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Nitric Oxide-Independent CTL Suppression during Tumor Progression: Association with Arginase-Producing (M2) Myeloid Cells[1]

Yuanqing Liu,[2] Jo A. Van Ginderachter, Lea Brys, Patrick De Baetselier, Geert Raes,[3] and Anja B. Geldhof[3,4]

Most of the mice bearing a s.c. BW-Sp3 lymphoma tumor mount a CD8$^+$ T cell-mediated response resulting in tumor regression. Nonetheless, tumor progression occurs in some of the recipients and is associated with CTL inactivity. We demonstrated that T cell-activating APC were induced in regressors whereas T cell suppressive myeloid cells predominated in the spleen of progressors. Indeed, in vitro depletion of either the adherent or the CD11b$^+$ populations restored T cell cytotoxicity and proliferation in these mice. This CTL inhibition was cell-to-cell contact-dependent but not mediated by NO. However, the same progressor suppressive cells prevented the activity of in vitro-restimulated CTLs derived from regressors in a cell-to-cell contact and NO-dependent fashion. Thus, either the NO-dependent or -independent suppressive pathway prevailed, depending on the target CTL population. In addition, the suppressive population expressed a high arginase activity, suggesting an association of the suppressive phenotype with alternatively activated (M2) myeloid cells. However, the high arginase activity is not directly involved in the suppressive process. Our results provide new insights for myeloid cell-mediated CTL inhibition during cancer progression. ***The Journal of Immunology,*** **2003, 170: 5064–5074.**

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The identification of tumor-associated Ags has provided evidence that antitumor responses can be generated in immune-competent individuals, resulting in tumor destruction (1). Since then, tremendous effort has been made to improve Ag-specific immune responses against cancer. Theoretically, optimal T cell activation requires at least two distinct signals (2, 3). Specific signal one is derived from the TCR interaction with the MHC-peptide complex and signal two is delivered to T cells via costimulatory molecules (such as CD28-B7 ligation) or cytokines (e.g. IL-2). In this context, genetically modified cancer cells with enhanced immunogenicity are able to elicit effective tumor rejection (4–11). But until now, little success has been achieved when treating hosts with an established tumor burden. Knowledge about the mechanisms underlying tumor-induced immune defects is still largely fragmental. Indeed, various types of immune components are implicated in the down-regulation of antitumor responses, such as CD4$^+$CD25$^+$ T regulatory cells (12), NKT cells (13), B cells (14), and myeloid suppressive cells (15–20). In parallel, different malignancies cause diverse defects in T cells, ranging from alteration of signal transduction (20, 21), induction of T cell tolerance/anergy (22), to triggering apoptosis in activated effector cells (23, 24).

Myeloid cells play critical roles in defending a host against intruding foreign pathogens and cancer (25, 26). More importantly, cells such as macrophages and dendritic cells are capable of bridging the innate to the acquired arm of the immune system through Ag presentation and release of cytokines (27–29). In contrast, immune antagonistic effects by cells of this lineage are broadly documented as well. Examples include splenic CD11b$^+$ and/or Gr-1$^+$ cells in mice treated with cyclophosphamide (30) or staphylococcal enterotoxin A (31), peritoneal exudate cells from mice challenged with parasites or parasite-derived oligosaccharide (32–34), monocytes in UV exposed skin (35), and myeloid cells obtained from tumor-bearing hosts (16, 17, 19, 36, 37). Based on whether the activated myeloid cells are operating in a type I- or type II-associated cytokine environment, two subtypes can be identified: the IFN-γ-dependent, classically activated myeloid cells (M1), and the IL-4- and/or IL-13-dependent, alternatively activated myeloid cells (M2) (38–43). IL-10 and TGF-β can possibly, in concert with IL-4/IL-13, further steer M2 differentiation (39, 40, 44–46). M1 cells metabolize L-arginine mainly via inducible NO synthase (iNOS)[5] and thus produce NO. NO and its reactive intermediates contribute to M1-mediated tumoricidal and immune suppressive activities (26, 47). In contrast, M2 cells are characterized by an alternative metabolic pathway for L-arginine, catalyzed by arginase, generating urea and polyamines. The latter nourish cell growth and promote tissue repair (48, 49). Activated myeloid cells with an increased arginase activity have been observed in several pathological conditions, such as type II response-associated parasite infections and tumor progression (42, 50). M2 cells are also

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[5] Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; L-NMMA, N^G-methyl-L-arginine; norNOHA, N-ω-hydroxy-nor-L-arginine.

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suggested to attenuate Th1 responses and induce peripheral tolerance (38, 51). However, how exactly these M2 cells exert suppression on functional T cells is virtually unknown.

Subcutaneously inoculated BW-Sp3 cells give rise to spontaneous tumor rejection in a fraction of the recipients and tumor progression in the others (7, 52). In the two types of tumor recipients, either CTL activation or immune down-regulation is observed, respectively. We searched to improve CTL responses in the BW tumor bearers by administrating genetically modified BW-Sp3 cells expressing IFN-γ, B7-1, B7-2, or CD40 ligand as therapeutic vaccines. Except for the IFN-γ/B7-1 double transfectants, none of these vaccines were able to counteract tumor progression (7, 52). Possibly, a profound immune suppression in progressors is hard to be rectified by only supplying additional tumor Ags and/or immunomodulatory factors. It was, thus, of our primary interest to determine the mechanisms underlying immune suppression during the course of in vivo tumor evolution. In the present investigation, we demonstrated a significant contribution of a splenic myeloid $CD11b^+$ population derived from tumor progressors in the down-regulation of CTL function. We analyzed the suppressive properties of these cells on different CTL populations obtained from regressors or progressors. Two distinct mechanisms were revealed: the inhibition of CTLs of regressors was cell-to-cell contact and NO-dependent whereas the inhibition of CTLs of progressors was cell-to-cell contact-dependent but NO-independent. In addition, the suppressive adherent population showed a high arginase activity suggesting that it contained M2 myeloid cells. The in vivo relevance of these results is discussed.

Materials and Methods

Mice and tumor growth

Pathogen-free, 6- to 9-wk-old female AKR mice (Thy 1.1, H-2^k), purchased from Harlan (Horst, The Netherlands), were used in all experiments. To obtain tumor bearers, mice were implanted s.c. into the right flank with 2×10^6 BW-Sp3 tumor cells.

Cell lines and media

The BW-Sp3 cell line was derived from the original spontaneous BW5147 (BW-O) T cell lymphoma (AKR origin; Salk Institute, La Jolla, CA) by in vitro and in vivo passages, as described previously (53). The generation of BW-Sp3 (B7-1) cells was described earlier (7). Tumor cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.03% L-glutamine, 100 mg/ml streptomycin, and 100 mg/ml penicillin (Life Technologies, Paisley, Scotland). To culture the splenocytes from tumor-bearing animals, the above RPMI 1640 medium was further supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate (Life Technologies) and 0.02 mM 2-ME (Merck, Schuchardt, Germany). Aminopterin and HT supplement (Life Technologies) were supplied to prevent the growth of BW cells. All cells were incubated at 37°C with 5% CO_2.

mAbs and reagents

The following mAbs were purchased from BD PharMingen (San Diego, CA): Abs (purified or labeled) recognizing mouse CD11b (M1/70), Gr-1 (Ly-6G, RB6-8C5), CD16/32 (FcγRII/III, 2.4G2), CD11c (HL3), H-$2D^k$ (15-5-5), I-A^k (11-5.2), I-E^k (17-3-3), and the isotype-matched controls used in flow cytometry, immunohistochemistry, and in vitro depletion. The PE-labeled anti-mouse F4/80 Abs were purchased from Serotec (Oxford, U.K.). The biotinylated rabbit anti-rat IgG, the Elite ABC kit, and the DAB substrate kit used in immunohistochemistry were purchased from Vector Laboratories (Burlingame, CA). The neutralizing rat-anti-mouse IFN-γ Ab (F3) was purchased from Innogenetics (Zwijndrecht, Belgium). The FITC-labeled hamster anti-mouse CD80 (B7-1) (2G10) and CD86 (B7-2) were kind gifts of Prof. K. Thielemans (Academisch Ziekenhuis, Vrije Universiteit Brussel, Brussel, Belgium).

