The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 01 2005

Restoration by IL-15 of MHC Class I Antigen-Processing Machinery in Human Dendritic Cells Inhibited by Tumor-Derived Gangliosides[1] FREE

Irina L. Tourkova; ... et. al

J Immunol (2005) 175 (5): 3045–3052.
https://doi.org/10.4049/jimmunol.175.5.3045

Restoration by IL-15 of MHC Class I Antigen-Processing Machinery in Human Dendritic Cells Inhibited by Tumor-Derived Gangliosides[1]

Irina L. Tourkova,* Galina V. Shurin,* Gurkamal S. Chatta,†§ Lori Perez,* James Finke,¶ Theresa L. Whiteside,*‡§ Soldano Ferrone,‖ and Michael R. Shurin[2]*‡§

We have recently reported that MHC class I Ag-processing machinery (APM) component expression in dendritic cells (DC) might be down-regulated by tumor cells. However, the tumor-derived factors responsible for inhibition of the APM component expression in DC generated in the tumor microenvironment as well as potential protective mechanism have not yet been investigated. In this article, we demonstrate that expression of several MHC class I APM components, including MB1 (β5), LMP2, LMP7, LMP10, and ERp57, is significantly down-regulated in human DC generated in the presence of primary oral squamous cell carcinoma cell lines or coincubated with purified gangliosides. Suppression of MHC class I APM component expression in DC generated in the presence of tumor cells was significantly attenuated by the inhibition of glucosyl transferase in tumor cells, suggesting that tumor-induced MHC class I APM component down-regulation in DC was mediated in part by oral squamous cell carcinoma-derived gangliosides. Furthermore, rIL-15 restored both tumor cell-induced and ganglioside-induced MHC class I APM component expression in DC, as well as their ability to present Ags to autologous Ag-specific T cells. These results demonstrate that IL-15 restores MHC class I APM component expression in DC down-regulated by tumor-derived gangliosides. *The Journal of Immunology,* **2005, 175: 3045–3052.**

Dendritic cells (DC)[3] are the most efficient APCs, capable of priming naive T lymphocytes. DC play a crucial role in the generation of tumor Ag (TA)-specific immune response by activating Ag-specific naive MHC class II-restricted $CD4^+$ T cells as well as MHC class I-restricted $CD8^+$ T cells (1, 2). DC are also capable of cross-presenting exogenous Ags via the MHC class I Ag-processing pathway (3, 4). To escape from immune recognition, tumor cells have evolved mechanisms that induce dysfunction of the DC system. Suppressed DC functions have been reported in various in vitro models simulating the tumor microenvironment, and in tumor-bearing animals, as well as in cancer patients (5–7). Several reports have described inhibitory effects of soluble tumor-derived factors such as IL-6, IL-10, TGF-β, vascular endothelial growth factor, and gangliosides on DC generation and function (8–11). Many tumor cells express membrane-associated gangliosides, which may be shed into the tumor microenvironment (12, 13). Purified or tumor-derived gangliosides inhibit the ability of human monocytes and DC to initiate MHC class II Ag-restricted T cell responses, by down-regulation of costimulatory and MHC class II molecules on DC. Gangliosides also reduce IL-12 and TNF-α production by DC (14–17). However, it is unknown whether gangliosides might have effects on Ag-processing machinery (APM) component expression. We have recently reported that MHC class I APM components in DC is down-regulated by tumor cells (18). However, the molecular mechanisms underlying these phenotypic and functional changes in DC have not yet been investigated.

APM components play a crucial role in the assembly of the trimolecular MHC class I complex, which consists of the MHC class I H chain, β2-microglobulin, and an Ag-derived peptide (19). APM components include the following: constitutive β subunits of the proteolytic delta (δ) (or β1) and MB1 (β5); inducible proteasome (immunoproteasome) β-type subunits LMP2, LMP7, and LMP10; peptide transporters TAP1 and TAP2; and endoplasmic reticulum chaperones calnexin, calreticulin, ERp57, and tapasin. Acting in concert, these components are responsible for generation of antigenic peptides, their translocation into endoplasmic reticulum, and loading of β2-microglobulin-associated MHC class I H chains with peptides (20). Defects in MHC class I APM component expression are likely to affect the cell surface expression of the MHC class I trimolecular complex and/or the repertoire of Ag-derived peptides presented by MHC class I Ags as well as DC interaction with T lymphocytes necessary for Ag presentation. Although the components of MHC class I Ag-processing and Ag presentation pathways have been well characterized in human DC (3, 21), it is not known how tumor-derived factors affect Ag-processing pathways in DC. Also, the ability of cytokines to interact with the components of these pathways in DC has not been evaluated to date.

IL-15 is produced by a variety of cells in response to environmental stimuli and has a broad spectrum of biological activities in

*Department of Pathology, †Department of Medicine, and ‡Department of Immunology, University of Pittsburgh Medical Center, and §University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213; ¶Department of Immunology, Cleveland Clinic Foundation, Cleveland, OH 44195; and ‖Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263

Received for publication December 29, 2004. Accepted for publication June 17, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Institutes of Health Grants RO1 CA 084270 (to M.R.S.) and RO1 CA 67108 (to S.F.).

[2] Address correspondence and reprint requests to Dr. Michael R. Shurin, Clinical Immunopathology, 5725 Children's Hospital of Pittsburgh, Main Tower, 200 Lothrop Street, Pittsburgh, PA 15213. E-mail address: shurinmr@upmc.edu

[3] Abbreviations used in this paper: DC, dendritic cell; TA, tumor Ag; APM, Ag-processing machinery; SCC, squamous cell carcinoma; CM, complete medium; rh, recombinant human; PPMP, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol; siRNA, small interfering RNA; SI, stimulation index; PFA, paraformaldehyde; RT, room temperature; MFI, mean fluorescence intensity.

0022-1767/05/$02.00

various types of cells, including T and B lymphocytes, NK cells, mast cells, granulocytes, monocytes/macrophages, and DC (22–25). Although IL-15 uses the common β and γ subunits of the IL-2R, it exerts different biological effects than IL-2 on most cell populations (26). IL-15 itself is able to activate DC in vivo and in vitro, and the exposure of splenic DC to IL-15 up-regulates costimulatory molecules, markedly increases IFN-γ production, and enhances the ability of DC to stimulate Ag-specific $CD8^+$ T cells (25, 27). Although the role of IL-15 in regulation of DC generation, function, and survival is well established, its effects on Ag-processing pathways in DC in the tumor microenvironment have not been characterized.

Using a unique set of APM component-specific mAb, we report that human DC generated in the presence of oral squamous cell carcinoma (SCC) cell lines down-regulate expression of several APM components, including MB1 (β5), LMP2, LMP7, LMP10, and ERp57. It appears that tumor-derived gangliosides are responsible for this effect and that incubation of DC with IL-15 reverses APM component down-regulation.

