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The Tumor Recall Response of Antitumor Immunity Primed by a Live, Recombinant *Listeria monocytogenes* Vaccine Comprises Multiple Effector Mechanisms[1]

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Listeria monocytogenes, a facultative intracellular bacterium, can induce a potent antitumor immune response if engineered to express a model tumor antigen also expressed by the tumor cells. The effectiveness of this approach is dependent on _L. monocytogenes_-induced tumor-specific CD4$^+$ and CD8$^+$ T-cells. CD8$^+$ T-cells may mediate tumor eradication largely through direct CTL activity, but the role of CD4$^+$ T-cells and other cells of the immune system is less clear. Here we investigate their role and the role of the cytokines they produce in the ability of _L. monocytogenes_-induced antitumor immunity to protect against tumor challenge. Our results suggest that a complex cytokine response, involving type 2 as well as type 1 cytokines, is responsible for the ability of Lm-NP-immunized mice to resist tumor challenge, potentially mediating tumor cell killing through multiple effector pathways.

***Key Words:* tumor immunity; immunotherapy; _Listeria monocytogenes;_ Th1/Th2; cytokines.**

INTRODUCTION

Listeria monocytogenes is a facultative intracellular pathogen which infects and activates macrophages and has the unusual ability to avoid phagolysosomal destruction by breaking out of this compartment and living in the cytoplasm of the cell. *L. monocytogenes* enters macrophages by phagocytosis and then escapes into the cytoplasm by secreting virulence factors, including a hemolysin (listeriolysin O; LLO) and phospholipase C, that induce lysis of the phagolysosome. This escape process, however, is inefficient, and about 90% of the entering bacteria are killed and degraded in the phagolysosomal compartment (1). Successful infiltration to the cytoplasm of infected cells enables bacterial replication. Infection of adjacent cells can subsequently occur, by a direct cell-to-cell spreading mechanism, which allows *L. monocytogenes* to avoid contact with the extracellular environment (2). Infected macrophages respond rapidly by secreting IL-1, IL-6, and TNF-α (3). In addition, IL-12 production is induced, leading to the secretion of IFN-γ by NK and $\gamma\delta$TCR$^+$ T-cells (4, 5). IFN-γ, in turn, activates macrophages and increases killing of *L. monocytogenes.* In addition to macrophages and NK cells, $\gamma\delta$ T-cells (6, 7) and neutrophils (8, 9) play an important role in the early control of infection, and neutrophils apparently kill infected hepatocytes (10). Although this innate immune response successfully limits infection, complete eradication of *L. monocytogenes* requires the acquisition of T-cell-mediated immunity, and both CD8$^+$ and CD4$^+$ T-cells are generated in response to infection. T-cells also mediate protection against subsequent challenge with *L. monocytogenes* (7, 11, 12). The memory response to *L. monocytogenes* is largely effected by CD8$^+$ T-cells (11, 12) and may be mediated by perforin as well as TNF-α and CD95-ligand expression (13, 14). CD4$^+$ cells, although less effective than CD8$^+$ cells, can also contribute to anti-*L. monocytogenes* immunity, inducing a delayed-type hypersensitivity response (11, 12).

Infection with *L. monocytogenes,* therefore, induces a predominantly type-1 (T1;[5] Th1)-like immune response, characterized by the expression of IL-12 and

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[5] Abbreviations used: Lm-Gag, recombinant *Listeria monocytogenes* strain that secretes a fusion protein consisting of Gag of HIV-1 plus part of the hemolysin of *L. monocytogenes;* Lm-NP, recombinant *L. monocytogenes* strain that secretes a fusion protein consisting of the nucleoprotein of influenza strain A/PR/8/34 plus part of the hemolysin of *L. monocytogenes;* T1, type 1 cytokine pattern; T2, type 2 cytokine pattern.

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IFN-γ (15, 16). Although the type 2 (T2; Th2)-associated cytokine IL-10 is also eventually produced in the course of the anti-*L. monocytogenes* immune response, the effect is downregulatory, functioning to limit both the production of IL-12 and the effect of IL-12 on NK cells (16). IL-4, another potentially important immunoregulatory T2-associated cytokine which can downregulate T1-associated immune responses and stimulate T2 immune responses, is expressed very early in the course of infection, functioning to induce secretion of the chemokine monocyte chemoattracting protein-1 (17–19). However, the IL-4 response is transient and begins to diminish as quickly as 3 h after infection, apparently as a result of the inactivation of $CD4^+$ $NK1^+$ $\alpha\beta$-$TCR^{intermediate}$ NKT cells by IL-12 and IFN-γ (20). The overall immunological relevance of this very early IL-4 production, however, is unclear, since ablation of IL-4 with anti-IL-4 mAb treatment prior to listerial challenge was shown to enhance the expression of anti-*L. monocytogenes* immunity, as measured by reduced numbers of *L. monocytogenes* subsequently recoverable from spleen and liver (21). This suggests that the net effect of IL-4, like IL-10, is inhibition of anti-listerial immunity.

