The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2004

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Sergei Kusmartsev; ... et. al

J Immunol (2004) 172 (2): 989–999.
https://doi.org/10.4049/jimmunol.172.2.989

Antigen-Specific Inhibition of CD8$^+$ T Cell Response by Immature Myeloid Cells in Cancer Is Mediated by Reactive Oxygen Species[1]

Sergei Kusmartsev, Yulia Nefedova, Daniel Yoder, and Dmitry I. Gabrilovich[2]

Tumor growth is associated with the accumulation of immature myeloid cells (ImC), which in mice are characterized by the expression of Gr-1 and CD11b markers. These cells suppress Ag-specific CD8$^+$ T cells via direct cell-cell contact. However, the mechanism of immunosuppressive activity of tumor-derived ImC remains unclear. In this study we analyzed the function of ImC isolated from tumor-free control and tumor-bearing mice. Only ImC isolated from tumor-bearing mice, not those from their control counterparts, were able to inhibit the Ag-specific response of CD8$^+$ T cells. ImC obtained from tumor-bearing mice had significantly higher levels of reactive oxygen species (ROS) than ImC isolated from tumor-free animals. Accumulation of H_2O_2, but not superoxide or NO, was a major contributor to this increased pool of ROS. It appears that arginase activity played an important role in H_2O_2 accumulation in these cells. Inhibition of ROS in ImC completely abrogated the inhibitory effect of these cells on T cells, indicating that ImC generated in tumor-bearing hosts suppress the CD8$^+$ T cell response via production of ROS. Interaction of ImC with Ag-specific T cells in the presence of specific Ags resulted in a significant increase in ROS production compared with control Ags. That increase was independent of IFN-γ production by T cells, but was mediated by integrins CD11b, CD18, and CD29. Blocking of these integrins with specific Abs abrogated ROS production and ImC-mediated suppression of CD8$^+$ T cell responses. This study demonstrates a new mechanism of Ag-specific T cell inhibition mediated by ROS produced by ImCs in cancer. ***The Journal of Immunology,*** **2004, 172: 989–999.**

There is now ample evidence that tumor growth in cancer patients and tumor-bearing mice is associated with an accumulation of immature myeloid cells (ImC)[3] (1–9). This was found in all tested animal tumor models and in patients with all tested types of cancer. In mice, these ImCs have the phenotype Gr-1$^+$CD11b$^+$. In contrast to immunosuppressive macrophages, these cells do not produce NO, do not affect CD4$^+$ T cells, and exert their suppressive effect via direct contact with CD8$^+$ T cells (10–12). ImC may be responsible for the failure of T cells from tumor-bearing hosts to effectively recognize and eliminate tumor cells in an Ag-specific manner, thus allowing the tumor to escape from immune system control (13, 14). The increased presence of ImC capable of inhibiting T cell responses could also be a major factor contributing to the development of previously described tumor-induced T cell tolerance in tumor-bearing hosts.

However, immature myeloid cells are also an intrinsic part of the normal process of myeloid cell differentiation, and they are present in relatively small numbers in naive hosts. The number of immature myeloid cells significantly increases during bacterial infection and immunization with potent immunogens, such as vaccinia virus encoding IL-2 (11, 15) and superantigen (15). Neither naive nor immunized hosts suffer from Ag-specific T cell unresponsiveness. Understanding the mechanisms used by ImCs to suppress the T cell response is critically important for the development of effective methods to counter this phenomenon.

In this study we asked whether ImC from tumor-free and tumor-bearing hosts differ in their ability to suppress CD8$^+$ T cells, and what could be a mechanism of Ag-specific inhibition of T cell response by ImC in cancer. We report, for the first time, that Ag-specific inhibition of CD8$^+$ T cells by ImC could result from up-regulation of reactive oxygen species (ROS) production by these cells, and that this process is possibly mediated by integrins.

Materials and Methods

Mice and tumors

Female BALB/c and C57BL/6 mice, aged 6–10 wk, were purchased from the National Cancer Institute (Frederick, MD). OT-1 TCR transgenic mice (C57BL/6-Tg(TCRαTCRβ)1100Mjb) and IFN-γ receptor (IFN-γR) knockout B6.129S7-Ifngr^{tm1Agt} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C3 fibrosarcoma was established in C57BL/6 mice by s.c. inoculation of 5×10^5 cells. Mice were immunized once s.c. with 0.3 mg of OVA protein emulsified in IFA at the base of tail. Isolation of Gr-1$^+$ cells from immunized mice was performed on day 10 after vaccination.

Media and reagents

RPMI 1640 medium was supplemented with 10% FCS, 20 mM HEPES, 200 U/ml penicillin, 50 μg/ml streptomycin, 0.05 mM 2-ME, and 2-mM glutamine (all from Life Technologies, Grand Island, NY). OVA-derived peptide (H-2K^b-restricted, aa 257–264, SIINFEKL) and C3-derived peptide RAHYNIVTF were purchased from SynPep (Dublin, CA), IFA was obtained from Sigma-Aldrich (St. Louis, MO), and dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethytium (DHE) were purchased from Molecular Probes (Eugene, OR). Superoxide dismutase (SOD), catalase, uric acid, and the arginase inhibitor, *N*-hydroxy-nor-L-arginine (nor-NOHA) were purchased from Calbiochem (La Jolla, CA). The following

H. Lee Moffitt Cancer Center, University of South Florida, Tampa, FL 33612

Received for publication July 2, 2003. Accepted for publication November 6, 2003.

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[1] This work was supported by National Institutes of Health Grant CA84488 (to D.I.G.).

[2] Address correspondence and reprint requests to Dr. Dmitry Gabrilovich, H. Lee Moffitt Cancer Center, University of South Florida, MRC-2E, Room 2067, 12902 Magnolia Drive, Tampa, FL 33612. E-mail address: dgabril@moffitt.usf.edu

[3] Abbreviations used in this paper: ImC, immature myeloid cell; DCFDA, dichlorodihydrofluorescein diacetate; DHE, dihydroethytium; IFN-γR, IFN-γ receptor; MPO, myeloperoxidase; nor-HOHA, *N*-hydroxy-nor-L-arginine; ROS, reactive oxygen species; SOD, superoxide dismutase.

0022-1767/04/$02.00

Abs were used for flow cytometry: Gr-1-allophycocyanin (clone RB6-8C5), CD11b-PE (clone M1/70), CD18-PE (clone C71/16), and CD29-PE (clone KM16; all from BD PharMingen, San Diego, CA).

Isolation of Gr-1$^+$ splenocytes

Spleens were harvested under sterile conditions. Single-cell suspensions were prepared, and RBC were removed using ACK lysing buffer (Biosource International, Camarillo, CA). Splenocytes were resuspended in PBS, and 5–6 $\times$ 10^6 cells were incubated with 5 μg of biotinylated anti-Gr-1 mAbs (BD PharMingen) for 15 min on ice. Cells were washed with cold PBS to remove unbound Abs, then incubated with streptavidin microbeads for 15 min at 4°C. The Gr-1$^+$ cell population was isolated on a MiniMACS column according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The purity of the Gr-1$^+$ cell population was evaluated by flow cytometry and exceeded 90%.

Morphology and cytochemistry

For morphologic characterization, Gr-1$^+$ cells were stained with H&E. Myeloperoxidase, α-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase activities in isolated Gr-1$^+$ cells were evaluated by cytochemical staining according to the manufacturer's instructions using kits obtained from Sigma-Aldrich.

ELISPOT assay

ELISPOT assay was performed as described previously (12). Briefly, MultiScreen-HA plates (Millipore, Berford, MA) were precoated with anti-IFN-γ mAbs (clone R4-A2; BD PharMingen, San Diego, CA) by overnight incubation in PBS at 4°C. Splenocytes isolated from OVA-immunized mice (2 $\times$ 10^5 cells/well) were cultured for 24 h at 37°C in the presence of the specific SIINFEKL or control RAHYNIVTF peptides (10 μg/ml). Cells were then washed out with PBS containing 0.1% Tween, and plates were incubated overnight at 4°C with biotinylated anti-IFN-γ mAbs (clone XMG1.2, BD PharMingen). Results were visualized using avidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma-Aldrich). The number of spots cells in each well was scored in a blind fashion by two investigators and then recalculated per 10^6 cells.

