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Augmentation of an Antitumor CTL Response In Vivo by Inhibition of Suppressor Macrophage Nitric Oxide

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Evidence is provided that inhibition of macrophage NO production can augment in vivo CTL responses. Specifically, administration of NO\textsubscript{G}-monomethyl-L-arginine (NGMMA) via osmotic pumps increases the tumor-specific CTL response against the P815 mastocytoma in the peritoneal cavity of preimmunized mice. Both the magnitude and duration of the CTL response were increased. That the augmented CTL response resulted from inhibition of the NO synthase pathway is supported by the finding that macrophage NO production from NGMMA-treated mice was reduced. Also, in vitro inhibition of NO production by peritoneal exudate cells from P815 tumor-challenged mice augmented the secondary CTL response observed. Cell proliferation was augmented by NGMMA in these cultures, suggesting that macrophage NO may suppress CTL by inhibiting clonal expansion. NO-mediated inhibition was observed in vivo in this experimental system, even though the CTL response is not suppressed, in that tumor rejection occurs. Therefore, the present results are consistent with the conclusion that macrophage-NO-mediated inhibition of the CTL response is a side effect of activating macrophages rather than resulting from the action of a distinct subset of what have long been termed suppressor macrophages. Most important, the results indicate that NO-mediated suppressor macrophage activity can be an important CTL immunoregulatory element in vivo. The Journal of Immunology, 1999, 163: 5877–5882.

Macrophages are a cornucopia of immunologic influence. As protagonists of immune responses, macrophages sometimes exert their influence early, providing signals necessary for lymphocyte activation (1–4). Other times, macrophages participate late, serving as the final effectors for the destruction of pathogens (5, 6). An opposing role for macrophages, as antagonists of immune responses, was first suggested in the late 1960s (7) and with increasing frequency in the 1970s (8–12). However, the physiologic importance of what have been termed suppressor macrophages is controversial. The controversy originated from the fact that macrophage suppressor activity was measured in vitro, and thus may not accurately reflect the in vivo circumstance. For example, on the one hand, the appearance of suppressor macrophage activity has temporally coincided with the decline in host resistance observed in many progressive diseases such as malaria and cancer (12). On the other hand, there have also been reports showing increased suppressor activity coexisting with increased disease resistance in vivo (12–15). The latter type of observations fueled opinions that macrophage suppressor activity is an artifact of in vitro culture, which in certain conditions it was. Perhaps the best known example was the report showing that macrophages secrete cold thymidine that results in an artifactual decrease in lymphocyte proliferation by decreasing the uptake of [\textsuperscript{3}H]thymidine (16). However, it has now become clear that macrophages can strongly inhibit lymphocyte proliferation in vitro. As for the mechanism involved, results from this laboratory (17) and others (18, 19) have demonstrated that NO is a prime mediator of suppressor macrophage activity. Also, the inhibition of NO in cultures of leukocytes from rejecting allografts increased the CTL response observed (20).

More recently, evidence has begun to accumulate that macrophage-derived NO can also inhibit lymphocyte responses in vivo. For example, treatment of mice with NO\textsubscript{G}-monomethyl-L-arginine (NGMMA) during antitumor vaccination increased the subsequent antitumor response (21). That NO could inhibit immune responses stands in contrast to the well-characterized role of NO as an effector molecule for the destruction of bacteria, parasites, and tumor cells (22–25). At the same time, it is not surprising that macrophage-derived NO can concomitantly serve roles in immune defense and in immune suppression, given that NO utilizes similar mechanisms to inhibit prokaryotic and eukaryotic cells (24). However, if the same molecule (NO) does mediate host defense and immune suppression, the most straightforward conclusion is that suppression is an outcome of macrophage activation rather than resulting from distinct subsets of macrophages, as sometimes suggested (12, 26). Evidence consistent with this postulate includes the recent observation that inhibition of NO production in Salmonella infection decreased suppression and decreased resistance, both mediated by macrophages (27).

