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High-Dose Granulocyte-Macrophage Colony–Stimulating Factor–Producing Vaccines Impair the Immune Response through the Recruitment of Myeloid Suppressor Cells

Paolo Serafini,[1] Rebecca Carbley,[1] Kimberly A. Noonan,[1] Gladys Tan,[1] Vincenzo Bronte,[2] and Ivan Borrello[1]

[1]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland; and [2]Department of Oncology and Surgical Sciences, Padova, Italy

ABSTRACT

Tumor vaccines have shown promise in early clinical trials. Among them, tumor cells genetically engineered to secrete biologically active granulocyte-macrophage colony–stimulating factor (GM-CSF) can generate a systemic antitumor immune response. Although the minimal required GM-CSF dose produced by modified tumor cells to achieve a measurable antitumor effect is well known, no data examined whether an upper therapeutic limit may exist for this vaccination strategy. Because recent data demonstrate an immunosuppressive effect of GM-CSF produced by growing tumors, we thus sought to determine whether high GM-CSF doses administered in a vaccine formulation could impair antitumor immunity. Using a vaccine strategy involving a GM-CSF–producing bystander cell line (B78H1-GM) admixed with autologous tumor, we assessed the impact of varying doses of GM-CSF while maintaining a constant antigen dose. Our results defined a threshold above which a GM-CSF–based vaccine not only lost its efficacy, but more importantly for its clinical implications resulted in substantial immunosuppression *in vivo*. Above this threshold, GM-CSF induced $Gr1^+/CD11b^+$ myeloid suppressor cells that substantially impaired antigen-specific T-cell responses and adversely affected antitumor immune responses *in vivo*. The dual effects of GM-CSF are mediated by the systemic and not local concentration of this cytokine. Myeloid suppressor cell-induced immunosuppression is mediated by nitric oxide production via inducible nitric oxide synthase (iNOS) because the specific iNOS inhibitor, L-NMMA, restored antigen-specific T-cell responsiveness *in vitro*. Taken together, our data demonstrated the negative impact of supra-therapeutic vaccine doses of GM-CSF and underscored the importance of identifying these critical variables in an effort to increase the therapeutic efficacy of tumor vaccines.

INTRODUCTION

Cytokine-secreting tumor vaccines have shown promise in preclinical models as well as in early clinical studies. Although many lymphokine genes have been transduced into tumor cells, granulocyte-macrophage colony–stimulating factor (GM-CSF) has been found to be more potent in generating a long-lived, specific, systemic antitumor response than any other single cytokine examined if GM-CSF production is above 35 ng per 10^6 cells per 24 hours (1, 2). Although the exact immunostimulatory action of GM-CSF is unclear, it is believed that it exerts its role by enhancing local recruitment of dendritic cells to the vaccine site and subsequently increasing antigen presentation. GM-CSF is a critical cytokine required in the differentiation of dendritic cells (3, 4).

High local GM-CSF concentrations may enhance dendritic cell presentation of tumor antigens. This is supported by the finding that the antitumor immunity was dependent on both $CD4^+$ (T-helper) and $CD8^+$ (T-cytotoxic) lymphocytes (5, 6). In addition, GM-CSF invokes both T helper 1 [Th1; interleukin (IL)-2– and γIFN-mediated cellular immunity] and T helper 2 (Th2; IL-4–, IL-5–, and IL-6–mediated humoral immunity and allergic responses) responses thus making it a potent immunostimulatory mediator (7). The injection of irradiated, GM-CSF–secreting tumor cells stimulates an intense local reaction consisting of dendritic cells, macrophages, and granulocytes (8). The accumulation of large numbers of professional antigen-presenting cells suggested that one function of GM-CSF in this model involved the augmentation of tumor antigen presentation (9).

Although a clear therapeutic benefit exists, an increasing body of literature demonstrates that the autocrine release of GM-CSF by growing tumors is capable of suppressing the immune response (10–12) and has correlated with spontaneous metastases (13). Moreover, as additional evidence of its immunosuppressive properties, GM-CSF treatment has been used in the treatment of experimental autoimmune thyroiditis in mouse models and has been shown to suppress autoreactive T cells through the generation of $CD4^+/CD25^+$ regulatory T cells (14). Considering the inhibitory effects generated by GM-CSF–producing tumors, we asked whether high GM-CSF doses delivered in a vaccine formulation would exert an equally negative impact on the generation of a tumor-specific immune response. Evidence of such a finding would have profound implications on the design of subsequent clinical studies.

The improvement in gene transfer technology and an increased understanding of the critical requirements for priming an effective antitumor response with GM-CSF–based vaccines permitted us to devise a vaccine formulation that substantially facilitates manufacturing with the development of a universal GM-CSF–producing bystander cell line (15). A formulation independently consisting of antigen and cytokine enables the production of a patient-specific vaccine with minimal inter-patient GM-CSF variability. Furthermore, varying the tumor to bystander ratio allows for the possibility to obtain a high-dose GM-CSF–releasing vaccine for clinical use. This possibility underscores the importance of determining whether a maximal dose limit exists and of examining the potential impact of the delivery of high cytokine doses in the vaccine setting.

