59 melanomas, nonadherent PEC from C471 immune mice were treated with anti-thymocyte serum or anti-Thy-1.2 and complement and then co-injected with B59 cells into naive mice. This treatment completely abrogated the capacity of immune nonadherent PEC to protect mice against B59 melanoma (Table IV). To confirm that the T cells alone were capable of inhibiting melanoma formation, immune T cells were purified by passage of C471-immune nonadherent PEC over a nylon wool column. Mice inoculated with a mixture of these T cells and B59 melanoma cells were protected against B59 melanomas; this protection was abolished by treatment of the immune T cells with anti-thymocyte serum or anti-Thy-1.2 and complement (Table IV). In contrast, treatment of C471 immune glass-adherent PEC with ATS or anti-Thy-1.2 and complement had no effect on the capacity of these leukocytes to inhibit B59 tumorogenesis (Table IV). These results indicate macrophages and thymus-derived lymphocytes are responsible for the in vivo tumor inhibitory effects of the corresponding glass-adherent and nonadherent C471 cell-immune PEC fractions; and that macrophages from C471 immunized mice retain their tumoricidal activity in the absence of immune donor T cells.

**Participation of Recipient ATS-sensitive Leukocytes in Rejection of B59 Melanoma Cells.** ATS-sensitive lymphocytes can be removed from either unfractionated or glass-adherent immune PEC without impairing their capacity to cause rejection of B59 cells in Winn assays in normal mice (Table IV). Therefore, both unseparated and adherent PEC should yield a high degree of protection if recipient T cells do not

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**Table IV**

Effects of In Vitro Treatment of C471-immune PEC With Anti-Thy-1.2 or Rabbit ATS on Their Tumor Inhibitory Properties

| C471-immune PEC subpopulation* | Treatment                                                                 | Percent lysis | Mice with tumor/ mice inoculated¶ |
|--------------------------------|--------------------------------------------------------------------------|---------------|----------------------------------|
|                                 | C’ Alone‡                                                                | 1.6 (0/6)     | 18.8 (1/6)                       |
|                                 | Anti-Thy-1.2§ + C’                                                      | 0.9 (0/4)     | 3.5 (0/5)                        |
|                                 | ATS¶ + C’                                                               | 1.1 (0/4)     | 47.1 (6/6)                       |
| Unfractionated                  |                                                                          | 0.9 (1/21)    | 94.5 (8/8)                       |
| Glass adherent                  |                                                                          |               |                                  |
| Glass nonadherent               |                                                                          |               |                                  |
| Nylon wool nonadherent          |                                                                          |               |                                  |

* PEC were obtained and fractionated as described in Materials and Methods. The nylon wool-nonadherent cells were obtained by passage of glass-nonadherent cells through nylon wool columns as described in Materials and Methods.

† Rabbit complement diluted 1:4

§ Monoclonal mouse anti-Thy-1.2 diluted 1:200.

¶ Rabbit anti-mouse thymocyte serum diluted 1:100.

‡ PEC inoculated with B59 melanoma cells at 25:1 ratio using 2 × 10^5 viable melanoma cells and 5 × 10^6 PEC.
participate in the rejection of B559 cells in Winn assays. To examine this question, naïve mice were treated with ATS before inoculation with C471 cell-immune PEC and B559 cells. ATS treatment was suspended 24 h before leukocyte transfer to minimize effects of circulating anti-T cell antibody on immune donor PEC. As expected, the glass-nonadherent fraction was unable to protect against tumor formation in the ATS-treated recipients. Surprisingly, however, neither unfractionated C471-immune PEC (containing macrophages and T cells), nor the glass-adherent subfraction (comprised primarily of macrophages), was able to convey protection against B559 melanoma upon adoptive transfer into ATS-treated syngeneic mice (Table V).