Materials and Methods

Cells

Primary oral SCC cell lines PCI-4B and PCI-38 established at the University of Pittsburgh Cancer Institute and described previously (28) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin sulfate, 10 mM HEPES, 5% FBS, 10 mM nonessential amino acids, and 1 mM sodium pyruvate, (Invitrogen Life Technologies). This medium is referred to as complete medium (CM).

DC were generated from PBMC of healthy donors. After gradient separation on Histopaque-1077 (Sigma-Aldrich) and lysis of RBC, PBMC were resuspended in CM (5×10^6cells/ml) and incubated for 1 h at 37°C. Nonadherent cells were removed, and adherent monocytes were cultured in CM with 1000 U/ml recombinant human (rh)GM-CSF and 1000 U/ml rhIL-4 (PeproTech). On day 7, nonadherent cells were collected and analyzed. Their morphology and phenotype corresponded to immature DC characterized previously (18, 29). Percentage ranges for various surface markers in DC obtained from 10 donors were 72–99% for HLA-DR, 60–87% for CD86, 16–25% for CD80, 12–30% for CD83, 54–88% for CD40, and 2–20% for CD14.

DC ($0.7–1.5 \times 10^6$cells/well) were coincubated with 40 μg/ml disialoganglioside GD_3 (tested at a range of concentrations from 20 to 80 μg/ml) (Matreya) reconstituted in DMSO (Sigma-Aldrich) and added once a day for the first 3 days in culture. DC were also incubated with SCC cell lines PCI-4B or PCI-38 (25×10^4cells/well) in CM separated by membrane inserts with 0.4-μm pore size (Transwell system) for the first 3 days in culture. As a control, DC were treated with medium alone, medium with DMSO, or coincubated with immortalized human keratinocytes HET-1A (CRL-2692; American Type Culture Collection) in the same way. To test the ability of IL-15 to restore the integrity of APM in DC in the tumor microenvironment, we added rhIL-15 (50 ng/ml) to DC generated in the presence of SCC cell lines and to control DC once per day for the last three consecutive days in culture.

To block ganglioside synthesis, tumor cells were cultured in the presence of inhibitor of glucosyl transferase D-threo-PPMP (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol · HCl) (5 μM) (Matreya) once a day for 3 days.

Antibodies

mAbs to individual APM components were generated and characterized according to the methodology we described previously (18). The anti-calnexin mAb TO-5, anti-calreticulin mAb TO-11, anti-ERp57 mAb TO-2, and anti-tapasin mAb TO-3 were developed and characterized as described previously (30). The anti-MB1 mAb SJJ-3, anti-low m.w. protein (LMP2) mAb SY-1, anti-LMP7 mAb SY-3, and anti-LMP10 mAb TO-7 were developed and characterized using the strategy described elsewhere (30, 31). Briefly, the mAb-secreting hybridomas were derived from BALB/c mice immunized with synthetic peptides derived from the amino acid sequence of the native protein and with recombinant proteins. Abs of the desired specificity were identified by their specific binding to the immunizing peptides in ELISA. The specificity of the selected mAb was proven by their reactivity with the corresponding Ags when tested with lymphoid cell lysates with the appropriate phenotype in Western blotting. The specificity of anti-LMP2 mAb SY-1 was corroborated further by their lack of reactivity with a lysate of the T2 cell line, which does not express these molecules. For additional controls, rabbit anti-LMP2 and anti-LMP7 polyclonal Ab were purchased from Affinity Research Products. mAb W6/32 was produced by Dr. A. DeLeo (University of Pittsburgh, Pittsburgh, PA) using a hybridoma obtained from American Type Culture Collection. Labeled mAbs for staining of surface Ags on DC (anti-HLA-DR, -HLA-ABC, -CD80, -CD83, -CD86, -CD40, and -CD14) by flow cytometry were purchased from BD Biosciences Pharmingen. FITC-conjugated goat anti-mouse and goat anti-rabbit IgG Ab were purchased from The Jackson Laboratory. Anti-human disialoganglioside GD3 Ab were purchased from BD Biosciences Pharmingen.

Small interfering RNAs (siRNA) and nucleofection

siRNA for LMP10 and MB1 were purchased from Dharmacon. Nucleofection efficiencies were determined with 5 μg of plasmid DNA encoding the GFP using Human Dendritic Cell Nucleofector Kit (Amaxa) and varied between 40 and 60%. Nucleofection was conducted according to the manufacturer-optimized protocol for human DC (Amaxa). Briefly, 6-day-old DC were centrifuged, and 2×10^6 cells were resuspended in Dendritic Cell Nucleofection Solution (Amaxa). Next, the cells were mixed with 5 μg of LMP10 or MB1 siRNA, transferred to the special cuvette, and inserted into the Nucleofector (program U-02). All of the samples were removed from the cuvette immediately after the program had been finished, transferred into the 6-well plates, and cultured in CM (0.25×10^6 cells/ml) with 1000 U/ml rhGM-CSF and 1000 U/ml rhIL-4 for 12–18 h at 37°C.

Western blot

The protein expression of APM components in nucleofected and control DC was assessed using a Western blot technique. Cells were collected, washed in HBSS, and lysed in 100 μl of extraction buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 0.6 mM PMSF, and 5 μg/ml leupeptin) for 30 min on ice. After centrifugation ($9000 \times g$, 15 min, at 4°C), the protein concentration was determined in the supernatants by the Bradford method using the BioRad protein kit (Bio-Rad). Fifty micrograms of total proteins were dissolved in electrophoresis sample buffer, separated by 4–12% PAGE (NuPAGE), and transferred to a nitrocellulose membrane (NOVEX). The membrane was blocked with 0.5% nonfat milk, 0.1% Tween 20 (Fisher Scientific) in 20 mM Tris-HCl buffer (pH 7.2). LMP10 and MB1 were detected using the anti-LMP10 mAb TO-7 and anti-MB1 mAb SJJ-3 in a final concentration of 100 μg/ml and goat anti-mouse secondary mAb (IgG (H+L), 1/150,000 dilution; Pierce). Expression of β-actin was evaluated as a housekeeper control protein using mouse anti-human mAb (IgG1, 1/100,000 dilution; Sigma-Aldrich). Secondary Abs were the same as for APM components detection. The membrane was processed and treated with chemiluminescent reagents (Pierce), and the bands were visualized on a Kodak film (Eastman Kodak).