Detection of surface Ag expression in flow cytometry

Adherent cells were recuperated from 5-day splenocyte cultures by gentle scraping after a 30-min treatment with 0.5 mM EDTA. Cells were resuspended in PBS and incubated with appropriately diluted FITC-or PE-conjugated Abs on ice for 30 min. After extensive washing with PBS, cells were measured with a FACSVantage station (BD Biosciences, San Jose, CA) to determine the expression of surface Ags. To analyze the amount of $CD11b^+$ and/or Gr-1^+ cells in the spleen, fresh cells from homogenized spleens were incubated with appropriately diluted FITC-conjugated anti-CD11b and PE-coupled anti-Gr-1 Abs at 4°C for 30 min. Positive cells were determined with a FACSVantage station and data were analyzed with CellQuest software.

Immunohistochemistry

Immunohistochemistry was conducted on acetone-fixed frozen sections cut at 7 μm. Specimens were treated with 10% normal rabbit serum before incubation with appropriately diluted anti-mouse CD11b or control Abs. Following an extensive rinse in PBS, appropriately diluted biotinylated rabbit anti-rat IgG Abs were applied as secondary Abs. Positive cells were then stained by sequential incubation with avidin-biotin-peroxidase complex and diaminobenzidine tetrahydrochloride according to manufacturer's instruction. Specimens were briefly counterstained with hematoxylin before microscopic analysis.

Depletion of the splenic $CD11b^+$ population

Spleens were homogenized in erythrocyte lysis buffer and cells were resuspended in HBSS buffer containing 0.1% BSA. The cells at 4×10^7/ml were incubated with PE-coupled anti-CD11b Abs or the isotype controls for 10^7 splenocytes per microgram of Ab. Following incubation at 4°C for 30 min on a rotator, cells were washed and mixed with prewashed sheep anti-rat IgG Ab-coated dynabeads (Dynal, Oslo, Norway) at six to eight beads per positive cell. After another 30-min incubation at 4°C on a rotator, samples were placed in a Dynal magnetic concentrator to remove the $CD11b^+$ cells. The washout, negative cell fraction was collected for CTL restimulation. Aliquots of cells before and after one round of dynabead treatment were examined with flow cytometry for $CD11b^+$ cells.

Evaluation of CTL activity

Spleens of BW-Sp3 tumor bearers (progressors and regressors) 5–7 wk postinoculation were homogenized in RPMI 1640 medium and filtered through a nylon membrane. Approximately 2×10^7/well of splenocytes were cocultured with 10^6 irradiated BW-Sp3(B7-1) cells in six-well plates (Falcon; BD Labware, Franklin Lakes, NJ). In vitro restimulation was conducted for 5 days and the nonadherent cells recuperated from the CTL cultures were loaded on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) for gradient centrifugation. Viable cells collected on the interface were tested for the cytotoxicity toward ^{111}In-labeled BW targets. To label targets, the tumor cells were brought to 5×10^5 cells in 100 μl of RPMI 1640 medium containing 5% FCS and incubated with a $^{111}InCl_2$ (Amersham, Buckinghamshire, U.K.)/tropolon mixture (5 μCi) for 10 min. Following extensive washing, the target cells were placed in 96-well round-bottom plates at 5×10^3 cells/well and mixed with effector cells at designated E:T ratios. The percentage of specific lysis of target cells was determined via ^{111}In release in the supernatant after a 4-h incubation and was calculated as follows: (experimental release $-$ spontaneous release)/(maximal release $-$ spontaneous release) $\times$ 100%. The spontaneous release was determined from labeled target cells incubated without effector cells and the maximal lysis was measured from target cells lysed in 2% SDS 1 h before measuring.

To evaluate the modulatory activity of adherent cells on CTL activity, total splenocytes were initially cultured for 2 days to let the adherent cells adhere. Then, the adherent and nonadherent fractions were separated. The adherent cells remained in the original wells, while the nonadherent cells were transferred into fresh wells or into wells that contained an appropriate adherent population. In another experimental set-up, adherent cells were recuperated from the plates by gentle scraping after a 30-min treatment with 1 mM cold EDTA-PBS and then cocultured with nonadherent CTL populations. To test whether cell-to-cell contact was required for CTL inhibition, nonadherent cells were transferred into transwells (0.4-μm pore size membrane; Costar, Cambridge, MA) and the transwells containing nonadherent cells were placed in wells with an adherent population of choice. Coculture was conducted for another 3 days and the CTL activity was tested with nonadherent populations. To block NO production in the CTL cultures, a final concentration of 0.5 mM N^G-methyl-L-arginine (L-NMMA) (Sigma-Aldrich, Steinheim, Germany) or 10 μg/ml neutralizing anti-IFN-γ Abs were added into the cultures at day 2 of restimulation. To inhibit arginase activity, a final concentration of 0.2 mM N-ω-hydroxy-nor-L-arginine (norNOHA) (Bachem, Bubendorf, Germany) was added into cultures when restimulation was initiated.

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Cell proliferation assay

To evaluate the proliferation of splenic CTLs, splenocytes were plated at 8×10^5/well in 96-well plates in the presence of 4×10^4 irradiated BW-Sp3(B7-1) cells. The transfer of CTL populations and the addition of L-NMMA (0.5 mM) or anti-IFN-γ mAbs (10 μg/ml) were conducted at day 2. Cells were pulsed by addition of 1 μCi [^{3}H]thymidine (Amersham Biosciences, Bergrand, The Netherlands) and the incorporated radioactivity was measured 18 h later.

NO measurement

NO was measured as nitrite using Griess reagent. Culture supernatant (100 μl) was mixed with 100 μl of 1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% H_3PO_4. Absorbance was measured at 540 nm in a microplate reader.

Determination of arginase activity

Arginase activity in the adherent splenocytes was measured as described by Munder et al. (40) with minor modifications. Briefly, 1 million adherent cells were lysed in 100 μl of 0.1% Triton X-100. After 30 min on a shaker, 100 μl of 25 mM Tris-HCl and 35 μl of 10 mM $MnCl_2$ were added and arginase was activated by heating at 56°C for 10 min. L-arginine hydrolysis was conducted by incubating 40 μl of cell lysate with 40 μl of L-arginine (0.5 M, pH 9.7) at 37°C for 60 min. The reaction was stopped with 320 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1 v/3 v/7 v). The urea production was quantified at 540 nm after addition of 8 μl of 2% α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute.

Results

Differential activity of splenic adherent cells from tumor progressors vs regressors on antitumor CTLs

All mice inoculated s.c. with BW-Sp3 cells exhibit a progressive tumor growth in the first 2–3 wk. Subsequently, ~70% of the animals reject the tumor and exhibit a $CD8^+$ T cell-mediated antitumor immunity. However, tumor elimination fails in 30% of the recipients resulting in a further progressive tumor growth and an impaired antitumor CTL activity (7, 52). To investigate the mechanisms underlying the CTL inhibition, 5–7 wk after tumor inoculation mice were sacrificed and a comparison was made between tumor regressors (mice that completely rejected the tumor) and progressors (mice that bore a 15–20 mm^2 s.c. tumor). To evaluate tumor-specific CTL activity, splenocytes were restimulated in vitro with irradiated BW-Sp3(B7-1) cells for 5 days before testing the cytotoxic activity. During CTL restimulation, an outgrowth of plastic adherent cells was observed in the CTL cultures of tumor progressors. As compared with naive cultures, not only the number but also the morphology of adherent splenocytes changed from a mixed "fried egg"/fibroblast-like morphology in naive spleens to a more pronounced fibroblast-like morphology in progressors (data not shown). To test whether the adherent cells in progressor CTL cultures could modulate lymphocyte activity, adherent cells were removed from CTL cultures after 2 days of restimulation, and after 3 days of further culture CTL activity in the nonadherent fraction was monitored on BW tumor targets. CTLs obtained via this restimulation protocol were not lytic toward the MHC class I-negative, NK-sensitive variant BW-O (Refs. 7 and 53; see also Fig. 7) demonstrating the true CTL nature of these cells. Because BW-Sp3(B7-1) are more susceptible targets than the parental BW-Sp3 cells (7), we show in this paper only the CTL lysis on BW-Sp3(B7-1). As shown in Fig. 1*a*, the total spleen cells of progressors exhibited a low CTL activity after in vitro restimulation. However, this low CTL activity (8–11%) increased in three independent experiments (E:T ratio, 100:1) to 30–40% target cell lysis after removing the adherent cells. In contrast, a significant CTL activity was always detected in the splenocytes of tumor regressors, irrespective of whether the restimulation was conducted in the total splenic population or in the nonadherent fraction (Fig. 1*b*). Upon coculture with the adherent cells of progressors but not of naive mice, CTL activity in the nonadherent splenocytes of regressors drastically declined from 45–58% to 20–31% target lysis (E:T ratio, 100:1) (Fig. 1*b*).

