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Identification of Tumor-Infiltrating Macrophages as the Killers of Tumor Cells After Immunization in a Rat Model System[1]

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Immunization can prevent tumor growth, but the effector cells directly responsible for tumor cell killing in immunized hosts remain undetermined. The present study compares tumor grafts that progress in naive syngeneic rats with the same grafts that completely regress in hosts preimmunized with an immunogenic cell variant. The progressive tumors contain only a few macrophages that remain at the periphery of the tumor without direct contact with the cancer cells. These macrophages do not kill tumor cells in vitro. In contrast, tumors grafted in immunized hosts and examined at the beginning of tumor regression show a dramatic infiltration with mature macrophages, many of them in direct contact with the cancer cells. These macrophages are strongly cytotoxic for the tumor cells in vitro. In contrast to macrophages, tumor-associated lymphocytes are not directly cytotoxic to the tumor cells, even when obtained from tumor-immune rats. However, $CD4^+$ and $CD8^+$ T cells prepared from the regressing tumors induce tumoricidal activity in splenic macrophages from normal or tumor-bearing rats and in macrophages that infiltrate progressive tumors. These results strongly suggest that the main tumoricidal effector cells in preimmunized rats are macrophages that have been activated by adjacent tumor-immune lymphocytes. *The Journal of Immunology,* **2001, 167: 5077–5083.**

The ability of specific T cells from tumor-immune animals to induce tumor regression is well-established in several experimental models of adoptive immunotherapy using T cell transfer. However, the ultimate mechanism(s) responsible for tumor cell killing are still unclear. It is often advanced that, in tumor-immune hosts, neoplastic cells are directly destroyed by $CD8^+$ T cells activated as CTL. However, this possibility was not substantiated by immunohistological data and analysis of tumoricidal properties of tumor-infiltrating lymphocytes (TIL) upon recovery from regressive tumors in the immunized hosts (reviewed in Ref. 1). Tumoricidal activity of TIL is usually low, even after in vitro restimulation, and requires relatively large E:T ratios (2). Furthermore, cytolytic activity of T cells, which requires direct contact with the tumor target cells, is limited by migration of activated $CD8^+$ T cells away from the tumor site (3). Recent studies have shown that tumor regression after adoptive transfer of $CD8^+$ T cells was independent of both perforin and Fas ligand pathways, suggesting that this effect was not related to a direct cytotoxic effect on the tumor cells (1). Thus, the prevailing role of CTL in tumor cell destruction remains questionable.

The experimental progressive/regressive (PRO/REG)[3] tumor cell system (4) is a well-suited rat model to determine the precise nature of the cells involved in tumor cell destruction in immunized hosts. PRO and REG tumor cell clones were established from a chemically induced colon carcinoma growing in a strain of inbred rats (4). These clones constitutively differ in immunogenicity and tumorigenicity in syngeneic hosts (4, 5). The PRO clones give rise to progressive, metastatic, and lethal tumors. The REG clones, which yield spontaneously regressive tumors, induce a strong tumor-specific immune response that fully and durably protects the rats against subsequent PRO cell or parental tumor injection (6). The spontaneous regression of the REG tumors is related to a T cell-dependent immune response because REG cells give rise to progressive tumors in the nude mice or cyclosporine-treated syngeneic rats (7). Induction of an efficient anti-tumor immune response by REG cells is now well documented (8, 9). We have demonstrated that tumor Ags liberated by early death of a part of these tumor cells following s.c. injection are endocytosed by phagocytic cells which differentiate into dendritic cells. These cells migrate to the draining lymph nodes where they are able to present these Ags and to stimulate specific anti-tumor T cells. However, REG tumor regression is not associated with the appearance of cytotoxic T cells or the increase in NK cell activity in the spleen and in tumor-associated lymphocytes (10).

A study designed to determine the precise nature of effector cells responsible for ultimate elimination of the REG tumors is limited by the small size of these tumors before complete regression. Furthermore, even though a tumor-specific immune response is the predominant mechanism of REG tumor rejection (7), other factors such as spontaneous death of REG cells (8) may contribute to it. To circumvent this problem, we grafted established PRO tumors in rats that have been previously immunized with REG cells. These tumors continue their progression in naive rats but are invariably rejected in immunized rats. In this rat model system, immunohistochemical analyses with complementary in vitro functional studies led us to identify macrophages as the direct effectors of tumor cell killing. Furthermore, tumor-associated lymphocytes that are unable to destroy tumor cells independently are instrumental for macrophage activation.

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[3] Abbreviations used in this paper : PRO, progressive; REG, regressive; NMMA, N^G-methyl-L-arginine; REGb, regressive variant DHD-K12/TSb; PROb, progressive variant DHD-K12/TRb; TIL, tumor-infiltrating lymphocyte.

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Materials and Methods

Materials

Recombinant rat IFN-γ, anti-rat IFN-γ, anti-rat TNF-α Abs, and a colorimetric ELISA kit for rat IFN-γ (Quantikine) were purchased from R&D Systems (Oxon, U.K.). LPS from *Escherichia coli*, serotype 0128:B12, and N^G-methyl-L-arginine (NMMA) were obtained from Sigma (St. Louis, MO).