The immunobiology of the host–pathogen interaction makes *L. monocytogenes* an attractive potential live vaccine vector. The first demonstration that a live *L. monocytogenes* recombinant expressing a transgenic antigen could be used to immunize mice (22) indicated that antigen-specific CTL could be generated following infection either orally or ip. The efficacy of this vaccination strategy was evaluated in a tumor system, using an *L. monocytogenes* strain (Lm-NP) engineered to secrete a fusion protein consisting of the first 420 amino acids of listeriolysin-O followed by the nucleoprotein (NP) of influenza (23, 24). Immunization with Lm-NP protected mice from subsequent challenge with either Renca or CT26 tumor cells retrovirally transduced to express the NP antigen. In addition, mice bearing established, macroscopic tumors could be effectively treated with Lm-NP, leading to substantial inhibition of tumor growth or even complete tumor regression in some mice. Immunohistochemical analysis of tumor nodules from Lm-NP-immunized mice revealed substantial infiltration of the tumors by both $CD8^+$ and $CD4^+$ T-cells, and depletion of either T-cell subset during the effector phase of the immune response inhibits the ability of *L. monocytogenes* to protect against tumor challenge. The $CD8^+$ T-cells may mediate tumor killing by CTL activity *in vivo,* since Lm-NP-immunized mice generate strong NP-specific CTL that will kill tumor cells transduced with NP *in vitro* (23, 24), but the role of $CD4^+$ T-cells is less clear.

There has been a recent burgeoning of interest in the role of $CD4^+$ T-cells in antitumor immunity against MHC class II-negative tumors (25). A cognate cytolytic effect by these cells is unlikely, since the tumors do not express the appropriate MHC-restriction element. A requirement for $CD4^+$ T-cells in the induction of antitumor CTL responses has been noted (26, 27). However, this is not an adequate explanation in our case, since abrogation of protection against tumor challenge by CT26-NP or Renca-NP when $CD4^+$ T-cells are depleted occurs at a time when the secondary population of $CD8^+$ T-cells has already been induced by immunization with *L. monocytogenes*. The two most likely explanations are that $CD4^+$ cells are required for the production of cytokines, such as IL-2, which may be required to maintain NP-specific T-cells in an activated state (28) or to enhance the ability of the $CD8^+$ population to expand when recalled by tumor-expressed antigen. Second, the presence of $CD4^+$ T-cells within the tumor suggests a role for bystander necrosis perhaps via inflammatory cytokines produced by these cells or other cells. Adoptively transferred $CD4^+$ T-cells have been demonstrated to slow tumor growth in the absence of transferred $CD8^+$ T-cells (29), although this may be due to their ability to facilitate the induction of $CD8^+$ T-cells to endogenous tumor antigens rather than a direct tumoricidal effect (30). Any postulated mechanism requires the presentation of tumor-associated antigens to $CD4^+$ T-cells which cannot be mediated by the tumor cells themselves, thus implying a role for professional antigen-presenting cells in tumor-expressed antigen recall of *L. monocytogenes*-induced T-cells.

In the present study, we wished to examine the requirement for the professional antigen-presenting cells, macrophages and dendritic cells, in addition to NK cells, in expression of Lm-NP-induced immunity against CT26-NP tumor cells. We also sought to determine the role of cytokines, particularly those that are produced only by $CD4^+$ T-cells, in this recall response. Other workers have used mice genetically engineered to eliminate single immune components for such studies (so-called “knockout” mice). However, the antitumor immunity induced by *L. monocytogenes* is the result of an active infection by a bacterial pathogen, the immune response to which is seriously modified in such animals. Accordingly, to allow for proper clearance of the live bacterial vaccine, we selectively depleted these cellular subsets after Lm-NP immunization, at the time of tumor challenge. Depletion of dendritic cells and NK cells greatly reduced protection against tumor challenge, whereas, surprisingly, macrophage depletion did not. Using a similar approach we also identified several cytokines, IL-12, GM-CSF, and IL-6, which are the products of these nonadaptive immune system cells, that are also required for maximal recall responses to NP. In addition, we examined the role of T-helper cell cytokines. Whereas antibodies against IFN-γ, TNF-α, IL-2, and IL-4 administered at the time of tumor challenge significantly inhibited protective antitumor immunity, neutralization of IL-10 or administration of a control antibody had no effect. Thus both T1 and T2 cytokines appear to be important in the recall response to NP induced by CT26-NP. Taken together, these results indicate that a complex cytokine response is responsible for the ability of Lm-NP-immunized mice to resist tumor challenge. The results will be discussed in the context of the multiple possible effector pathways that may be utilized for tumor cell killing.

MATERIALS AND METHODS

Plasmids and Bacterial Strains

The *L. monocytogenes* strain principally used in this study is Lm-NP (DP-L2028) (31). This strain is derived from a *prfA* (virulence regulon transcription factor)-defective mutant of the wild-type strain 10403S that has been stably transfected with a plasmid containing *prfA* and a fusion protein gene encoding the first 420 amino acids of LLO, a secreted *L. monocytogenes* hemolysin, coupled to the NP gene of influenza strain A/PR/8/34. Lm-NP secretes large amounts of LLO-NP fusion protein, which is expressed under the control of the LLO promoter. The LD_{50} of Lm-NP is 1–2 × 10^8 colony-forming units upon intraperitoneal injection. For reference, mice in control groups were infected with a recombinant strain of *L. monocytogenes* expressing a fusion protein consisting of the promoter and signal sequence of the LLO gene and the Gag gene of HIV-1 (strain Lm-Gag); the construction of this strain has been previously described (32). Lm-Gag has approximately the same LD_{50} as Lm-NP in BALB/c mice (5 × 10^7 pfu), allowing us to deliver approximately the same number of bacteria to control groups.

Lm-NP was prepared for injection by culture at 37°C in brain–heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 10 μg/ml chloramphenicol. Lm-Gag, which does not require antibiotic selection to retain expression of the viral transgene, was grown under similar conditions without chloramphenicol. After overnight growth, the broth culture was aliquoted and stored at −70°C; the culture density was determined by optical density and enumeration of colony-forming units following platings of dilutions of the broth culture medium. Individual aliquots were thawed as needed and washed three times in PBS prior to injection.