OVA-specific presentation by DC

To evaluate the capacity of DC to present Ags, 6-day-old DC, which had been incubated for the first 3 days with tumor cells, and control DC incubated with immortalized keratinocytes were pulsed overnight with OVA (Sigma-Aldrich) (500 μg/ml). Next, DC were washed twice and cultured for 96 h at 37°C, 5% CO_2 in 96-well plates in CM with autologous OVA-specific $CD8^+$ T cells (1×10^4 cells/well) at different DC:T ratios (1:100 to 1:1). At the end of incubation, T cell proliferation was measured by uptake of [^{3}H]thymidine (1 μCi/well, 5 Ci/mmol; PerkinElmer Life Science) added for the last 16–18 h. Cultures were harvested on GF/C glass fiber filter paper (Whatman) using a MACH III microwell harvester (Tomtec). [^{3}H]Thymidine incorporation was determined in a MicroBeta TriLux liquid scintillation counter (Wallac). Counts are expressed as cpm and presented as stimulation index (SI). SI was calculated as stimulated cpm divided by spontaneous cpm.

To generate autologous OVA-specific T cells, $CD8^+$ T cells were isolated from PBMC using MACS paramagnetic purification system (Miltenyi Biotec) and cultured with weekly addition of irradiated autologous PBMC (1:1), OVA (Sigma-Aldrich; 500 μg/ml), and IL-2 (10 IU/ml) for 4 wk.

Flow cytometry analysis of DC and SCC cells

Intracellular staining for detection of APM protein expression in DC was performed with modifications, as described earlier (30). Briefly, DC were washed three times in PBS/1% BSA, fixed with 2% paraformaldehyde (PFA) at room temperature (RT) for 20 min, washed three times in PBS/1% BSA, resuspended in 10 ml of PBS containing 0.5% BSA, transferred to glass flasks, and treated in a microwave. Cells were then immediately

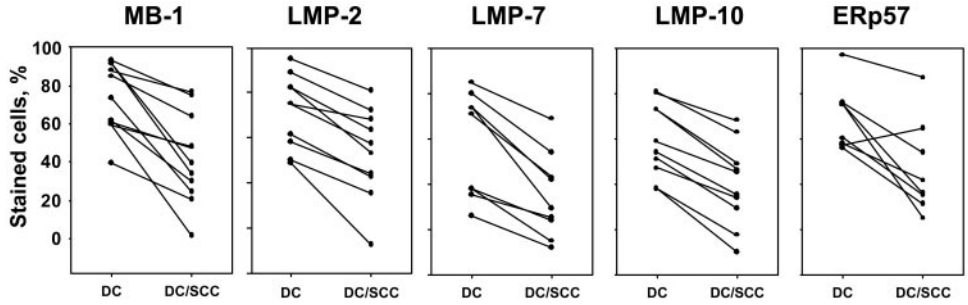

FIGURE 1. HLA class I APM component down-regulation in human DC coincubated with oral SCC cell lines in vitro. DC precursors were cocultured with SCC cell lines PCI-4B or PCI-38 in a Transwell system for 72 h. Tumor-treated and control (keratinocyte-treated) DC were intracytoplasmatically stained with APM component-specific mAb to MB1, LMP2, LMP7, LMP10, ERp57, tapasin, calnexin, and calreticulin, and analyzed by flow cytometry as described in *Materials and Methods*. Expression of MB1, LMP2, LMP7, LMP10, and ERp57 was significantly down-regulated in SCC-treated DC ($p < 0.05$). Data represent the percentage of positive cells from 10 independent donors.

chilled on ice for 10 min, washed three times in PBS-1% BSA, permeabilized at RT for 30 min with 100 μl/sample of PBS containing 1% BSA and 0.1% saponin (Sigma-Aldrich), and stained with an optimal amount of primary mAb at RT for the next 30 min. Murine IgG were used as a control. Cells were then washed three times with PBS containing 1% BSA and 0.1% saponin, and incubated with FITC-conjugated goat anti-mouse IgG Ab (1:100) (Jackson ImmunoResearch Laboratories) at RT for 30 min. Cells were washed three times again and fixed with 0.5% PFA.

For evaluation of ganglioside expression, tumor cells (PCI-4B and PCI-38) were trypsinized, washed in FACS medium (PBS containing 0.1% BSA and 0.1% NaN_3), fixed in 1% PFA (15 min on ice), followed by washing with FACS medium containing 0.1% saponin and staining with murine anti-human disialoganglioside GD3 Ab (BD Biosciences Pharmingen) for 30 min at 4°C. Murine IgG was used as a control. Next, tumor cells were washed with FACS medium containing 0.1% saponin and incubated with FITC-conjugated goat anti-mouse IgG Ab (1:100) (Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Finally, cells were washed in FACS medium and fixed with 0.5% PFA.

Fluorescence was measured using a FACScan flow cytometer (BD Biosciences), and the data analysis was performed using the CellQuest Software (BD Biosciences). Results are expressed as percentage of positive cells and as mean fluorescence intensity (MFI).

HPLC for gangliosides in PCI-4B cells

To confirm ganglioside expression in SCC cells, total lipid extraction of tumor cells (10^6 cells) was performed using chloroform:methanol, and gangliosides were isolated from the lipids by a diisopropyl ether/1-butanol partition and subsequent Sephadex G-25 gel filtration step (32). The water-soluble gangliosides were further fractionated by HPLC using a LiChrosorb-NH2 column (Merck). Gangliosides were eluted using a linear flow rate of 0.5 ml/min throughout, where solvent A was Acetonitrile-5 mM phosphate buffer, pH5.6 (83:17) and solvent B was Acetonitrile-20 mM phosphate buffer, pH5.6 (50:50). Individual bovine brain-derived gangliosides were also subjected to HPLC analysis to establish the retention times of known gangliosides (GM2, GM1, GD3, GD1a, GD1b, and GT1b).

Statistical analysis

The differences between groups were determined using the Student's *t* test or the nonparametric Mann-Whitney *U* test after evaluation for normality. For multiple group comparison, ANOVA was used. For all statistical analyses, a *p* value of 0.05 was considered significant. Data are presented as mean ± SEM. All of the experiments were repeated at least three times.