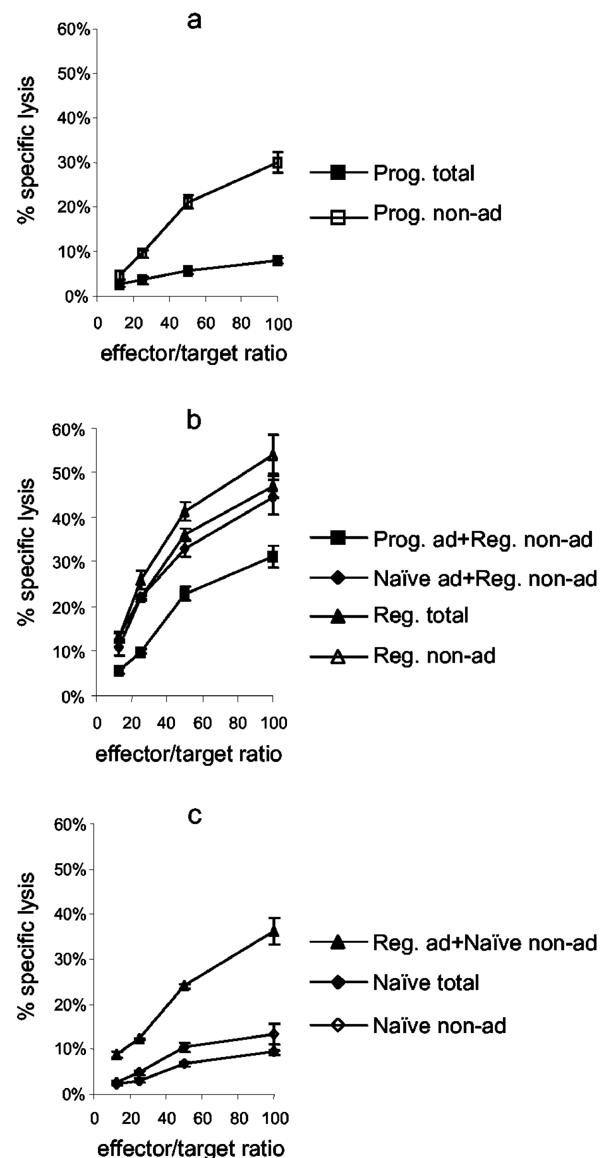

FIGURE 1. Splenic adherent populations modulate tumor-specific CTL activity in vitro. Homogenized splenocytes of naive mice, tumor progressors or regressors, 5–7 wk postinoculation, were restimulated in vitro with irradiated BW-Sp3(B7-1) cells for a total of 5 days. To test the function of adherent cells (ad), adherent and nonadherent cells (non-ad) were separated on day 2. The nonadherent cells were subsequently further cultured for 3 days, either in a fresh plate or on top of an adherent population of another origin as indicated. The cytotoxic activity was tested in the nonadherent cell fraction using ^{111}In-labeled BW-Sp3(B7-1) cells as targets. Results show the average ^{111}In release ± SD of triplicates. Similar results were obtained from at least three independent experiments. *a*, Modulation of CTL activity in splenocytes of progressors by adherent cells of progressors; *b*, modulation of CTL activity in nonadherent splenocytes of regressors by adherent cells of progressors; *c*, priming of naive splenocytes by adherent cells of regressors.

In contrast to progressors, tumor regressors had few splenic adherent cells with a total absence of the fibroblast-like morphology (data not shown). These cells were highly capable of inducing

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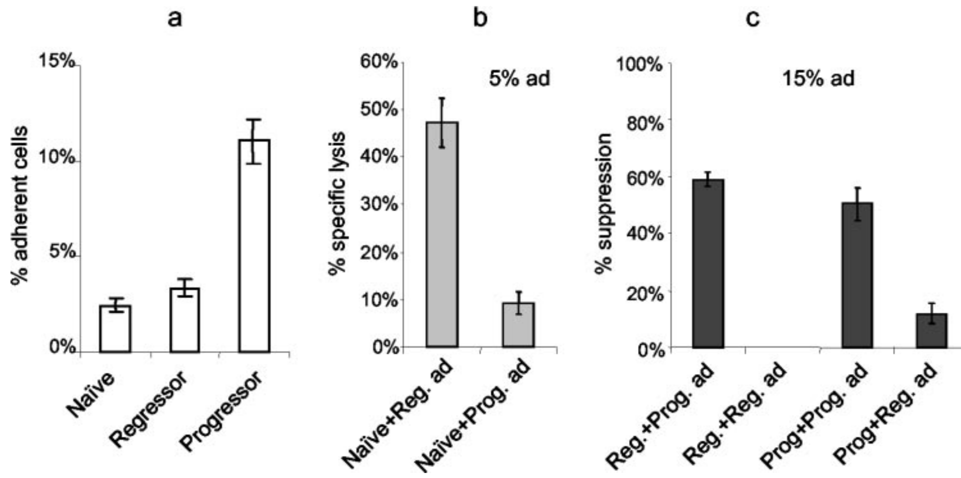

FIGURE 2. Distinct CTL modulatory activity of adherent populations from progressors vs regressors is not due to a quantitative difference in culture. CTL cultures were initiated with progressor or regressor splenocytes. On day 2, nonadherent cells were removed and adherent cells were recuperated by gentle scraping after 30-min treatment with 0.5 mM EDTA. Subsequently, 10^7 nonadherent cells were cocultured with different adherent cells at indicated ratios for another 3 days. The CTL activity was tested on BW-Sp3(B7-1) targets. *a*, Mean percentage ± SD of adherent cells in day 2 CTL cultures of five independent experiments. *b*, Nonadherent naive splenocytes were cocultured with 5% adherent splenocytes of regressors or progressors for 3 days, and the CTL activity in the nonadherent fraction was tested. *c*, Nonadherent regressor or progressor CTLs were cocultured with 15% adherent cells of either progressors or regressors for 3 days. The CTL activity in the nonadherent fraction was tested. Control cultures contained only the nonadherent CTLs. Percentage suppression was calculated as ((lysis of control culture − lysis of coculture)/lysis of control culture) × 100%.

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tumor specific cytotoxicity in naive splenocytes (Fig. 1*c*). Indeed, while stimulating cultured naive splenocytes (total or nonadherent fraction) with irradiated BW-Sp3(B7-1) cells did not yield a significant CTL activity, a strong tumor-specific CTL activity (reaching 36–43% target lysis at E:T ratio, 100:1) was induced in the nonadherent naive splenocytes in the presence of adherent splenocytes of regressors. This CTL activity was a result of in vitro priming of naive T cells and not of residual CTLs from regressors, because no CTL activity could be detected in cultures that only contained adherent cells (data not shown).