Flow cytometry

One million cells were incubated for 30 min on ice in 100 μl of PBS with 1 μg of the relevant Abs and then washed twice with cold PBS. FACS data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and were analyzed using CellQuest software (BD Biosciences). During the analysis of ROS in myeloid cells, several groups of cells with different levels of fluorescence were present. In this situation we used the geometric mean of fluorescence to measure fluorescence intensity.

ROS production

The oxidation-sensitive dyes DCFDA and DHE were used for the measurement of ROS production by Gr-1$^+$ cells. Cells were incubated at 37°C in DMEM in the presence of 2 μM DCFDA for 30 min or 2 μM DHE for 60 min, washed twice with cold PBS, and then labeled with allophycocyanin-conjugated anti-Gr-1 Ab and PE-conjugated anti-CD11b Abs. After incubation on ice for 20 min, cells were washed with cold PBS and analyzed by three-color flow cytometry using a FACSCalibur.

To block ROS production, Gr-1$^+$ cells were incubated for 10 min at 37°C with different antioxidants, followed by 20-min incubation at 37°C with DCFDA. After that time cells were washed in cold PBS and kept on ice before analyzing by flow cytometry. The following reagents purchased from Calbiochem were used: SOD, 200 U/ml; SOD mimetic (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride), 20 μM; myeloperoxidase (MPO), 30 nM; catalase, 1000 U/ml; peroxynitrite scavenger (uric acid), 0.5 mM; and arginase inhibitor (nor-NOHA, diacetate salt), 2 μM.

Activation of ROS in vitro with immobilized Abs

Purified anti-CD11b, -CD18, -CD29, and -H-2D^b Abs were immobilized by affinity binding of the Fc portion of the Abs to protein G-coated surfaces. Briefly, Reacti-Bind, protein G-coated, 96-well plates (Pierce, Rockford, IL) were washed with PBS. Fifty microliters of mAb at 10 μg/ml in blocking buffer (containing 5% of dry milk and 0.1% Tween 20 in PBS) was added to each well. After incubation for 2 h at room temperature, wells were gently washed three times with PBS. Freshly isolated Gr-1$^+$ cells preloaded with DCFDA (3 $\times$ 10^5 cells in 50 μl of DMEM) were added to each well with immobilized Abs. Cells were incubated for 15 min at 37°C, and then the level of DCFDA oxidation was analyzed using a Wallac 1420 plate fluorometer (Wallac Oy, Turku, Finland). Readings were taken at 488/538 nm every 5 min for a total of 60 min.

MPO activity

MPO activity in Gr-1$^+$ cells was determined as described by Grisham et al. (16). Freshly isolated Gr-1$^+$ cells (1 $\times$ 10^6) were washed in 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl. Pellets were resuspended in a solution containing 0.5 ml of hexadecyltrimethyl ammonium bromide in 10 mM potassium buffer and centrifuged at 8000 $\times$ *g* for 15 min at 4°C. MPO activity was assessed in supernatants by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. The absorbance at 655 nm was measured using a microplate spectrophotometer (Bio-Rad, Hercules, CA), and the results were normalized to protein content.

Western blotting

Gr1$^+$ cells were isolated from spleen of naive or tumor-bearing mice, washed twice with ice-cold PBS, and lysed in RIPA buffer. An equal amount of total protein was loaded on a 10% SDS-PAGE gel. After electrophoresis and transferring, membranes were blocked in 5% nonfat milk in TBS/Tween 20 for 1 h and then probed with primary goat anti-mouse MPO Ab (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with corresponding rabbit anti-goat secondary Ab conjugated with HRP. Membranes were developed using an ECL detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL). Equal loading was assessed using anti-β-actin Ab (Santa Cruz Biotechnology).

Statistical analysis

The statistical significance between values was determined by Student's *t* test. All data were expressed as the mean $\pm$ SD of triplicate determinations. Values of $p > 0.05$ were considered nonsignificant.

Results

Inhibition of CD8$^+$ T cell response by ImC isolated from tumor-bearing, but not control, mice

To compare the inhibitory potential of ImC from tumor-free and tumor-bearing mice, we isolated Gr-1$^+$ cells from naive C57BL/6 mice, tumor-free C57BL/6 mice immunized with OVA, and C3 tumor-bearing C57BL/6 mice using the magnetic beads separation technique. As responders in an ELISPOT assay we used splenocytes from C57BL/6 mice immunized with the OVA-derived, MHC class I-restricted peptide SIINFEKL. Splenocytes were restimulated in vitro with control or specific MHC class I-bound peptides. As expected, specific peptide induced a significant increase in the number of IFN-γ-producing T cells in the ELISPOT assay. Gr-1$^+$ cells derived from tumor-bearing, but not from tumor-free, immunized or naive mice inhibited this response (Fig. 1). The experiments shown in Fig. 1 were performed at a 1/4 Gr-1$^+$ cell/splenocyte ratio. Thus, ImC represented 20% of the total population of splenocytes. This proportion is substantially higher than observed in tumor-free naive mice ($<$5%) or immunized tumor-free mice (8–12%) (12, 17). The lack of suppressive activity in ImC from tumor-free mice at that concentration indicates that ImC isolated from tumor-bearing mice have a unique ability to suppress the CD8-mediated T cell response.

What could be the reason for the immunosuppressive activity of ImC isolated from tumor-bearing mice? The level of MHC class I expression was equal in ImC obtained from control and tumor-bearing mice (data not shown), and neither control nor tumor-derived ImC produced detectable levels of NO (data not shown). Next, we investigated the enzymatic activity of and ROS production by these cells.

Enzymatic activity and ROS production by ImC

Gr-1$^+$ cells were isolated from spleens of naive, immunized, and tumor-bearing mice using the magnetic bead separation technique. We evaluated the activity of three enzymes specific for myeloid cells: MPO, α-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase. In three experiments performed, 42–45% of

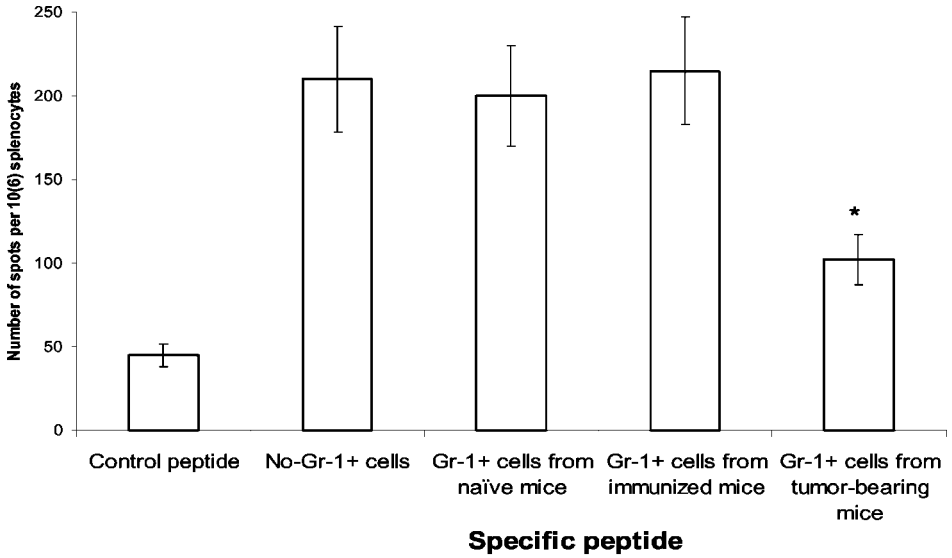

FIGURE 1. Freshly isolated Gr-1$^+$ splenocytes from tumor-bearing, but not tumor-free, mice inhibited the Ag-specific response of CD8$^+$ T cells. Gr-1$^+$ splenocytes were isolated from naive, immunized, and C3 tumor-bearing C57BL/6 mice using the magnetic bead separation technique. Splenocytes from C57BL/6 mice immunized with SIINFEKL (MHC class I-restricted peptide) were used as responders. Gr-1$^+$ cells were incubated in triplicate with responder cells (cell ratio, 1:4) in the presence of specific or control peptides for 24 h, and the number of IFN-γ-producing cells was scored in an ELISPOT assay as described in *Materials and Methods*. Results are presented as the average ± SD. Two experiments were performed, yielding the same results. *, Statistically significant differences between the group of cells treated with the specific peptide ($p < 0.05$).