Recombinant (12) or tumor-secreted GM-CSF (16, 17) can elicit an inhibitory myeloid population characterized as $CD11b^+/Gr1^+$ cells. We further examined the impact of GM-CSF dose in the vaccine formulation using a fixed antigen dose and varying amounts of GM-CSF produced by the MHC-negative, B16-derived cell line, B78H1-GM. This line is derived from a C57/Bl6 strain and as such is allogeneic to BALB/c mice (15). Here, we demonstrate the negative impact of high-dose GM-CSF vaccines and highlight the mechanisms whereby such a formulation suppresses T-cell immune responsiveness and impairs overall anti-tumor efficacy. This is the first description of an upper GM-CSF threshold that increases our understanding of the requirements to optimize vaccine-mediated antitumor immunity and sheds new light on the controversial activating or repressing role of GM-CSF on the immune system.

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Requests for reprints: Ivan Borrello, 1650 Orleans Street, Room 453, Baltimore, MD 21231. E-mail: borreiv@jhmi.edu.

MATERIALS AND METHODS

Mice

Six- to 8-week-old BALB/c mice were obtained from the NIH (Frederick, MD). TCR transgenic mice (6.5) expressing an $\alpha\beta$ T-cell receptor specific for influenza hemagglutinin peptide (amino acids 110–120) presented by I-E^d were a gift from Harald von Boehmer (Basel, Switzerland) (18). These 6.5 mice were crossed to a BALB/c background for more than 10 generations. The transgenic mice used in these experiments were heterozygous for the transgene and Thy1.1+. All experiments involving the use of mice were in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Tumor Cells

A20 cells were obtained from the American Type Culture Collection (Rockville, MD). A20HA was generated by electroporation-mediated plasmid transfection, and transfected cells were selected and grown as previously reported (19). B78H1-WT cells are a class I and II negative melanoma cell line described elsewhere (15). B78H1-GM was generated by electroporation-mediated plasmid transfection and grown in hygromycin selection (1200 ng/mL). B78H1-GM secrete up to 6000 ng of GM-CSF/10^6 cells/24 hours as determined by ELISA.

Adoptive Transfer

Single-cell suspensions were made from peripheral lymph nodes and spleens collected from TCR transgenic mice. The percentage of double-positive lymphocytes for CD4 and the clonotypic TCR (6.5) was determined by flow cytometry as described below. Cells were washed three times in sterile HBSS and injected into the tail vein of BALB/c donors such that a total of 2.5×10^6 $CD4^+$ anti-hemagglutinin TCR^+ T cells were transferred to each recipient. A20HA tumor cells (1×10^5 per mouse) were washed three times in sterile HBSS and given to mice by i.v. injection in a volume of 0.2 mL into the tail vein.

Vaccination and Tumor Inoculation

Bystander Vaccination. A20WT or A20HA was admixed with B78H1-GM and/or B78H1-WT cells. The mixture was prepared in different ratios according to the desired dose of GM-CSF in each vaccine formulation. The cells were then washed three times with HBBS, γ-irradiated (5000 rad), and injected s.c. in 100 μL of HBSS.

Vaccinia Vaccination. A recombination vaccinia virus encoding hemagglutinin (VaccHA) from the 1034 strain of influenza virus was a generous gift of Frank Guarnieri (Johns Hopkins University). VaccHA was expanded on Hu-TK^- cells in the presence of 5-bromo-2′-deoxyuridine (Sigma, St. Louis, MO) at 25 μg/mL and purified from the cellular lysate by sucrose banding and titered by plaque assay on BSC-1 cells. Mice were given 1×10^7 plaque-forming units of VaccHA by s.c. injection or i.p. injection, in a total volume of 0.1 mL.

A20WT tumor cells were washed three times in sterile HBSS and given to the mice by i.v. injection in a volume of 0.2 mL into the tail vein (1×10^5 tumor cells per mouse).

Flow Cytometric Analysis

Splenic T cells were obtained by passing splenocytes through nylon wool. Clonotypic hemagglutinin-specific CD4 cells were stained with the biotinylated rat anticlonotypic TCR Mab 6.5 followed by phycoerythrin-conjugated streptavidin. They were then stained with FITC-conjugated antimouse Thy1.1 (Thy1.1-FITC; BD-PharMingen San Jose, CA), cychrome-conjugated antimouse CD4 (CD4-Cy; BD-PharMingen), and CD8 cells were stained with FITC-conjugated antimouse Thy1.1 and cychrome-conjugated antimouse CD8.

In another experiment, allophycocianine-conjugated antimouse CD4 and cychrome-antimouse CD3 were used. A total of 35,000 events were collected for each sample on a FACScan flow cytometer, and the data were analyzed using Cell Quest software (Becton Dickinson, San Jose, CA). Data represent the mean ± SE of the percentage of cells expressing the clonotypic TCR.

Proliferation

Splenocytes (4×10^5/well) were collected from BALB/c mice containing (2.5×10^6) 6.5 adoptively transferred transgenic T cells that were vaccinated with VaccHA (10^7 plaque-forming units) and sacrificed 3 days later. The splenocytes were then stimulated with 5 μg/mL of the MHC class II-restricted hemagglutinin peptide (amino acids 110–120; SFERFEIFPKE) in a 96-flat well plate (BD-PharMingen). Three days later, the cells were harvested, labeled for 6.5, CD4, and CD3, and analyzed by fluorescence-activated cell sorting. The number of clonotypic T cells was calculated by multiplying the total number of cells recovered by the percentage of triple-positive cells. Values are displayed as the mean ± SE of triplicate wells. Data are derived from one experiment representative of a total of three separate experiments.