Because the CTL cultures of progressors contained 3- to 4-fold more adherent cells (11.06 ± 1.15%) than the regressor cultures (3.34 ± 0.46%) (Fig. 2*a*), functional differences between progressor and regressor adherent populations could be quantitative rather than qualitative. Therefore, adherent cells were harvested from cultures on day 2, and different amounts (5%, 10% and 15%) of

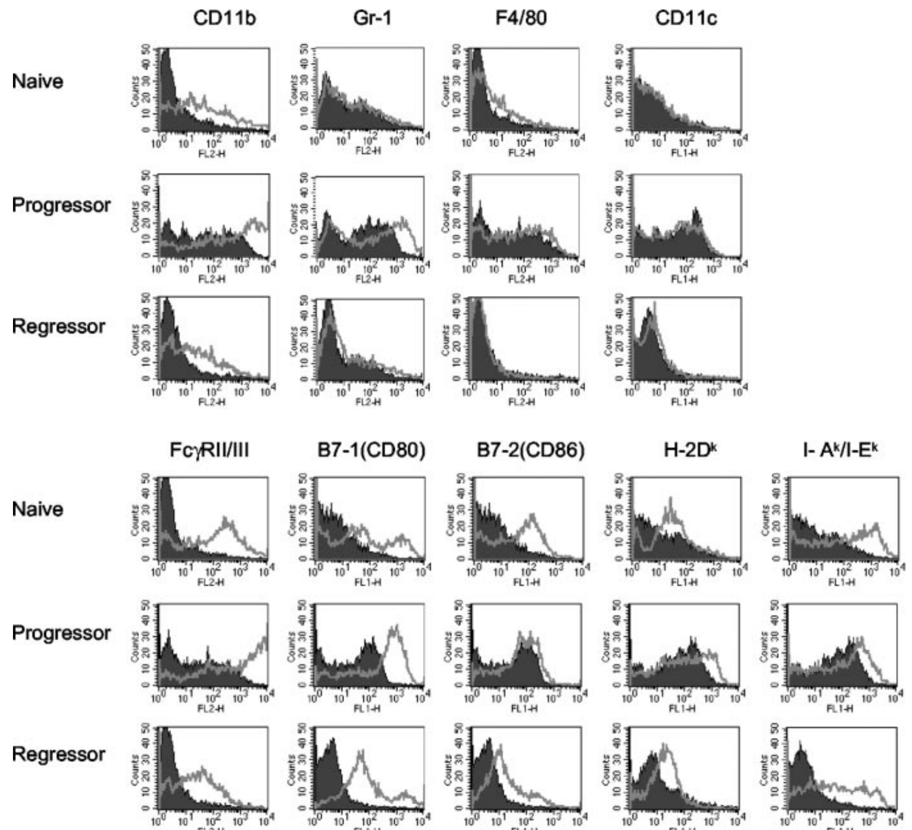

FIGURE 3. Characterization of splenic adherent populations. Adherent cells were recuperated from 5 day splenocyte cultures by gentle scraping after 30-min treatment with 0.5 mM EDTA. Cells were directly labeled with PE-or FITC-conjugated mAbs recognizing surface Ags or isotype-matched control Abs for FACS analysis. Living cells were gated according to forward scatter and side scatter profiles. Expression levels of these Ags are presented in histograms. Positive staining (open areas) was overlaid on the staining with isotype controls (filled areas).

adherent cells were cocultured with 10^7 nonadherent cells. As shown in Fig. 2*b*, strong antitumor CTL activity could already be induced in naive splenocytes by 5% of regressor adherent cells, while the same amount of progressor adherent cells failed to exert this CTL priming. In contrast, suppressive activity required 10–15% of progressor (data shown for 15%), but not regressor, adherent cells to cause 50–60% decrease of CTL activity in both regressor and progressor nonadherent cells (Fig. 2*c*). It should be remarked that 5% of adherent cells was not sufficient for suppression (data not shown), identifying 10–15% as a minimum concentration threshold for suppression. Altogether, these data demonstrate that BW-Sp3 tumors are capable of inducing distinct types of splenic accessory populations: induction or suppression of CTL activity in regressors vs progressors, respectively, related to the presence of stimulating vs inhibitory splenic adherent cells.

Characterization of inhibitory spleen cells in tumor progressors

To better characterize the adherent splenocytes from tumor progressors and regressors, we performed FACS analysis with a wide panel of mAbs. These experiments revealed a marked increase of CD11b and Gr-1-positive cells in progressors. These progressor adherent cells, but not the regressor adherent cells, also contained a minor population of F4/80-positive cells. In contrast, CD11c, a dendritic cell marker, was barely detectable in all three adherent populations. Furthermore, progressor adherent population strongly up-regulated FcγRII/III expression as compared with naive and regressor adherent populations. In addition, the B7-1 and B7-2 costimulatory molecules and the MHC Ags were generally detectable in all three adherent populations, although the expression level might differ somewhat from one population to the other (Fig. 3). These data linked the suppressive adherent population to splenic myeloid cells expressing CD11b and Gr-1 Ags. We then analyzed the presence of CD11b and Gr-1-positive cells in fresh splenocyte populations. As shown in Fig. 4, progression of BW-Sp3 tumors was indeed accompanied by an expansion of the splenic $CD11b^+/Gr-1^+$ population. The CD11b and Gr-1 double-positive splenic cells increased from 1.73 $\pm$ 0.37% (average $\pm$ SD of four experiments) in naive mice to 18.88 $\pm$ 2.31% in progressors. The tumor regressors, in contrast, contained ~2.76 $\pm$ 0.53% of $CD11b^+/Gr-1^+$ cells in the spleen, a level nearly comparable to that in naive mice (Fig. 4*a*). Immunohistochemistry on splenic cryosections further illustrated that the $CD11b^+$ cells in tumor progressors selectively aggregated in the splenic perilymphoid zones, in contrast to naive mice and regressors where $CD11b^+$ cells were dispersed over the red pulp (Fig. 4*b*).

To further evaluate the contribution of $CD11b^+$ cells in the suppressive activity of adherent cells in CTL cultures of progressors, splenocytes were depleted of $CD11b^+$ cells via Dynal magnetic beads before CTL restimulation. After only one round of dynabead treatment, the depletion efficiency was ~85–90% for $CD11b^+/Gr-1^+$ cells and ~75% for total $CD11b^+$ cells (Fig. 5*a*). And as shown in Fig. 5*b*, this depletion resulted in an optimal CTL induction in the spleen cells of progressors. Concomitantly, the amount of arginase-producing adherent cells in the CTL cultures was dramatically reduced (data not shown). The treatment with isotype-matched control Abs could slightly improve CTL activity, possibly due to aspecific binding of Abs to the FcRs resulting in a partial loss of suppressive cells. Collectively, a splenic $CD11b^+$ cell population, induced or mobilized during BW-Sp3 tumor progression, is functionally involved in impairing antitumor CTL response at least at the level of the spleen.