Hybridomas and mAb Preparation

C17.8, with specificity for mouse IL-12 p40, and XT22.11, with specificity for mouse TNF-α, were a generous gift from Giorgio Trinchieri (Wistar Institute, Philadelphia, PA). Ascites from the Xmg1.2 hybridoma, specific for mouse IFN-γ, was a generous gift from Drew Pardoll (Johns Hopkins University School of Medicine, Baltimore, MD). 33D1, specific for a subset of dendritic cells, was a generous gift from B.-D. Sheng (University of Pennsylvania School of Medicine, Philadelphia, PA). SH-34, specific for asialo-GM-1, was a generous gift from Robert Stout (Eastern Tennessee State University, Johnson City, TN). JES5-2A5, specific for IL-10, was a generous gift from Robert Coffman (DNAX Research Institute, Palo Alto, CA). 11B11, which produces an anti-mouse IL-4 mAb, and S4B6, which produces an anti-mouse IL-2 mAb, were obtained from the American Type Culture Collection (ATCC; Rockville, MD). MP1-22E9, specific for granulocyte–macrophage colony-stimulating factor; MP5-20F3, specific for IL-6; and GL117.41, which produces an anti-*Escherichia coli* β-galactosidase, were obtained from the ATCC with permission from DNAX Research Institute. The mAb used for *in vivo* depletion experiments were produced as ascites, following the protocol of Yokoyama (33). Briefly, SCID mice (Charles River Laboratories, Wilmington, MA) were injected with 5 × 10^6 hybridoma cells at least 7 days after ip treatment with 0.5 ml of pristane (Sigma Chemical Co., St. Louis, MO). Ascites was collected aseptically and antibody was affinity-purified on protein G–Sepharose columns (Pharmacia Biotech, Inc., Piscataway, NJ). *In vivo* ascites production was performed according to guidelines provided by the University of Pennsylvania Institutional Animal Care and Use Committee.

Tumor Cell Lines

CT26, an *N*-nitroso-*N*-methylurethane-induced murine colon carcinoma syngeneic in BALB/c mice (34), is MHC class I positive and MHC class II negative. The NP gene of influenza strain A/PR/8/34 was inserted into the tumor line by retroviral transduction using a replication-incompetent Moloney leukemia virus system, generating CT26-NP, as previously described (35). The expression of NP does not alter the tumorogenicity of the tumors: the minimal lethal tumor dose for both CT26-NP and CT26 is 5 × 10^3.

Tumor Challenge Experiments

BALB/c mice, 6–8 weeks of age, were obtained from Charles River Laboratories. The animal use protocol for these studies has been approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The mice were immunized with Lm-NP or Lm-Gag by ip inoculation with 0.1× LD_{50}, followed by a second ip inoculation of 0.1× LD_{50} 2 weeks later. After an additional 2 weeks, 5×10^5 CT26-NP tumor cells were injected sc on the right hind flank. Depletion of specific cytokines and cell subsets was started 1 day prior to tumor challenge as described below. Eight mice were used for each experimental group. Mice were followed for tumor growth, and tumor sizes were determined as the average of the longest and shortest diameters, as measured by calipers. Tumor-bearing mice were sacrificed when tumor sizes reached about 20–25 mm in average diameter or if animals showed signs of ill health or distress.

Cell and Cytokine Depletion

Macrophages were depleted by iv treatment with liposomes containing the cytotoxic drug clodronate (dichloromethylene bisphosphonate), which induces macrophage apoptosis (36). Clodronate was generously provided by Boehringer Mannheim GMBH (Mannheim, Germany). The liposomes containing clodronate solution were made following the method of van Rooijen and Sanders (37). Cholesterol (8 mg) and phosphatidylcholine (0.86 mg) were dissolved in 10 ml of chloroform and evaporated onto the sides of a round-bottom flask. The resulting phospholipid film was then dispersed in a 0.6 M clodronate solution in PBS and rotated at approximately 150 rpm for 10 minutes. After a 2-h incubation at room temperature, the liposome suspension was sonicated in a water bath sonicator, incubated overnight at 4°C, washed thrice by centrifugation, and then resuspended in 4 ml of PBS. As a control, liposomes were also prepared with PBS vehicle alone. Initial liposome injections consisted of 0.25 ml of this suspension delivered iv 1 day before tumor cell challenge; subsequent treatments of 0.1 ml were administered every 5 days thereafter. The effectiveness of macrophage depletion was evaluated by flow cytometric analysis for expression of F4/80 in mice treated with clodronate-containing liposomes compared to PBS-containing liposomes. Four days after treatment with the initial dose of liposomes, more than 75% of the F4/80-expressing cells were typically removed from the spleen. Spleens from mice evaluated at the conclusion of the experiment by FACS analysis had 62% fewer $F4/80^+$ splenocytes than controls.