Animals, cell lines, and tumorigenicity assays

Animals used in these experiments were BD-IX strain rats bred in our laboratory by brother-sister mating. Animal use and handling were approved by the local Veterinary Committee and were performed according to the French laws for animal experimentation. Two cell lines, the regressive variant DHD-K12/TSb (REGb) and the progressive variant DHD-K12/TRb (PROb) were established from the tumor DHD, a colon adenocarcinoma induced by 1–2 dimethylhydrazine in a BD-IX strain female rat (4). GV1A1 cell line was established from a glioma induced by *N*-ethyl-*N*-nitrosourea in a BD-IX rat (11). These tumor cells were cultured in Ham's F10 medium (BioWhittaker, Verviers, Belgium) complemented with 10% FBS (Life Technologies, Paisley, Scotland), as previously described (5). For the tumorigenicity assays, 1×10^6 PROb tumor cells in 100 μl of serum-free Ham's F10 medium were injected s.c. into the anterolateral thoracic wall of syngeneic BD-IX rats. Four weeks later, the resulting PROb tumor was resected, and tumor pieces weighing ~50 mg were immediately grafted s.c. into the thoracic wall, at distance from the site of REGb cell-immunizing injections. Tumor volume was evaluated weekly, using a caliper to measure two perpendicular diameters.

Histological study of the tumor cell injection site

Immunized and naive rats were sacrificed 7, 14, or 21 days after the tumor graft. The tumor was resected, embedded in Tissue-Tek (Miles, Elkhart, IN), and snap-frozen in methylbutane cooled in liquid nitrogen. An immunohistochemical study of tumor-infiltrating inflammatory cells was performed on acetone-fixed 5 μM cryostat sections. Murine mAbs to rat leukocyte markers, obtained from Serotec (Oxford, U.K.), were used on serially cut sections. Tumor cells were also labeled with a specific mAb (12C) raised against PROb cells. After incubation with specific mAbs, sections were incubated with biotinylated sheep Ab to mouse IgG (Amersham, Arlington Heights, IL), then with streptavidin-peroxidase and stained with aminoethylcarbazole.

Cytotoxicity studies

Rats were immunized with three s.c. injections of 1×10^6 REGb cells, 1 mo apart. Macrophages were prepared from spleens of one or two naive or immunized rats, with or without 14-day-old PROb tumor grafts. Tumor grafts were performed 2 wk after the last immunizing injection. A spleen cell suspension was prepared by pressing the spleen through a stainless-steel wire mesh into RPMI 1640 culture medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% FBS. Tumor-infiltrating cells were purified from tumor grafts resected from groups of five to eight rats, pooled, cut in small fragments, and dissociated with 0.5 mg/ml collagenase (type II; Sigma) and 0.05 mg/ml DNase (type II; Sigma) for 1 h at 37°C in 5% CO_2 in poly(2-hydroxymethyl methacrylate)-coated vessels (Sigma) to prevent macrophage attachment. The subsequent steps for purifying tumor-infiltrating cells were performed at 4°C. Tumor cells were depleted by incubating cell suspensions with tumor cell-specific mAb 12C, then harvesting the mAb-labeled cells on anti-mouse IgG-coated magnetic beads (Dynabeads M450; Dynal Biotech, Oslo, Norway). Macrophage concentrations in spleen and tumor cell suspensions were estimated by counting adherent cells after a 1-h attachment period on a hemocytometer glass surface at 37°C in 5% CO_2. One to 2×10^5 macrophages could be recovered from each individual tumor. For preparing macrophage culture, tumor-infiltrating cells containing 1×10^6 macrophages/ml were incubated in flat-bottom 96-well plates (Nunclon, Roskilde, Denmark). After 2 h of incubation at 37°C in 5% CO_2, nonadherent cells were harvested and the plates were washed twice with PBS to remove all the nonadherent cells. Most (>95%) adherent cells were shown to be macrophages according to their morphology and labeling with mAb ED2. Most harvested nonadherent cells were lymphocytes. In some experiments, $CD4^+$ and $CD8^+$ T cells were positively selected, following manufacturer's instructions, using magnetic cell sorting after marking the nonadherent cell suspension with MicroBeads (Miltenyi Biotec, Paris, France) conjugated with mouse mAb anti-rat CD4 and CD8α-chain, respectively. The enrichment in $CD4^+$ and $CD8^+$ cells was 90 and 98%, respectively, when assayed on rat spleen cells in preliminary experiments. Macrophages with or without lymphocytes were cocultured in triplicate with tumor cells (1×10^5 cells/ml) in RPMI 1640 medium supplemented with 10% FBS for 48 h at 37°C in a 5% CO_2 atmosphere. The lymphocyte/macrophage/tumor cell ratio was 30:10:1 in these experiments. At the end of this incubation period, culture medium was harvested, centrifuged, and immediately frozen at −20°C until cytokine assays were performed. The wells were gently washed twice with PBS, then the cultures were fixed for 15 min with methanol. The plates were allowed to dry, and labeled for 30 min at room temperature with 1% crystal violet, a protein-labeling dye that stains tumor cells that remain attached to the wells after the incubation period. The plates were carefully washed under tap water and dried, and the dye staining the residual tumor cells was eluted in 100 μl of 33% acetic acid in water (v/v). The absorbance of the eluted dye in each well was read at 570 nm on a Labtec scan (Bio-Advance, Emerainville, France). Triplicate wells were used to determine mean and SD. We checked that absorbance correlated linearly with the number of remaining viable tumor cells. Cell survival was determined by comparing cell viability in PROb cells in mixed culture with that of PROb cells alone. Macrophages did not contribute to the absorbance as they poorly fixed crystal violet.