Chemicals

L-Norvaline and L-NMMA were purchased from Calbiochem (San Diego, CA) and used as described previously (20).

Enzyme-Linked Immunosorbent Assay

GM-CSF ELISA was performed on serum from B78H1-GM–vaccinated mice and supernatant of the cultured B78H1-GM cells using the mouse GM-CSF ELISA kit (Endogen/Pierce Biotechnologies, Rockford, IL) following the manufacturer's instructions.

Cell Purification

CD11b purification was performed with the Mouse CD11b MicroBeads (Miltenyi Biotec, Bergish-Gladbach, Germany), and positive and negative fractions were sorted with the LV columns following the manufacturer's instructions. In experiments requiring negative selection of 6.5^+ cells, B220, anti-class II, and anti-CD8 biotin-conjugated antibodies were used with the Dynabeads M-280 Streptavidin kit (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed with Epi Info v 3.01[3] for survival Mann-Whitney/Wilcoxon analysis and with Sigma plot v 8.0[4] for Student's *t* test for graph and area under the curve calculations.

RESULTS

High-Dose Granulocyte-Macrophage Colony–Stimulating Factor Vaccines Fail to Generate Antitumor Immunity in A20-Bearing Mice. We sought to explore the impact of escalating GM-CSF doses on overall survival using the bystander vaccine with a fixed concentration of antigen in the vaccine formulation (15). Mice were challenged with 10^5 A20WT cells i.v. Five days later, they were vaccinated with 10^6 A20WT-irradiated cells admixed with irradiated B78H1-GM and B78H1-WT titered to release the desired GM-CSF dose. With this design, we were able to maintain a constant antigen dose of A20WT and vary the GM-CSF dose released by the whole vaccine. Furthermore, by adding B78H1-WT as the number of B78H1-GM cells was reduced in the vaccine formulations containing less GM-CSF, we maintained a fixed bystander to tumor ratio that enabled us to determine whether ineffective antitumor efficacy at the higher vaccine doses was due to steric hindrance generated by non-tumor antigen cells preventing effective capture of the tumor antigens or to the immune suppressive effects of high GM-CSF levels. As previously reported (21), we confirmed the efficacy of GM-CSF–based vaccines in imparting measurable antitumor efficacy when administrated at low doses. As shown in Fig. 1*A* the groups vaccinated with 30 or 300 ng of GM-CSF–producing vaccine had a significant

[3] Internet address: http://www.cdc.gov/epiinfo.
[4] Internet address: http://www.sigmaplot.com.

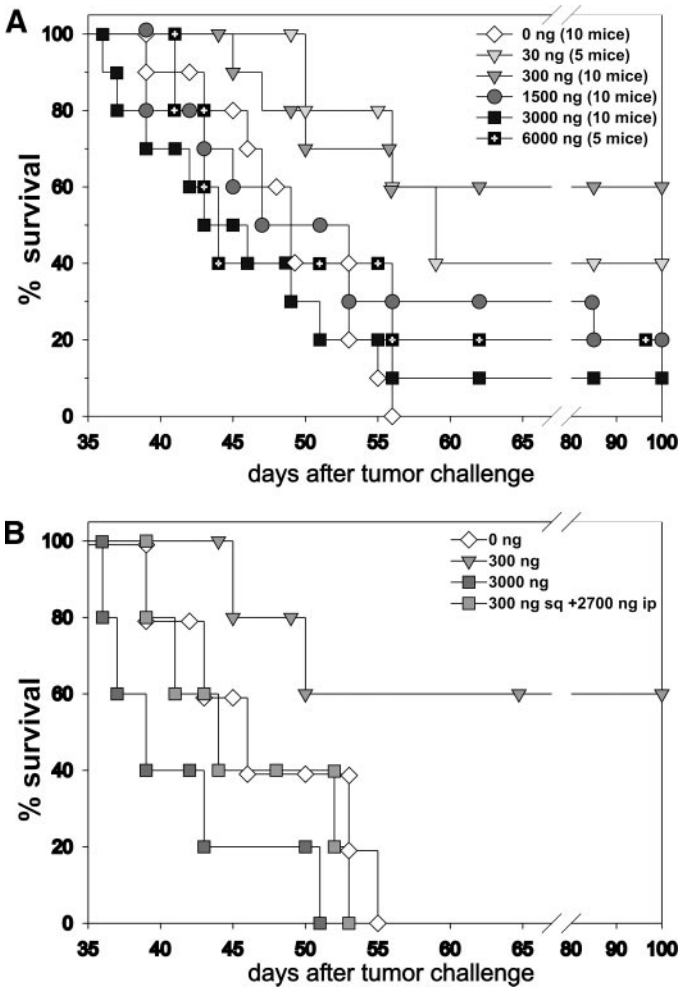

Fig. 1. High dose GM-CSF vaccines impair the immune response in tumor bearing mice. *A*. A20WT tumor-bearing mice (1×10^5 cells i.v.) were immunized with vaccines composed of γ-irradiated A20WT cells (10^6) admixed with different ratios of B78H1-GM/B78H1-WT bystander cells 5 days later as described in Materials and Methods. Each vaccine secreted the GM-CSF concentration as reported in the legend. Mice were followed for tumor-free survival. Data derived from two independent experiments. Statistical differences: 300 *versus* 0, $P < 0.01$; 300 *versus* 1500, $P = 0.062$; 300 *versus* 3000, $P < 0.01$; 300 *versus* 6000, $P = 0.04$. *B*. A20WT-bearing mice were immunized 5 days after tumor challenge as described in *A*. One group (300 ng + 2700 ng) received the 300-ng GM-CSF vaccine s.c. and 2700 ng of GM-CSF–secreting cells i.p. (2700 + 300 *versus* 3000, $P = 0.44$; 2700 + 300 *versus* 300, $P = 0.05$).