All other *in vivo* depletions were performed using antibodies specific for that immune component essentially as previously described (38). Each experiment was performed at least twice with reproducible results, summarized in Table 1. Briefly, mice received mAb treatments ip on days −1, 0, 1, 4, 8, and 12 relative to tumor challenge, at a dose of 1.2 mg (unless otherwise noted) per treatment. To control for nonspecific effects other groups of mice received either 1.2 or 3.0 mg of anti-β-galactosidase antibody of the same isotype. The efficacy of mAb depletion of NK cells with SH-34 and dendritic cells with 33D1 was determined by FACS analysis of splenocytes on four individual mice. Although complete depletion of either of these subsets was not achieved, 42 to 72% of NK cells and approximately 50% of 33D1-positive dendritic cells were depleted by this treatment. It is known that some cell surface markers are not as cell specific as originally described. For example RB6-8C5, which binds to the granulocyte marker Ly6-G, was reported to specifically delete granulocytes and to not bind to monocyte–macrophage or lymphocytic cells (38). However, we (Pan and Paterson, unpublished observation) and others (9) have observed that *in vivo* administration of this antibody at the levels required to deplete granulocytes will also deplete T-cells. In addition, the NK cell marker asialo-GM1 (39) has been shown to be expressed on virus-specific $CD8^+$ T-cells (40). We were careful, therefore, to examine the effects of the 33D1 and SH-34 antibody-depletion protocols on the $CD4^+$ and $CD8^+$ T-cell compartments in the spleens of treated mice by FACS analysis and found no reduction in either of these T-cell subsets.

Statistics

For comparisons of tumor diameters, the mean and standard deviation of tumor size for each treatment group were determined, and statistical significance was determined by Student's *t* test (41). In all experiments, a *P* value less than or equal to 0.05 was considered significant.

RESULTS

Effect of Depletion of Macrophages and Dendritic Cells on Lm-NP-Induced Protective Antitumor Immunity

In light of the observation that $CD4^+$ T-cells accumulate in the CT26-NP tumor site of Lm-NP-treated mice (23), even though the tumor cells express little or no MHC class II, we sought to determine the importance of macrophages as professional antigen-presenting cells of NP for Lm-NP-induced antitumor immunity. To deplete macrophages, a group of Lm-NP-immunized mice was treated with clodronate-containing liposomes iv (37) beginning 1 day prior to tumor cell challenge. For reference, groups of immunized mice were also treated with PBS-containing liposomes or were left untreated. As shown in Fig. 1, clodronate treatment of immunized mice did not significantly affect the growth of the CT26-NP tumors, compared to immunized mice treated with PBS-containing liposomes. Treatment of naive tumor-bearing mice with clodronate-containing liposomes did not affect the growth rate of the CT26-NP tumors compared to untreated naive mice. Clodronate treatment does not completely deplete the entire macrophage population; however, only 25% of F4/80-expressing cells remain in the spleen. Although we cannot rule out the possibility that this residual macrophage population plays a role in antitumor immunity, the fact that the depletion of the majority of F4/80-expressing cells has a complete lack of impact on tumor growth suggests that macrophages do not appear to be essential for the protective antitumor immunity induced by immunization with Lm-NP.

$CD4^+$ T-cells clearly play a role in the expression of protective antitumor immunity following immunization with Lm-NP, because depletion of this subset at the time of tumor challenge reduces the ability of immunized mice to resist tumor challenge (23). If macrophages are not required for the expression of antitumor immunity, it is conceivable that a different MHC class II-expressing antigen-presenting cell is necessary for antitumor immunity. Accordingly, we depleted Lm-NP-immunized mice of dendritic cells using mAb 33D1 (42) administered at the time of tumor challenge. 33D1 recognizes a marginal zone marker that was originally reported to be present on 80 to 90% of splenic dendritic cells (43). It is now thought that the proportion of dendritic cells expressing this marker may be somewhat lower in other populations (44). Nevertheless, despite this and the fact that complete depletion of dendritic cells was also not achieved, in marked contrast to the depletion of macrophages, depletion of $33D1^+$ dendritic cells significantly reduced the ability of Lm-NP-immunized mice to resist tumor cell challenge (Fig. 2a and Table 1).

Effect of Depletion of Asialo-GM-1-Bearing Cells on Lm-NP-Induced Protective Antitumor Immunity

NK cells are a prominent component of the innate immune response to *L. monocytogenes*. To evaluate the role of NK cells in antitumor immunity, mice were immunized twice with Lm-NP and then treated with the anti-asialo-GM-1 mAb SH34, beginning 1 day before tumor cell challenge. Following challenge with 5×10^5 CT26NP tumor cells, mice that had received immunization with Lm-NP exhibited strong protective antitumor immunity, with six of eight mice tumor free (Fig. 2b). However, treatment with anti-asialo-GM-1 substantially impaired the antitumor immune response, and only two of eight mice resisted challenge. Although the tumor size in anti-asialo-GM-1 mAb-treated mice was not statistically different from that of untreated mice at day 26 ($P \leq 0.1$, Table 1), by day 40 the difference in the average size of the tumors between the two groups was significant ($P \leq 0.01$). Growth of the tumor cells does not appear to be affected by anti-asialo-GM-1, since tumor sizes were not significantly different in naive (unimmunized) mice in the presence or absence of antibody treatment. Thus, asialo-GM-1-expressing cells appear to be an important component of the antitumor immune response induced by immunization with Lm-NP.