Results

Down-regulation by oral SCC cells and restoration by IL-15 of APM component expression in DC

Previously, we observed that the expression of some APM components was down-regulated in DC incubated with SCC cells. To confirm these findings, we evaluated the expression of MB1 (β5), LMP2, LMP7, LMP10, calnexin, calreticulin, ERp57, and tapasin in DC, which had been coincubated with SCC cells in a Transwell system, intracytoplasmatically stained with APM component-specific mAb, and analyzed by flow cytometry. As a control, DC were treated with medium alone or coincubated with immortalized keratinocytes. In 10 independent experiments with DC from 10 donors, expression of MB1, LMP2, LMP7, LMP10, and ERp57 was significantly and reproducibly decreased in DC generated in the presence of the PCI-4B or PCI-38 cell lines (Fig. 1). For instance, the tumor-induced inhibition of APM components expression in DC was 44.8 ± 9.6% for MB1, 39.4 ± 8.7% for LMP2, 43.3 ±

Table I. *Restoration by IL-15 of HLA class I APM component expression in DC preincubated with oral SCC cells*[a]

	MFI			
APM Components[b]	DC	DC + IL-15	DC + SCC	DC + SCC + IL-15
HLA ABC	241	361	176	264
MB1	36	40	19	28
LMP2	21	28	11	24
LMP2[c]	14	15	8	16
LMP7	40	48	22	36
LMP7[c]	20	26	13	22
LMP10	59	65	45	58
ERp57	21	26	12	20
Tapasin	24	26	25	25
Calnexin	91	92	90	94
Calreticulin	89	125	94	101

[a] DC were cocultured with SCC (PCI-38 and PCI-4B) cells in a Transwell system for 72 h. Then, recombinant human IL-15 (50 ng/ml) was added once per day for the last three consecutive days in culture. Expression of APM components in control and tumor-treated DC was determined by flow cytometry. The results of one representative donor among seven studied are shown.

[b] Direct comparison of commercial ([c]) (Affinity Research Products) and in-house anti-APM component Abs detects similar alterations of expression of APM components in control and tumor-treated human DC.

6.2% for LMP7, 36.6 ± 6.7% for LMP10, and 39.8 ± 9.8% for ERp57. Similar results were obtained by analyzing the corresponding MFI values (Table I). In contrast, expression of the calnexin, calreticulin, and tapasin was not significantly altered in the tumor cell-treated human DC.

Because IL-15 has been shown to promote DC activation (25) and increased DC function and survival in the tumor microenvironment (33), we hypothesized that IL-15 might restore the integrity of APM in DC generated in the presence of tumor cells. To test this possibility, we added rhIL-15 (50 ng/ml) to DC generated in the presence of SCC cell lines and to control DC once per day for the last 3 consecutive days in cultures and measured the level of APM component expression in tumor cell-treated DC. Treatment with IL-15 restored expression of MB1, LMP2, LMP7, LMP10, and ERp57 in tumor cell-treated DC in the range from 53 to 98%, depending on the donor (Table I). These results demonstrate that not all APM components but only specific components of the constitutive proteasome and immunoproteasome are selectively decreased in expression in DC coincubated with tumor cells. Restoration by IL-15 of APM component expression suggests the ability of this cytokine to regulate APM in DC.

Depletion of APM component mRNA in DC correlates with significant reduction of APM protein expression in DC and with reduced expression of surface MHC class I molecules

To prove that anti-APM component Abs specifically detect up- or down-regulation of Ag-processing pathway components in DC, using commercially available siRNA for LMP10 and MB1, we assessed expression of LMP10 and MB1 in DC nucleofected with specific APM component siRNA, control RNA encoding GFP and empty vector. Our results revealed that the level of DC transfection using DC protocol developed by the manufacturer (Amaxa) was between 40 and 60% based on GFP expression assessed by flow cytometry. Next, we have demonstrated that depletion of LMP10 mRNA in DC correlates with significant reduction of LMP10 protein expression in the same cells, assessed by Western blot with specific anti-APM Abs (Fig. 2*A*). Importantly, down-regulation of LMP10 protein in DC induced by siRNA was associated with reduced expression of surface MHC class I molecules (Fig. 2*B*). Similar results were obtained for DC nucleofected with MB1 siRNA.

Restoration by IL-15 of suppressed OVA-specific Ag presentation by DC generated in the presence of SCC cells

To test whether APM component down-regulation in DC generated in the presence of tumor cells has functional consequences of poor Ag presentation, we evaluated the ability of DC to present OVA to OVA-specific autologous $CD8^+$ T cells. Six-day-old tumor cell-treated and control DC were pulsed with OVA (500 μg/ml) overnight, washed, and coincubated with OVA-specific autologous $CD8^+$ T cells, and T cell proliferation was measured by uptake of [^{3}H]thymidine. The ability of DC to induce proliferation of $CD8^+$ T cells was significantly decreased when DC had been coincubated with PCI-4B cells, in comparison to DC coincubated with keratinocytes or in medium alone (Fig. 3). For instance, at the E:T ratio 1:10, [^{3}H]thymidine uptake was reduced by 76 ± 12% ($p < 0.05$) and at the ratio 1:1 × 60 ± 8% ($p < 0.05$). The ability of DC to induce proliferation of autologous $CD8^+$ T cells was HLA class I restricted, because it was inhibited by the addition of anti-HLA class I mAb W6/32. Thus, coincubation of DC with SCC cells impairs DC ability to present OVA-derived peptides to autologous OVA-specific $CD8^+$ T cells. Because it has been shown that DC generated in the presence of GM-CSF plus IL-15 prime potent $CD8^+$ Tc1 responses in vivo (27), and because of the ability of IL-15 to reverse tumor cell-induced down-regulation of APM component expression in DC, we hypothesized that IL-15 might also restore DC Ag-presenting function. To test this possibility, IL-15 (50 ng/ml) was added to DC generated in the presence of SCC cell lines and to control DC once per day for the last 3 consecutive days in culture, and the ability of DC to present OVA Ag to OVA-specific autologous $CD8^+$ T cells was measured. The addition of IL-15 caused a complete recovery of suppressed DC Ag-presenting activity (Fig. 3). These results suggest that IL-15 is able to restore the SCC cell-induced inhibition of APM component expression and Ag-presenting function of DC.

Down-regulation by gangliosides and restoration by IL-15 of APM component expression in DC

It has been previously shown that gangliosides down-regulate maturation and function of APC (13–17). To test whether gangliosides are responsible for modifying APM component expression in DC, disialoganglioside GD_3 (40 μg/ml) was added once daily for the first 3 days in culture. As a control, DC were treated with DMSO,

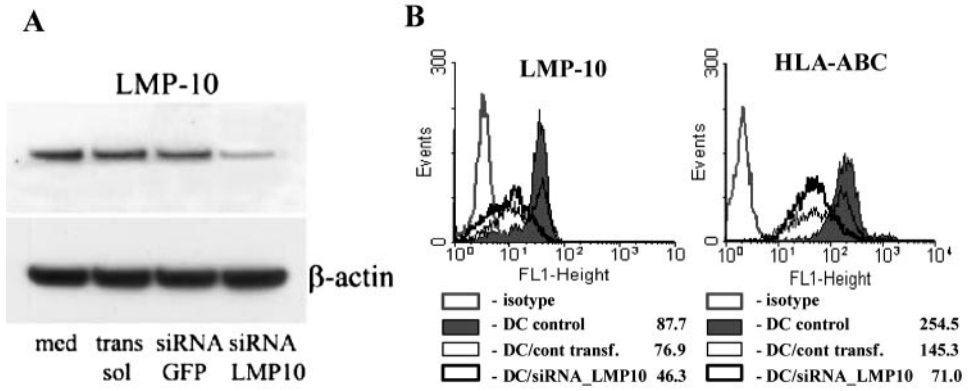

FIGURE 2. Down-regulation of expression of APM components in DC nucleofected with siRNA correlates with reduction of the corresponding protein expression in DC and reduced expression of surface MHC class I molecules. The transfection efficiencies of DC nucleofection ranged between 40 and 60% based on GFP expression. *A*, The protein expression of APM components in DC nucleofected with LMP10 siRNA, control RNA encoding GFP, and empty vector was assessed using Western blot technique as described in *Materials and Methods*. *B*, LMP10 protein expression and expression of surface MHC class I molecules in nontreated DC, control transfected DC, and DC nucleofected with LMP10 siRNA was determined by flow cytometry as described in Fig. 1 legend. Results are expressed as MFI. The results of one representative of three independent experiments are shown.