Results

Regression of tumor grafts in immunized animals

PRO tumor pieces weighing ~50 mg were invariably accepted when engrafted in naive syngeneic hosts and continued to grow as progressive tumors. In contrast, rats immunized by three monthly s.c. injections of 1×10^6 REGb cells systematically rejected PROb tumors grafted 2 wk after the last REGb cell injection. In these rats, tumors began to shrink from day 12 until complete disappearance 4–8 wk later (Fig. 1).

Regressing PROb tumors were massively infiltrated by macrophages

To analyze the regression mechanisms, immunohistological analyses were performed on regressing tumors in the immunized animals as well as progressive tumors in the naive rats on days 7, 14, and 21 postgrafting. The most significant differences were observed on day 14, at the beginning of tumor regression in immunized rats. At this time, tumor cells were easily identifiable according to their typical morphology, 12C positivity (Fig. 2*A*), and the formation of poorly differentiated dense nodules. Progressive tumors in naive rats showed a peripheral halo of different inflammatory cells including $ED1^+$ monocytes and immature dendritic

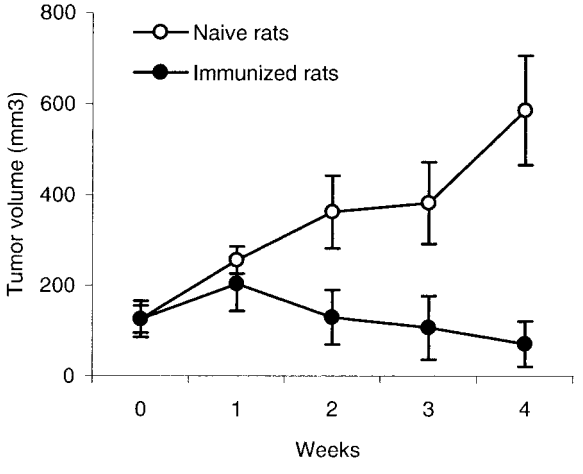

FIGURE 1. Growth of the tumor grafts in naive and immune rats. PROb tumor pieces (~50-mg weight) were s.c. grafted in naive animals (○) and animals immunized by three injections of REGb cells, 1 mo apart, 2 wk before the PROb tumor graft (●). The curves represent the mean ± SD of two independent experiments on a total of 10 rats in each group. All the tumors that were grafted in naive rats progressed, and rats were sacrificed at the 4th wk. All the tumors that were grafted in immunized rats completely disappeared by the 8th wk.

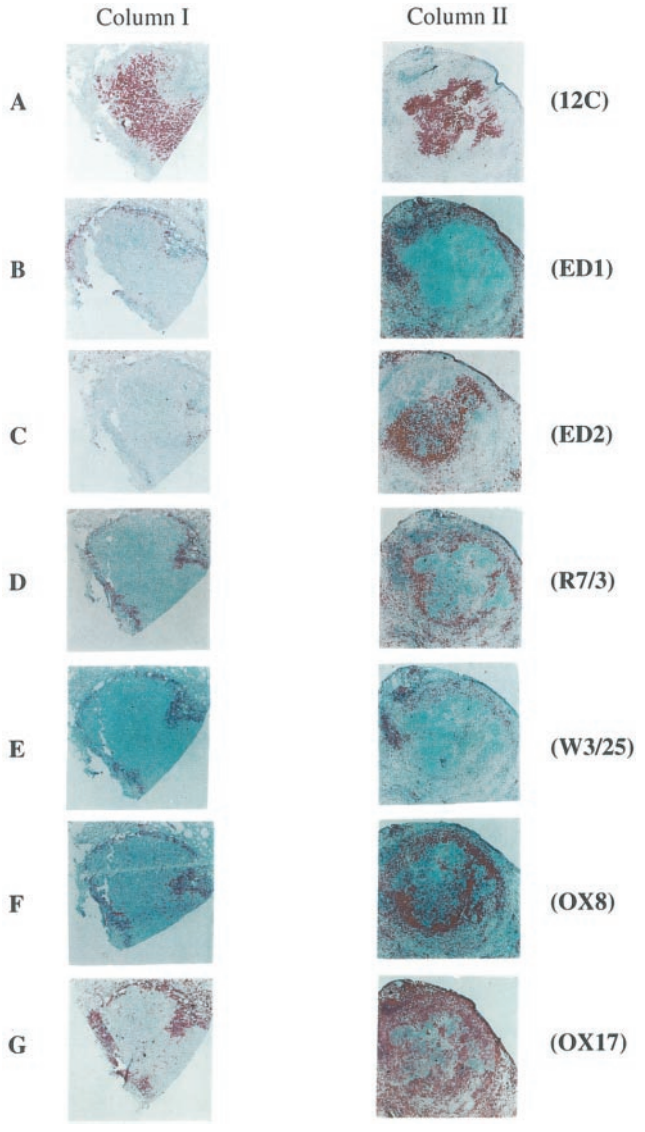

FIGURE 2. Immunohistological analysis of 14-day tumor grafts in naive and immune rats. Immunohistochemical analyses of tumors grafted in naive (*column I*) or immune (*column II*) rats were performed on serial sections by using mAbs that label tumor cells (12C), monocytes and dendritic cells (ED1, CD68-like glycosylated lysosomal Ag), mature macrophages (ED2, membrane glycoprotein), $TCR^+\alpha\beta$ T cells (R7/3), $CD4^+$ T cells (W3/25), $CD8^+$ T cells and macrophages (OX8), and MHC class II^+ cells (OX-17, RT1-D) (×25). The tumor periphery was heavily surrounded and invaded by a dense infiltrate of inflammatory cells in the immunized animals. Similar results were observed in six pairs of tumor grafts from naive or immunized animals.