The inability of the macrophage-enriched fraction to protect ATS-treated recipients against B559 melanoma suggested that recipient T cells participate in tumor neutralization by C471-immune macrophages. This hypothesis is tenable only if the ATS used in these experiments did not inhibit macrophage recruitment, viability, or tumoricidal effector functions. Two lines of evidence show that it did not. First, mice immunized intraperitoneally with C471 cells for 3 successive wk were treated with ATS as described in Materials and Methods. Recovery of total PEC from these mice was invariably nearly twice that from untreated immune mice (7.6 × 10^6 vs. 4.5 × 10^6), and Wright-Giemsa-stained cytocentrifuge preparations of these PEC contained 65% macrophages, 2% lymphocytes, 16% neutrophils, and 17% eosinophils. PEC from ATS-treated immune mice contained more macrophages than PEC from immune mice (4.9 × 10^6 vs. 2.25 × 10^6). Second, ATS in the presence (Table IV) or absence of complement (Tables VI and VII) did not inhibit the tumoricidal activity of immune macrophages in vitro (Table VI) or in vivo (Tables IV and VII). That the concentration of ATS used exceeded that in the serum of ATS pretreated mice at the time of Winn assay was determined as follows: ATS at a dilution of 1:100 lysed 95% of C471-immune nylon wool-purified T cells (Table IV), whereas undiluted serum from mice treated with ATS (as in Table V) plus complement lysed only 30-37% of these T cells (data not shown). No detectable lysis of C471-immune macrophages was observed when these cells were incubated with undiluted serum from ATS treated

### Table V

**Treatment of Recipient Mice with ATS Blocks the Capacity of C471-Immune PEC to Inhibit B559 Melanoma Formation in Winn Assays**

| C471-immune subpopulation* | Mice with tumors/mice inoculated | MLP ± SD | Mice with tumors/mice inoculated | MLP ± SD |
|-----------------------------|---------------------------------|---------|---------------------------------|---------|
| Unfractionated              | 0/11                            | —       | 14/14                           | 10.4 ± 2.0 |
| Glass adherent              | 1/6                             | 19.0 ± 0| 7/7                             | 14.5 ± 1.7 |
| Glass nonadherent           | 1/7                             | 26.0 ± 0| 7/7                             | 15.7 ± 3.8 |
| B559 Controls               | 11/11                           | 9.4 ± 2.4| 12/12                           | 8.3 ± 3.1 |

* As in Table IV.

† Normal C57BL/6 mice.

§ Normal C57BL/6 mice pretreated with rabbit ATS. These mice received five 0.2-cc inoculations of ATS intraperitoneally on days −6, −5, −4, −2, and −1 before subcutaneous inoculation of immune PEC with melanoma cells for Winn assay.
**Table VI**

*ATS Does Not Inhibit Killing of B559 Melanoma Cells by C471-immune Adherent PEC In Vitro*

| Number of adherent PEC*/well | ATS‡ | Percent B559 cells viable§ |
|-----------------------------|------|---------------------------|
| 5 × 10⁶                      | −    | 0.01%                     |
| 5 × 10⁶                      | +    | 0.01%                     |
| 2 × 10⁶                      | −    | 0.5%                      |
| 2 × 10⁶                      | +    | 0.5%                      |
| −                           | −    | 100%                      |
| −                           | +    | 100%                      |

* Glass-adherent PEC from C471-immunized mice harvested as described in Materials and Methods.
‡ ATS at a 1:100 concentration in MEM with 10% FBS.
§ Percentage of B559 melanoma cells killed is based upon the number of colony-forming units of B559 cells remaining after 24 h incubation at 37°C of a mixture of 1 × 10⁵ B559 cells and the indicated number of C471-immune PEC. B559 cells were harvested and plated; colonies were enumerated as described in Materials and Methods. 100% represents the average number of B559 colonies recovered from control wells.

**Table VII**

*Co-Injection of ATS With C471-immune Macrophages Does Not Inhibit Their Tumoricidal Activity*

| Mice with tumors/mice inoculated | MLP ± SD |
|----------------------------------|----------|
| C471-immune adherent PEC*       | 0/4      |
| C471-immune adherent PEC + ATS‡ | 0/6      |
| B559 controls                    | 5/6      |

* Each mouse was inoculated subcutaneously with 0.5 ml MEM containing 5 × 10⁶ viable glass-adherent PEC from C471-immunized mice and 2 × 10⁵ B559 melanoma cells.
‡ Each mouse was inoculated subcutaneously with 0.2 ml MEM containing 5 × 10⁶ viable glass-adherent PEC from C471-immunized mice, 2 × 10⁵ B559 melanoma cells, and 2 μl ATS. The final ATS concentration was 1:100.