Gr-1$^+$ cells were positive for α-naphthyl acetate esterase, and the same 42–45% cells were positive for naphthol AS-D chloroacetate esterase. These proportions remained the same in all tested groups of mice (Fig. 2*A*). Gr-1$^+$ cells derived from mice with tumors showed a profound deficit of MPO activity, a phenomenon not found in tumor-free immunized and naive mice (Fig. 2*A*). Only 7.0 ± 2.2% of the total Gr-1$^+$ splenocytes from tumor-bearing mice were positive for MPO, whereas 43.5 ± 2.7% of Gr-1$^+$ cells from immunized and 33.0 ± 3.0% of Gr-1$^+$ cells from naive mice showed activity of this enzyme ($p < 0.05$; Fig. 2, *A* and *B*). MPO activity was also measured in the total cell lysates using a spectrophotometer. Gr-1$^+$ cells from tumor-bearing mice demonstrated substantially lower MPO activity than Gr-1$^+$ cells isolated from tumor-free control mice (Fig. 2*C*). We asked whether decreased activity of MPO was due to a lack of that protein. Gr-1$^+$ cells were isolated from spleens of naive and tumor-bearing mice, whole cell lysates were prepared, and the presence of MPO was evaluated using Western blotting. Gr-1$^+$ cells isolated from tumor-bearing mice had the same level of MPO as Gr-1$^+$ cells from tumor-free animals (Fig. 2*C*).

Previous studies have shown that oxidative stress by activated granulocytes in cancer patients (18) or by tumor-derived macrophages in mice (19) contributes to the suppression of T cell function. To evaluate the level of ROS in Gr-1$^+$ cells we used two dyes: DHE and DCFDA. DHE is selectively oxidized by superoxide anion, whereas the fluorescence of DCFDA indicates oxidation by hydrogen peroxide, peroxynitrite, or hydroxyl radical. Superoxide anions also can contribute to DCFDA oxidation, albeit to a lesser degree. Intracellular ROS oxidize DCFDA and form the fluorescent product 2′,7′-dichlorofluorescein, which can be monitored by measurement of fluorescence emission using flow cytometry (20). No difference in superoxide production (DHE oxidation) was found between the two groups of cells (data not shown). Gr-1$^+$ splenocytes isolated from tumor-bearing mice, then loaded with DCFDA, demonstrated a >2-fold higher proportion of the cells with bright fluorescence and a 3-fold higher intensity of total fluorescence than Gr-1$^+$ cells isolated from immunized mice, even without additional stimulation with PMA (Fig. 3). Activation of cells with PMA increased the observed differences. Thus, Gr-1$^+$ ImC from tumor-bearing mice had substantially higher levels of ROS production than ImC from tumor-free mice.

Nature of ROS produced by ImC and their role in the suppression of CD8$^+$ T cells

The pool of ROS may include different types of molecules, ranging from singlet oxygen to hydrogen peroxide. We examined what type of ROS are produced by ImC. Gr-1$^+$ cells were isolated from tumor-bearing mice, and different oxygen species were neutralized using specific inhibitors or scavengers. The effects of these compounds were evaluated by flow cytometry using DCFDA. Catalase reduced ROS levels in ImCs >4-fold, indicating that H_2O_2 contributed greatly to the overall level of ROS in these cells (Fig. 4*A*). Uric acid had similar effects, suggesting that peroxynitrite could be a substantial part of the ROS pool. However, the most noticeable differences were found in the effect of the arginase inhibitor nor-NOHA. It decreased ROS levels in ImCs >10-fold (Fig. 4*A*). This strongly suggests that arginine metabolites play a critical role in the generation of ROS in tumor-bearing mouse-derived ImCs. SOD did not significantly affect the levels of ROS (Fig. 4*A*), suggesting a rather minor contribution of superoxide to the total ROS pool, results consistent with the lack of change in DHE oxidation.

Next, we asked whether increased ROS production was responsible for ImC-mediated suppression of CD8$^+$ cells. Control mice were immunized twice with C3-specific peptide in IFA. Ten days after the last immunization, splenocytes were isolated and stimulated with either control (SIINFEKL) or specific C3-derived (RAHYNIVTF) peptides. Gr-1$^+$ ImC were isolated from C3-bearing mice (3 wk after tumor inoculation) and added to splenocytes at a 1/4 ratio. In parallel, cells were cultured in the presence of ROS scavengers or inhibitors. Selected doses of the compounds did not affect cell viability (data not shown). The Ag-specific response was evaluated in an ELISPOT assay. Inhibitors of arginase (nor-NOHA) and peroxynitrite scavenger (uric acid) did not affect the