survival advantage (Mann-Whitney/Wilcoxon, $P = 0.01$ and 0.004, respectively) compared with the control group vaccinated with A20WT and the non-GM-CSF–producing bystander cell B78H1-WT. Surprisingly, the groups immunized with higher GM-CSF–producing vaccines (1500, 3000, or 6000 ng/10^6 cells/24 hours) showed no survival advantage over the control group (Mann-Whitney/Wilcoxon, $P > 0.5$). These data suggest for the first time that there is an upper GM-CSF limit above which GM-CSF–based vaccines impair the generation of tumor-specific immune responses. To understand whether this effect was due to the local secretion of GM-CSF at the vaccine site or a result of the systemic effect of high serum levels of GM-CSF, we repeated the experiment in A20WT tumor-bearing mice comparing the antitumor effect of a "nontherapeutic" vaccine consisting of A20WT and B78H1-GM (3000 ng/10^6 cells/24 hours) administered as a single vaccine s.c. with a "therapeutic" vaccine of A20WT and B78H1-GM (300 ng/10^6 cells/24 hours) s.c. with the additional administration of B78H1-GM bystander cells i.p. at 2700 ng/10^6 cells/24 hours to achieve a total GM-CSF systemic dose of 3000 ng/10^6 cells/24 hours. Tumor-bearing mice vaccinated with A20WT and with GM-CSF at 0 or 300 ng/10^6 cells/24 hours served as negative or positive controls, respectively. The data shown in Fig. 1*B* suggest that the systemic and not local concentration of GM-CSF is responsible for the impaired antitumor response observed with high GM-CSF–producing vaccines.

High-Dose Granulocyte-Macrophage Colony–Stimulating Factor–Secreting Vaccines Fail to Induce the Expansion of a Preexisting Population of Tumor-Specific T Cells. $CD4^+$ T cells are critical effectors of the antitumor immune responses (6). To examine the impact of escalating doses of GM-CSF on $CD4^+$ mediated antitumor immunity, we used the A20 lymphoma model modified to express the influenza hemagglutinin (HA) protein, which serves as a model tumor antigen (22). Hemagglutinin-specific transgenic $CD4^+$ cells specific for the I-E^d–restricted hemagglutinin$_{110-120}$ peptide (6.5 cells) were adoptively transferred into BALB/c mice on day 0. On days 3 and 9, mice were immunized with 0, 300, or 6000 ng of GM-CSF–secreting vaccine consisting of B78H1-GM cells (or B78H1-WT for the 0 ng group) and A20HA cells. As shown in Fig. 2, only the mice vaccinated with the therapeutic dose of GM-CSF vaccine exhibit a substantial expansion of hemagglutinin-specific $CD4^+$ T cells. In contrast, mice vaccinated with the higher GM-CSF dose demonstrated $CD4^+$ T-cell levels equivalent to the vaccinated group without GM-CSF in terms of clonotypic expansion. This lack of T-cell expansion with high-dose GM-CSF reflects a reduced antigen-specific proliferation during both the priming and boost phases of vaccination, leading to an overall blunted T-cell response (data not shown).

High-Dose Granulocyte-Macrophage Colony–Stimulating Factor–Secreting Vaccines Induce the Transient Appearance of Myeloid Suppressor Cells Early Post-Vaccination. A key to successful GM-CSF–based vaccination is the ability to recruit large numbers of antigen-presenting cells (23). The failure to achieve measurable antitumor protection with the high-producing GM-CSF vaccine could be due to inadequate antigen-presenting cell recruitment, processing, or presentation, or to the appearance of a population of suppressor cells. We have previously shown that GM-CSF secreted by

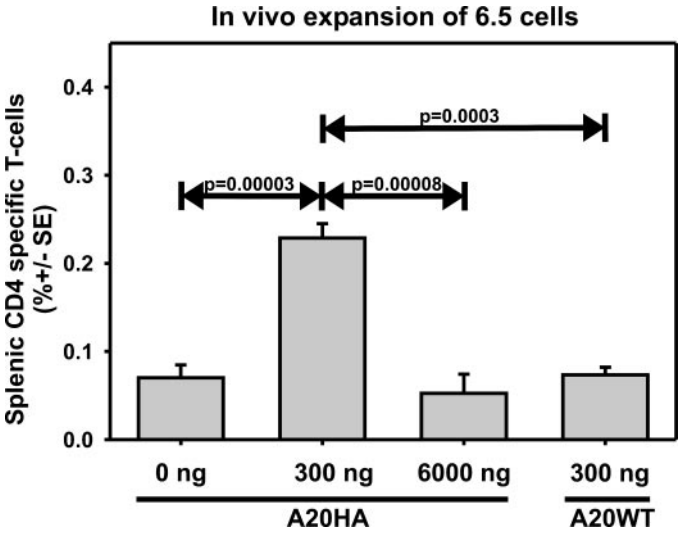

Fig. 2. A preexisting population of antitumor CD4+ T cells fails to expand after immunization with a 6000-ng GM-CSF–secreting vaccine. Hemagglutinin-specific 6.5 $CD4^+$ T cells (2.5×10^6) were injected i.v. on day 0. On days 3 and 9, the mice were vaccinated with γ-irradiated A20HA admixed with 0 ng (B78H1-WT), 300 ng, or 6000 ng per vaccine B78H1-GM cells. As a negative control, a group was vaccinated with A20WT cells with the therapeutic dose of GM-CSF (300 ng of B78H1-GM). On day 14, the mice were sacrificed, and clonal expansion in the spleen was analyzed by fluorescence-activated cell sorting as described in Materials and Methods. Data are derived from two independent experiments consisting of three mice per group.