**Table VIII**

*Effect of ATS Treatment In Vivo on Tumoricidal Activity of PEC of C471-Immunized Mice*

| C471-immune subpopulation       | Mice with tumors/mice inoculated |
|----------------------------------|----------------------------------|
| From untreated immune donors‡    | From ATS-treated immune donors§  | From NRS-treated immune donors||
| Unfractionated                   | 2/29                             | 29/30                          | 0/5                             |
| Glass adherent                   | 0/10                             | 14/16                          | 0/8                             |
| Glass nonadherent                | 0/12                             | 11/12                          | 0/8                             |
| B559 Controls§                   | 35/36                             |

* 5 × 10⁶ PEC harvested from C471-immune mice, prepared as described in Materials and Methods, were inoculated together with 2 × 10⁵ B559 cells into naive C57BL/6 mice.
‡ PEC from untreated C471-immunized mice as in Table IV.
§ PEC from C471-immunized mice as above harvested 24 h after the last inoculation of rabbit ATS. Mice were treated with ATS for 3 d (days −6, −5, and −4) after their last immunization with C471 cells, and again on the 2 d (days −2 and −1) before harvest of their PEC as described in Materials and Methods.
|| Mice were treated with NRS following the schedule described above ‡.
§ 2 × 10⁵ B559 cells inoculated subcutaneously into naive C57BL/6 mice.
**Table IX**

**Activation of Macrophage Tumoricidal Activity by C471-Immune Lymphocytes**

| Source of glass-adherent PEC used in Winn Assays* | Cells incubated with glass adherent PEC | Mice with tumors/mice inoculated | MLP ± SD |
|--------------------------------------------------|----------------------------------------|----------------------------------|---------|
| ATS-treated, C471-immunized                      | Glass nonadherent from normal C57BL/6 mice | 7/7                              | 11.4 ± 3.5 |
|                                                  | Nylon wool eluate from normal C57BL/6 mice | 5/5                              | 18.3 ± 7.9 |
|                                                  | Glass nonadherent from C471-immunized mice | 2/21                             | 17.0 ± 14  |
|                                                  | Nylon wool eluate from C471-immunized mice | 1/5                              | 26.0      |
| Normal C57BL/6 mice                              | Glass nonadherent from normal C57BL/6 mice | 3/3                              | 22.3 ± 9.8 |
|                                                  | Nylon wool eluate from normal C57BL/6 mice | 4/4                              | 15.8 ± 5.3 |
|                                                  | Glass nonadherent from C471-immunized mice | 0/5                              | --        |
|                                                  | Nylon wool eluate from C471-immunized mice | 1/4                              | 18.0      |
| B59 cells                                        | None                                   | 13/14                            | 12.6 ± 4.1 |

* Glass-adherent PEC were harvested from either naïve, untreated C57BL/6 mice or from C471-immunized mice injected intraperitoneally with rabbit ATS after the last inoculation of C471 cells and before harvest of PEC as in Table VIII and as described in Materials and Methods. The nonadherent cells were incubated with the adherent PEC for 24 h and then removed by washing. The adherent cells were removed from the monolayer, mixed with B59 cells, and inoculated into naïve mice at a ratio of 5 × 10⁶ viable adherent cells: 2 × 10⁶ B59 cells as described in Materials and Methods.

**ATS-sensitive T Lymphocytes are Required for the Maintenance of Tumoricidal Macrophages in C471 Cell-immune Mice.** To determine whether T cell functions are required for the maintenance of macrophage cytotoxic effector functions in vivo C471 cell-immunized mice were treated with ATS after their last immunization with C471 cells. Their PEC were then harvested and co-inoculated together with B59 cells into naïve C57BL/6 mice. ATS treatment of the immunized donor mice resulted in abrogation of the tumor protective capacity of their PEC; both adherent and nonadherent PEC were inactive. Normal rabbit serum had no effect (Table VIII). These results suggested that the inhibitory action of ATS on the maintenance of macrophage tumoricidal activity was an indirect one, a consequence of ATS-mediated destruction of T lymphocytes. If so, then addition of C471-immune lymphocytes to these macrophages should restore their tumoricidal activity. To test this hypothesis, glass-adherent PEC harvested from ATS treated immune mice, were washed to remove nonadherent leukocytes, and incubated for 18 h with nonadherent or nylon wool-purified leukocytes from C471-immunized donors or from nonimmunized C57BL/6 mice. Nonadherent PEC were removed by extensive washing. 92% of the glass-adherent cells ingested IgG-coated glutaraldehyde-fixed sheep erythrocytes, showing that the washing procedure was sufficient to remove virtually all added immune lymphocytes. The macrophages were harvested and injected together with
B59 melanoma cells into naive C57BL/6 mice. Macrophages incubated with either glass nonadherent or nylon wool-purified leukocytes from immunocompetent C3471-immune mice, conferred a high degree of protection against B59 tumor formation to naive Winn assay recipients (Table IX). Macrophages incubated with either nonadherent or nylon wool-purified cells from unimmunized C57BL/6 mice provided no protection against tumor formation (Table IX). These results confirm that T lymphocytes from C3471-immune mice are essential for the generation and maintenance of the tumor inhibitory properties of macrophages.

