Resistance to Apoptosis

Lag-3 (CD223) Contributes to Melanoma Resistance to Apoptosis

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Malignant melanomas express tumor-associated and tumor-specific Ags but also often the MHC class II (MHC II) molecules (1–4), which could make these tumors detectable and eliminable by the immune system. However, there is a lack of immune-mediated eradication. The lymphocyte activation gene-3 (LAG-3) is a natural ligand for MHC II that is substantially expressed on melanoma-infiltrating T cells including those endowed with potent immune-suppressive activity. Based on our previous data showing the signaling capacity of MHC II in melanoma cells, we hypothesized that LAG-3 could contribute to melanoma survival through its MHC II signaling capacity in melanoma cells. In this study, we demonstrate that both soluble LAG-3 and LAG-3–transfected cells can protect MHC II-positive melanoma cells, but not MHC II-negative cells, from FAS-mediated and drug-induced apoptosis. Interaction of LAG-3 with MHC II expressed on melanoma cells upregulates both MAPK/Erk and PI3K/Akt pathways, albeit with different kinetics. Inhibition studies using specific inhibitors of both pathways provided evidence of their involvement in the LAG-3–induced protection from apoptosis. Altogether, our data suggest that the LAG-3–MHC II interaction could be viewed as a bidirectional immune escape pathway in melanoma, with direct consequences shared by both melanoma and immune cells. In the future, compounds that efficiently hinder LAG-3–MHC II interaction might be used as an adjuvant to current therapy for MHC II-positive melanoma.

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Abbreviations used in this article: C<sub>e</sub>, cycle threshold; ΔOD<sub>cyc</sub>, 3,3'-diethyloxacarbo-cyanine; LAG-3, lymphocyte activation gene-3; MHC II, MHC class II; PI, propidium iodide; sLAG-3, soluble LAG-3.

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LAG-3 induces melanoma resistance to apoptosis via MHC II


Materials and Methods

Patients

Upon obtaining written informed consent, PBMCs were obtained from blood samples collected from stage III/IV melanoma patients (n = 14), as well as from metastatic (n = 5) and healthy (n = 3) lymph nodes obtained by surgery from stage IV melanoma patients. Melanoma patients were untreated at time of blood sampling (i.e., no immunomodulatory treatments at least 1 mo before blood sampling). The experimental procedures were approved by the local ethical committee (Comité consultatif pour la protection des personnes dans les recherches biomédicales, Hôpital Saint-Louis, Paris, France). PBMCs were isolated from peripheral blood samples obtained from patients treated with surgery from stage IV melanoma patients. Melanoma cell lines A375, Mel-1, M74, and 429/5 were kindly provided by E. Tartour (Hôpital Européen Georges-Pompidou, Assistance Publique des Hôpitaux de Paris, Paris, France). Melanoma cell lines WM983A and WM983B were kindly provided by A. Mauriel (Curie Institute, INSERM U1021/Centre National de Recherche Scientifique Unité Mixte de Recherche Scientifique 3347, Orsay, France) and the Meljuso cell line by N. Mooney (INSERM Unité Mixte de Recherche Scientifique 940, Hôpital Saint-Louis, Paris, France). The melanoma cell lines HM11 were obtained from a primary cutaneous melanoma tumor and were provided along with the Skmel5 melanoma cell line by N. Dumaz (INSERM U976, Hôpital Saint-Louis, Paris, France). SLM-8 and WAC melanoma cells were obtained from a lymph node metastasis after patients provided signed informed consent following human ethics committee approval (Comité consultatif pour la protection des personnes dans les recherches biomédicales, Hôpital Saint-Louis, Paris, France). Briefly, single-cell suspensions were generated by enzymatic digestion of the biopsy in 2 mg/ml collagenase and 0.5 mg/ml hyaluronidase (Sigma-Aldrich, Saint-Quentin Fallavier, France) in DMEM/F12 medium for 30 min at 37°C and cultured under the conditions mentioned for melanoma cell lines. Positive staining using anti-pan melanoma (HBMB45+M2-7C10+M2-9E3+T311) Abs mixture (Novus Biological Inc., Littleton, CO) confirmed the melanoma cell phenotype. All melanoma cells were seeded at 5 × 10^5 cells/cm^2 and grown in DMEM/F12 medium (Life Technologies) supplemented with 10% FBS (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (DMEM complete medium) at 37°C with 5% CO_2 and were analyzed for MHC II expression using specific Abs and flow cytometry as described under the later section Phenotype analysis.