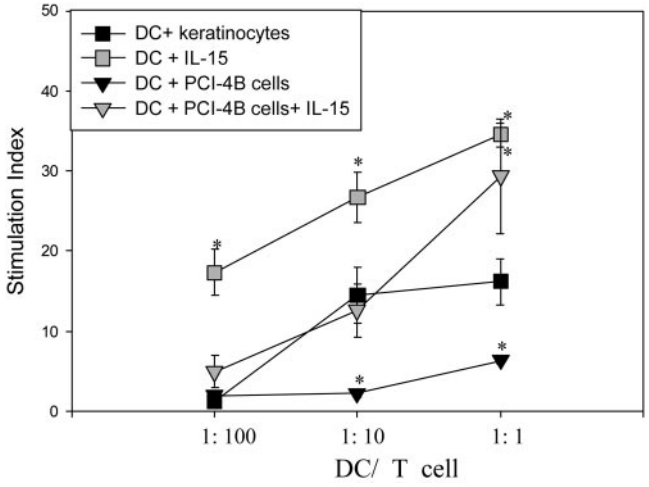

FIGURE 3. IL-15 restores DC Ag-presenting capacity suppressed by oral SCC cells. DC were cocultured with SCC cell line PCI-4B (▼) or keratinocytes (■) in Transwell system for 72 h. Then rhIL-15 (50 ng/ml) was added to DC generated in the presence of SCC cell lines (▽) and control DC (▨) once per day for the last 3 consecutive days in culture. Tumor-treated and keratinocyte-treated 6-day-old DC were loaded with OVA overnight, then washed and cultured in 96-well plates in CM with autologous OVA-specific $CD8^+$ T cells (1×10^4 cells/well) for 96 h. Proliferation of T cells was measured by uptake of [^{3}H]thymidine added for the last 16–18 h. Inhibition of the proliferation with anti-HLA class I W6/32 mAb indicates that DC-induced activation of $CD8^+$ T cells was HLA class I restricted. The counts are presented as SI. Similar results were obtained with PCI-38 oral SCC cell line. Data represent the mean ± SEM of triplicate measurements from three independent experiments. *, $p < 0.05$.

used as a solvent for gangliosides. Synthetic gangliosides significantly ($p < 0.05$) suppressed MB1, LMP2, LMP7, LMP10, and ERp57 expression in DC (Table II). For instance, MB1 was down-regulated by 37 ± 7%, LMP2 × 53 ± 10%, LMP7 × 60 ± 14%, LMP10 × 41 ± 8%, and ERp57 × 21 ± 6% in DC treated with gangliosides vs control DC. Other tested APM components were not significantly inhibited. Furthermore, the treatment of DC with IL-15 after coincubation with gangliosides reversed the inhibitory effect of gangliosides on MB1, LMP2, LMP7, LMP10, and ERp57 expression in the range from 40 to 100%, depending on the donor and specific APM components (Table II). Because synthetic gangliosides down-regulated similar APM components in DC as the SCC cell lines, and because IL-15 can restore these components expression in DC coincubated both with tumor cells or gangliosides, we hypothesized that tumor cell-induced APM component down-regulation in DC could be due to tumor cell-derived gangliosides.

GD_3 ganglioside expression by SCC cell lines PCI-4B and PCI-38

To test the hypothesis that tumor cell-induced APM component down-regulation in DC may be mediated by tumor cell-derived gangliosides, we first measured GD_3 ganglioside expression in the SCC cell lines PCI-4B and PCI-38. Neuroblastoma cell lines were used as a positive control (14). Flow cytometry and HPLC analysis showed that both cell lines express GD_3 ganglioside (Figs. 4 and 5).

Role of tumor cell-derived gangliosides in APM component down-regulation in DC

To prove that tumor cell-derived gangliosides are indeed responsible for APM component down-regulation in DC coincubated with SCC cells, we blocked synthesis of gangliosides in tumor cells using enzymatic inhibition. Tumor cells were pretreated with an inhibitor of glucosyl transferase D-threo-PPMP for 3 days to block glycosphingolipid synthesis, including ganglioside synthesis (14). APM component down-regulation in DC coincubated with SCC cells pretreated with PPMP was significantly ($p < 0.05$) less than down-regulation in DC coincubated with nontreated SCC cells (Fig. 6). Similar data were obtained for DC coincubated with both SCC cell lines PCI-4B and PCI-38. Thus, inhibition of ganglioside synthesis in tumor cells significantly decreased their inhibitory effect on APM component expression in DC, suggesting that the observed down-regulation of APM components is, at least in part, mediated by tumor cell-derived gangliosides.

Discussion

Tumor cell-induced suppression of DC function is an important mechanism of tumor escape from immune recognition. It has been shown that the expression of HLA-DR, CD40, and CD80 molecules on DC obtained from cancer patients as well as DC ability to stimulate T lymphocytes were markedly suppressed explaining, in part, why tumor-infiltrating T cells fail to eliminate tumor cells (5, 34, 35). Troy et al. (36) have demonstrated that DC are not recruited in large numbers into renal cell carcinoma lesions and that DC extracted from these tumors are minimally activated and have reduced allostimulatory activity. Similar data were obtained with DC infiltrating prostate cancer (36) and basal cell carcinoma (37) lesions. Furthermore, tumor cells may also inhibit other functions of DC in patients with cancer and in tumor-bearing animals. For instance, Tas et al. (38) noticed a decreased ability of peripheral blood DC to form clusters with T cells in patients with head and neck cancers. Using neuroblastoma and colon adenocarcinoma as models, we have recently shown that DC obtained from tumor-bearing mice had a significantly decreased ability to produce IL-12 upon CD40 ligation (10). Thurnher et al. (39) observed that DC obtained from renal cell carcinoma lesions had reduced potential to capture soluble Ag, as shown by the exclusion of FITC-labeled

Table II. *Restoration by IL-15 of HLA class I APM component expression in DC pretreated with gangliosides*[a]

APM Components	MFI			
	DC	DC + IL-15	DC + GD_3	DC + GD_3 + IL-15
HLA ABC	224	317	148	196
MB1	36	40	19	28
LMP2	31	38	11	26
LMP7	50	58	21	46
LMP10	59	64	40	56
ERp57	20	26	12	20

[a] Disialoganglioside GD_3 (40 μg/ml) was added to DC once daily for the first 3 days in cultures. As a control, DC were treated with DMSO used as a solvent for gangliosides. Then, rhIL-15 (50 ng/ml) was added once per day for the last three consecutive days in cultures. APM component expression in ganglioside-treated and control DC was determined by flow cytometry. The results of one from four independent experiments are shown.