To determine whether lymphocytes from C3471 cell-immune animals could stimulate macrophage tumoricidal activity in the absence of tumor antigens in the adherent cell population, macrophages from normal, nonimmunized C57BL/6 mice were incubated with glass nonadherent or nylon wool eluate PEC from C3471-immunized mice. At the end of the incubation, the nonadherent cells were removed by extensive washing. (Over 90% of the remaining adherent leukocytes were macrophages as measured by their ingestion of IgG-coated glutaraldehyde-fixed erythrocytes [IgG-GRBC].) The adherent leukocytes were detached from the plates and coinjected with B59 melanoma cells into naive mice. These adherent cells prevented tumor formation in eight of nine recipients (Table IX). All animals receiving macrophages that had been incubated with glass nonadherent or nylon wool eluate cells from unimmunized mice developed lethal melanomas (Table IX).

**Acquisition of Tumor Immunity by Winn Assay Recipients.** B59 melanoma cells are not immunogenic. B59 tumor formation is not prevented by inoculation of mitomycin-treated or radiation-killed B59 cells (1, 29), or by repeated inoculation of crude membranes prepared from cultured B59 cells (29) into syngeneic mice. Also, animals bearing B59 tumors do not exhibit concomitant tumor immunity (3). Efforts to transfer immunity against B59 melanoma to naive syngeneic mice with C3471-immune leukocytes have been unsuccessful. Neither 10^7 peritoneal nor 10^7 spleen cells from C3471-immunized mice conferred any protection against B59 tumor formation even when administered on multiple occasions, subcutaneously, or intravenously to normal or sublethally irradiated naive C57BL/6 mice. In these experiments 27 of 28 normal mice and 22 of 23 irradiated mice receiving C3471-immune leukocytes developed lethal melanomas when challenged with 2 × 10^5 B59 cells. However, when animals that had received 5 × 10^6 unfractionated C3471-immune PEC together with the melanoma cells in a Winn assay were tested by a second injection of B59 cells 40–60 d later, 12 of 21 animals remained free of tumors 40 d after this second challenge. Thus, a significant degree of systemic immunity to B59 melanoma can be adoptively transferred by immune peritoneal leukocytes in the presence of tumor cells.

**Discussion**

We have examined the mechanism of rejection of clone B59 of B16 melanoma by syngeneic C57BL/6 mice immunized with an immunogenic variant (clone C3471) of the parental melanoma. Immunization with C3471 cells results in systemic immunity to B59 melanoma (1, 2). The protective effect of immunization is most pronounced when immunization and subsequent challenge are within the same tissue compartment (the peritoneal cavity); nonetheless, significant systemic resistance to tumor challenge is observed when the sites of immunization and/or tumor challenge lie outside the peritoneum (Fig. 1). Treatment of naive mice with ATS before C3471
inoculation permits the development of C3471 tumors (20), and established immunity to B559 cells can be abrogated by ATS treatment (Tables II and V) or X irradiation of the immune mice. These findings indicate that intact T cell function is required for the establishment and maintenance of immunity to this melanoma. This conclusion was substantiated when leukocyte subpopulations from immune mice were coinjected with B559 cells into naïve or ATS-treated recipients. Macrophage or T cell enriched populations prevented tumor growth in naïve mice but not in ATS-treated mice (Table V).

There are two distinct pathways via which immune T cells might act in this system. T cells might function as cytotoxic effector cells or as amplifier and helper cells. We do not know whether C3471-immune T cells are themselves cytotoxic. Further work is needed to resolve this issue. However, our data do indicate that these T cells act as amplifiers of the immune reaction, promoting the activation of mononuclear phagocytes that are tumoricidal in vivo (Table IX). A requirement for cooperation between mononuclear phagocytes and immune T cells is further emphasized by the inability of macrophages from C3471-immunized mice to prevent tumors in ATS-treated Winn assay recipients (Table V).