Given this background, it was considered of interest to directly determine whether NO produced during macrophage activation in vivo influences specific lymphocyte responses. In this connection, results from this laboratory (28) showed that specific regression of the P815 tumor in preimmunized mice is associated with a marked, but brief, intratumor CTL response. Concomitant with the CTL response, there is IFN-\gamma-mediated up-regulation of intratumor macrophage NO production. Therefore, this model system afforded

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3 Abbreviations used in this paper: NGMMA, NO\textsubscript{G}-monomethyl-L-arginine; mit-C, mitomycin-C; PEC, peritoneal exudate cell.
the opportunity to test the hypothesis that the brevity of the CTL response results, in part, from macrophage NO production. The results will show that inhibition of macrophage NO in vivo does augment CTL production, suggesting that there is potential to increase or decrease lymphocyte responses by modulating the level of this even more fascinating gas.

**Materials and Methods**

**Animals**

(C57BL/6 × DBA/2)F1 (B6D2F1) female mice were purchased from Tac-toxic Farms (Germantown, NY). Mice used for experiments were between 9 and 14 wk old. They were routinely tested for common murine pathogens by the University of Minnesota Research Animal Resources. Mice were euthanized with carbon dioxide.

**Tumors**

The P815 mastocytoma employed is syngeneic to DBA/2 mice and was the same tumor employed in previous studies (28–30). Tumor cells to be used for immunization or secondary tumor challenge were passaged weekly as an i.p. ascites in irradiated B6D2F1 mice. B6D2F1 were employed because they allow tumors of either the C57BL/6 or DBA/2 origin to be investigated, and previous studies have shown that B6D2F1 generate a tumor-specific response to the P815 tumor (29). Tumor stocks were cryopreserved in liquid nitrogen in RPMI 1640 containing 20% FBS and 10% DMSO (J. T. Baker, Phillipsburg, NJ). A new passage was initiated every 3 mo from these stocks. The L5178Y lymphoma, another tumor syngeneic to DBA/2 mice, was passaged and preserved as with the P815 tumor. The L5178Y and the P815 tumors were free of common viral pathogens according to the results of routine serological screening. For in vivo use, tumor cells were harvested in PBS containing antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin), washed, and resuspended to an appropriate volume of PBS. P815 tumor cells were mitotically inactivated by incubation in mit-C at 50 μg/ml (Sigma, St. Louis, MO) for 1 h at 37°C. For in vitro use, P815 and L5178Y tumors were grown and passaged weekly in RPMI 1640 containing 10% FBS and antibiotics.

**Alzet continuous infusion pumps**

Mice were anesthetized by i.p. injection of Nembutal (75 mg/kg). Alzet osmotic minipumps (Model 1007D; Alza, Palo Alto, CA) were implanted in the peritoneal cavity, and the incision was closed in two layers with 6-0 Dexon suture. The pumps delivered 6 μg of NGMMA in 14 μl PBS/mouse/day or 14 μl of the vehicle alone (PBS)/mouse/day in another group (three to five mice per group). Pumps were explanted at the time of peritoneal exudate cell (PEC) extraction (days 5 and 7), and appropriate delivery of NGMMA or PBS was affirmed by measuring the volume of liquid remaining in the pumps.

**Tumor immunization and secondary challenge**

Mice were immunized against the P815 tumor by intradermal injection with 2 × 106 P815 tumor cells admixed with 50 μg of *Corynebacterium parvum* in a volume of 0.05 ml into B6D2F1 mice, as in preceding studies (28, 31). This protocol results in complete and permanent regression of the intradermally implanted tumor in 80–90% of mice. Mice were used for experiments 3 wk after tumor plus *C. parvum* injection. P815-immune mice were injected i.p. with 5 × 105 P815 tumor cells, or with 2 × 106 mit-C inactivated P815 tumor cells in a volume of 0.5 ml PBS. For cell transfer, PEC were collected 3 days after mit-C tumor challenge, and 4 × 107 cells were injected i.p. into mice that had been irradiated (750 R) 3 days before and implanted i.p. with Alzet pumps containing NGMMA or PBS 2 days before.

**PEC collection and culture**

PEC were harvested by three injections of 5 ml PBS containing antibiotics, washed, and resuspended in RPMI 1640 supplemented with antibiotics, 1 × 10–2 M morpholinopropane sulfonic acid (buffer), 5 × 10–3 M 2-ME, and 10% FBS (complete medium). Cells were plated at 3 × 105 or 1 × 106/ml in 0.1 ml in triplicate flat-bottom microtiter wells. Adherent and nonadherent cells were separated by repeated washing after a 2-h incubation at 37°C in an atmosphere of 7% CO2. PEC recovered from mice rechallenged with 5 × 105 P815 tumor cells in the peritoneal cavity 3 wk after the initial vaccination. Reimplantation of the P815 tumor triggers the development of a tumor-specific secondary CTL response that peaks at day 5 and is accompanied by up-regulation of macrophage NO production (28). Complete tumor rejection occurs by 5 days postchallenge.