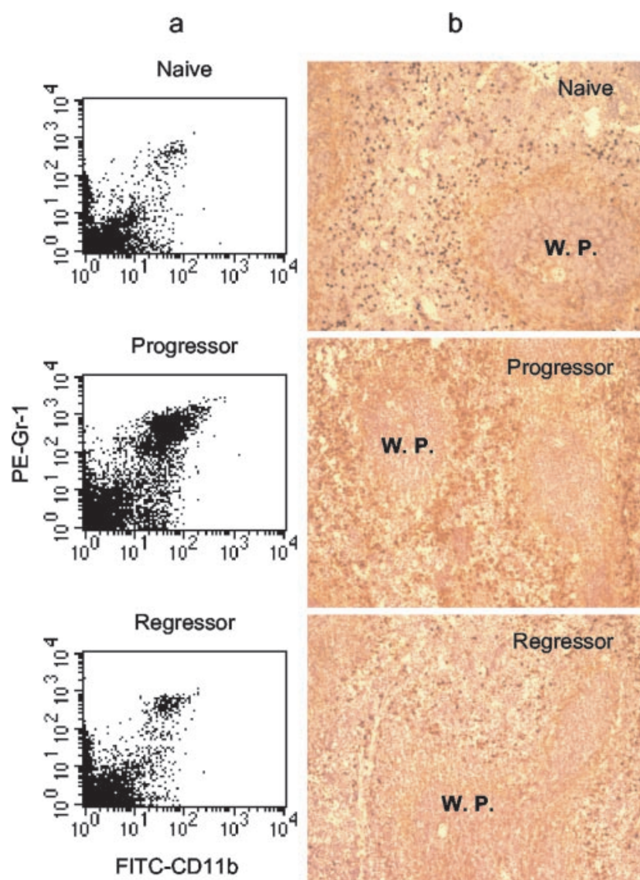

FIGURE 4. $CD11b^+$ and/or $Gr-1^+$ cells are expanded in the spleen of tumor progressors. *a*, Fresh splenocytes, homogenized in erythrocyte lysis buffer, were stained with FITC-labeled anti-mouse CD11b and PE-labeled anti-mouse Gr-1 mAbs for flow cytometric analysis. Dot plots show a drastic increase in $CD11b^+/Gr-1^+$ cells in tumor progressors. *b*, Splenic cryosections were acetone-fixed and incubated with rat anti-mouse CD11b mAbs as primary Ab and biotinylated rabbit anti-rat mAb as secondary Ab. Positive staining was developed with a Vector peroxidase kit and specimens were briefly counterstained with hematoxylin. Representative pictures for naive mice, progressors, and regressors show splenic white pulp (W.P., lymphoid zones) and perilymphoid areas. Cells stained in brown were $CD11b^+$ cells.

Modulation of CTL activity by the splenic adherent cells is cell-to-cell contact-dependent

Several mechanisms, relying either on cell-to-cell contact or soluble factors, might be used by the adherent cells to regulate CTL activity. To test whether cell-to-cell contact was required, the CTL restimulation was partially conducted in transwells where cocultured adherent and nonadherent cells were separated by a 0.4-μm porous membrane. The CTL restimulation was initiated with total splenocytes and 2 days later the nonadherent cells were transferred into transwells while the adherent cells remained in the original wells. The transwells containing nonadherent cells were then placed on top of an adherent population, as indicated in Fig. 6, for 3 days of further incubation. After a total of 5 days of restimulation, the CTL activity in the nonadherent fraction was tested toward the BW-Sp3(B7-1) target. Cell-to-cell contact between nonadherent splenocytes of either progressors or regressors and the adherent cells of progressors was necessary for the inhibition of CTL activity (Fig. 6, *a* and *b*). Applying an identical transwell

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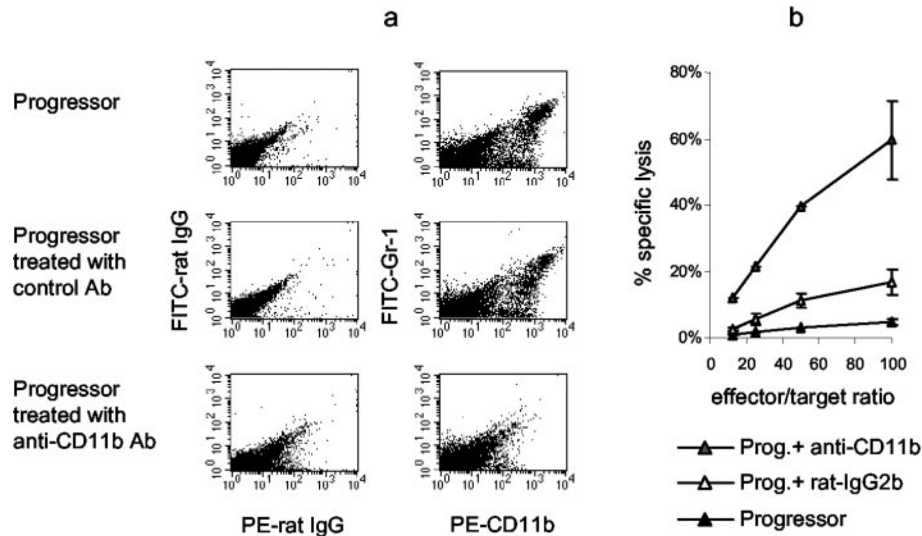

FIGURE 5. Depletion of the splenic CD11b^{+} cells restores the antitumor CTL activity in progressors. Fresh splenocytes were first incubated with PE-conjugated rat anti-mouse CD11b or isotype-matched control Abs, washed, and then incubated with sheep anti-rat IgG-coated dynabeads. The dynabead-bound CD11b^{+} cells were then removed by placing samples in a Dynal magnetic devise. The CD11b^{-} cells were collected and restimulated in vitro with irradiated BW-Sp3(B7-1) cells for 5 days as described previously. *a*, Aliquots of splenocytes before and after the treatment were labeled by incubation with PE-anti-mouse CD11b mAb and biotinylated anti-mouse Gr-1 mAb, followed by incubation with FITC-streptavidin. Positive cells were determined in flow cytometry. *b*, After in vitro restimulation, the CTL activity in dynabead-treated progressor splenocytes was tested toward ^{111}In-labeled BW-Sp3(B7-1) targets. Representative results from one of three experiments are shown.

experimental set-up, we furthermore showed that the CTL inducing capacity of the adherent splenic cells of regressors, is also strictly cell-to-cell contact-dependent (Fig. 6*c*).

The suppressive adherent cells inhibit CTL activity by NO-dependent and -independent mechanisms

According to the above-described experiments, cell-to-cell contact is essential for the adherent progressor splenocytes to suppress the CTL activity. However, the experimental setting in transwells does not exclude a potential role for soluble mediators such as NO in the observed CTL suppression. Indeed, NO-mediated suppressive activity can be short-lived and only active over short distances (26). In this context, NO was detected in the total splenocyte cultures of progressors (±20–25 μM) but not in the cultures of nonadherent cells (Fig. 7*a*). To investigate the involvement of NO in the suppressive process, equal amounts (10^{7}) of viable nonadherent cells from either progressors or regressors were cultured on top of the same population of progressor adherent cells, with or without reagents to prevent the NO secretion by macrophages. L-NMMA was added to block the iNOS activity, and neutralizing anti-IFN-γ Abs were used to prevent classical macrophage activation. However, although addition of the two reagents abolished the NO production, these treatments did not

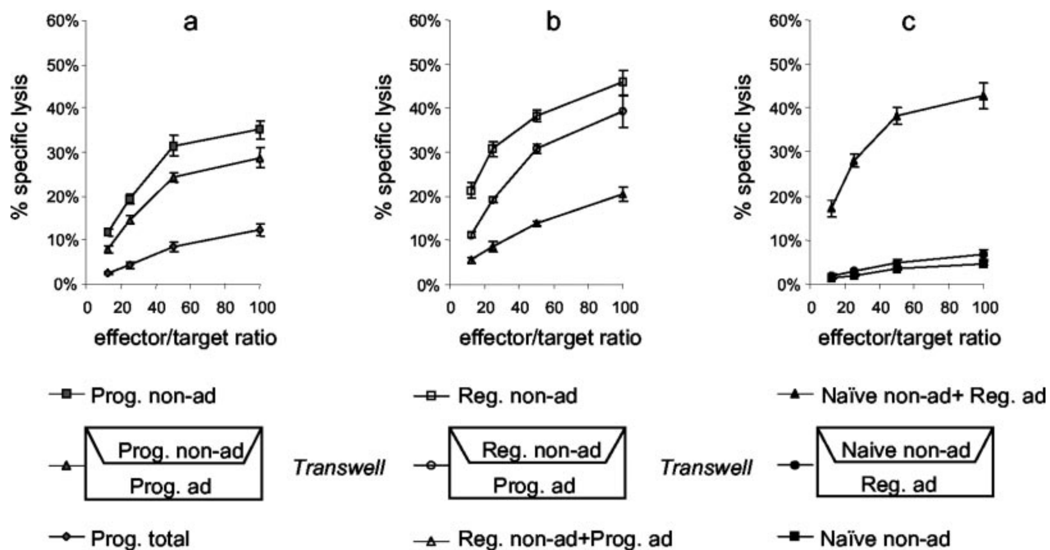

FIGURE 6. The modulatory activities of adherent populations on tumor-reactive CTLs are prevented in transwells. CTL cultures were initiated with total splenocytes as previously described. To test whether a cell-to-cell contact was required for the CTL modulatory activity of adherent cells, the nonadherent CTL fractions were transferred into transwells on day 2, whereas the adherent cells remained in the original wells. The transwells containing nonadherent cells were then placed on top of the adherent populations as indicated. At day 5, viable cells collected from transwells were tested for their CTL activity toward ^{111}In-labeled BW-Sp3(B7-1) targets. Results show average ^{111}In release ± SD of triplicates. Similar results were obtained from two independent experiments.