cells (Fig. 2*B*), and few, if any, $ED2^+$-stained mature macrophages (Fig. 2*C*). TCR^+ T cells were also restricted to the tumor periphery (Fig. 2*D*) and were either $CD4^+$ (Fig. 2*E*) or $CD8^+$ (Fig. 2*F*). A few cells, also limited to the tumor periphery, expressed MHC class II molecules (Fig. 2*G*). In contrast, regressing tumors in immunized rats showed a spectacular infiltration by $ED2^+$ mature macrophages within the tumor nodule (Fig. 2*C*). $ED1^+$-staining cells (Fig. 2*B*), as well as TCR^+, $CD4^+$, and $CD8^+$ T cells (Fig. 2, *D–F*), which were more abundant in these regressive tumor nodules, remained at the periphery of the tumor. TCR^-CD8^+ cells infiltrating the tumor nodules (Fig. 2, *D* and *F*) were observed to be large, irregular cells with a cytoplasmic staining. These cells are likely to be macrophages as they correspond in morphology and location to $ED2^+$ tumor-infiltrating macrophages, and rat macrophages were shown to express CD8 molecules (12). To determine whether the $CD8^+$ cells found inside the tumor nodules were $CD8^+$ lymphoid cells (CTL or NK cells) or $CD8^+$ macrophages, another mAb to rat CD8α, G28, was used. In contrast to OX8 mAb, which labels a rat CD8α hinge region epitope expressed by both macrophages and lymphoid cells, G28 labels an Ig variable-like region epitope, which is expressed only by CTL and NK cells (13). Both OX8 and G28 Abs labeled cells at the periphery of the tumor nodules. Only OX8, but not G28, labeled $CD8^+$ cells that infiltrated tumor nodules (Fig. 3). These $CD8^+G28^-$ cells were also characterized by their elongated morphology and cytoplasmic staining, in contrast to the $G28^+$ cells surrounding the tumor nodules that were characterized by a round shape and a predominantly cell surface labeling. Like TCR^+ T cells, $3.2.3^+$ NK cells were more abundant in regressive than in progressive tumors but remained confined to the periphery of the tumor nodules (data not shown).

Macrophages but not lymphocytes that infiltrate regressive tumors dramatically kill tumor cells in vitro

Cytotoxicity assays were performed in vitro to determine the effects of tumor-infiltrating and splenic macrophages (adherent

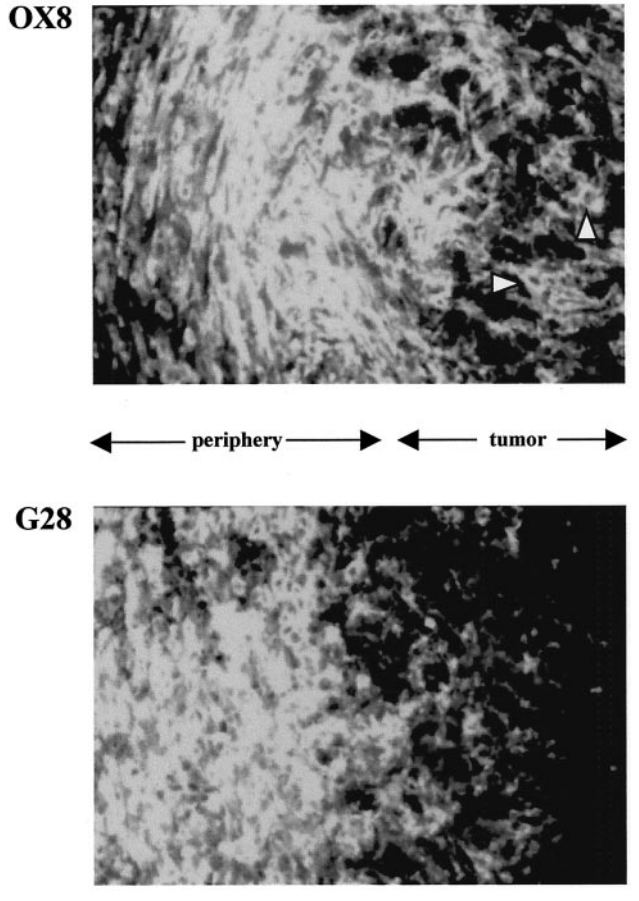

FIGURE 3. CD8 expression by cells infiltrating the regressive PROb tumor grafts. $CD8^+$ cells were labeled using either an Ab (OX-8) that binds a CD8α epitope expressed by both myeloid (macrophages) and lymphoid cells (CTL and NK cells), or an Ab (G28) that labels only $CD8^+$ T cells and NK cells. Deep-frozen sections were stained with OX-8 or G28, a biotinylated anti-mouse IgG Ab, then avidin-FITC, were subsequently added. $CD8^+$ cells surrounding the tumor nodules were labeled by both mAbs, whereas elongated $CD8^+$ cells infiltrating the tumor nodules were predominantly labeled with OX-8 mAb, suggesting their myeloid origin (arrowheads).