Previous work with macrophages elicited by BCG immunization showed that coinjection of BCG-activated macrophages together with B559 cells inhibited melanoma formation in normal, X-irradiated and nude mice (3). Thus it was surprising to find that demonstrably tumoricidal macrophages from C3471-immune mice did not inhibit B559 melanoma formation in mice treated with ATS (Table V). Careful examination of the effects of ATS showed that this serum did not inhibit macrophage recruitment in vivo, or macrophage viability or tumoricidal activity in vitro (Tables IV, VI, and VII). These findings indicate that the inhibitory effect of ATS on macrophage tumoricidal activity in vivo is not due to a direct action of this serum on the macrophages. We conclude that T cell functions are required for the full expression of macrophage tumoricidal activity in vivo.

In addition to a requirement for T cells and macrophages, other cell types may be involved in the rejection of B559 cells by C3471 cell-immunized mice. Beige mice immunized with C3471 cells fail to reject B559 melanoma (38), suggesting that natural killer and/or other leukocyte subpopulations that are defective in beige mice (39-41) may be required.

Approximately half of the animals that reject B559 cells after co-injection of these melanoma cells with immune PEC developed long lasting immunity to B559 cells as measured by their ability to reject a challenge with the tumorigenic melanoma cells. In contrast, as we reported previously (3), mice that did not develop tumors after co-injection of BCG-immune PEC with B559 cells remained completely susceptible to subsequent challenge with B559 cells. Thus, the ability of the mice that received C3471-immune peritoneal leukocytes and B559 melanoma cells to resist a second challenge 40–60 d later appears to be due to stimulatory effects on the host immune system of adoptively transferred immune leucocytes in the presence of antigen (B559 cells).

The BrdUrd-induced changes responsible for the immunizing properties of C3471 cells have not been defined. However, virus production by C3471 cells seems to play a key role. C57BL/6 mice immunized with nontumorigenic feral mouse cells chronically infected with C3471 cell-derived retrovirus are protected against challenge with
B559 melanoma cells (38).

In summary, three novel findings emerge from this work. First, that an intact T lymphocyte system is needed to elicit tumoricidal macrophages by immunization with the nontumorigenic BrdUrd-grown melanoma variant. Second, that the tumoricidal macrophages elicited are unable to mediate rejection of B559 melanoma cells in a Winn assay in the absence of intact recipient T lymphocyte function, further emphasizing a requirement for cooperation of T cells with other leukocyte populations. Third, that a significant proportion of naïve C57BL/6 mice can be immunized against B559 melanoma when these tumor cells are co-injected with peritoneal cells from C471 cell-immune mice. This last finding suggests one approach to immunization against nonimmunogenic syngeneic tumors.

Summary

The mechanisms by which various leukocyte subpopulations elicited by an immunogenic, nontumorigenic subclone (C471) of B16 melanoma caused rejection of the tumorigenic parental melanoma (B559), were investigated. Leukocytes from C471-immune mice were co-injected with B559 tumor cells in Winn assays into normal syngeneic recipients. Tumor formation by B559 cells was prevented when C471-immune (a) unfractionated peritoneal leukocytes, or (b) glass-adherent peritoneal cells (90% macrophages), or (c) nylon wool purified nonadherent cells (95% Thy-1.2+) were used in the Winn assays. If the C471-immunized mice were treated with antithymocyte serum before harvest of their peritoneal cells, none of these leukocyte populations were effective in the Winn assay. However, macrophages from these immunologically compromised donors regained their tumoricidal activity after incubation in vitro with T lymphocytes from untreated C471-immune donors; similarly, C471-immune lymphocytes rendered normal resident peritoneal macrophages tumoricidal in Winn assays. When C471-immunized mice were irradiated or treated with antithymocyte serum before direct challenge with B559 cells, melanomas developed, thus providing additional evidence for the need for intact T cell function to establish immunity against the melanoma. Furthermore, when Winn assay recipients were treated with antithymocyte serum, neither C471-immune macrophages nor T cells were able to prevent tumor formation. These findings indicate that antithymocyte serum-sensitive (Thy-1.2+) lymphocytes are necessary both for the generation of tumoricidal leukocytes in C471-immunized mice, and for the rejection of B559 melanoma by demonstrably tumoricidal macrophages in Winn assay recipients. In addition, long-lasting immunity developed in 50% of the normal mice that had received both C471-immune peritoneal cells and B559 tumor cells, as manifested by their capacity to reject a second challenge with B559 cells 40–60 d later.

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