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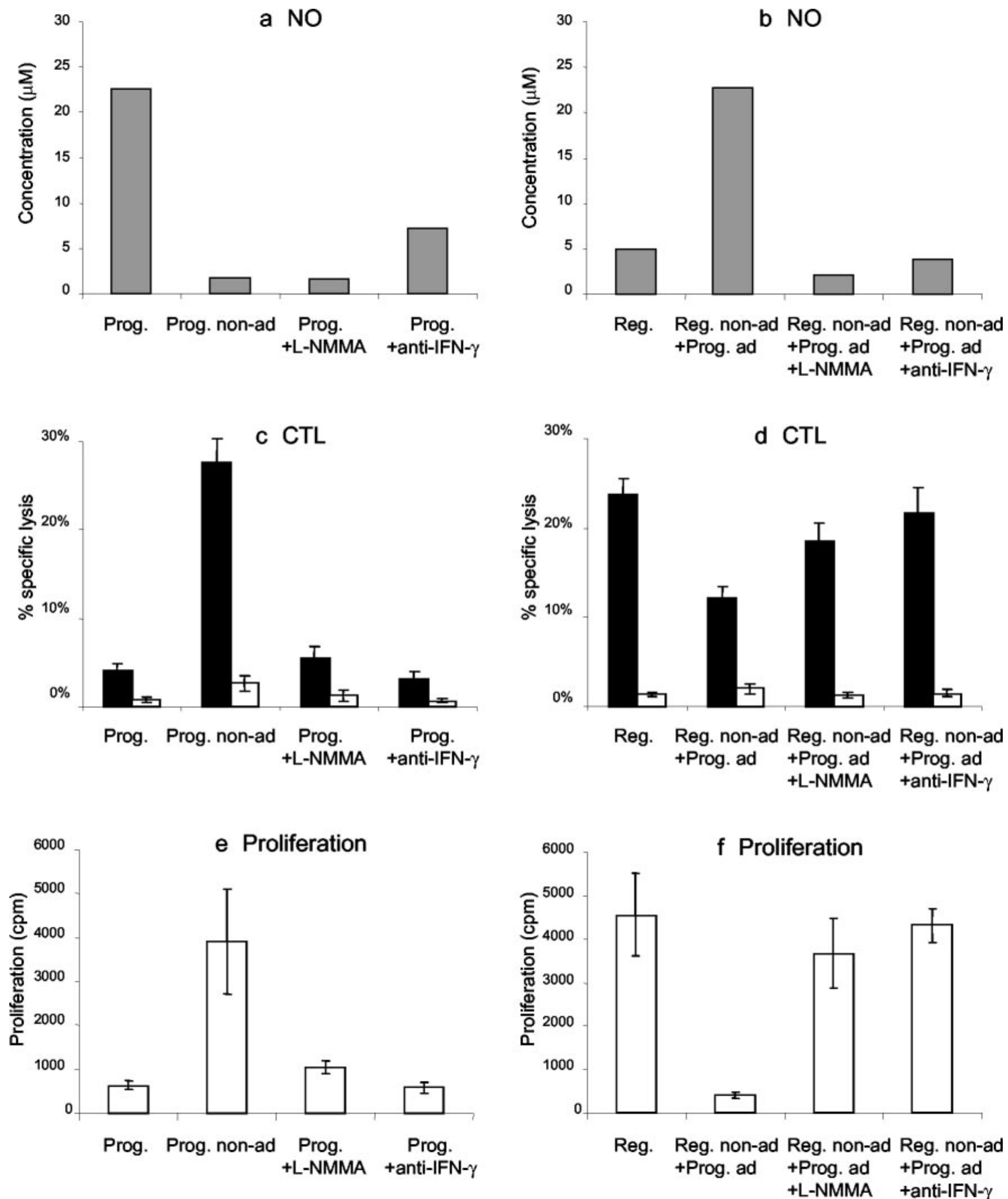

FIGURE 7. Inhibition of progressor CTLs by adherent cells of progressors is NO-independent, while the suppression of regressor CTLs is NO-mediated. CTL cultures were initiated as previously described. On day 2, equal amounts (10^7) of viable nonadherent cells from either progressors or regressors were cultured on top of the same population of progressor adherent cells. To block NO production in CTL cultures, L-NMMA was added at a final concentration of 0.5 mM and neutralizing anti-IFN-γ Abs were added at 10 μg/ml. Both reagents were added on day 2 of restimulation. *a* and *b*, The nitrite concentration was measured in day 5 CTL supernatants. *c* and *d*, The CTL activity was monitored on BW-Sp3(B7-1) (■) or BW-O (□) targets after 5 days of incubation. Target lysis is shown for an E:T ratio of 100:1 (average ± SD of triplicates). *a–d*, Results from the same experiment. Similar results were obtained from three independent experiments. *e* and *f*, To test whether cell proliferation was affected by NO, in an additional experiment CTL cultures were proportionally downscaled to 96-well plates. Cultures were pulsed at day 4 with [^{3}H]thymidine and the incorporated radioactivity was measured 18 h later. Results show an average ± SD of triplicates.

lead to a recovery of CTL activity in the cultures of total progressor splenocytes (Fig. 7*c*), excluding a major role for NO in this suppressive process. In contrast, the inhibition on CTLs of regressors by the same adherent population of progressors depended largely on NO. As shown in Fig. 7*d*, coculture of the splenocytes of regressors with the adherent cells of progressors caused a 50% reduction in tumor-specific CTL activity. This CTL impairment correlated with enhanced NO production in the cocultures (Fig. 7*b*). Moreover, inhibition of NO production, upon treatment with L-NMMA or anti-IFN-γ mAb, resulted in 78 or 91% recovery of CTL activity, respectively (Fig. 7, *b* and *d*). To exclude a direct negative effect of anti-IFN-γ mAb on CTL generation, control experiments were performed by supplying anti-IFN-γ mAb to nonadherent CTLs of progressors or regressors at day 2 and this treatment did not influence their cytotoxic activity (data

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not shown). In conclusion, because equal amounts of nonadherent cells were cocultured with the same progressor adherent population, the two different mechanisms of CTL suppression (NO-dependent vs NO-independent) reflect a qualitatively different responsiveness in the respective CTL populations.