growing tumors induced substantial immunosuppression by recruitment of an immature suppressive myeloid population. These myeloid suppressor cells were characterized as $CD11b^+$ and $Gr1^+$ (24, 25). Another recent observation correlated GM-CSF treatment with the recruitment of $CD4^+CD25^+$ T regulatory cells (14). To determine whether these mechanisms are also involved in the immune suppression observed in the nontherapeutic, high-dose GM-CSF vaccine, we performed a time-course experiment to examine the induction of myeloid suppressor cells post-vaccination. Three cohorts of mice were vaccinated with the A20WT/B78H1-GM vaccine at the 300-ng (therapeutic vaccine), 1500-ng, or 6000-ng (supra-therapeutic vaccine) dose. Mice were sacrificed at different time points, and blood, spleen, and draining as well as nondraining lymph nodes were analyzed for the presence of varying suppressive populations (regulatory T cells, $CD4^+/CD25^+$; myeloid suppressor cells, $CD11b^+/Gr1^+$). The only suppressive cell population directly correlating with increased GM-CSF doses and inversely with survival was the myeloid suppressor cell population (Fig. 3). Interestingly, in all groups, the serum GM-CSF kinetics peaked at 24 to 36 hours. To determine the overall impact of varying GM-CSF vaccine doses, we examined the area under the curve as a measurement of the biological concentration of GM-CSF. The GM-CSF areas under the curve were 7723, 206, and 58 pg for vaccine doses of 6000, 1500, and 300 ng per 10^6 cells per 24 hours, respectively. The maximal myeloid suppressor cell concentration peaked by day 3 and was most pronounced in the spleens reaching 18 to 20% of the total cellular population. The draining lymph nodes reached a total concentration of 1.1% *versus* 0.45% in the nondraining in those mice vaccinated with the high-dose GM-CSF vaccine. In contrast, no detectable increases over baseline were observed in mice that received the therapeutic vaccine.

By comparing the 1500-ng with the 6000-ng cohort of mice, it seems that the serum GM-CSF concentration does not directly correlate with the amount of myeloid suppressor cells in the spleens or in the lymph nodes. Instead, GM-CSF imparts an all-or-none signal to the immune system that is dictated by the intrinsic upper threshold, which in our model was seen with an area under the curve of 206 (1500 ng per 10^6 per 24 hours).

$CD11b^+$ but not $CD11b^-$ Cells Suppress the Proliferation of Activated $CD4^+$ Cells. To evaluate the suppressive capacity of the vaccine-generated myeloid suppressor cells, mice were vaccinated with either a nontherapeutic (6000 ng) or therapeutic (300 ng) GM-CSF–secreting vaccine composed of B78H1-GM bystander cells admixed with 10^6 A20 lymphoma cells transfected with the hemagglutinin gene (HA; A20HA). The mice were sacrificed 3 days later at the time of maximal myeloid suppressor cell expansion (Fig. 3), and myeloid suppressor cells were magnetically sorted from splenocytes with CD11b antibodies conjugated to magnetic beads to greater than 95% purity (data not shown). To test their suppressive ability, we examined the proliferative capacity of hemagglutinin-specific 6.5 $CD4^+$ cells previously primed *in vivo* with VaccHA to proliferate in response to the nominal peptide *in vitro*. Hemagglutinin-specific proliferation was completely abrogated by the addition of $CD11b^+$ 6000-ng–derived cells but not by $CD11b^-$ cells obtained from the same mice or $CD11b^+$ or $CD11b^-$ cells obtained from the 300-ng GM-CSF–vaccinated mice (Fig. 4). These cells were added to the splenocytes containing hemagglutinin-specific T cells at a final concentration of 20%—the same percentage of myeloid suppressor cells found in the spleens of mice vaccinated with the high nontherapeutic dose of the GM-CSF–secreting vaccine. From this experiment, we conclude that high-dose GM-CSF vaccines not only recruit a $CD11b^+$ $GR1^+$ population, but these cells are able to functionally impair $CD4^+$ hemagglutinin-specific T cells. In contrast, the $CD11b^+$ population from a therapeutic GM-CSF vaccine dose shows no suppressive activity.