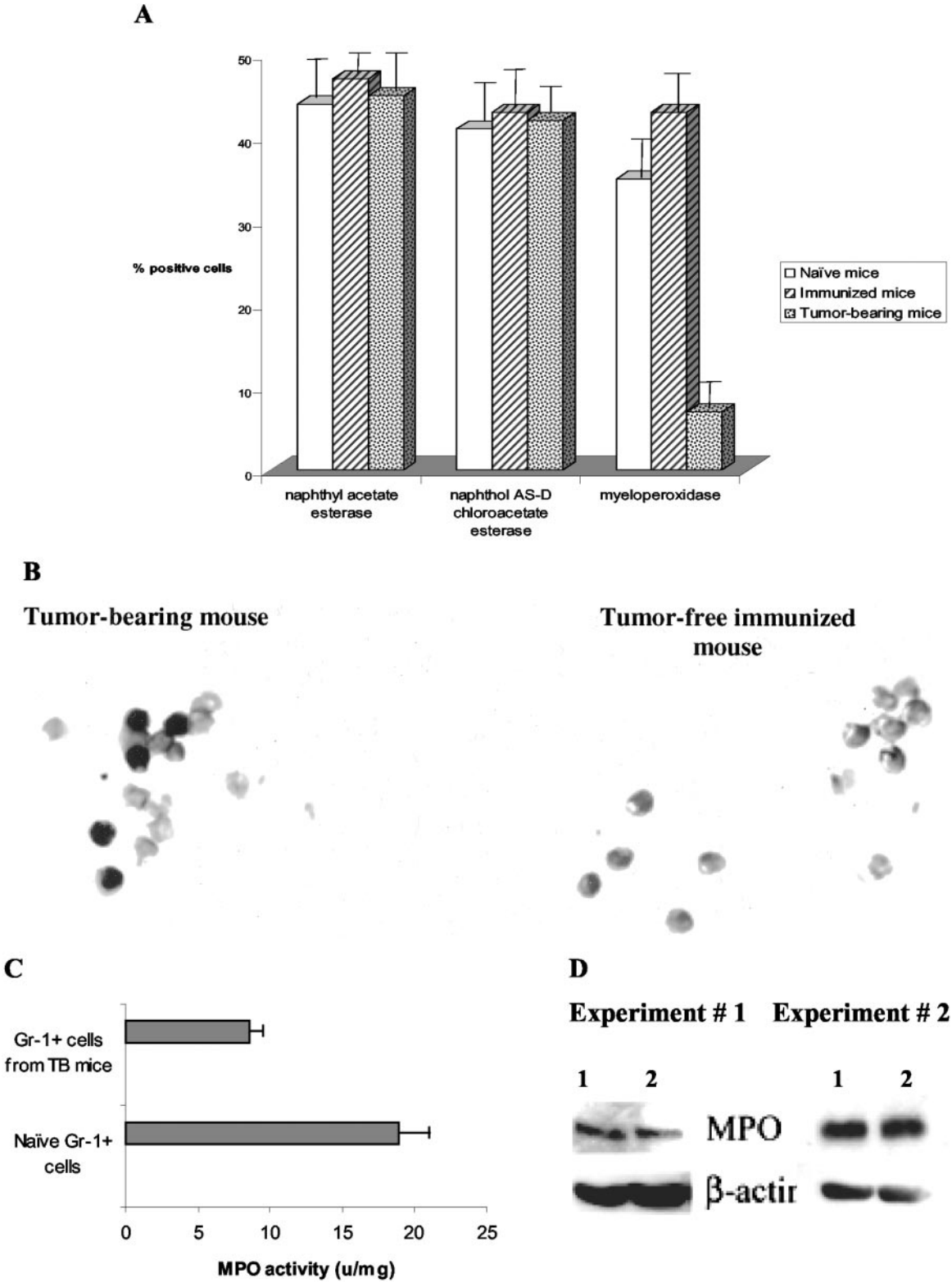

FIGURE 2. Enzymatic activity of Gr-1$^+$ splenocytes from different groups of mice. *A*, Enzymatic activity in Gr-1$^+$ ImC isolated from different groups of mice. Gr-1$^+$ cells were isolated from different groups of mice using the magnetic bead separation technique. Cytospin slides were prepared and stained using detection kits as described in *Materials and Methods*. At least 200 cells were calculated independently by two investigators, and the proportion of positive cells was calculated within the total population of ImC. The average ± SD from three experiments are shown. Differences in MPO activity between tumor-free and tumor-bearing mice were statistically significant. *B*, Typical example of MPO activity (magnification, ×400). Cells were spun down on slides and stained using an MPO-specific kit according to the manufacturer's protocol. MPO activity appears as a dark color inside the cells. Cells were counterstained with hematoxylin. *C*, MPO activity in whole cell lysates of Gr-1$^+$ ImCs. Gr-1$^+$ cells were isolated from tumor-bearing mice (TB) and tumor-free control mice (naive). MPO activity was measured in cell lysates as described in *Materials and Methods*. Experiments were performed in triplicate. MPO activity in units per milligram of protein is shown. The differences between groups were statistically significant ($p < 0.01$). *D*, Results of two experiments evaluating the presence of MPO in ImC. Gr-1$^+$ cells were isolated from tumor-free immunized (1) or tumor-bearing mice (2), whole cell lysates were prepared, and Western blotting was performed as described in *Materials and Methods*.

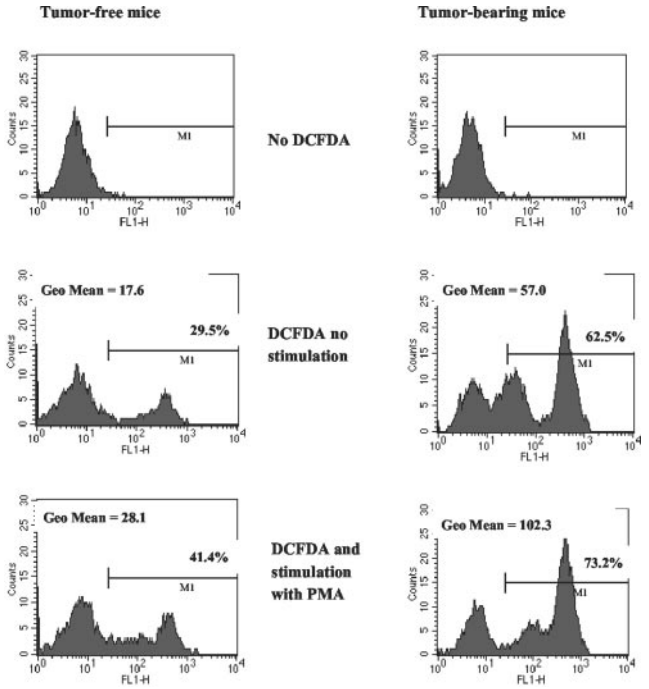

FIGURE 3. Production of ROS by Gr-1$^+$ splenocytes. Purified Gr-1$^+$ splenocytes were incubated with 2 μM DCFDA for 15 min at 37°C, then washed twice with PBS. In the case of stimulation with PMA, cells were incubated at 37°C for 5 min with 30 ng/ml PMA and then washed again with cold PBS. The intensity of fluorescence was measured by flow cytometry. Typical results from one of three experiments performed are shown. The proportion of cells with bright fluorescence was calculated. As several groups of cells had different levels of fluorescence, we used geometric mean (Geo Mean) to calculate the total intensity of fluorescence.

background level of IFN-γ-producing cells, but completely abrogated the Ag-specific inhibitory effect of ImC (Fig. 4*B*). Catalase increased the background level of IFN-γ production, but also abrogated the effect of ImC (Fig. 4*B*).

Ag-specific activation of ROS production in ImC

These data demonstrate a critical role for ROS in ImC-mediated suppression of T cells. We and others have shown that this suppression requires direct cell-cell contact and is Ag specific. The necessity of direct cell-cell contact is evident from the fact that ROS are short-lived substances operating through very short distances. However, the Ag-specific nature of the suppression may have two potential mechanisms. First, Ag-specific interaction between ImC and T cells may simply provide prolonged contact between these cells, which may be necessary for ROS to exert their effects. Second, Ag-specific interaction between T cells and ImC may affect the level of ROS produced by ImC. To test these possibilities, Gr-1$^+$ cells isolated from C3 tumor-bearing or tumor-free naive C57BL/6 mice were loaded with either the C3-derived peptide RAHYNIVTF or the OVA-derived peptide SIINFEKL, then incubated for different times, either alone or with T cells isolated from transgenic OT-1 mice bearing TCR specific for SIINFEKL. The Gr-1$^+$ cell/T cell ratio was 1/1. To distinguish Gr-1$^+$ cells during the analysis, cells were labeled with anti-Gr-1 Ab conjugated with allophycocyanin. Only Gr-1$^+$ cells were analyzed. A typical example of such analysis is shown in Fig. 5*A*. Neither of the two peptides alone affected the levels of ROS in ImCs from control or tumor-bearing mice (data not shown). After a 1-h incubation with OT-1 T cells, ImC isolated from tumor-bearing mice and loaded with specific peptide (SIINFEKL) had >2-fold higher levels of ROS than ImCs loaded with control C3-derived peptide. No such effect was observed in ImC isolated from control mice (Fig. 5*A*). These data suggest that interaction of Gr-1$^+$ cells with Ag-specific T cells in the presence of the specific Ag may stimulate ROS production by ImCs from tumor-bearing, but not control, mice.

We have investigated whether increased ROS production by Gr-1$^+$ ImC could result in apoptosis in these cells. ImC isolated from naive tumor-free or C3 tumor-bearing mice were incubated with T cells and control or specific peptide as described above. Cells were then labeled with anti-Gr-1 Ab, annexin V, and propidium iodide. The proportion of apoptotic cells among Gr-1$^+$ ImC was evaluated. The level of apoptosis in Gr-1$^+$ cells was 6–9%. No differences were found among any of the tested groups of Gr-1$^+$ ImC (data not shown).

We also investigated the type of ROS produced after ImC incubation with T cells and specific peptide. ImC were incubated with OT-1 T cells and specific peptide as described above in the presence of different inhibitors of ROS. The level of ROS in Gr-1$^+$ ImC was evaluated using DCFDA. The results showed that the same molecules contributed to the ROS pool in ImC after their contact with T cells as in nonstimulated ImC (Fig. 5*B*).