cells) and lymphocytes (nonadherent cells) on PROb tumor cells. Tumor-infiltrating macrophages isolated 14 days after the graft in immune rats were strongly cytotoxic as they killed all tumor cells in a 48-h coculture assay at a 10:1 E:T ratio. In comparison, tumor-infiltrating macrophages isolated 14 days after the graft in nonimmune rats were also cytotoxic, but to a considerably lower extent, reducing tumor cell viability by only 32–45%. Splenic macrophages from naive and immune rats, either tumor-bearing or not, showed no significant cytotoxicity (Fig. 4*A*). Nonadherent cells, which were predominantly lymphocytes, were isolated from the spleen and tumor of naive or immunized rats. These cells showed no significant cytotoxicity to tumor cells even when used at a 30:1 E:T ratio (Fig. 4*B*). Similar results were obtained in five independent experiments. This macrophage-mediated cytotoxic effect was not restricted to cells from the colon cancer from which the PRO/REG cell variants originated. Macrophages from regressing PROb tumors also demonstrated cytotoxic activity toward a rat glioma cell line (Fig. 5*A*). We investigated also the mechanisms used by tumor-infiltrating macrophages for killing PROb cells. Addition of

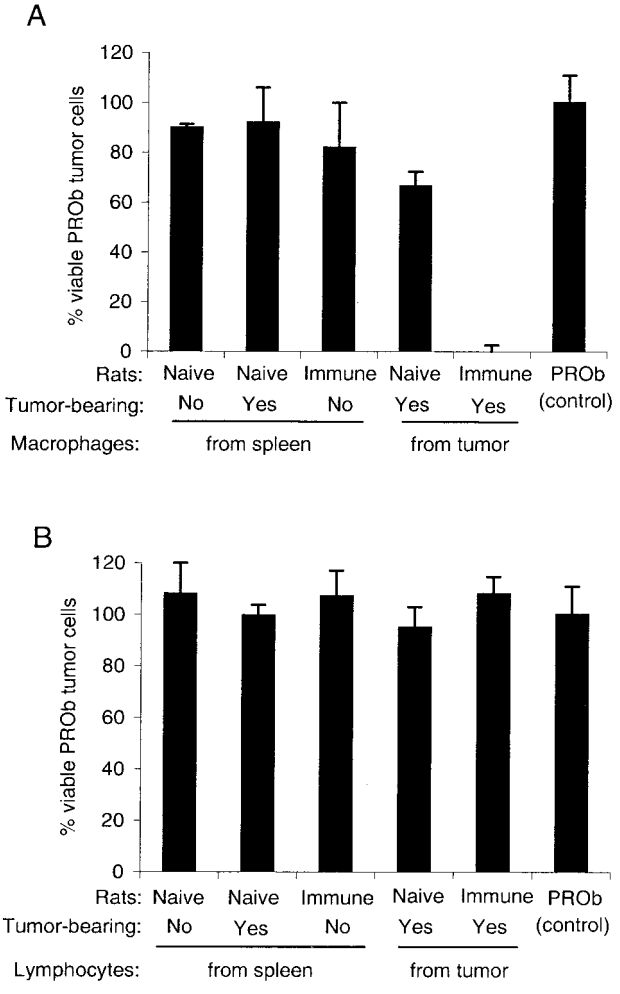

FIGURE 4. Macrophages but not lymphocytes that infiltrate regressive tumors kill PROb tumor cells in vitro. Macrophages and lymphocytes were isolated from the spleen of tumor-free naive, tumor-bearing naive, and tumor-free immunized rats, and from PROb tumors 14 days after their graft in naive and immunized rats. These cells were assayed for their capacity to kill PROb cells. Columns indicate the percentage of adherent PROb cells relative to PROb cells cultured alone (*column 6*) at the end of a 48-h mixed culture assay. *A*, Effect of macrophages. *B*, Effect of lymphocytes. E:T ratios were 10:1 and 30:1 for macrophages and lymphocytes, respectively (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures).