**Inhibition of NO production in vivo augments the antitumor CTL response**

To investigate whether macrophage-derived NO plays a role in down-regulating CTL production, groups of four P815-preimmunized mice were implanted with Alzet pumps containing the NO proliferation was measured by adding 1 μCi of [3H]thymidine to micro-wells 18 h before cell harvest on day 3 of culture.

**Culture reagents and chemicals**

Unless indicated, media and additives were purchased from Life Technolo-gies (Gaithersburg, MD). FBS was purchased in large lots from HyClone Laboratories (Logan, UT), and contained less than 0.027 ng/ml endotoxin. NGMMA was purchased from Calbiochemical (San Diego, CA). NGMMA is a competitive inhibitor of NO synthase enzymes (32). *C. parvum* was purchased from Wellcome Laboratories (Research Triangle Park, NC). Sodium chromate (51Cr) was purchased from DuPont-NEC (Boston, MA).

**51Cr release assay**

The details of the assay have been described previously (28, 30). Briefly, P815 or L5178Y tumor cells to be used as target cells were harvested during log phase growth, and 5 × 107 tumor cells in 0.4 ml tumor growth medium were labeled with 0.1 mCi 51Cr at 37°C for 1 h. The assay was performed in triplicate with wells containing 5 × 105 labeled tumor cells and 3 × 104 PEC or 1 × 105 PEC, to obtain E:T cell ratios of 60:1 or 20:1, respectively. The total volume contained in each well was 0.2 ml. Micro-titer plates were centrifuged for 3 min at 250 g to sediment the target cells. After 4-h incubation at 37°C in an atmosphere of 5% CO2, 0.5 ml of supernatant was removed from each well and counted in a 1470 Wizard automatic gamma counter (Wallac Oy, Turku, Finland). Total 51Cr release was the amount of 51Cr released from the target cells by treatment with 0.5% Triton-X. Total release was >97%, and spontaneous release ranged from 8–12% of the total release. The percent specific 51Cr release was calculated as follows: [(experimental cpm−spontaneous cpm)/(total cpm−spontaneous cpm)] × 100. A cytotoxic unit was defined as the number of cells required to cause 20% 51Cr release from 5 × 105 P815 target cells. In turn, cytotoxic units per mouse were obtained by the formula: number of PEC per mouse/number of PEC for one cytotoxic unit.

**Nitrite assay**

NO production by PEC was measured in supernatants collected after 20 h of culture, as described previously (33). Briefly, 5 μl of Griess reagent (prepared with reagents from Sigma) was added to 50 μl of supernatant, and absorbance was read at 550 nm using an automated plate reader. Nitrite concentration was calculated from a NaNO2 standard curve.

**Data presentation**

Data reported are means ± 1 SD from a representative experiment. All of the experiments reported in this study were repeated two to three times with an identical pattern of results, except for results in Fig. 5, which are from one experiment. ANOVA and Newman-Keuls were used to analyze statistical significance.

**Results and Discussion**

**Properties of experimental model used to investigate the role of macrophage-derived NO in suppressing CTL production in vivo**

As described in a previous study (28), mice were first vaccinated against the P815 tumor by injecting 2.5 × 106 tumor cells admixed with *C. parvum* in the intradermal region of B6D2F1 mice (Fig. 1). It is known from previous studies (29) that this type of vaccination stimulates a vigorous CTL response that peaks as tumor regression commences, and then declines to undetectable levels as complete tumor regression occurs (about 14 days). Mice are then rechallenged with 5 × 105 P815 tumor cells in the peritoneal cavity 3 wk after the initial vaccination. Reimplantation of the P815 tumor triggers the development of a tumor-specific secondary CTL response that peaks at day 5 and is accompanied by up-regulation of macrophage NO production (28). Complete tumor rejection occurs by 5 days postchallenge.