Mechanism of Ag-specific up-regulation of ROS by Gr-1$^+$ ImC

Thus, it appears that ROS production significantly increased after ImC incubation with T cells in the presence of the specific peptide. What could cause this up-regulation? IFN-γ is a major cytokine produced by activated T cells, and it has been implicated in the up-regulation of ROS production in hepatocytes, fibroblasts, and macrophages (21–24). We suggested that the release of IFN-γ by T cells could stimulate ROS production by ImC. To test this hypothesis we incubated Gr-1$^+$ ImC isolated from tumor-bearing mice for 1 h with different concentrations of IFN-γ (1–300 ng/ml). IFN-γ did not affect the level of ROS at any tested concentration (data not shown). It was possible that the addition of exogenous IFN-γ did not have the same effect on ImC as endogenous IFN-γ. To test this possibility we used Gr-1$^+$ ImC isolated from tumor-bearing mice lacking IFN-γR. These ImC were incubated with T cells from OT-1 mice at a 1/1 ratio in the presence of control (RAHYNIVTF) or specific (SIINFEKL) peptides, and the level of ROS production was evaluated in Gr-1$^+$ cells as described above. In parallel, ImC from wild-type C57BL/6 tumor-bearing mice were used. Peptides alone did not affect ROS production in Gr-1$^+$ cells (data not shown). The presence of the specific peptide during incubation of IFN-γR$^{+/+}$ ImC with T cells resulted in a 2-fold increase in ROS production compared with control peptide. Almost 50% of all ImCs expressed high levels of ROS (Fig. 6*A*). IFN-γR$^{-/-}$ ImC incubated with T cells and control peptide had substantially lower levels of ROS than their wild-type counterparts. However, specific peptide induced a similar 2-fold increase in ROS production in ImC from IFN-γR$^{-/-}$ mice as in those from wild-type mice. We also evaluated the possible immunosuppressive effect of ImC from IFN-γR$^{-/-}$ mice on T cells. ImC isolated from IFN-γR$^{-/-}$ tumor-bearing mice had the same ability to suppress an Ag-specific T cell response as ImC from IFN-γR$^{+/+}$ mice (Fig. 6*B*). Thus, taken together, these data suggested that IFN-γ production by Ag-specific T was not a factor affecting ROS production in ImC and was probably not involved in ImC-mediated T cell suppression.

We then tested the potential roles of several surface molecules by exposing ImC to immobilized Abs against MHC class I and adhesion molecules. Abs against adhesion molecules CD11b, CD18, or CD29 significantly increased ROS production by ImCs, whereas no effect was observed for anti-MHC class I Ab (Fig. 7*A*).

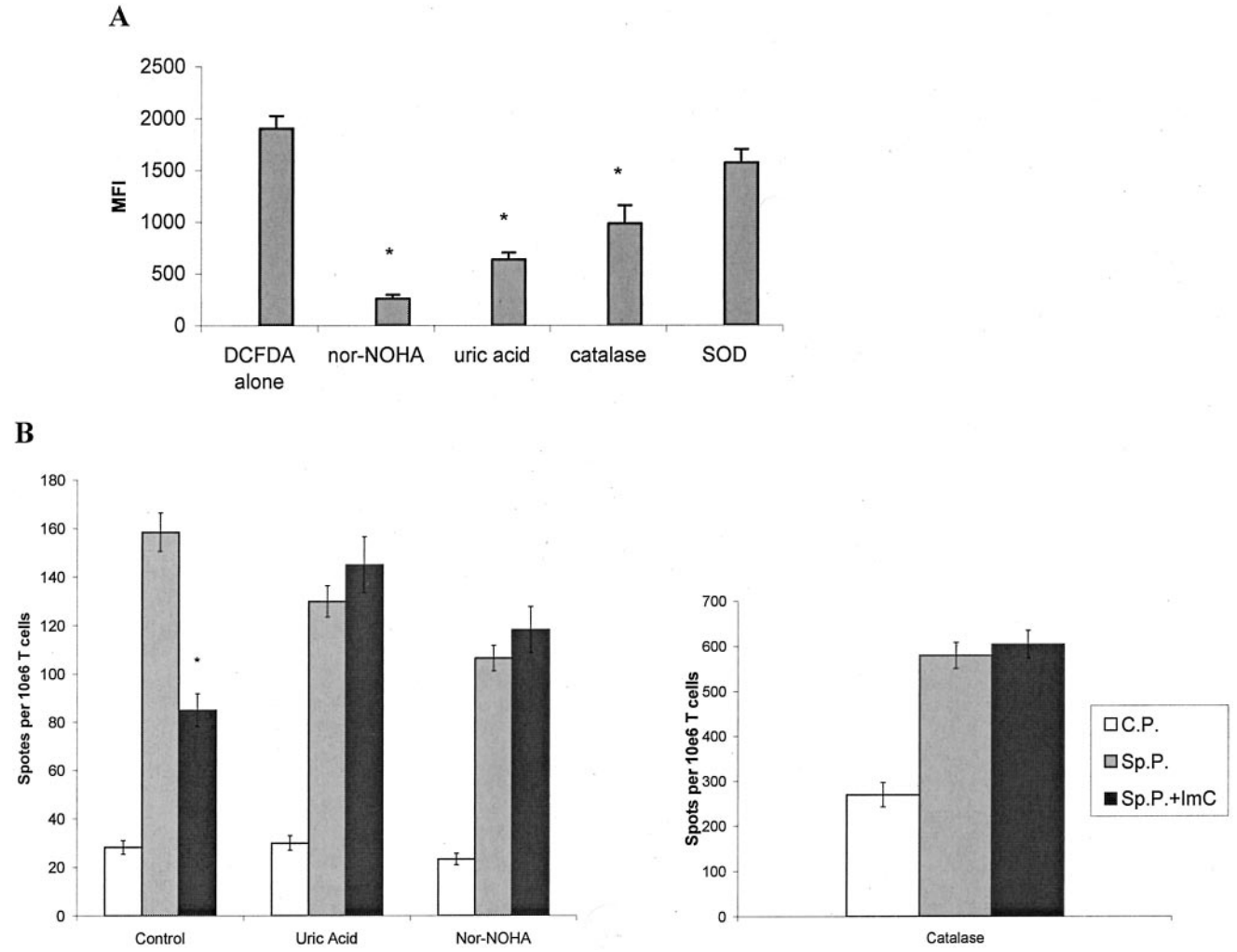

FIGURE 4. Contributions of different oxygen species to the total level of ROS in ImC and T cell inhibition. *A*, Gr-1$^+$ cells were isolated from control and tumor-bearing mice as described in *Materials and Methods*. The oxidative-sensitive dye DCFDA was used for the measurement of ROS production by these cells. To block ROS production, Gr-1$^+$ cells were incubated for 10 min at 37°C with different inhibitors, followed by 20-min incubation at 37°C with DCFDA. The following reagents purchased from Calbiochem were used: SOD, 200 U/ml; catalase, 1000 U/ml; peroxynitrite scavenger (uric acid), 0.5 mM; and arginase inhibitor (nor-NOHA), 2 μM. After incubation, cells were washed in cold PBS and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of ImC is shown. Combined results of two experiments are shown. $*$, Statistically significant differences from the group of untreated cells (DCFDA alone; $p < 0.05$). *B*, Splenocytes were isolated from mice immunized with C3-derived peptide. Gr-1$^+$ ImC were isolated from C3 tumor-bearing mice. Splenocytes (2×10^5 cells) were cultured alone or in the presence of ImC (0.6×10^5 cells). Cells were stimulated with 10 μg/ml control (C.P.; SIINFEKL) or specific (Sp.P.; RAHYNIVTF) peptide. Cells were cultured for 24 h either without ROS inhibitors (control) or with the indicated ROS inhibitors or scavengers. The number of IFN-γ-producing cells was evaluated in quadruplicate with ELISPOT as described in *Materials and Methods*. $*$, Statistically significant difference from cells stimulated with specific peptide without ImC ($p < 0.05$).