Effect of Depletion of Cytokines on Lm-NP-Induced Tumor Eradication

To identify the contribution of cytokines to the protective antitumor immunity that results from immunization with Lm-NP, mice were treated ip with neutralizing monoclonal antibodies beginning 1 day before tumor cell challenge and continuing until 12 days after challenge. The data from a single cytokine depletion experiment are shown in Fig. 3. The results summarized in Table 2 include replicate experiments for each cytokine depletion. The protective effect of Lm-NP immunization was effectively eliminated by depletion of IL-12 or IFN-γ (Fig. 3a, Table 2) and tumor sizes were not significantly different from those of naive tumor-bearing mice. Depletion of TNF-α (Fig. 3b, Table 2) also reversed the ability of Lm-NP-immunized mice to resist tumor cell challenge, although the inhibition of antitumor immunity was less complete, compared to naive mice, than with anti-IL-12 or anti-IFN-γ treatment. Likewise, depletion of either IL-2, GM-CSF, IL-6, or, surprisingly, IL-4 substantially inhibited the ability of Lm-NP-immunized mice to resist challenge, although the inhibition of antitumor immunity appeared incomplete, since Lm-NP-immunized mice treated with these antibodies were still able to effect tumor growth (Figs. 3c–3e, Table 2). In contrast, depletion of IL-10 (Fig. 3f, Table 2) did not significantly affect the level of protection exhibited by Lm-NP-immunized mice. Likewise, treatment of Lm-NP-immunized mice with equivalent doses of a control mAb of irrelevant specificity did not affect tumor growth relative to Lm-NP-immunized mice not treated with mAb (Fig. 3b, Table 2). To verify that mAb treatments were not directly affecting the growth of the tumor cells, naive (unimmunized) CT26-NP tumor-bearing mice were treated with the same dose of the anti-cytokine mAbs; no statistically significant effect was observed (data not shown). Thus, cytokines associated with both T1 and T2 immune responses contribute to the expres-

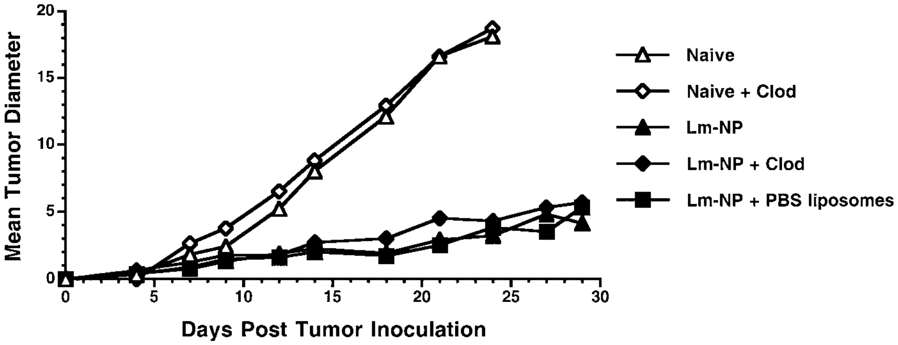

FIG. 1. Effect of macrophage depletion on the ability of Lm-NP-immunized mice to resist subsequent tumor challenge. Beginning 13 days after the second immunization, at a time when the live Lm-NP infection would have been cleared, the mice were treated iv with clodronate liposomes to deplete macrophages. Results are presented as the mean of tumor sizes for each group.

sion of the protective antitumor immunity induced by treatment with Lm-NP.

DISCUSSION

We have previously shown that immunization of mice with a live recombinant *L. monocytogenes* vaccine expressing the model tumor antigen NP induces a potent cell-mediated immune response capable of protecting mice from subsequent tumor challenge in a CD4$^+$ and CD8$^+$ T-cell-dependent manner. CD8$^+$ T-cells were previously found to be essential to the antitumor immune response induced by Lm-NP, and spleen cells from Lm-NP-immunized mice were found to generate potent CTL activity against CT26-NP tumor cells (23, 24), but the role of CD4$^+$ T-cells was less clear. In this study, we identify seven cytokines and two non-T-cell immune cells, dendritic cells and NK cells, which are important mediators of antitumor immunity.

CT26-NP recall of NP-specific CD4$^+$ T-cells induced by Lm-NP must involve presentation of the NP tumor antigen by professional MHC class II-bearing cells since CT26 is MHC class II$^-$. The studies described here clearly indicate that dendritic cells may play a role in that process since even their partial depletion has a significant impact on antitumor immunity recalled by CT26-NP. However, dendritic cells also play an important role in the cross-talk between the adaptive and the innate immune systems. They are potent secretors of IL-12, which itself activates NK cells to secrete interferon-γ. We have shown that all of these factors, NK cells, IL-12, and IFN-γ, are required for maximal antitumor immunity recalled by tumor challenge; thus we cannot rule out the possibility that dendritic cells also act via innate immune mechanisms through IL-12 secretion. In contrast, depletion of the other major subset of antigen-presenting cells, macrophages, made no impact on tumor growth. This sur-