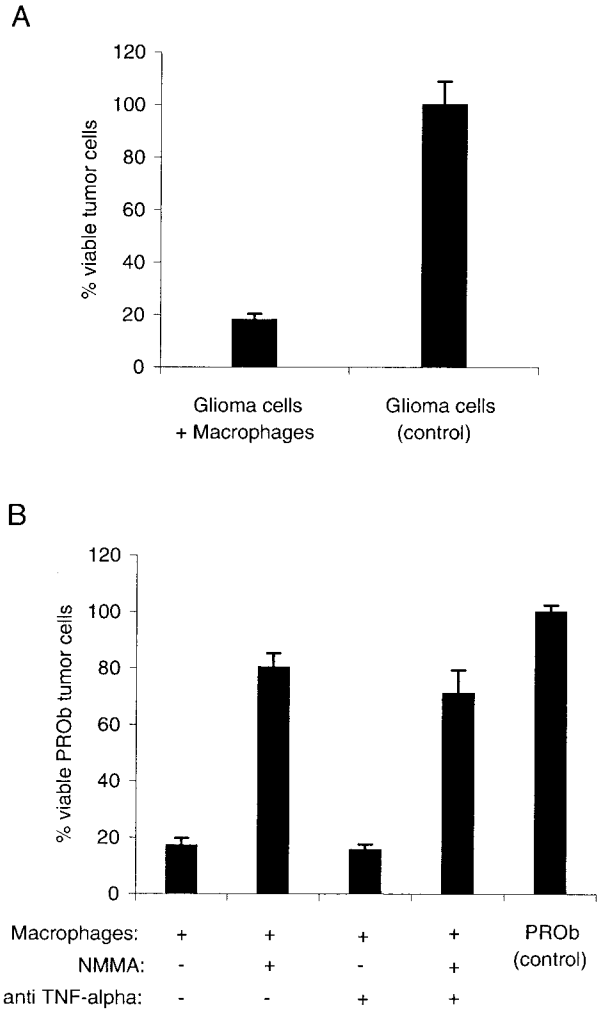

FIGURE 5. Macrophages that infiltrate regressive tumors kill tumor cells unrelated to the immunizing cell line. The cytotoxicity involves a NO-mediated pathway. *A*, Macrophages were isolated from regressive PROb grafts, and their ability to kill nonspecifically glioma cells was investigated. Columns indicate the percentage of adherent glioma cells relative to glioma cells cultured alone (*column 2*) at the end of a 48-h mixed culture assay, at a macrophage-glioma cell ratio of 10:1 (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures). *B*, Tumor-infiltrating macrophages from regressive PROb grafts were assayed for their capacity to kill PROb cells in the presence of the NO production inhibitor NMMA (1 mM), or with anti-TNF-α mAb (5 μg/ml). Results are the percentage of adherent PROb cells relative to PROb cells cultured alone (*column 5*) at the end of a 48-h mixed culture assay, at a macrophage-PROb cell ratio of 10:1 (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures).

1 mM NMMA, an inhibitor of NO generation from arginine, strongly inhibited PROb cell killing by macrophages from regressing PROb grafts, whereas macrophage-induced cytotoxicity was not influenced by an anti-TNF-α mAb (5 μg/ml) (Fig. 5*B*).

Lymphocytes associated with regressive tumors in immune hosts induced macrophage activation

When nonadherent cells infiltrating PROb tumors grafted in immune animals were added to splenic macrophages from normal, immune, or tumor-bearing rats, toxicity against tumor cells was dramatically increased and all the tumor cells were eliminated (Fig. 6*A*). Interestingly, when nonadherent cells infiltrating PROb tumors grafted in immune animals were added to macrophages