Myeloid Suppressor Cells Impair 6.5 Expansion by a Nitric Oxide-Dependent Mechanism. We and others have reported previously that myeloid suppressor cells can impair the immune response by the selective activation of enzymes involved in L-arginine metabolism (26, 27). In a mixed Th-1–Th-2 environment, myeloid suppressor cells impair CTL function via arginase 1 and iNOS coexpression

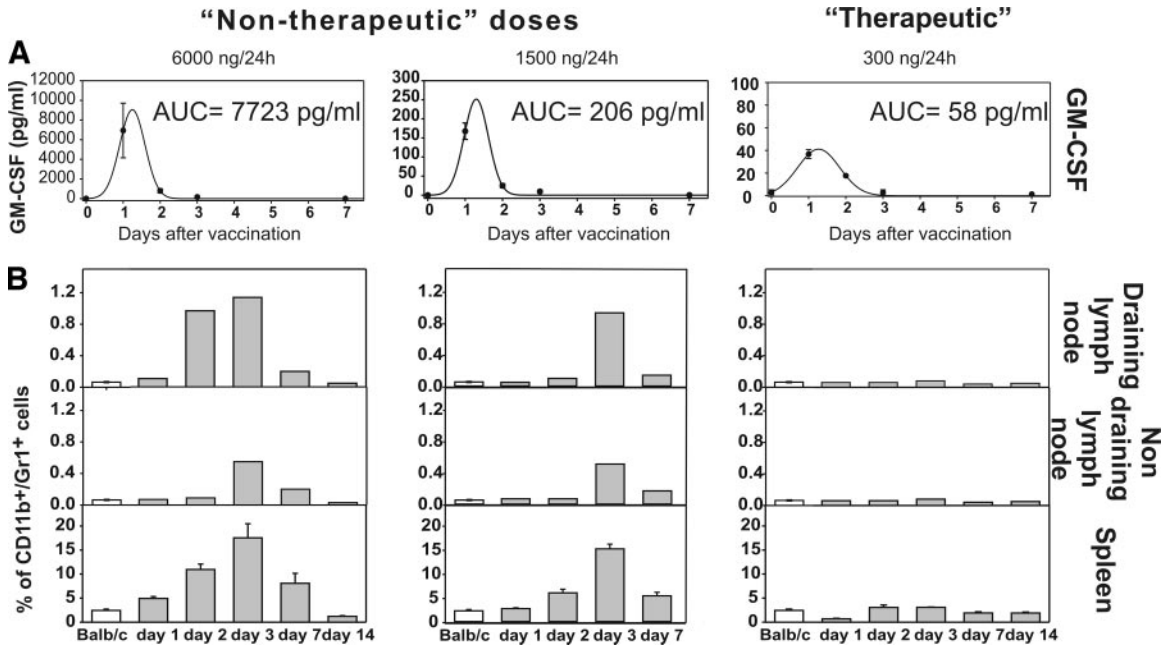

Fig. 3. High-dose GM-CSF vaccines induce myeloid suppressor cells. Mice were vaccinated s.c. with γ-irradiated A20WT cells admixed with different amounts of bystander cells secreting the indicated doses of GM-CSF. *A*. At different time points post-vaccination, mice were sacrificed, and the GM-CSF concentration in the serum was determined by ELISA. *B*. At the same time points, the spleens, draining, and nondraining lymph nodes were collected and stained with CD11b and Gr1 and analyzed by fluorescence-activated cell sorting. Data (mean ± SE) are derived from three mice for each time point. The spleens were analyzed separately, whereas the lymph nodes were pooled for analysis. The area under the curve (*AUC*) was calculated on the three-variable Gaussian curve that best fit the experimental data points.

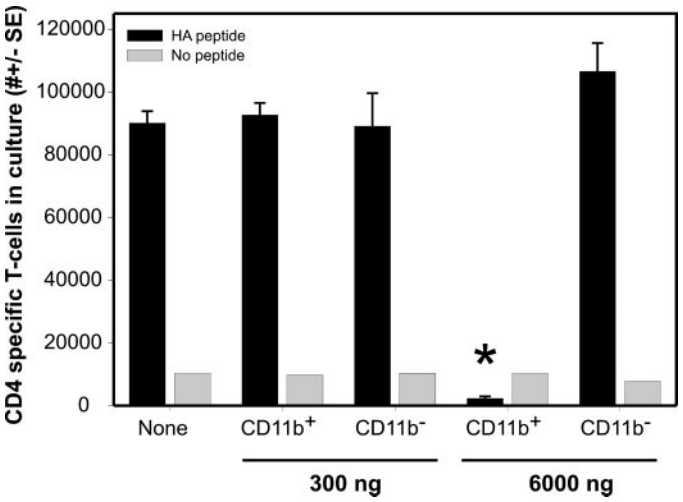

Fig. 4. CD11b$^+$ cells impair the antigen-specific CD4$^+$ T-cell proliferation. Mice were vaccinated with A20HA (10^6) admixed with B78H1-GM (10^6) producing 6000 ng of GM-CSF. They were sacrificed 3 days later. The splenocytes were pooled, and CD11b$^+$ cells were magnetically sorted as described in Materials and Methods. A mixed lymphocyte peptide culture was performed as described with the addition of the 6000 ng of vaccine-"primed" CD11b$^+$ or CD11b$^-$ cells to a final concentration of 20% of total cells. As a control, no additional cells or 300 ng of vaccine-primed CD11b$^+$ or CD11b$^-$ were added at the same concentration. Proliferation was measured as the clonotypic expansion by fluorescence-activated cell sorting on day 3 of 6.5$^+$/CD4$^+$ T cells. *, statistically different from all other groups; $P < 0.01$.