Experimental design

Apoptosis was studied in serum-starved melanoma cells by incubation with 0.1 μg/ml anti-Fas Ab (clone 711; Beckman Coulter Immunotech, Marseille, France) or 20 μg/ml etoposide [stock solution (20 mg/ml); Dakota Pharm, Le Plessis Robinson, France] for up to 48 h, as indicated in the legends to the figures of this article. For studying resistance to apoptosis, melanoma cells were incubated with sLAG-3 (IMP321; Immune, Chate-.”

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Proliferation by flow cytometry analysis of lymphocyte CFSE labeling

Freshly purified lymphocytes were resuspended in PBS containing 0.1% FBS at 2 × 10^6 cells/ml and incubated with CFSE (Invitrogen, Cergy Pontoise, France) (stock solution, 5 mM in DMSO; final concentration 1 μM) for 8 min at 37°C. Cells were washed and resuspended in cold complete RPMI medium for 10 min at 4°C to stabilize the CFSE labeling. After a final wash step, cells were resuspended in complete RPMI medium at 1 × 10^6 cells/ml then cocultured with subconfluent melanoma cells at 1:1 and 1:5 ratios. After 4 d of culturing, cells were harvested, and analyzed by flow cytometry using FACS calibur and CellQuestPro software (BD Biosciences, Le Pont de Claix, France) to determine the percentage of live lymphocytes in each division peak. Data were expressed as the sum of the percentage of lymphocytes displaying low CFSE (dividing cells).

Apoptosis assays

Apoptosis was evaluated by both measurement of mitochondrial membrane potential (ΔΨ_m) modifications and sub-G1 analysis as described (36). For measurement of ΔΨ_m modifications, 5 × 10^5 cells were incubated in 10% FBS-DMEM complete medium with addition of 0.1 μM 3,3′-diethylox-acarbocyanine (DiOC_6) fluorescent probe ( Molecular Probes, Eugene, OR) for 30 min at 37°C. Cells were then washed and resuspended in DMEM complete medium containing 10% FBS in the microscope chamber. After 30 min, cells were fixed in cold ethanol 70% for 1 h at 4°C. Cells were then washed twice in ice-cold PBS and fixed in ice-cold ethanol 70% for 1 h at 4°C. Cells were then washed twice in ice-cold PBS, pH 7.5, and incubated for 30 min at 37°C.
200 U/ml DNAse-free RNase A (Sigma-Aldrich). After centrifugation, cells were resuspended in 1 ml PBS with 10 μg/ml PI and stored in the dark on ice until flow cytometry analysis of the DNA content within the following hour. PI fluorescence emission (15,000 events) was detected using FACSCalibur (BD Biosciences), and the quantification of cells in the sub-G1 phase was performed with Modfit software (BD Biosciences).

**Phenotype analysis**

PBMCs (5 × 10⁶/sample) were stained with PerCP- or allophycocyanin-conjugated anti-CD3, PE-conjugated anti-CD8, PerCP- or allophycocyanin-conjugated anti-CD4, PE-conjugated anti-CD25, and PE-conjugated anti-CD56 (BD Biosciences, Le Pont de Clair, France), or FITC-conjugated anti-CD4 (clone 17B4). Immunofluorescence double staining was conducted using FITC-conjugated anti-HLA-DR (L243; BD Biosciences), unconjugated HLA-DP (B7/21), and HLA-DQ (33;1); these later Abs were affinity-purified from ascites using protein A–Sepharose column (Amersham Pharmacia Biotech). Unconjugated primary Abs were detected with PE-conjugated goat anti-mouse IgG1 (H+L) Ab (BD Biosciences). Intracellular staining for FOXP3 was conducted using allophycocyanin-conjugated FOXP3 staining assay kit (eBioscience, Interchim, Montluçon, France). Cells were analyzed by FACSCalibur and CellQuestPro software (BD Biosciences). The expression of MHC II molecules by the various melanoma cell lines and melanoma cells is displayed in Table I.

**Immunostaining**

Immunofluorescence double staining was conducted on frozen sections. Sections were fixed in cold acetone for 10 min and blocked in a solution of 30% BSA in PBS 1 × for 20 min. Slides were incubated with mouse anti-CD4 1 μg/ml or anti-CD8 1 μg/ml Abs (DAKO, Trappes, France) for 1 h, washed twice with PBS, and incubated with Texas red-conjugated anti-mouse Ab (Jackson ImmunoResearch, Interchim, Montrouge, France) for 1 h.Slides were then incubated with FITC-conjugated mouse anti–LAG-3 mAb (clone 17B4, 10 μg/ml; Immunet). Mounting was done using Vectashield medium (Vector Laboratories, Abycs, Paris, France) including 4',6-diamidino-2-phenylindole fluorochrome for nuclei staining. Images were acquired on a Zeiss LSM510 META laser scanning confocal microscope equipped with a Zeiss Plan Apochromat 63×1.4 numerical aperture oil immersion objective using the LSM510 software (version 3.2) (Carl Zeiss, Oberkochen, Germany).