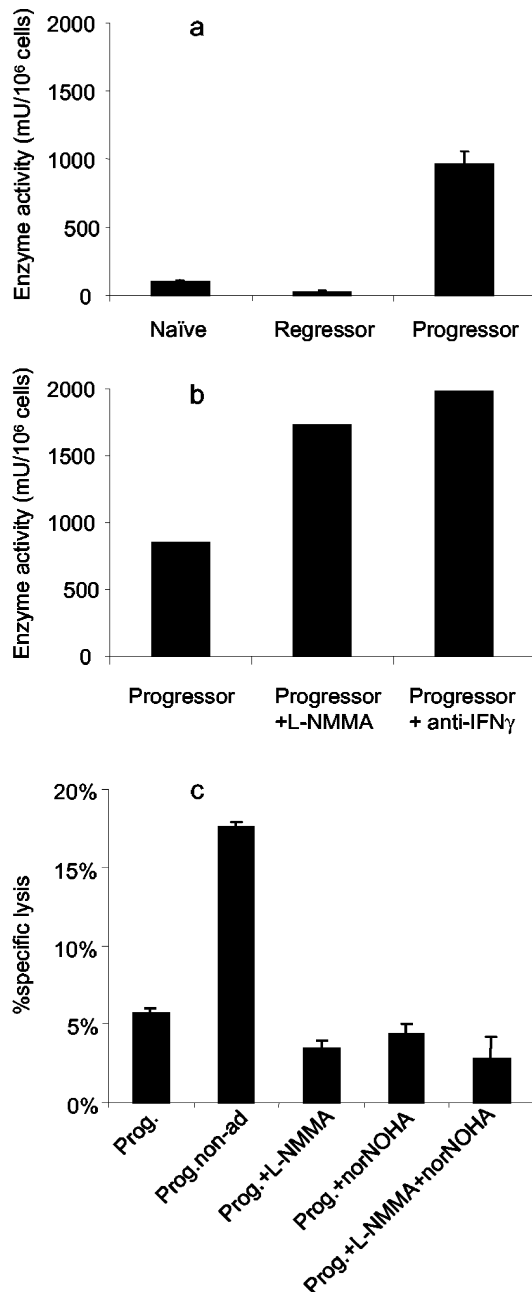

FIGURE 8. Splenic adherent cells of tumor progressors express high arginase activity, that is however not involved in CTL suppression. Adherent cells were recuperated from CTL cultures after 5 days of incubation. Cells were counted and lysed in Triton X-100 and the arginase activity was measured as described in *Materials and Methods*. *a*, Arginase activity in adherent cells from naive mice, regressors, and progressors. *b*, Effect of L-NMMA or anti-IFN-γ Abs on arginase activity of progressor adherent cells. *c*, Spleens of progressors were homogenized in erythrocyte lysis buffer. In vitro CTL restimulation was then initiated with irradiated BW-Sp3(B7-1) cells. At the same time, norNOHA was added in CTL cultures at a final concentration of 0.2 mM, alone or combined with L-NMMA (0.5 mM). CTL activity was monitored on BW-Sp3(B7-1) targets after 5 days of incubation. Target lysis is shown for the E:T ratio of 100:1 (average ± SD of triplicates).

Finally, it should be remarked that all described experiments were performed in the presence of erythrocytes, which are known to block the action of NO from suppressive macrophages (54). Therefore, we tested whether the NO-independent suppression of progressor CTLs could be reproduced in erythrocyte-depleted cultures. As will be illustrated below (Fig. 8*c*), the NO-independent T cell inhibition in progressors remained unaltered in such culture conditions, despite the presence of high NO concentration in these cultures (30–45 μM).

To assess whether the impaired CTL activity was due to the inhibition on lymphocyte proliferation, CTL cultures were downscaled into 96-well plates and the proliferation of cultured cells during the last 18 h of incubation (day 4 to day 5) was tested. As shown in Fig. 7, *e* and *f*, adherent cells of progressors inhibited the proliferation of nonadherent splenocytes of both progressors and regressors. Similar to the inhibition of CTL activity, the adherent cell-mediated antiproliferative effect on progressor splenocytes was not affected by addition of the NO inhibitors. But treatment with either L-NMMA or anti-IFN-γ mAb drastically recovered the cell proliferation in the regressor cultures. Collectively, the suppression of CTL activity by progressor adherent cells requires cell-to-cell contact. Depending on whether the CTL population is derived from regressors or progressors, the suppression is NO-dependent or -independent, respectively.

The adherent cells of progressors exhibit high arginase activity that is, however, dispensable for CTL suppression

According to current literature, myeloid cells can be activated in two different ways resulting in classically activated M1 cells and alternatively activated M2 cells. Both M1 and M2 cells are capable of down-regulating immune reactions under certain circumstances. M1 cells are typically characterized by high iNOS-catalyzed NO production, while M2 cells can instead be identified by high arginase activity. In this regard, we screened arginase activity in the adherent populations to assess their activation status. Naive adherent splenocytes had an average arginase activity of 95.9 ± 15.7 mU/10^6 cells (average ± SD of 10–12 individuals) while adherent regressor spleen cells showed a barely detectable arginase level (26.2 ± 3.94 mU/10^6 cells). In contrast, adherent splenocytes of progressors exhibited extremely high arginase activity (959.6 ± 90.9 mU/10^6 cells) supporting the notion that alternatively activated myeloid cells might be involved in the suppressive process (Fig. 8*a*). Interfering with M1 activation by anti-IFN-γ mAb or blocking iNOS activity by L-NMMA further elevated arginase activity in the adherent progressor cells (Fig. 8*b*). It should be noted as well that the concentration of NO in CTL cultures was 5- to 12-fold higher in the presence of, as compared with the absence of, adherent progressor cells (see Fig. 7, *a* and *b*). Together, these findings suggested a coexistence of two enzymatic pathways (iNOS and arginase) in adherent progressor cells.

Arginase activity has been shown in earlier reports to contribute to myeloid cell-mediated T cell suppression, either alone (55) or in cooperation with iNOS (56). Hence, such a mechanism could be responsible for the NO-independent CTL suppression by adherent progressor cells. To test this hypothesis, norNOHA, a potent and specific inhibitor for arginase (57), was added to the CTL cultures, either alone or in combination with the iNOS inhibitor L-NMMA. As shown in Fig. 8*c*, used either alone or together with L-NMMA, norNOHA did not diminish the CTL suppression. Hence, though increased arginase activity indicated a M2 differentiation in progressor adherent cells, the arginase pathway itself did not participate in CTL inhibition.

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Discussion

In previous work, we demonstrated that s.c. BW-Sp3 tumors induced a CTL response in syngeneic AKR/OlaHsd hosts, resulting in tumor rejection. Tumor progression, in contrast, was associated with significantly decreased CTL activity (7, 52). In this study, we investigated the importance of splenic myeloid cells in modulation of the CTL activity and revealed that two adherent populations, generated either in tumor regressors or progressors, were respectively stimulating or inhibiting the CTLs. Focusing on the suppressive myeloid population from progressors, we could further demonstrate that the suppressive cells resided in the splenic $CD11b^+$ population. Removal of the adherent or $CD11b^+$ cells from the progressor splenocyte cultures restored the CTL activity, indicating that the inhibition exerted on the CTLs was reversible. Hereby, two suppressive mechanisms were identified in the adherent progressor splenocytes, depending on whether the target CTLs were derived from regressors or progressors. Indeed, although in vitro inhibition of CTLs of regressors was as well cell-to-cell contact as NO-restricted, the suppression on CTLs of progressors was cell-to-cell contact-dependent but not mediated by NO. Finally, we found that the suppressive population expressed high arginase activity, suggesting a state of alternative activation in the progressor splenic myeloid cells.

Myeloid cells not only take part in the host innate immunity, but they are also crucial both in positive and negative regulation of the adaptive immune responses. APCs of myeloid origin, such as dendritic cells, are well-characterized for their capacity to induce T cell responses (29, 58). Ligation of the MHC-peptide complex on APC to the TCR on T cells, together with proper costimulatory signals (e.g., ligation of B7-CD28), leads to optimal T cell activation. Accordingly in tumor regressors, we could correlate effective CTL activity with the presence of potent APCs. As presented in this work, the adherent fraction of regressor splenocytes could induce tumor-specific CTL activity in cultured naive splenocytes in a cell-to-cell contact-dependent manner, illustrating that regressor-associated splenic myeloid cells are fully matured APCs, actively presenting tumor Ags to the immune system.