leading to peroxynitrite formation and the induction of apoptosis (20). A nontherapeutic vaccine consisting of A20HA and a 6000-ng bystander vaccine significantly reduces the number of CD8$^+$ hemagglutinin-specific T cells on a per cell basis, although no reduction in their proliferative response or γ-IFN production to the nominal peptide is observed (data not shown). In a Th-2 environment, arginase 1 alone prevents CTL proliferation by depleting the microenvironment of the semi-essential amino acid L-arginine (27). Finally, in a Th-1 environment, myeloid suppressor cells impair the downstream signaling of the IL-2 receptor and thus impair CTL proliferation through nitric oxide production via iNOS overexpression (28). To understand which of these mechanisms was responsible for CD4+ T-cell inactivation in this model, we used well-characterized *in vitro* inhibitors of iNOS (L-NMMA) and arginase-1 (L-norvaline). Splenocytes were obtained from mice vaccinated 3 days before with the 0-ng, 300-ng, or 6000-ng GM-CSF–secreting vaccine (B78H1-GM admixed with A20HA) and were used as antigen-presenting cells in a proliferation assay. Hemagglutinin-specific 6.5 CD4$^+$ cells were adoptively transferred into another cohort of mice, *in vivo* primed with VaccHA, and sacrificed 2 days later. The CD4$^+$ T cells were then purified by depletion of CD8$^+$ cells, B cells, and MHC class II-expressing cells. Hemagglutinin-specific expansion to the nominal peptide was used as the readout of T-cell response in culture with antigen-presenting cells isolated from the different vaccine groups either in the presence or absence of iNOS or arginase-1 inhibitors. L-Norvaline was unable to restore the proliferation of 6.5$^+$ cells (data not shown). However, L-NMMA alone completely abrogated the myeloid suppressor cell-mediated T-cell inhibition (Fig. 5). These data strongly suggest that the high-dose GM-CSF vaccine mediates myeloid suppressor cell immunosuppression via an iNOS-dependent pathway, thus identifying a potential mechanism that can be targeted to enhance vaccine efficacy.

DISCUSSION

Using a murine model, we report the existence of an upper therapeutic limit of GM-CSF when administered in a vaccine formulation. Furthermore, we demonstrate that GM-CSF–producing vaccines can either stimulate or suppress tumor-specific immunity in a dose-dependent manner. This duality of biological efficacy of GM-CSF has been extensively reported. GM-CSF is an essential growth factor in the maturation of dendritic cells and likely augments the antitumor immune response of vaccination through the recruitment of antigen-presenting cells to the vaccination site, which is the underlying rationale for the subsequent clinical development of GM-CSF–based vaccine strategies (4, 29, 30). In contrast, GM-CSF also plays a prominent role in the negative regulation of the immune response through the induction of myeloid suppressor Gr1$^+$/CD11b$^+$ cells (31, 32). The inhibitory effect of GM-CSF vaccines is determined by the systemic and not local secretion of GM-CSF. Myeloid suppressor cells are only observed in conditions in which the systemic levels of GM-CSF exceed a certain threshold and mediate T-cell unresponsiveness. Furthermore, the immunosuppression associated with the induction of myeloid suppressor cells can be reversed with iNOS-specific inhibitors.

Because the initial observation that transduction of GM-CSF into tumor cells could potentiate the immune response of less immunogenic tumors, a minimal GM-CSF dose required to generate a measurable antitumor response was identified as 36 ng per 10^6 cells per 24 hours (1). Although several early phase I vaccine studies show evidence of antitumor activity, the direct contribution of antigen dose *versus* GM-CSF dose cannot be dissected for the following reasons. First, most vaccines used clinically to date consist of a tumor cell (autologous or allogeneic) that is also the source of GM-CSF (33–37). Second, the total amount of GM-CSF in many of these vaccine formulations was less than 100 μg per vaccination. The bystander vaccine strategy not only facilitates vaccine manufacturing and pro-

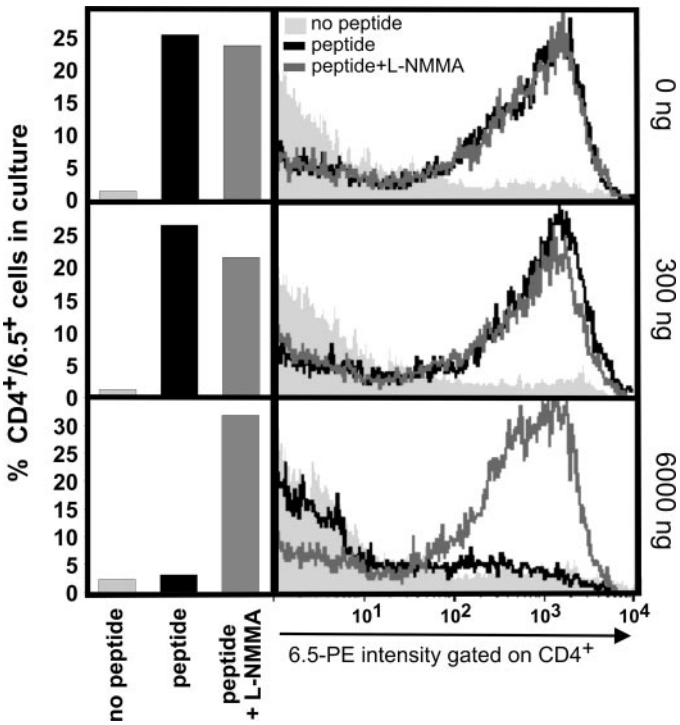

Fig. 5. L-NMMA reverts the myeloid suppressor cell-mediated immunosuppression. Mice were vaccinated with 10^6 γ-irradiated A20HA admixed with B78H1 bystander cells secreting 0, 300, or 6000 ng of GM-CSF and sacrificed 3 days later. The splenocytes from each group were harvested and added to *in vivo* VaccHA-primed hemagglutinin-specific CD4$^+$ T cells stimulated in the presence or absence of the nominal peptide. L-NMMA was added as indicated. After 3 days, the cells were stained with the anti-CD4 and anti-6.5 hemagglutinin-specific clonotypic antibody. Data are from one of three representative experiments.