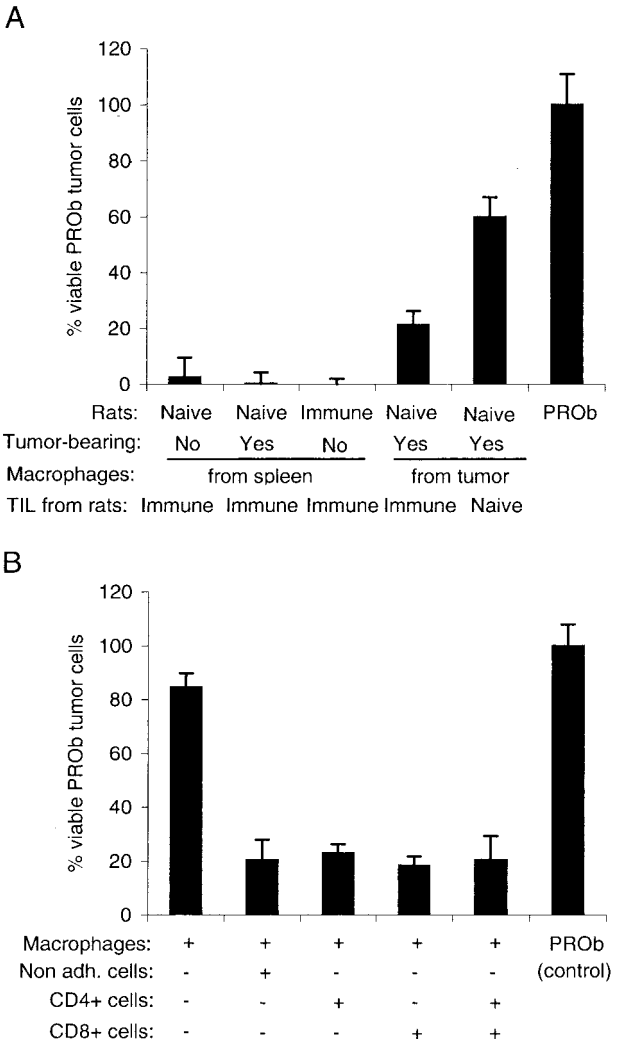

FIGURE 6. Cytotoxicity to PROb tumor cells of a coculture of macrophages + lymphocytes isolated from tumor grafts in immunized animals. Evidence for an effect of both $CD4^+$ and $CD8^+$ T cells. *A*, Various combinations of TIL from PROb tumors grafted in immunized or naive rats, and splenic or tumor-infiltrating macrophages were assayed for their capacity to kill PROb cells in a 48-h mixed culture assay at a lymphocyte-macrophage-PROb cell ratio of 30:10:1. Columns indicate the percentage of adherent PROb cells relative to PROb cells cultured alone (*column 6*) at 48 h (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures). Lymphocytes associated with progressive tumors from naive rats (*column 5*) were less efficient than lymphocytes associated with regressive tumors from immunized rats (*column 4*) for inducing tumoricidal activity of macrophages isolated from progressive tumors in naive rats. *B*, Unseparated nonadherent cells (3×10^5/well) or their content in selected $CD4^+$ cells (1.05×10^5/well) and/or $CD8^+$ cells (0.55×10^5/well) were added to 1×10^3 splenic macrophages from a naive rat and 1×10^4 PROb cells for a 48-h coculture assay (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures).

isolated from PROb tumors grafted in naive rats, cytotoxicity to PROb tumor cells was still observed, although this cytotoxic effect was more limited (Fig. 6*A*). In contrast, nonadherent cells isolated from PROb tumors grafted in naive animals or from spleen of naive rats did not increase the low cytotoxic effect of macrophages isolated from progressive tumor grafts in naive rats (Fig. 6*A* column 5, compared with Fig. 4*A* column 4). These results were reproduced in three independent experiments. T cells were responsible for the macrophage-activating effect of nonadherent cells isolated from regressing tumor grafts because $CD4^+$ or $CD8^+$ T lymphocytes isolated from these nonadherent cells activated macrophages as efficiently as the whole nonadherent cell fraction, without any significant difference between $CD4^+$ and $CD8^+$ cells (Fig. 6*B*). Surprisingly, addition of both $CD4^+$ and $CD8^+$ cells had no more macrophage-activating activity than addition of $CD4^+$ or $CD8^+$ cells alone. This could indicate that each T cell subpopulation maximally activated splenic macrophages in this experiment. We did not determine whether immune T cells activated macrophages through a direct cell contact or through production of cytokines in the tumor microenvironment.

IFN-γ is a lymphokine known for its capacity for inducing rat macrophages to become cytotoxic against cancer cells. We found that lymphocytes isolated from PROb tumors grafted in immune rats secreted significant amounts of IFN-γ (455 ± 78 pg/ml) in the culture medium when cultured for 48 h with PROb cells and splenic macrophages.

Tumoricidal activity of tumor-infiltrating and splenic macrophages from rats bearing progressive tumors can be induced by exposure to IFN-γ and bacterial LPS

As neither splenic macrophages nor macrophages that infiltrated progressive tumors in nonimmunized rats were cytotoxic to tumor cells, we investigated whether tumoricidal activity could be induced by exogenous activators. Addition of IFN-γ and/or LPS strongly increased macrophage cytotoxicity to PROb cells (Fig. 7). IFN-γ used alone did not induce a cytotoxicity sufficient to destroy all the tumor cells established in a monolayer, whereas LPS alone was almost as effective as LPS + IFN-γ for inducing macrophage-mediated cytotoxicity.

Discussion

Destruction of tumor cells in immunized hosts is generally attributed to a direct cytotoxic effect of $CD8^+$ CTL (14–18). However, the lack of correlation between CTL induction and tumor rejection in immunotherapy trials calls into question the relevance of $CD8^+$ T cell cytotoxic activity as measurement of anti-tumor immune

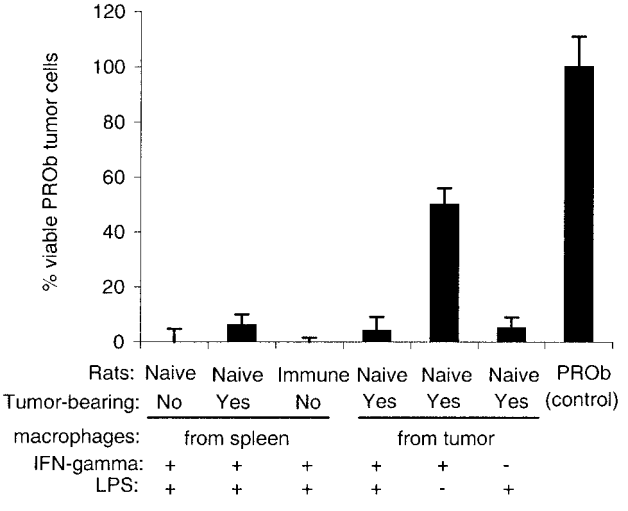

FIGURE 7. IFN-γ and LPS induce macrophage cytotoxicity to PROb tumor cells. Splenic macrophages from tumor-free naive, tumor-bearing naive, and tumor-free immunized rats, and tumor-infiltrating macrophages from PROb tumor grafts in naive rats were activated with IFN-γ (5 ng/ml) and/or LPS (100 ng/ml) and tested in cytotoxic assays for killing PROb cells. Columns indicate mean percentage of adherent PROb cells at the end of a 48-h mixed culture assay at a 10:1 E:T ratio (crystal violet cytotoxicity assay; mean ± SD from triplicate cultures).

activity (19). In the present study using a rat model system we demonstrate that the effector cell directly responsible for tumor cell killing and tumor graft rejection in the immunized host is not a lymphoid cell, but an activated mature macrophage. Regressing tumor grafts in preimmunized hosts were heavily infiltrated by inflammatory cells that were labeled with ED2 mAb, a marker of mature rat macrophages (20). These tumor-infiltrating macrophages also express CD8 recognized by OX8 mAb but not G28 mAb, which specifically labels a CD8α epitope expressed only by lymphoid cells (13). These macrophages were in direct contact with tumor cells and strongly cytotoxic to them in vitro.