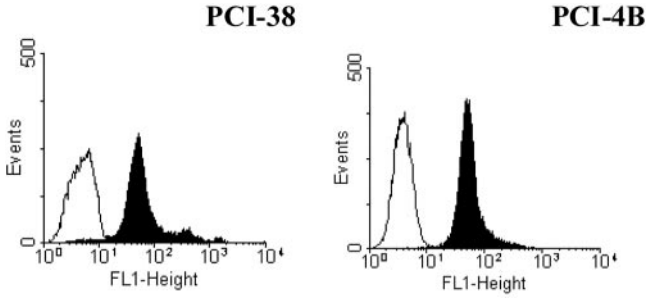

FIGURE 4. GD_3 ganglioside expression on oral SCC cell lines. FACScan analysis was performed on PCI-38 and PCI-4B cells, using staining with primary purified mouse anti-human disialoganglioside GD_3 Ab and secondary (FITC)-conjugated goat anti-mouse IgG Ab. No differences between both cell lines were detected. The results of one representative of three independent experiments are shown.

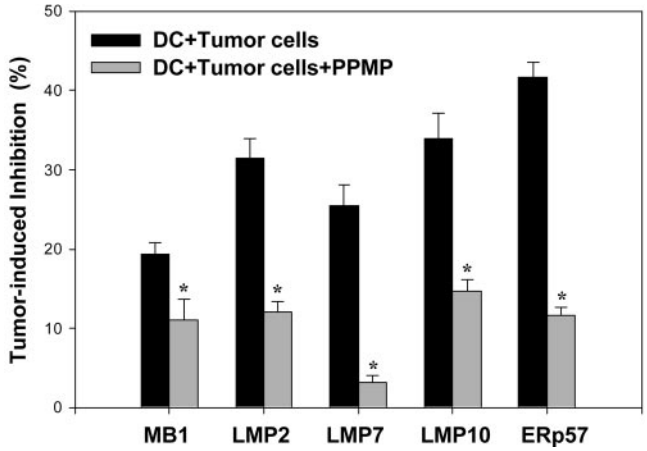

FIGURE 6. Suppression of ganglioside synthesis in oral SCC cells significantly abolished tumor-induced inhibition of APM component expression in DC. SCC cell line PCI-4B was treated with PPMP for 3 days to block ganglioside synthesis. DC were coincubated with PPMP-treated (▨) and nontreated (■) SCC cells in a Transwell system for 72 h. APM component expression in DC coincubated with treated and nontreated SCC cells was determined by flow cytometry as described in Fig. 1 legend. The results are presented as the percentage of inhibition of APM component expression in DC by PCI-4B cells. Similar results were obtained with PCI-38 cell line. Data represent the mean ± SEM from three independent experiments. *, $p < 0.01$.

dextran. Down-regulation of DC functional activity in the presence of tumor cells has been also reproduced using tumor cell lines in vitro. This provides a model to investigate the identity of inhibitory factors and mechanisms responsible for suppression of DC in cancer. We used this in vitro model to investigate the effect of SCC cells on APM in human DC. In our previous observations, we have demonstrated down-regulation of several APM components in human DC coincubated with primary oral SCC cell lines (18). In this study, we have evaluated different APM component expression in DC and have found that SCC cells induced significant down-regulation of constitutive proteasome subunit MB1, immunoproteasome subunits LMP2, LMP7, and LMP10, and thiol oxidoreductase ERp57 in DC. These data might explain reduced ability of DC to present Ag(s) to T cells in cancer. However, the specific role of each APM component in DC failure to present TA(s) to T cells in cancer remains to be characterized.

Tumor cell-induced down-regulation of APM components might be in part associated with DC maturation status. It has been shown that cross-presentation in DC is developmentally regulated (40, 41). However, the data concerning the level of proteasome expression in immature and mature DC are not consistent. For instance, it has been reported that expression of immunoproteasome subunits LMP2, LMP7, and LMP10 is down-regulated, whereas expression of their activators PA28α and PA28β, as well as of TAP, tapasin, and MHC class I molecules is up-regulated during maturation of human DC (41). Other investigators have

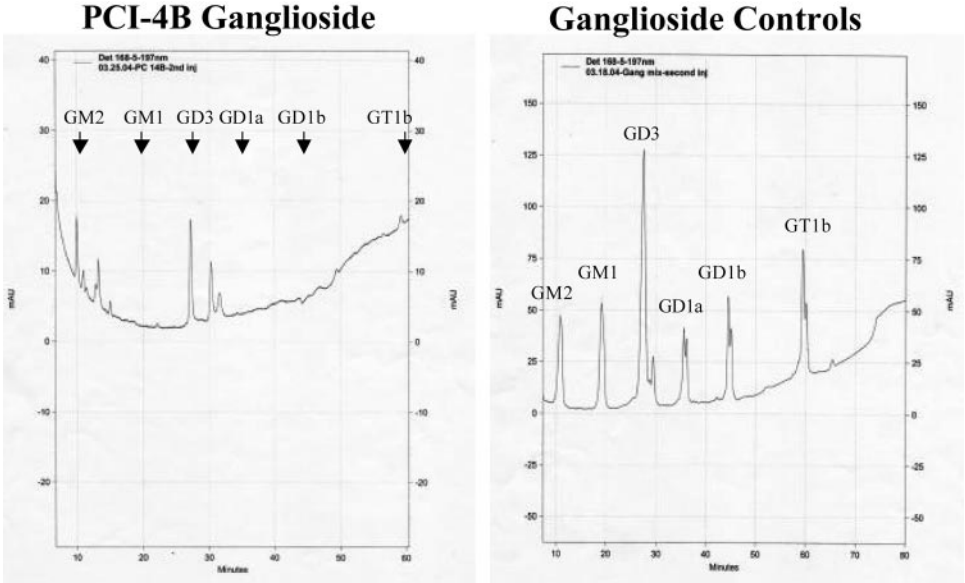

FIGURE 5. HPLC detection of ganglioside expression in PCI-4B cells. A total lipid extraction of tumor cells was performed using chloroform:methanol, and gangliosides were isolated from the lipids by a diisopropyl ether/1-butanol partition and subsequent Sephadex G-25 gel filtration step. The water-soluble gangliosides were further fractionated by HPLC using a LiChrosorb-NH2 column. Gangliosides were eluted using a linear flow rate of 0.5 ml/min throughout, where solvent A was Acetonitrile-5 mM phosphate buffer, pH 5.6 (83:17) and solvent B was Acetonitrile-20 mM phosphate buffer, pH5.6 (50:50). Individual bovine brain-derived gangliosides were also subjected to HPLC analysis to establish the retention times of known gangliosides (GM2, GM1, GD3, GD1a, GD1b, and GT1b).