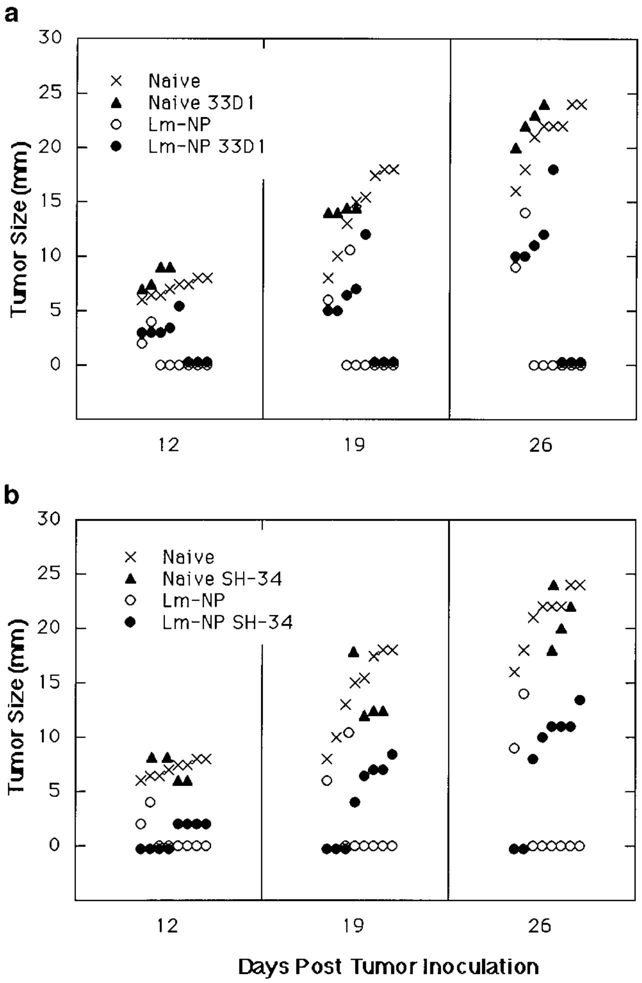

FIG. 2. Effect of depletion of dendritic cells and asialo-GM-1-expressing cells on the ability of Lm-NP-immunized mice to resist tumor challenge. Lm-NP-immunized mice were treated with 1.2 mg of anti-dendritic cell mAb 33D1 (a) or anti-asialo-GM-1 mAb SH-34 (b). Symbols represent tumor size in one mouse; data are depicted for representative time points.

TABLE 1

Summary of *in Vivo* Cell Depletion Studies

Treatment group	Cell type depleted	Days post-tumor challenge	Mean of individual tumor diameters (mm ± SD)	Significance relative to Lm-NP alone
Lm-NP + clodronate liposomes	Macrophages	32	4.1 ± 3.9	$P \leq 0.9$
Lm-NP + PBS liposomes	Macrophages	32	4.7 ± 4.5	$P \leq 0.9$
Lm-NP alone	None	32	4.4 ± 4.6	
Lm-NP + antibody 33D1	Dendritic cells	26	11.0 ± 8.45	$P \leq 0.01$
Lm-NP alone	None	26	2.56 ± 4.99	
LM-NP + antibody SH-34	NK cells	26	8.06 ± 5.2	$P \leq 0.1$
	NK cells	40	15.06 ± 6.36	$P \leq 0.01$
Lm-NP alone	None	26	2.88 ± 6.36	
	None	40	4.0 ± 8.49	

prising result suggests that macrophages may not act as antigen-presenting cells in the recall response. The role of macrophages in modulating tumor growth, however, is complicated. Although macrophages are found in tumors and surrounding stroma, their impact on tumor growth can be both positive and negative depending on their state of activation and the cytokines that they secrete. Macrophages are an important source of proinflammatory cytokines such as IL-12, IFN-γ, and TNF-α which can be tumoricidal. However, tumor-infiltrating macrophages can also be the source of immunosuppressive cytokines, angiogenic factors, and prostaglandins (reviewed in 45). Indeed, macrophages isolated from various murine and human tumor cells are known to enhance the proliferation of tumor cells (45, 46). Thus it is possible that depletion of macrophages could have an impact on presentation of tumor-associated antigen, in addition to other innate effects that control tumor growth, but that these are compensated by removal of macrophage-dependent mechanisms that enhance tumor growth.

We reported previously that immunization with Lm-NP induces infiltration of the tumors by both CD4$^+$ and CD8$^+$ cells in an antigen-specific manner, since Lm-NP-immunized mice bearing tumors from the parental cell line (not transduced to express NP) do not exhibit T-cell infiltration (23). It is likely that at least some of the cytokine production on tumor recall is the direct or indirect result of NP-specific T-cell activation. In addition, the variety of other leukocytes infiltrating the tumor, including dendritic cells and NK cells, may also contribute to the cytokine milieu, either due to activation by T-cell-derived cytokines or as a consequence of prior immunization with the live *L. monocytogenes*. Additionally, the detection of CD4-expressing cells does not necessarily distinguish between conventional MHC-restricted CD4$^+$ T-cells versus NKT cells, which express $\alpha\beta$ TCR as well as NK markers such as NK1 and which can be CD4$^{\text{intermediate}}$, CD4$^{\text{low}}$, or CD4$^-$. The production of a variety of cytokines, including IL-4 and IFN-γ, has been attributed to NKT cells (47), and NKT cells can mediate tumor clearance *in vivo* in an IL-4- or IL-12-dependent fashion (48–51).

Of the cytokines identified, IFN-γ, IL-2, and TNF-α are generally considered to be products of T1 CD4$^+$ helper cells and/or CD8$^+$ T-cells. The importance of these cytokines for protection against tumor cell challenge is not surprising, because sterilizing anti-*Listeria* immune responses are thought to be classically T1 CD4$^+$ and CD8$^+$ dependent (3, 52). It has been suggested (53) that IL-2 and IL-12 are required for the recall of a memory CD8$^+$ T-cell response in the absence of an active infection, as is the case upon CT26-NP tumor cell challenge of mice previously immunized with Lm-NP. In addition, expression of either TNF-α or IL-2 can competitively overcome the effect of TGF-β (54–56), a potentially immunosuppressive cytokine capable of downregulating CTL responses (54, 55, 57), which is secreted by CT26 (Beatty and Paterson, unpublished observation). Thus, the requirement for IL-2, IL-12, IFN-γ, and TNF-α is consistent with the contribution of CTL to tumor eradication.