**Immunohistochemistry analysis**

Frozen sections (7 μm) were prepared from metastatic lymph node, and immunohistochemical single staining was performed using standard polymer-peroxidase technique LSAB + System-AP (Dako Cytomation). After fixation with acetone for 10 min, the sections were blocked with serum-free protein buffer for 20 min. Slides were then incubated with monoclonal mouse anti–LAG-3 17B4 Ab 10 μg/ml (Immutep) for 30 min, followed by biotinylated anti-mouse Ab for 15 min, then with streptavidin alkaline phosphatase for another 15 min. Alkaline phosphatase activity was developed using Fuchsin plus Substrate-Chromogen System (Dako Cytomation) for 10 min.

**Reverse transcription and quantitative PCR**

Total RNAs were extracted using RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen, Courtaboeuf, France), and 1 μg of total RNA was converted to cDNA using ThermoScript reverse transcriptase (Invitrogen, Cergy, France) and oligonucleotide primers according to the manufacturer’s instructions. The cDNA was used at 1:10 for real-time PCR using SYBR Green with the ABI PRISM 7300 sequence detection system (Applied Biosystems, Courtaboeuf, France) according to the manufacturer’s instructions. The sets of primers were designed with the primer design software Primer Express version 3.0 (Applied Biosystems). The sequence of the forward primer for LAG-3 mRNA was 5’-AGGCTCTAATGCCCACTGTCACA-3’ and that of the reverse primer was 5’-CACGGTGATCAGGTGGTG-3’. For GUS mRNA, the forward primer was 5’-GAAAATATGTTGGTGAGACCTATT-3’ and the reverse primer was 5’-CCGAGTTGAAGATCCCCTTTTTA-3’. The conditions for PCR were as follows: 10 min at 95°C, then 40 cycles of amplification at 95°C for 15 s and 60 s at 60°C, and finally 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C. All samples were normalized to the expression of the housekeeping GUS gene. Quantification of the target gene expression was done using the comparative cycle threshold (Ct) method according to the manufacturer’s instructions (Applied Biosystems). Ct was normalized to GUS (ΔCt = Ct sample – Ct GUS). The ΔCt values were then compared between different cell populations.

**Immunoblot analysis**

Cells (2 × 10⁶) were lysed in HEPES 10 mM pH 7.8, KCl 10 mM, MgCl₂ 2 mM, EDTA 0.1 mM, protease inhibitor mixture (Boehringer, Reims, France), Nonidet P-40 1%, and DTT 1 mM buffer. Protein extracts (50 μg) were loaded onto a 10% polyacrylamide gel containing SDS, subjected to electrophoresis, and transferred to polyvinylidene fluoride membrane. Blots were hybridized with anti-p-Erk (E-4; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p-Akt (Ser-473; Cell Signaling Technologies, Ozyme, Saint-Quentin-en-Yveline, France) Abs. Immunoblots were stripped and reprobed with Abs to the corresponding unphosphorylated proteins using anti-Erk2 (C-14; Santa Cruz Biotechnology) or anti-Akt (Cell Signaling Technologies) to ensure equal loading. Blots were visualized using an HRP-conjugated secondary Ab followed by ECL detection (Amersham Pharmacia Biotech, Orsay, France).

**Statistical analyses**

Statistical significance for all experiments was assessed using the two-sided Student t test. Differences were considered as significant at p < 0.05.

**Results**

**LAG-3 is expressed by various subsets of tumor-infiltrating leukocytes**

Staining of frozen melanoma metastatic lymph node sections with specific anti–LAG-3 Ab identified cells positive for LAG-3 (Fig. 1A) and for LAG-3 and CD4 (Fig. 1B, left panel) and LAG-3 and CD8 (Fig. 1B, right panel), confirming previously reported data that described LAG-3–positive T cells among tumor-infiltrating leukocytes of patients with melanoma (12, 26). Real-time PCR analysis showed that CD4⁺ and CD8⁺ T cells that infiltrate metastatic lymph nodes express higher levels of LAG-3 mRNA than those purified from PBMCs of the same melanoma patients (Fig. 1C). Phenotypic analysis by flow cytometry confirmed these results by identifying significantly higher percentages of LAG-3⁺CD8⁺ (up to 46%, p < 0.01) and LAG-3⁺CD4⁺ (up to 10%, p < 0.001) T cells among tumor-infiltrating leukocytes from metastatic lymph nodes than those among leukocytes isolated from healthy lymph nodes or from peripheral blood of the same melanoma patients (Fig. 1D). A significant increase in the percentage of LAG-3⁺CD4⁺CD25⁺Foxp3⁺ (up to 12%, p < 0.001) T cells was also found among leukocytes that infiltrate metastatic lymph node nodes compared with those among leukocytes from healthy lymph nodes and peripheral blood (Fig. 1D). Leukocytes from metastatic lymph nodes also contained higher percentages of LAG-3⁺CD56⁺ CD3⁺ (up to 48%, p < 0.01) and LAG-3⁺CD56⁺CD3⁻ (up to 38%, p < 0.001) cells compared with those of leukocytes from healthy lymph nodes and from peripheral blood.