duction, but also permits a detailed analysis of the individual contribution of the two components of the vaccine: antigen and cytokine dose. Whereas most studies examining the inhibitory effect of GM-CSF focused on the endogenous release of this cytokine from tumors during the course of disease progression, this is the first description of the inhibitory role of high-dose GM-CSF administered in a vaccine formulation. The GM-CSF–bystander approach enabled us to dissect the role of the cytokine as an independent variable in the vaccine formulation and to examine the impact of varying GM-CSF doses coadministered with a fixed dose of antigen.

Our data demonstrate how the fate of the immune response generated by the vaccination is strictly dependent on the GM-CSF dose. Whereas a linear relationship exists between the GM-CSF dose delivered by the vaccine and the systemic cytokine levels achieved, we show the existence of a maximal therapeutic threshold. Above this limit, GM-CSF induces the recruitment of myeloid suppressor cells, leading to the inhibition of the T-cell response and ultimately decreased vaccine efficacy (Figs. 2 and 4; ref. 24). In contrast, lower GM-CSF levels do not induce a measurable increase in myeloid suppressor cells and can prime effective immune responses leading to measurable tumor regression. These data demonstrate that GM-CSF–based vaccines can exert either an immunostimulatory or immunosuppressive function mediated by the induction of myeloid suppressor cells that is completely determined by the amount of GM-CSF delivered in the vaccine formulation. The discovery of an upper dose limit of GM-CSF in this murine model will have a substantial impact on the design of future GM-CSF–based vaccine trials.

Although we have clearly identified an upper therapeutic GM-CSF dose in our model and demonstrated the relationship between the vaccine-mediated induction of myeloid suppressor cells and immunosuppression, the identification of the equivalent dose and/or variables to follow in the clinical setting presents several challenges. Interspecies differences of cytokine efficacy, half-life, and catabolism do not permit the use of a simplified calculation to identify the upper therapeutic limit in humans. Furthermore, the murine model is characterized by genetic uniformity that minimizes differences in serum GM-CSF levels within each group. In contrast, humans who received the same recombinant GM-CSF dose demonstrate significant differences in peak serum concentrations (38). For this reason, it is difficult to identify a precise dose of GM-CSF to be used in a vaccine formulation. Other biological variables need to be identified and used to define vaccine efficacy. One possible variable is the induction of myeloid suppressor cells. As shown in Fig. 3, no myeloid suppressor cells were observed at baseline or at the therapeutic vaccine dose but were detectable with the nontherapeutic vaccination. Their presence is clearly associated with the ability to impair T-cell responses (39) and likely represents a biological marker that could be used to monitor vaccine efficacy. Some reports have identified a $CD34^+$ population in humans with properties similar to murine myeloid suppressor cells (17), however these cells have not been phenotypically well characterized, and thus their use as a prognostic marker to assess vaccine efficacy is premature.

An alternative approach to maximize vaccine efficacy could be strategies aimed at reducing the immunosuppressive effects of myeloid suppressor cells. Although the effector mechanisms of myeloid suppressor cells are complex, L-arginine metabolism and nitric oxide have clearly been implicated in this immunosuppression and thus represent a potential therapeutic target (28). In addition to its immunosuppressive activity on T cells, constitutive expression of iNOS has also been implicated in tumor growth, angiogenesis, metastasis, and drug resistance (40) as well as T-cell–mediated antitumor immunity (6). In our experiments, antigen-specific proliferation in the presence of myeloid suppressor cells was completely restored by the addition of L-NMMA, suggesting that under these experimental conditions, the suppressive mechanism is iNOS dependent. Considering the confounding roles of iNOS on tumor growth and antitumor immunity, the impact of pharmacological inhibitors of iNOS will be primarily dependent on the prevalent mechanisms mediating immunosuppression.

Taken together, our data show the dual face of GM-CSF able to either enhance or restrain the immune response in a dose-dependent manner that implies a central role of this cytokine as a mediator of immune homeostasis. Furthermore, the inhibitory response induced by high-dose GM-CSF vaccines presents a new variable that will need to be considered in the future design of clinical trials. For this reason, subsequent GM-CSF–based trials need to be designed to maximize the antitumor efficacy and to minimize the possibility of immunosuppression. In addition, the development of effective biological markers of vaccine responsiveness as well as inhibitors of myeloid suppressor cell function will likely augment the efficacy of this approach, thereby establishing tumor vaccines as an important therapeutic strategy in the treatment of malignancies.

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High-Dose Granulocyte-Macrophage Colony-Stimulating Factor-Producing Vaccines Impair the Immune Response through the Recruitment of Myeloid Suppressor Cells

Paolo Serafini, Rebecca Carbley, Kimberly A. Noonan, et al.

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