In contrast, myeloid suppressive cells have been observed in a wide range of immunopathological situations of intense immune stress, including cancer (16, 17, 19, 30–35, 37, 59–62). However, the nature of these myeloid suppressive cells and the mechanisms by which they exert immune suppression remain controversial. A large body of literature has described these suppressive cells as immature myeloid cells expressing CD11b and/or Gr-1. A similar population was amplified and indeed suppressive to the CTLs in BW tumor progressors. One well-documented suppressive mechanism for $CD11b^+/Gr\text{-}1^+$ cells was described to be mediated by IFN-γ-dependent NO production (17, 30–33, 63). However, it is noteworthy that this type of suppressive activity was often tested toward the primary T cell proliferation induced by mitogens (30, 63), anti-CD3 Abs combined with anti-CD28 or IL-2 (17, 30, 32, 33), or unrelated Ags (63), but not toward the relevant Ag-specific T cells that the $CD11b^+/Gr\text{-}1^+$ cells regulate in vivo as in our study. Hence, the physiological relevance of these in vitro testing systems needs to be carefully evaluated. Indeed, in our tumor model, we demonstrated that the IFN-γ-dependent NO production largely contributed to the suppressive process, provided that the suppressive adherent population of progressors was cocultured together with the regressor CTLs. But in the more physiologically relevant condition, where both the suppressive and CTL populations were derived from the same hosts, NO inhibitors could not reverse the CTL suppression. Inhibition of progressor CTLs was strictly NO-independent even when the NO concentration was augmented by the depletion of erythrocytes. Thus, evidence is provided for the first time that within the suppressive cells under study two suppressive mechanisms coexist, and that the effective mechanism depends on relevant target CTL populations.

This different responsiveness in the two CTL populations (regressors vs progressors) toward suppressive signals is intriguing. We speculate that this could be due to the different activation status of the two T cell populations. Regressors are free of tumor load and likely provide largely tumor-specific memory T cells that undergo vigorous clonal expansion upon in vitro restimulation. In contrast, the CTLs derived from progressors encounter a persistent tumor load in vivo and are consequently chronically activated. Although NO can act as an antiproliferative molecule secreted by activated myeloid cells (64), its effect will depend on the status of activated T cell populations. For instance, the IFN-γ and NO-dependent inhibition by tumor host-derived $Gr\text{-}1^+$ cells was only observed in anti-CD3/CD28-stimulated naive T cells but not in T cells that were preactivated by these stimuli (17). In another study, Mills and colleagues (65) reported that 1) preimmunized $B6D2F_1$ mice could reject subsequently inoculated P-815 tumors, 2) detection of antitumor CTL activity in preimmunized animals was accompanied by IFN-γ-dependent macrophage NO production, and 3) blocking NO in vivo at the time of second tumor inoculation further augmented the following CTL response. These findings corroborate our hypothesis that expanding memory CTLs are susceptible to NO-mediated inhibition. Yet, the same group observed earlier that the low antitumor CTL activity in nonimmunized mice, in which tumors progressed, correlated rather with high arginase activity and low NO production in local macrophages (50). Given that CTLs from BW progressors are not susceptible to NO-dependent suppression, it is possible that NO is not the major relevant mediator for CTL suppression in P-815 tumor progressors and a second, NO-independent, suppressive mechanism might be involved.

In our model system, the NO-dependent and -independent types of CTL suppression were found to be present within the same myeloid population, obtained from tumor progressors. At this point, it is still unclear whether the NO-dependent and -independent inhibition in our system was exerted by the same cells or by coexisting but different myeloid populations. Mechanistically, both types of CTL suppression required cell-to-cell contact. For the NO-dependent suppression, NO and its intermediates could be only effective within a short range and thus separating the nonadherent from the adherent cells in transwells was sufficient to abolish their reactivity. Secondly, cell-to-cell contact might be needed for the suppressive cells to produce NO (18, 63). The latter hypothesis seems unlikely in our system, because in the presence of progressor adherent cells we could detect equal amounts of nitrite in transwells as in regular cultures (data not shown). The cell-to-cell contact-dependent, NO-independent T cell inhibition could involve membrane-bound molecules, whereby some yet to be identified membrane molecules directly deliver negative signals to T cells. Alternatively, a direct contact with T cells might trigger the myeloid cells to produce inhibitory factors (other than NO). The downstream mechanisms underlying cell-to-cell contact-dependent T cell suppression so far are not fully understood. However, cell cycle arrest in the responder T cells is plausible. As described in two studies in which IL-4- or M-CSF-activated macrophages were implicated in cell contact-dependent lymphocyte suppression, the suppressed responder cells were found to accumulate in the G_1, G_2/M, or G_1/S stages (34, 66). It is important to mention that the disrupted CTL function in BW progressors is not due to T cell deletion or anergy but rather to an active suppression by myeloid cells, because the tumor-specific CTL activity could be recovered upon in vitro removal of suppressive cells. Thus, in situations

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similar to the BW model attempts to augment CTL responses via vaccination or adoptive T cell transfer are unlikely to succeed.

It is documented that, in addition to the IFN-γ-dependent myeloid suppressive cells, some CD11b$^+$/Gr-1$^+$ cells acquired suppressive ability upon exposure to IL-4 (37, 67, 68). This distinction between IFN-γ- and IL-4-dependent CD11b$^+$/Gr-1$^+$ suppressive cells is reminiscent of the recently described existence of classically activated M1 and alternatively activated M2 myeloid cells (38, 69). The suppressive adherent CD11b$^+$ population in BW progressors indeed manifested a high arginase activity, which was further enhanced after blocking iNOS activity. Hence, it is evident that in BW progressors the suppressive splenic population contains M2 cells. However, we have yet to establish a direct link between M2 cells and the CD11b$^+$ suppressors on a single cell basis. We also need to investigate whether the CD11b$^+$ splenocytes, when freshly purified from progressors, express arginase as high as the cultured adherent cells. M2 cells are considered to induce Th2 differentiation of CD4$^+$ T cells and cause Th1 tolerance (38, 51, 69). They may have potential roles in immune suppression (34, 62). Loke et al. (34) reported that the IL-4-induced alternatively activated macrophages reversibly inhibited T cell proliferation by a cell-to-cell contact mechanism, similar to the suppression we observed in tumor progressors. In addition, arginase activity appeared to be a reliable cellular maker to monitor the activation status in myeloid populations. Furthermore, arginase has been shown to mediate T cell suppression in some models via different mechanisms: 1) high arginase activity might deplete L-arginine from the culture, starving the T cells (55); or 2) arginase increases superoxide production in myeloid cells through a pathway that likely uses the reductase domain of iNOS (56). However, this enzymatic pathway did not contribute to the CTL inhibition in our tumor system, as manifested by specific blockade of its activity using norNOHA, either alone or in combination with L-NMMA. We reported recently that in early stages of BW tumor growth, the induction of efficient CTL activity correlates with polarized induction of type 1-associated cytokines and M1. In that study, we demonstrated that manipulation of the immune system, tilting the balance in favor of a type 2-associated environment, results in an induction of M2 and tumor progression. Our results further showed that the activated NK cell population is one critical factor in controlling this process in BW-Sp3 tumor bearers. Indeed, in the absence of fully activated NK cells, upon depletion or in tumor progressors (where NK activity is severely reduced), Ag-specific CTL activity is impaired and can be correlated with the presence of M2 and myeloid suppressive cells (70). Tumor-secreted GM-CSF was another factor proposed to be involved in the induction of myeloid suppressors (37, 71). However, GM-CSF is not likely a major stimulator of the CD11b$^+$ suppressors in our system, because 1) BW-Sp3 cells do not produce this cytokine and 2) the splenocytes of progressors do not produce more GM-CSF than the splenocytes of regressors (data not shown). In contrast, BW-Sp3 cells do express TGF-β, at least at the mRNA level (data not shown). Whether TGF-β affects the myeloid differentiation in BW tumor hosts is currently under investigation.

Collectively, our BW-Sp3 tumor model allowed us to dissect immune mechanisms operating during tumor regression vs progression. Indeed, the effectiveness of CTL responses was closely associated with the presence of differentially activated myeloid cells. Although mature APC induced tumor-reactive CTL activity in regressors, a M2-containing splenic myeloid population inhibited the CTL activity in progressors by a cell-to-cell contact-dependent, NO-independent mechanism. According to our results, appropriate CTL populations should be used to unravel the in vivo relevant mechanisms underlying myeloid cell-mediated T cell inhibition.

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