**Inhibition of NO production in vivo augments the antitumor CTL response**

To investigate whether macrophage-derived NO plays a role in down-regulating CTL production, groups of four P815-preimmu-

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The cytotoxic activity observed in this model is mediated by tumor-infiltrating lymphocytes (28, 30). It can also be seen in Fig. 2 that the cytotoxic activity observed is specific for the P815 tumor. These results are consistent with the conclusion that inhibition of NO production does augment the total cytotoxic response observed. Indeed, if anything, analysis of the results in this way magnifies the difference between the NGMMA- and the PBS-treated or untreated groups.

Properties of NGMMA-augmented CTL response

Results in the foregoing section indicated that inhibition of NO production augmented the CTL response observed 5 days following implantation of tumor cells. Additional experiments were undertaken to characterize in more detail the properties of the NGMMA-augmented CTL response. In this regard, live tumor cells were injected in the experiments in the preceding section. Therefore, to investigate whether inhibition of NO production also augments the CTL response to a nonreplicating Ag, preimmunized mice were injected i.p. with 5 x 10^5 P815 tumor cells. It can be seen first in Fig. 2 that PEC from mice treated with NGMMA exhibited significantly more cytotoxic activity toward the P815 tumor than PEC from PBS-treated mice. That the augmented response observed resulted from CTL is supported by the additional finding in Fig. 2 that the cytotoxic activity observed is specific for the P815 tumor. These results are in keeping with those seen in a preceding publication showing that inhibition of macrophage NO production in vivo can augment the antitumor CTL response.

Inhibition of NO production in vitro increases the magnitude and duration of the antitumor CTL response

Results in the foregoing sections indicated that inhibition of NO production in vivo with NGMMA augments the antitumor CTL response. Also, macrophages recovered from NGMMA-treated mice produced less NO than macrophages from control-treated mice. Although these results are consistent with the conclusion that inhibition of macrophage NO production in vivo can augment the antitumor CTL response, it was considered important to determine whether inhibition of NO production in vivo by NGMMA also increased the total CTL response in the peritoneal cavity. To do this, cytotoxic units per mouse were determined by dividing cytotoxic activity into the number of leukocytes recovered from the peritoneal cavity of the different groups. Fig. 3C shows that inhibition of NO production does augment the total cytotoxic response observed. Indeed, if anything, analysis of the results in this way magnifies the difference between the NGMMA-treated and the PBS-treated or untreated groups.

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NGMMA augments the CTL response in vivo by inhibiting suppressor macrophage NO production, the possibility remained that the CTL response was enhanced because of some other physiological effect of inhibiting NO production. Therefore, experiments were undertaken to determine whether the ability of NGMMA to augment the CTL response could be directly attributed to macrophage NO production. To do this, PEC were harvested from preimmunized mice at 0, 1, 3, 5, and 7 days following P815 tumor implantation and cultured for 3 days in the presence or absence of NGMMA. Tumor implantations were staggered so that PEC were all removed for culture on the same day. No Ag was added to culture. It can be seen first in Fig. 4 that the CTL activity of the PEC when assayed directly (before culture) reached a peak 5 days following tumor implantation, as observed in a previous publication (28). When the PEC (pooled from the same groups of mice) were cultured for 3 days, it can be seen that the CTL activity that was present in day 5 PEC declines, and the CTL activity of PEC collected at other time points remains low. In marked contrast, duplicate cultures of day 3 PEC containing NGMMA (0.5 mM) exhibit markedly increased CTL activity, and the CTL activity of day 5 PEC was maintained. Cytotoxic activity after culture was measured by adding $^{51} \text{Cr}$-labeled target cells to wells identical to those used to initially measure cytotoxic activity. Therefore, the increased cytotoxic activity observed are a net increase rather than simply an increase on a cell per cell basis. Finally, the increased cytotoxic activity observed after culture in NGMMA was again specific for the P815 tumor (day 3, L5178Y 515% lysis; day 5, L5178Y 56% lysis). That NGMMA did inhibit NO production is indicated by the quantity of NO$_2$ present in the supernatants (shown in the corresponding histobars). As for the

**FIGURE 3.** Properties of NGMMA-augmented CTL response. A, Cytotoxic activity of PEC from NGMMA-treated, PBS-treated, or control preimmunized mice 5 or 7 days following injection of $2 \times 10^7$ mit-C-treated P815 tumor cells. B, Specificity of day 5 PEC cytotoxic activity from NGMMA-treated, PBS-treated, or control mice for the P815 tumor. C, Total cytotoxic units per peritoneal cavity of NGMMA-treated, PBS-treated, or control mice 5 and 7 days following injection of mit-C-treated tumor cells.