Interestingly, the protective antitumor immune response induced by Lm-NP is also abrogated by depleting the T2-associated cytokine IL-4. The anti-IL-4 mAb treatment does not seem to directly affect the growth of the CT26-NP tumor cells, since treatment of unimmunized mice with the same dose of mAb does not significantly affect tumor growth. Moreover, neither the anti-IL-4 mAb nor the added IL-4 affects the *in vitro* growth of CT26-NP tumor cells (data not shown). The importance of IL-4 for tumor eradication following immunization with Lm-NP is surprising because IL-4 is not believed to significantly enhance the anti-*L. monocytogenes* immune response. In the experiments reported here, CT26-NP tumor challenge took place 4 weeks after initial induction of the NP-specific T-cell response. Thus, even though Lm-NP infection induces a classic T1-type immune response, the expression of a memory response to the NP antigen expressed by CT26

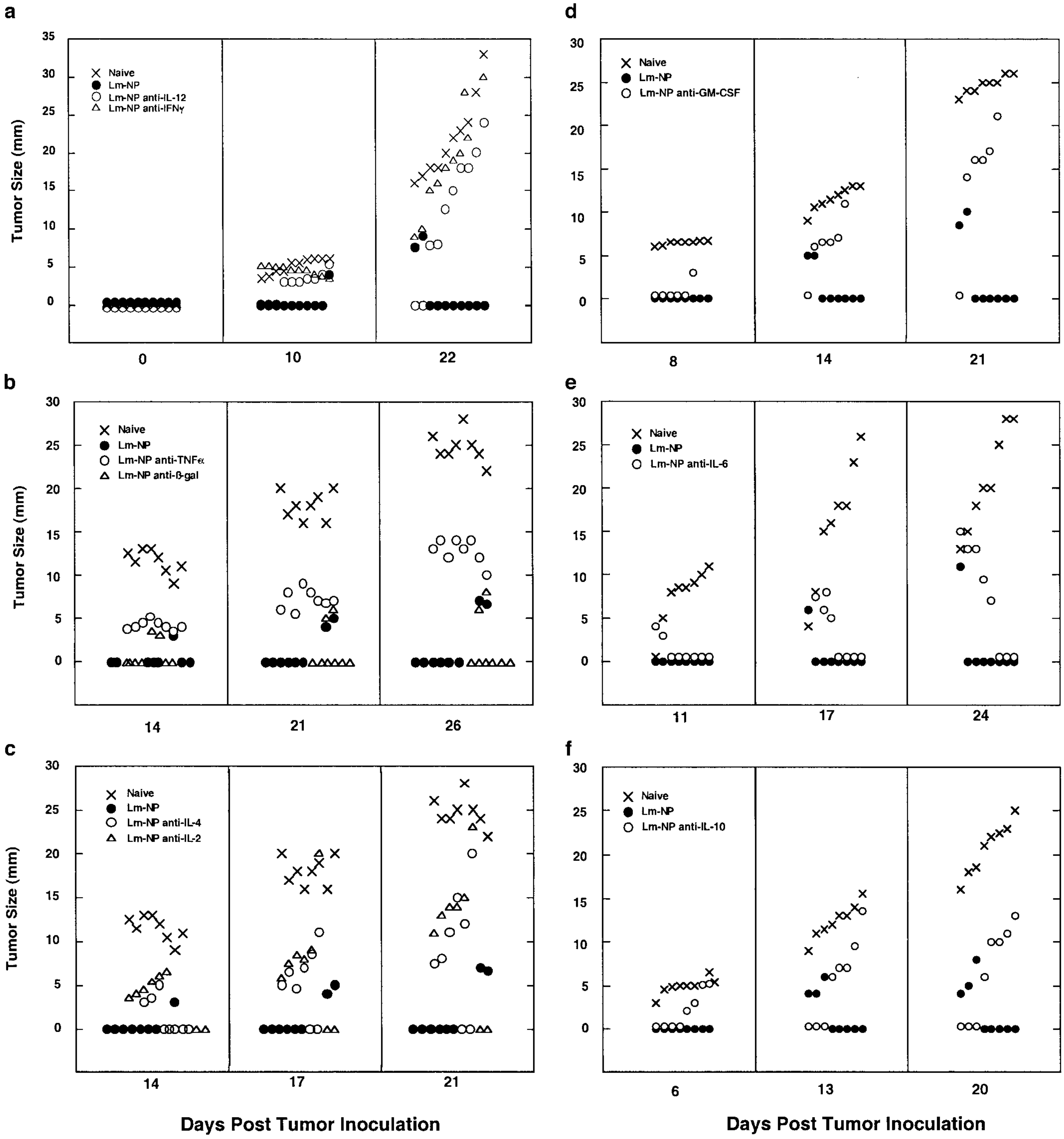

FIG. 3. Effect of anti-cytokine mAb treatment on the ability of Lm-NP-immunized mice to resist subsequent CT26-NP tumor challenge. Mice were preimmunized with 0.1 LD_{50} of live Lm-NP and then challenged with CT26-NP; mAb treatments of 1.2 or 3.0 mg in PBS were administered on days −1, 0, 1, 4, and 8 relative to tumor inoculation. (a) Treatment with 1.2 mg of anti-IL-12 or anti-IFN-γ mAb. (b) Treatment with 3.0 mg of anti-TNF-α or irrelevant control mAb (anti-β-galactosidase). (c) Treatment with 1.2 mg of anti-IL-2 or anti-IL-4. (d) Treatment with 1.2 mg of anti-GM-CSF. (e) Treatment with 1.2 mg of anti-IL-6. (f) Treatment with 1.2 mg of anti-IL-10. For reference, a group of Lm-NP-immunized mice challenged with CT26-NP tumor cells was not treated with mAb (filled circles). The rate of tumor growth in naive (unimmunized) mice is also shown. Symbols represent tumor size in one mouse; data are depicted for representative time points.