demonstrated up-regulation of the immunoproteasomes in mature DC (40). The observed LMP2, LMP7, and LMP10 down-regulation in tumor cell-treated DC could be interpreted as induction of DC maturation in the presence of the tumor. However, this speculation conflicts with the reports demonstrating down-regulation of the costimulatory molecules CD80 and CD86, CD40 and MHC class I and class II molecules in DC coincubated with tumor cell lines (42). It is also possible, that the blunted Ag processing, and low APM component expression in tumor cell-treated DC may simply reflect the immature status of treated DC. However, whereas immature DC display high endocytotic activity, DC coincubated with tumor cells display also a reduced endocytotic activity (M. R. Shurin, unpublished data). Thus, it is unlikely that DC are simply immature in the tumor microenvironment. Moreover, our data reveal down-regulation of the constitutive proteasome molecules MB1, as well as of the immunoproteasome subunits LMP2, LMP7, and LMP10 in tumor cell-treated DC. Suppression of both proteasome types in DC coincubated with tumor cells is likely to be associated with a profound dysregulation of APM components and cannot be explained by the effect on DC maturation status. Furthermore, tumor cell-induced down-regulation of APM component expression in DC has been accompanied by impaired Ag-presenting function. Thus, our results suggest that tumor cells do not simply delay or prevent DC maturation, but actively suppress DC activity and function, including Ag processing and presentation.

One of the mechanisms used by tumor cells to induce alteration in APM components in DC and impaired DC function could be the release of gangliosides (13–16). In fact, Ladisch and his coworkers (43) showed that purified FBL-3 erythroleukemia cell-derived gangliosides inhibited syngeneic TA-specific immune response in murine models both in vivo and in vitro. They also noticed that the lack of NF-κB activity might be a mechanism contributing to ganglioside-mediated inhibition of monocyte and monocyte-derived DC Ag-presenting function (17). Using an in vitro model of DC generation in the presence of tumor cells, we have shown here for the first time that tumor-derived gangliosides can down-regulate expression of several APM components in human DC.

In terms of the mechanism(s) responsible for the tumor cell- and ganglioside-induced APM component down-regulation in DC, restoration of APM component expression by IL-15 suggests an important role of exogenous and probably endogenous IL-15 in APM regulation in DC. It has been shown that IL-15 is expressed by DC in response to different stimuli and promotes DC activation (25). An alternative, but not an exclusive mechanism is reduced interaction of IL-15 with its receptor. IL-15 is known to play a critical role in Ag-presenting cell functions and in the innate immune responses (44). However, intracellular pathways involved in APM regulation in DC by IL-15 in the tumor microenvironment are yet to be determined.

In summary, our data demonstrate that tumor cell-derived gangliosides can down-regulate APM component expression in DC, which has potential functional consequences of interfering with DC Ag-presenting function. Treatment of DC with IL-15 can restore both the APM component expression and the Ag-presenting capacity of DC. Therefore, our results suggest a new mechanism for the ability of IL-15 to enhance the efficacy of DC-based vaccines for cancer.

Disclosures

The authors have no financial conflict of interest.

References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245–252.
2. Delamarre, L., H. Holcombe, and I. Mellman. 2003. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J. Exp. Med.* 198: 111–122.
3. Brossart, P., and M. J. Bevan. 1997. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 90: 1594–1599.
4. Larsson, M., J. F. Fonteneau, and N. Bhardwaj. 2003. Cross-presentation of cell-associated antigens by dendritic cells. *Curr. Top. Microbiol. Immunol.* 276: 261–275.
5. Orsini, E., A. Guarini, S. Chiaretti, F. R. Mauro, and R. Foa. 2003. The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. *Cancer Res.* 63: 4497–4506.
6. Sharma, S., M. Stolina, S. C. Yang, F. Baratelli, J. F. Lin, K. Atianzar, J. Luo, L. Zhu, Y. Lin, M. Huang, et al. 2003. Tumor cyclooxygenase 2-dependent suppression of dendritic cell function. *Clin. Cancer Res.* 9: 961–968.
7. Yang, A. S., and E. C. Lattime. 2003. Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. *Cancer Res.* 63: 2150–2157.
8. Gabrilovich, D., T. Ishida, T. Oyama, S. Ran, V. Kravtsov, S. Nadaf, and D. P. Carbone. 1998. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* 92: 4150–4166.
9. Kiertscher, S. M., J. Luo, S. M. Dubinett, and M. D. Roth. 2000. Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. *J. Immunol.* 164: 1269–1276.
10. Shurin, M. R., Z. R. Yurkovetsky, I. L. Tourkova, L. Balkir, and G. V. Shurin. 2002. Inhibition of CD40 expression and CD40-mediated dendritic cell function by tumor-derived IL-10. *Int. J. Cancer* 101: 61–68.
11. Kao, J. Y., Y. Gong, C. M. Chen, Q. D. Zheng, and J. J. Chen. 2003. Tumor-derived TGF-β reduces the efficacy of dendritic cell/tumor fusion vaccine. *J. Immunol.* 170: 3806–3811.
12. Ladisch, S., Z. L. Wu, S. Feig, L. Ulsh, E. Schwartz, G. Floutsis, F. Wiley, C. Lenarsky, and R. Seeger. 1987. Shedding of GD2 ganglioside by human neuroblastoma. *Int. J. Cancer* 39: 73–76.
13. Peguet-Navarro, J., M. Sportouch, I. Popa, O. Berthier, D. Schmitt, and J. Portoukalian. 2003. Gangliosides from human melanoma tumors impair dendritic cell differentiation from monocytes and induce their apoptosis. *J. Immunol.* 170: 3488–3494.
14. Shurin, G. V., M. R. Shurin, S. N. Bykovskaia, J. Shogan, M. T. Lotze, and E. M. J. Barksdale. 2001. Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. *Cancer Res.* 61: 363–369.
15. Shen, W., and S. Ladisch. 2002. Ganglioside GD1a impedes lipopolysaccharide-induced maturation of human dendritic cells. *Cell. Immunol.* 220: 125–133.
16. Wolfl, M., W. Y. Batten, C. Posovszky, H. Bernhard, and F. Berthold. 2002. Gangliosides inhibit the development from monocytes to dendritic cells. *Clin. Exp. Immunol.* 130: 441–448.
17. Caldwell, S., A. Heitger, W. Shen, Y. Liu, B. Taylor, and S. Ladisch. 2003. Mechanisms of ganglioside inhibition of APC function. *J. Immunol.* 171: 1676–1683.
18. Whiteside, T. L., J. Stanson, M. R. Shurin, and S. Ferrone. 2004. Antigen-processing machinery in human dendritic cells: up-regulation by maturation and down-regulation by tumor cells. *J. Immunol.* 173: 1526–1534.
19. Cresswell, P., N. Bangia, T. Dick, and G. Diedrich. 1999. The nature of the MHC class I peptide loading complex. *Immunol. Rev.* 172: 21–28.
20. Antoniou, A. N., S. J. Powis, and T. Elliott. 2003. Assembly and export of MHC class I peptide ligands. *Curr. Opin. Immunol.* 15: 75–81.
21. Ackerman, A. L., and P. Cresswell. 2003. Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. *J. Immunol.* 170: 4178–4188.
22. Trentin, L., R. Zambello, M. Facco, R. Sancetta, C. Agostini, and G. Semenzato. 1997. Interleukin-15: a novel cytokine with regulatory properties on normal and neoplastic B lymphocytes. *Leuk. Lymphoma* 27: 35–42.
23. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype $CD8^+$ T cells in vivo by IL-15. *Immunity* 8: 591–599.
24. Waldmann, T. A., and Y. Tagaya. 1999. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu. Rev. Immunol.* 17: 19–49.
25. Mattei, F., G. Schiavoni, F. Belardelli, and D. F. Tough. 2001. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. *J. Immunol.* 167: 1179–1187.
26. Girard, D., M. E. Paquet, R. Paquin, and A. D. Beaulieu. 1996. Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils: modulation of phagocytosis, cytoskeleton rearrangement, gene expression, and apoptosis by IL-15. *Blood* 88: 3176–3184.
27. Pulendran, B., S. Dillon, C. Joseph, T. Curiel, J. Banchereau, and M. Mohamadzadeh. 2004. Dendritic cells generated in the presence of GM-CSF plus IL-15 prime potent $CD8^+$ Tc1 responses in vivo. *Eur. J. Immunol.* 34: 66–73.
28. Wang, J., L. Xi, J. L. Hunt, W. Gooding, T. L. Whiteside, Z. Chen, T. E. Godfrey, and R. L. Ferris. 2004. Expression pattern of chemokine receptor 6 (CCR6) and

CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. *Cancer Res.* 64: 1861–1866.
29. Aalamian, M., G. Pirtskhalaishvili, A. Nunez, C. Esche, G. V. Shurin, E. Huland, H. Huland, and M. R. Shurin. 2001. Human prostate cancer regulates generation and maturation of monocyte-derived dendritic cells. *Prostate* 46: 68–75.
30. Ogino, T., X. Wang, and S. Ferrone. 2003. Modified flow cytometry and cell-ELISA methodology to detect HLA class I antigen processing machinery components in cytoplasm and endoplasmic reticulum. *J. Immunol. Methods* 278: 33–44.
31. Wang, X., B. Liang, V. Rebmann, J. Lu, E. Celis, T. Kageshita, H. Grosse-Wilde, and S. Ferrone. 2003. Specificity and functional characteristics of anti-HLA-A mAbs LGIII-147.4.1 and LGIII-220.6.2. *Tissue Antigens* 62: 139–148.
32. Ladisch, S., and R. Li. 2000. Purification and analysis of gangliosides. *Methods Enzymol.* 312: 135–145.
33. Tourkova, I. L., Z. R. Yurkovetsky, A. Gambotto, V. P. Makarenkova, L. Perez, L. Balkir, P. D. Robbins, M. R. Shurin, and G. V. Shurin. 2002. Increased function and survival of IL-15-transduced human dendritic cells are mediated by up-regulation of IL-15Rα and Bcl-2. *J. Leukocyte Biol.* 72: 1037–1045.
34. Gabrilovich, D. I., J. Corak, I. F. Ciernik, D. Kavanaugh, and D. P. Carbone. 1997. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin. Cancer Res.* 3: 483–490.
35. Ratta, M., F. Fagnoni, A. Curti, R. Vescovini, P. Sansoni, B. Oliviero, M. Fogli, E. Ferri, G. R. Della Cuna, S. Tura, et al. 2002. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* 100: 230–237.
36. Troy, A., P. Davidson, C. Atkinson, and D. Hart. 1999. Renal cell carcinoma and prostate cancer inhibit dendritic cell activation. *Aust. NZJ Surg.* 69: A111.
37. Nestle, F. O., G. Burg, J. Fah, T. Wrone-Smith, and B. J. Nickoloff. 1997. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell costimulatory molecules and are impaired as antigen-presenting cells. *Am. J. Pathol.* 150: 641–651.
38. Tas, M. P., P. J. Simons, F. J. Balm, and H. A. Drexhage. 1993. Depressed monocyte polarization and clustering of dendritic cells in patients with head and neck cancer: in vitro restoration of this immunosuppression by thymic hormones. *Cancer Immunol. Immunother.* 36: 108–114.
39. Thurnher, M., C. Radmayr, R. Ramoner, S. Ebner, G. Bock, H. Klocker, N. Romani, and G. Bartsch. 1996. Human renal-cell carcinoma tissue contains dendritic cells. *Int. J. Cancer* 68: 1–7.
40. Macagno, A., M. Gilliet, F. Sallusto, A. Lanzavecchia, F. O. Nestle, and M. Groettrup. 1999. Dendritic cells up-regulate immunoproteasomes and the proteasome regulator PA28 during maturation. *Eur. J. Immunol.* 29: 4037–4042.
41. Li, J., B. Schuler-Thurner, G. Schuler, C. Huber, and B. Seliger. 2001. Bipartite regulation of different components of the MHC class I antigen-processing machinery during dendritic cell maturation. *Int. Immunol.* 13: 1515–1523.
42. Shurin, G. V., Z. R. Yurkovetsky, and M. R. Shurin. 2003. Tumor-induced dendritic cell dysfunction. In *Mechanisms of Tumor Escape from the Immune Response.* A. Ochoa, ed. Taylor and Francis, London and New York, p. 112–138.
43. McKallip, R., R. Li, and S. Ladisch. 1999. Tumor gangliosides inhibit the tumor-specific immune response. *J. Immunol.* 163: 3718–3726.
44. Ohteki, T., K. Suzue, C. Maki, T. Ota, and S. Koyasu. 2001. Critical role of IL-15-IL-15R for antigen-presenting cell functions in the innate immune response. *Nat. Immunol.* 2: 1138–1143.