**FIGURE 4.** A, Inhibition of NO augments in vitro CTL response. In vivo CTL: initial cytotoxic activity of PEC harvested from P815-preimmunized mice (‘0’) or 1, 3, 5, or 7 days following rechallenge with $5 \times 10^5$ P815 tumor cells. Assay with $3 \times 10^5$ PEC and $5 \times 10^3$ labeled P815 target cells (E:T = 60:1). After 3-day culture: CTL activity of replicate wells was measured after 3 days of culture or after 3 days of culture in 0.5 mM NGMMA. NO$_2$ in the culture supernatants is shown in the histobars. B, Inhibition of NO or depletion of macrophages augments proliferation of PEC harvested 5 days postrechallenge with tumor cells.
Adoptive transfer of PEC into NGMMA-treated irradiated recipients reveals potent augmentation of the CTL response

Results in Fig. 3 suggested that the presence of the Alzet pump in the peritoneal cavity may have suppressed the CTL response there. In turn, the ability of NGMMA to augment the CTL response may have been somewhat masked. Separately, results in the preceding section showed that if PEC are removed 3 days postrechallenge and cultured in NGMMA, there is a dramatic enhancement of the CTL response. Therefore, to more fully assess the potential for augmenting the CTL responses by inhibiting NO, the in vivo experimental system was modified. Specifically, mice that had been preirradiated 3 days before were implanted i.p. with Alzet pumps delivering NGMMA or PBS. The next day, these mice were injected i.p. with $4 \times 10^7$ PEC from immune mice that had been challenged 3 days before with mit-C P815 as in Fig. 4. Because the irradiated recipients contained very few PEC (less than $2 \times 10^3$), they were very useful for studying the CTL response without the confounding variable of nonspecific inflammation caused by the Alzet pumps. The results in Fig. 5 show that the recipient mice receiving NGMMA exhibited a markedly enhanced CTL response. Specifically, 3 days following cell transfer, PEC from three NGMMA-treated recipients exhibited $29 \pm 10.3\%$ $^{51}$Cr release, while the three control pump recipients exhibited $14.5 \pm 7.7\%$ $^{51}$Cr release. By 5 days posttransfer, the enhancement of the CTL response became more pronounced in the NGMMA-treated group ($36.5 \pm 13.4\%$ vs $6.7 \pm 9.4\%$), indicating that inhibiting NO can augment both the magnitude and the duration of the CTL response.

In conclusion, the results in this communication provide evidence that macrophage-derived NO can inhibit a tumor-specific CTL response in vivo. The potential impact of NO-mediated immunoregulation is complex. In many diseases, macrophages (and NO, in particular) serve as one of the effector mechanisms (34). For example, in the experimental system reported in this study, macrophages probably act in concert with CTL to cause tumor rejection. In this type of circumstance, if the CTL response is more effective at causing tumor rejection than macrophages, then it may be possible to increase host resistance by inhibiting NO. Experiments are underway to test this hypothesis by determining if the augmentation of the CTL response that results from inhibiting NO observed in this study also augments overall host antitumor immunity. Recently published results (21) suggest that inhibiting NO can increase antitumor immunity. In other circumstances, in which macrophages are critically required for disease eradication, one would predict that inhibiting NO would be detrimental. Experimental evidence showing that inhibition of NO exacerbates *Leishmania* (35) and other infectious diseases (34) is consistent with this prediction. Even in circumstances in which macrophage NO is necessary, however, an excess production of NO could still have a negative effect by inhibiting the ability of T lymphocytes to optimally activate macrophages. In this connection, inhibiting NO has been reported (36) to enhance the primary immunologic response to *Listeria*, even though macrophages are known to be a key host defense mechanism (6). Relatedly, many of the descriptions of suppressor macrophages (12) have been with bacterial and parasitic infections involving macrophage-dependent host resistance. Of course, if the lymphocyte response is undesirable, such as in autoimmunity, then macrophage NO could serve a protective role (25). Recent evidence showing that treatment of mice with IL-12 before induction of experimental autoimmune uveitis lessens disease severity (37) is consistent with this postulate. Together, the present results, combined with these previous observations, suggest that macrophage-derived NO can be an important and exploitable down-regulatory element for lymphocyte responses in vivo.

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References