TABLE 2

Summary of *in Vivo* Cytokine Neutralization Studies

Treatment group	Days post-tumor challenge	Mean of individual tumor diameters (mm ± SD)	Significance relative to Lm-NP alone
Lm-NP + anti-IL-12	22	14.9 ± 9.9	$P \leq 0.01$
Lm-NP + anti-IFN-γ		23.9 ± 5.2	$P \leq 0.001$
Lm-NP alone		2.6 ± 4.1	
Lm-NP + anti-IL-2	21	10.4 ± 6.6	$P \leq 0.01$
Lm-NP + anti-IL-4		9.9 ± 8.4	$P \leq 0.05$
Lm-NP alone		2.1 ± 4.1	
Lm-NP + anti-TNF-α	21	11.1 ± 1.2	$P \leq 0.001$
Lm-NP + anti-β-gal		1.75 ± 3.3	$P \leq 0.9$
Lm-NP alone		2.9 ± 4.2	
Lm-NP + anti-GM-CSF	21	14.5 ± 7.8	$P \leq 0.01$
Lm-NP alone		2.3 ± 4.3	
Lm-NP + anti-IL-6	24	7.2 ± 6.4	$P \leq 0.05$
Lm-NP alone		1.4 ± 3.9	
Lm-NP + anti-IL-10	20	6.3 ± 5.5	$P \leq 0.1$
Lm-NP alone		2.1 ± 3.1	

tumor cells apparently requires IL-4. In addition, it has been shown that neutralization of IL-4 by treatment with mAb enhances clearance of *L. monocytogenes* infection (21), suggesting that IL-4-secreting cells actually are produced during infection even though a T1 response ultimately predominates. A recent study has demonstrated that IL-4 maintains the balance between T2 and T1 cells *in vivo* by enhancing IL-12 production by dendritic cells (58). Given that both IL-12 and dendritic cells are also required for maximal antitumor recall responses induced by Lm-NP, it is quite possible that IL-4 mediates antitumor immunity indirectly by this mechanism.

In addition to a potential role for IL-4 in promoting IL-12 production by dendritic cells during the tumor recall response, IL-4 may play a direct role in the antitumor recall response. It is now recognized that effective antitumor immune responses, unlike anti-*Listeria* immune responses, can involve a combination of T1 and T2 cytokines. Indeed, studies with knockout mice illustrate the essential role of IL-4 for the antitumor protective effectiveness of immunization with irradiated tumor cell vaccines (27, 59): in several tumor models, IL-4-deficient mice fail to develop protective antitumor immunity following vaccination. Hung and associates have shown that the potent anti-tumor immunity induced by GM-CSF-expressing irradiated B16F10 melanoma cells is dependent on the production of IL-4 and eosinophil infiltration at the site of tumor challenge (27). The protective antitumor immunity induced by immunization with irradiated TS/A mammary adenocarcinoma cells or CT26 tumor cells also is dependent on the presence of IL-4, apparently for the induction of an antitumor CTL response (59). Interestingly, Schuler *et al.* found a requirement for IL-4 at the vaccine site, but not at the challenge site (59). Taken together, these studies indicate that IL-4 can directly play a critical role either at the induction stage or at the expression stage of the antitumor immune response.

The cytokines found to be important in the Lm-NP system could be involved in either the induction or the activation of effector functions. The protective antitumor immunity induced by Lm-NP was previously shown to be critically dependent on the participation of $CD8^+$ T-cells, which exert potent CTL activity *in vitro* (23) and presumably also *in vivo*. Here, we present evidence that several other cell types and a number of cytokines also contribute to protective antitumor immunity. A variety of cytolytic effector cells, potentially including NK cells and macrophages, could conceivably be activated by the various cytokines determined to be important in the anti-NP memory response. A contribution of NK cells to protection against tumor challenge is suggested by the studies presented here. Given that CT26 tumor cells transduced to express NP eventually lose antigen expression *in vivo* (23), it is possible that nonspecific antitumor mechanisms may be required for protection against tumor challenge by eliminating NP antigen-loss variant tumor cells. Evidence for the existence of antigenically nonspecific effector mechanisms in the tumor nodules of Lm-NP-immunized mice comes from "innocent bystander" killing studies (Z.-K. Pan and Y. Paterson, unpublished observations): Lm-NP-immunized mice, when challenged with an admixture of the renal cell carcinoma-derived tumor line Renca-NP and the parental CT26 tumor cells, did not develop tumors, whereas injection of the two tumor lines into opposite sites did result in formation and progression of CT26 tumors, but not Renca-NP tumors.

In addition to a possible role in activating various effector cell populations, the cytokines may have other more direct effects on tumor growth. For example, IL-12 and IFN-γ are potent inhibitors of angiogenesis (60, 61), and we have shown that IFN-γ produced by CD4$^+$ T-cells infiltrating CT26 tumors plays an important role in the early control of tumor growth (Beatty and Paterson, *J. Immunol.*, in press). In addition, TNF-α, in combination with IFN-γ, can directly kill CT26-NP tumor cells (Beatty and Paterson, unpublished observation). At the same time, it is possible that these cytokines also facilitate the induction of T-cell responses against additional tumor-associated antigens during the expression of the anti-NP immune response against the CT26-NP tumor cell challenge. Lm-NP immunized mice that successfully resist CT26-NP challenge can sometimes resist a subsequent rechallenge with parental CT26 tumor cells, suggesting that recognition of endogenous tumor antigens can be induced (Weiskirch, Pan, and Paterson, unpublished observation). It is possible that some of the cytokines required during tumor cell challenge, perhaps including IL-4, facilitate the activation of naïve T-cells recognizing these tumor antigens. The importance of these additional T-cell responses induced during tumor cell challenge is currently under investigation.

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