The capacity of activated macrophages to destroy tumor cells in vitro has been known for a long time (21–23). However, to the best of our knowledge, the role of macrophages in tumor rejection observed in specifically tumor-immunized hosts has never been analyzed. The mechanisms by which activated macrophages kill neoplastic cells include NO production through activation of inducible NO synthase, and TNF-α secretion (21–23). We demonstrate here that the cytotoxic effect of activated macrophages isolated from regressing tumors is suppressed by NMMA, an inhibitor of NO production, whereas this cytotoxic effect is not influenced by a blocking anti-TNF-α mAb, probably because PRO cells have no receptor to TNF-α (24). On paraffin-embedded sections, the damaged tumor cells were not stained after terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL), demonstrating that macrophage-induced tumor cell death does not imply DNA internucleosomal cleavage (result not shown).

Tumoricidal activity was restricted to macrophages infiltrating tumors engrafted in immunized hosts. Splenic macrophages from immunized rats were not cytotoxic. Macrophages infiltrating tumors in naive rats were few and sparse. Most of them were not in contact with the cancer cells and demonstrated only a limited cytotoxic effect toward tumor cells in vitro. These observations suggest that macrophages have to undergo in situ activation to exert tumoricidal function. Interestingly, tumor-associated lymphocytes from immunized hosts induce the tumoricidal activity of inactive splenic macrophages from normal or tumor-bearing rats, as well as in macrophages isolated from PROb tumors grown in naive rats. This observation strongly suggests that lymphocytes that surround tumor nodules in immunized rats are stimulated in this local microenvironment, then deliver activation signals to macrophages that infiltrate these tumors.

In contrast with macrophages, T lymphocytes were located at the periphery of the regressing tumor nodules, were only infrequently observed to be in contact with the tumor cells, and had no direct cytotoxic effect against tumor cells, even at E:T ratios as high as 30:1. Passively transferred $CD8^+$ T cells from tumor-immunized donors have been shown to induce complete, Th1 cytokine-dependent tumor regression in the recipient in the absence of any direct cytotoxic effect (1). Furthermore, the therapeutic effectiveness of tumor-infiltrating $CD8^+$ T lymphocyte cultures correlates with their ability to secrete lymphokines rather than their cytotoxic capacity in vitro (25–27). $CD8^+$ T cells, as well as $CD4^+$ Th1 cells (28), are a source of cytokines such as IFN-γ, GM-CSF, and TNF-α, which can stimulate the tumoricidal activity of host cells, including tumor-associated macrophages. In agreement with these findings, IFN-γ levels measured in 48-h supernatants of macrophage, tumor cell, and TIL cocultures were higher for TIL isolated from immunized than from naive animals. Moreover, addition of IFN-γ, as well as endotoxin, to splenic or tumor-associated macrophages from naive animals generated significant cytotoxic activity in these otherwise noncytotoxic macrophages. In contrast to mouse macrophages that require two signals (a priming signal from IFN-γ and a progression signal from LPS) to become cytotoxic against cancer cells (29), a single exogenous stimulus, IFN-γ or LPS, activates rat resident macrophages for NO production and tumor cytotoxicity (30).

Progressive human tumors are frequently infiltrated by macrophages (31). However, these macrophages are not potent effectors of tumoricidal activity in the absence of stimulation and could even enhance tumor growth (reviewed in Ref. 32). Tumor cells could adversely alter macrophage tumoricidal activity (33). Interestingly, tumoricidal activity of macrophages isolated from progressive PROb tumors in naive animals could be restored in vitro by either exposure to lymphocytes isolated from the regressive tumors of immune rats, or exogenous stimuli such as IFN-γ and bacterial endotoxins. The effect of endotoxins is particularly interesting because peritoneal carcinomatosis that results from PROb cell i.p. injections into naive rats can be definitely cured upon multiple administrations of lipid A, an endotoxin component (34, 35).

We have previously demonstrated that tumor proteins released as a consequence of initial events following injection of REG cells in naive syngeneic rats are engulfed by inflammatory cells and transported to lymph node T cell areas (9). However, the protective immune response following REG tumor regression was not accompanied by a detectable increase in the specific or nonspecific cytotoxic activity of splenic and tumor-associated T lymphocytes and NK cells (10). In the present study, which was performed on the same rat tumor model, we clarified the downstream events and defined the effector cells involved in the final elimination of the tumors in immunized hosts. These events involve migration of activated tumor-specific T cells to the tumor site, local production of IFN-γ and possibly other cytokines that activate tumor-associated macrophages, and subsequent elimination of tumor cells by these macrophages. The present work also demonstrates that macrophages that infiltrate progressive tumors have the potential to destroy tumor cells, provided they receive appropriate T cell-mediated activation signals, such as cytokines, or nonimmune stimuli, such as bacterial endotoxins.

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