These data suggested that adhesion molecules, separately or together, could contribute to ROS-mediated immunosuppression. We clarified this possibility in experiments in which these Abs were used to neutralize the immunosuppressive effect of ImC. Gr-1$^+$ cells isolated from tumor-bearing mice were pretreated with Abs against CD11b, CD18, or CD29 molecules for 30 min at 4°C. Excess unlabeled Abs were washed off, and cells were incubated for an additional 1 h at 37°C to provide for internalization of the surface receptors. After that time, ImC were cultured with splenocytes from OT-1 mice in the presence of control or specific peptides. IFN-γ production was evaluated 24 h later in an ELISPOT assay. Preincubation of Gr-1$^+$ ImC with each adhesion molecule abrogated the suppressive effect of these cells on CD8$^+$ T cells (Fig. 7*B*).

These experiments indicated that signaling via adhesion molecules on the ImC surface could be a major mechanism of ImC-mediated immunosuppression. However, it was unclear why Ag-specific up-regulation of ROS is taking place only in ImC isolated from tumor-bearing, but not tumor-free, mice. To address this question we compared the levels of surface expression of these adhesion molecules on Gr-1$^+$ cells isolated from tumor-bearing and tumor-free mice. Gr-1$^+$ from tumor-free mice had substantially lower levels of CD11b, CD18, and CD29 molecules than Gr-1$^+$ cells from tumor-bearing mice (Fig. 7*C*). Thus, high expression of these molecules on tumor-bearing mouse-derived ImC may contribute to the unique ability of these cells to up-regulate ROS production after contact with Ag-specific T cells and suppress CD8$^+$ T cells.

Discussion

Recent data from a number of groups have demonstrated that ImC accumulating in tumor-bearing hosts play an important role in tumor nonresponsiveness by suppressing Ag-specific T cell responses (reviewed in Refs. 25 and 26). These cells may also be an

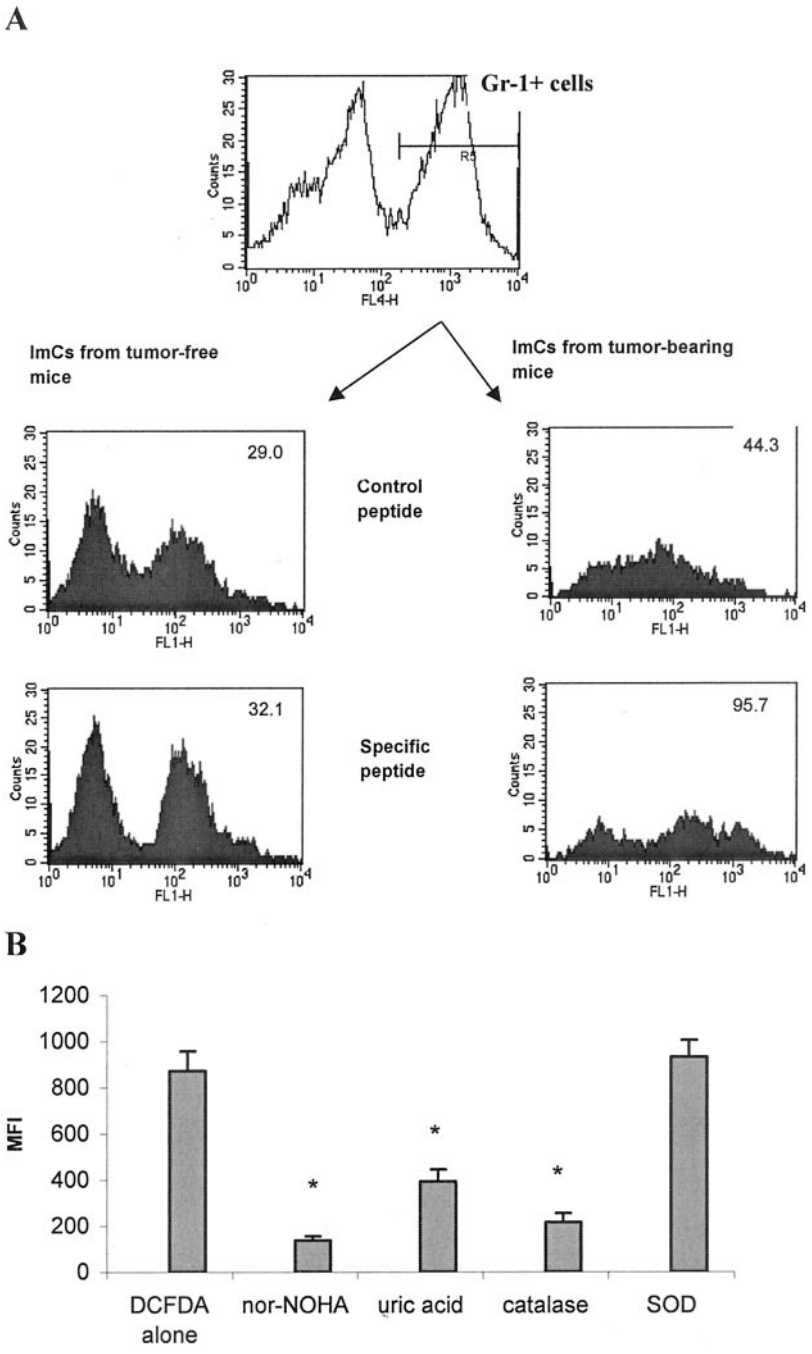

FIGURE 5. Ag-specific activation of ROS production in ImC during their interaction with T cells. *A*, Gr-1$^+$ cells were isolated from tumor-free and C3 tumor-bearing C57BL/6 mice, pulsed for 2 h with 10 μg/ml of either control (RAHYNIVTF) or OVA-derived specific peptide (SIINFEKL). After that time, cells were washed and loaded with 2 μM DCDFA. Loaded Gr-1$^+$ cells were incubated with T lymphocytes isolated from transgenic OT-1 mice bearing TCR specific for SIINFEKL. The Gr-1$^+$ cell/T cell ratio was 1:1. After 1-h incubation, cells were collected, stained with allophycocyanin-conjugated anti-Gr-1 Ab, and analyzed for ROS by flow cytometry. Only Gr-1$^+$ cells were analyzed as illustrated in the *top panel*. The two experiments with similar results were performed. The intensity of fluorescence (Geo Mean) for each histogram is shown. *B*, Gr-1$^+$ cells isolated from C3 tumor-bearing mice were pulsed for 2 h with the OVA-derived peptide SIINFEKL, washed, and then incubated for 1 h with T cells isolated from OT-1 mice. The Gr-1$^+$ cell/T cell ratio was 1:1. Different inhibitors of ROS were added as described in Fig. 4*A*. ROS production was measured using DCFDA. After incubation with DCFDA, cells were labeled with allophycocyanin-conjugated anti-Gr-1 Ab. The level of ROS was measured in gated populations of Gr-1$^+$ cells. The mean fluorescence intensity (MFI) of ImC is shown. The combined results of two experiments are shown. *, Statistically significant differences from the group of untreated cells (DCFDA alone; $p < 0.05$).

important factor contributing to the failure of tumor vaccines in patients with advanced cancer. In mice, ImC were characterized as Gr-1$^+$ or Gr-1$^+$CD11b$^+$ cells. Myeloid lineage differentiation Ag Gr-1 (Ly 6G) is expressed on myeloid precursor cells and granulocytes and transiently on monocytes (27). In normal mice, Gr-1$^+$ cells can be found primarily in the bone marrow (25–35%) and at low levels in the spleen (2–4%). Numerous reports have demonstrated that tumor growth is associated with systemic expansion of Gr-1$^+$ myeloid cells coexpressing the CD11b (Mac-1) marker (7, 8, 12, 28). An increase in the Gr-1$^+$ splenocyte population was also demonstrated in infected tumor-free mice (29) and after the administration of potent Ag (11, 15).

These data raise questions about the biological role of ImCs. Indeed, these cells are an intrinsic part of myeloid cell differentiation and are present in healthy individuals. Their production is significantly increased in response to vaccination or bacterial infection. However, neither healthy nor vaccinated individuals suffer from systemic immunosuppression. Moreover, tumor-bearing mice and cancer patients do not display signs of systemic immunosuppression until the tumor becomes bulky or in the late stages of metastatic disease.

As we demonstrated previously, highly purified ImCs from tumor-bearing mice suppress CD8$^+$ T cell response. This suppression was NO independent and Ag specific, required direct cell-cell contact, and was dependent on MHC class I (12). However, ImC obtained from neither naive nor vaccinated mice were able to suppress CD8$^+$ T cells. Our experiments revealed striking differences between Gr-1$^+$ ImC isolated from tumor-bearing and tumor-free mice. Tumor-bearing mouse-derived ImC had 5-fold fewer MPO-positive cells than the population of ImC obtained from tumor-free mice. This was associated with a >3-fold higher level of ROS. These differences were not restricted to only one type of tumor or

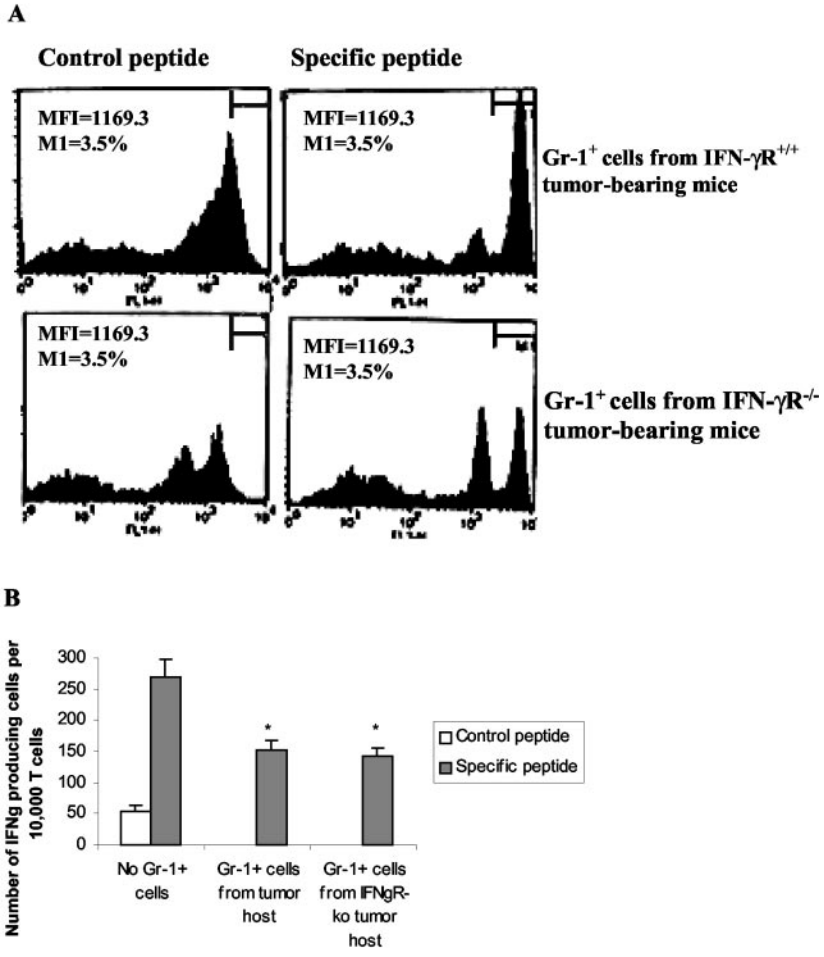

FIGURE 6. IFN-γ is not responsible for up-regulation of ROS in ImC during the contact with Ag-specific T cells. *A*, Gr-1$^+$ cells were isolated from C3-bearing wild-type or IFN-$\gamma R^{-/-}$ mice, pulsed with control (C3-derived) or specific (OVA-derived) peptides and incubated for 1 h with T cells isolated from OT-1 mice. ROS production in ImC was measured as described in Fig. 5. MFI, mean fluorescence of all cells; M1, proportion of cells with a high level of ROS. Two experiments with the same results were performed. *B*, Gr-1$^+$ cells were isolated from C3-bearing wild-type or IFN-$\gamma R^{-/-}$ mice, and T cells were isolated from OT-1 mice. Irradiated splenocytes from control C57BL/6 mice were used as APC. Each well contained 10^4 T cells, 10^5 irradiated splenocytes, and 10^3 Gr-1$^+$ cells. Cells were incubated in quadruplicate for 24 h in the presence of 10 μg/ml control (RAHYNIVTF) or OVA-derived specific peptide (SIINFEKL). The number of IFN-γ-producing cells was evaluated in an ELISPOT assay. $*$, Statistically significant differences from the results obtained in the absence of ImC ($p < 0.05$).

one strain of mouse. We observed this in CT-26 tumor-bearing BALB/c mice as well as in C3 tumor-bearing C57BL/6 mice. MPO catalyzes a reaction between hydrogen peroxide and chloride to generate hypochlorous acid, a potent oxidant with strong microbicidal activity (30). In addition, a new important function for MPO has recently been demonstrated to play a buffering role to protect the granule proteases from oxidative degradation by high levels of H_2O_2 (31). We hypothesized that the lack of MPO activity might affect the levels of ROS in these cells because MPO is a main consumer of hydrogen peroxide (32).

It appears that H_2O_2 is important component of ROS in ImCs. This was confirmed by the fact that catalase, which converts H_2O_2 to oxygen and water, decreased ROS levels in these cells >4-fold. However, it appears that the largest contribution to the ROS pool is arginase activity. The arginase inhibitor nor-NOHA decreased the total level of ROS in ImC derived from tumor-bearing mice by >10-fold. Arginase catalyzes the hydrolysis of L-arginine to urea and L-ornithine. L-arginine is used by NO synthase as a substrate for the generation of NO (33). However, low concentrations of L-arginine result in low NO formation and high generation of superoxide ($O_2^{\cdot -}$) (reviewed in Ref. 34). Thus, high arginase activity in tumor-bearing mouse-derived ImC may have lowered the level of L-arginine and resulted in increased production of $O_2^{\cdot -}$ instead of NO. Superoxide itself is very unstable and is converted to H_2O_2 and oxygen. This is consistent with our data showing that in ImC, ROS accumulates primarily in the form of H_2O_2, not $O_2^{\cdot -}$. There are several reports showing increased arginase activity in squamous cell and basal cell carcinomas of the skin as well as in prostate cancer (35, 36). Consistent with our findings, overexpression of arginase in macrophages promoted tumor growth (37). Several known tumor-derived factors, such as TGF-β and IL-10, are able to increase arginase activity in macrophages (38, 39), and a number of other cytokines and growth factors produced by tumor can induce ROS production, including IL-6, IL-3, platelet-derived growth factor, GM-CSF, and fibroblast growth factor (reviewed in Ref. 40). Constant production of these factors in tumor-bearing mice could lead to the different levels of ROS observed in ImC from tumor-bearing and tumor-free mice.

It appears that the main target for arginase and oxygen species on T cells is CD3ζ. L-arginine starvation results in a decrease in CD3ζ expression in Jurkat cells, due in part to decreased mRNA stability (41). Otsuji et al. (10) found that oxidative stress, caused by tumor-derived macrophages (Mac-1$^+$ splenocytes that coexpressed Gr-1) suppressed ζ-chain expression in T cells. Granulocyte-derived H_2O_2 has been shown to be involved in the inhibition of IFN-γ production and the suppression of CD3ζ-chain expression by T cells in advanced cancer patients (18).

Thus, increased ROS production by ImC derived from tumor-bearing mice seems to be a major factor responsible for inhibition of the CD8$^+$ T cell response. ROS are short-lived substances, exerting their effect over a very short distance, which could explain the finding that ImC need direct contact with T cells to suppress their response and also explain the Ag-specific nature of the inhibition. Previous studies in vitro and in vivo have indicated that the Ag-specific interaction between T cells and APCs is much more stable and lasts much longer than interaction in the absence of the Ag (42, 43). Gr-1$^+$ ImC express MHC class I molecules, but a low or undetectable level of MHC class II (12). This lack of MHC class

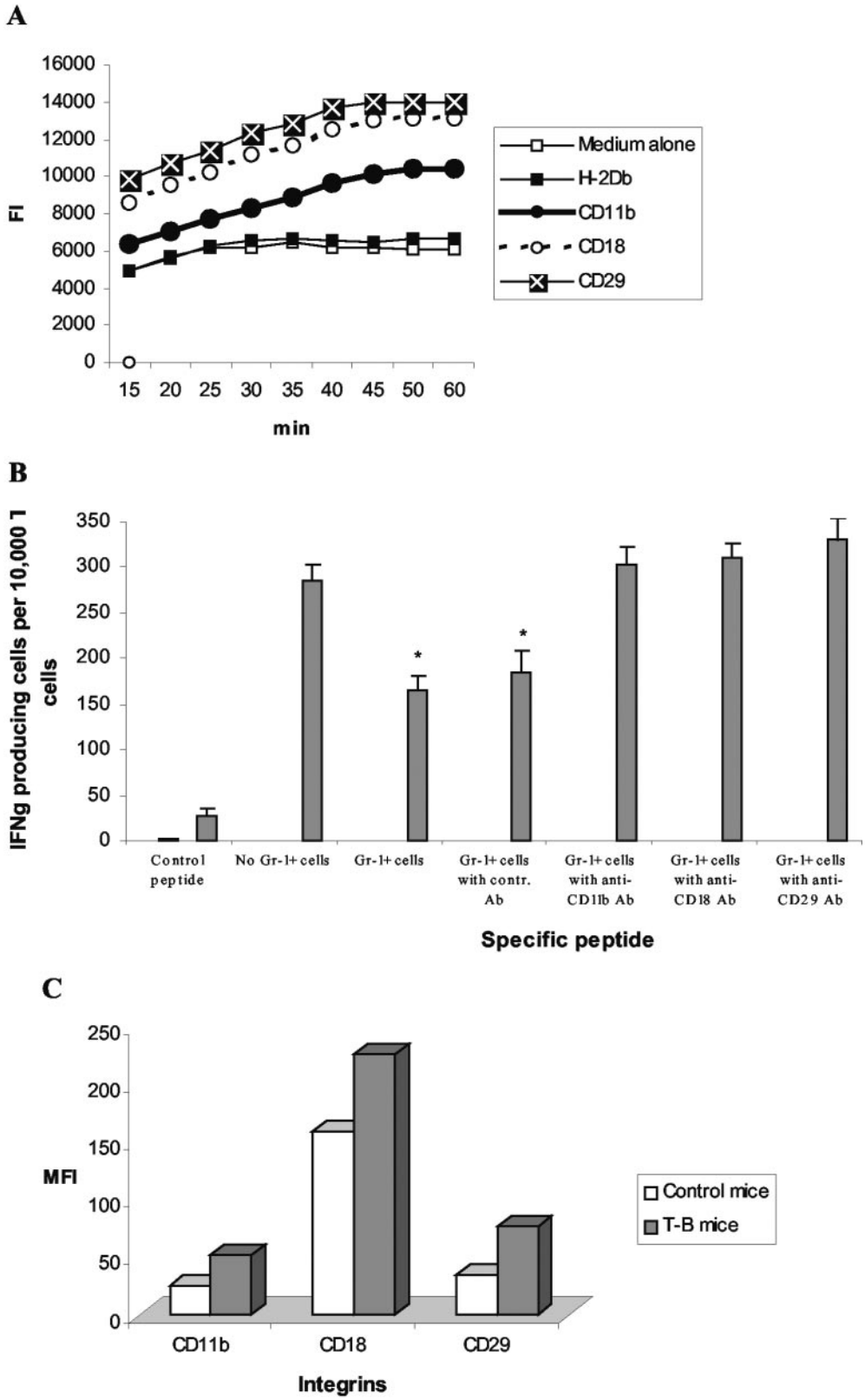

FIGURE 7. Role of integrins in ROS production by ImC and T cell suppression. *A*, Purified anti-CD11b, CD18, CD29, and H-2D^b Abs were immobilized in 96-well plates by affinity binding of the Fc portion of the Abs to protein G-coated surfaces. Gr-1$^+$ cells (3×10^5) isolated from C3 tumor-bearing mice were loaded with DCFDA and incubated at 37°C in triplicate in plates precoated with different Abs. The kinetics of DCFDA oxidation were measured in a Wallac 1420 plate fluorometer. Readings (fluorescence intensity (FI)) were taken at 488/538 nm every 5 min for a total of 60 min. *B*, Gr-1$^+$ cells isolated from C3 tumor-bearing mice were labeled for 30 min at 4°C with 5 μg of Abs as indicated. Excess Abs were washed away, and cells were incubated for an additional 60 min at 37°C to allow for internalization of the receptors. Gr-1$^+$ cells were then used to inhibit CD8$^+$ T cells exactly as described in Fig. 6*B*. ∗, Statistically significant differences from the results obtained in the absence of ImC ($p < 0.05$). *C*, Gr-1$^+$ cells were isolated from naive, tumor-free, and C3 tumor-bearing C57BL/6 mice. Cells were labeled with Abs against indicated integrins, and the levels of expression were measured by flow cytometry. MFI, mean fluorescence intensity. Two experiments with the same results were performed.

II molecules may preclude them from the formation of Ag-specific interactions with $CD4^+$ cells, which may explain the lack of suppression of $CD4^+$ T cells (12).

Ag-specific interactions between ImC and T cells resulted in a significant increase in ROS production by ImC, possibly further contributing to inhibition of the T cell response. This effect was observed only in ImC obtained from tumor-bearing, not tumor-free, mice. Our initial hypothesis was that T cells activate ROS production via IFN-γ, because activation of ROS production by IFN-γ has been described previously (21, 23). To test this hypothesis we used ImC isolated from tumor-bearing IFN-γR knockout mice, which should not react to IFN-γ released by T cells. Despite the fact that the background level of ROS in IFN-$\gamma R^{-/-}$ ImC was lower than that in wild-type cells, they up-regulated ROS after interaction with Ag-specific T cells to the same degree as their wild-type counterparts. In addition, IFN-$\gamma R^{-/-}$ ImC inhibited the $CD8^+$ T cell response in a similar manner as IFN-$\gamma R^{+/+}$ ImC. This indicates that IFN-γ probably does not play a major role in that process. Adhesion molecules, and integrins in particular, have been recently implicated in ROS production by macrophages and fibroblasts (44–46). Adhesion molecules also play an important role in the interaction between T cells and APCs. All ImC isolated from tumor cells express high level of integrin α_M-chain (CD11b). Our experiments have shown that CD11b as well as integrin β_2-chain (CD18) and integrin β_1-chain (CD29) are actively involved in up-regulation of ROS production in ImC. Moreover, neutralization of these molecules resulted in abrogation of the immunosuppressive effect of ImC. Therefore, the lack of up-regulation of ROS production and immunosuppression in ImC obtained from tumor-free mice could be explained by the significantly lower level of integrins compared with ImCs from tumor-bearing mice.

In conclusion, our data suggest a new mechanism for Ag-specific, ImC-mediated suppression of $CD8^+$ T cells in cancer. Tumor-derived factors constantly stimulate the production of activated ImC. These cells have high level of arginase activity, which results in accumulation of ROS, mainly in the form of H_2O_2. Inhibition of T cell activity by ImC requires the presence of Ag and direct contact with Ag-specific T cells. Specific Ag stabilizes cell contact between ImC and Ag-specific T cells, which results in increased ROS production via signaling through integrins. Freshly isolated ImC do not express MHC class II, but are positive for MHC class I, which may explain the inhibition of $CD8^+$, but not $CD4^+$, T cells by these ImCs. The requirement for direct contact with T cells in the presence of specific Ags also explains the lack of systemic immunosuppression in tumor-bearing hosts despite the abundant presence of immature myeloid cells.

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