Altogether, these results clearly show that the leukocytes infiltrating melanoma metastatic lymph nodes contain not only T cells but also a substantial percentage of NKT and NK cells that express LAG-3.

**MHC class II molecules on melanoma cells functionally interact with LAG-3**

Upon its engagement with MHC II, signaling via LAG-3 in T cells downregulates their proliferation. Therefore, to ensure that MHC II expressed by melanoma cells can functionally interact with LAG-3, we analyzed the effect of anti–LAG-3 17B4 Ab, suggested as a blocker of LAG-3–MHC II interactions (13), on the proliferation of LAG-3–positive T cells in an allogenic setting as previously described (20, 22). Knowing that only activated T cells express LAG-3, allogenic PBMCs from healthy donors were activated by PHA for 48 h, and expression of LAG-3 was detected on ∼20% of T cells (data not shown). These PHA-activated allogenic lymphocytes were stained with CFSE and then cocultured with MHC II-positive melanoma cell line A375 for 4 d in the presence of...
irrelevant IgG1 or anti–LAG-3 17B4 mAb. The presence of the anti–LAG-3 17B4 mAb modestly though significantly enhanced the MHC II-positive melanoma cell-induced proliferation of allogenic PHA-activated lymphocytes at 1:5 and 1:1 ratios (by 1.15- and 1.22-fold increase, respectively) compared with that of irrelevant IgG1 (p < 0.01 and p < 0.02, respectively) (Fig. 2). Although modest as previously reported (22), this effect indicates that MHC II on melanoma cells could interact with LAG-3 on T cells.

LAG-3 protects MHC II-positive A375 melanoma cells from Fas-mediated apoptosis

We analyzed the effect of LAG-3 on apoptosis of melanoma cells induced by Fas ligation using the MHC II-positive melanoma cell line A375 and the MHC II-negative melanoma cell line M74; both express substantial levels of Fas and are sensitive for Fas-mediated apoptosis (10, 37, 38). A375 melanoma cells were preincubated or not with sLAG-3 for 1 h and were then treated with anti-Fas mAb (mAb 7C11). Measurement of ∆Ψm modifications indicated that treatment with anti-Fas mAb for 24 or 48 h induced apoptosis of A375 cells (Fig. 3A). This apoptosis was significantly decreased in the presence of sLAG-3, with a reduction of 24% at 24 h (p < 0.05) and 23% at 48 h (p < 0.05), respectively, compared with Fas-mediated apoptosis at the same time. Analysis of sub-G1 also showed that the presence of sLAG-3 decreases by 30% the Fas-mediated apoptosis of A375 melanoma cells (Fig. 3B).

Given that in our hands, anti–LAG-3 17B4 did not show high efficiency in antagonizing the effect of LAG-3 in lymphocytes, and given the lack of clear consensus on its antagonistic activity (18, 22, 26), we used instead the Fas-sensitive MHC II-negative M74 melanoma cell line as a control to ensure that the observed LAG-3–induced protection from Fas-mediated apoptosis occurs through MHC II molecules. In contrast to A375, preincubation of M74 cells with sLAG-3 did not affect their Fas-mediated apoptosis as evaluated by ∆Ψm modifications (Fig. 3C), indicating that the observed protective effect of LAG-3 is through MHC II. Similar results were obtained when Fas-mediated apoptosis of

![FIGURE 1. LAG-3 expression by tumor-infiltrating leukocytes in lymph node metastatic melanoma. A. The expression of LAG-3 in frozen sections of metastatic lymph node from a patient with stage IV melanoma was determined by immunohistochemistry using a specific anti–LAG-3 Ab as described in Materials and Methods. Arrows indicate LAG-3–positive cells. B. The expression of LAG-3 by tumor-infiltrating CD4 (left panel) and CD8 (right panel) cells in frozen sections of metastatic lymph node from a patient with stage IV melanoma using specific anti-CD4 and anti-CD8 Abs followed by Texas red-conjugated anti-mouse Ab (red), then FITC-conjugated mouse anti–LAG-3 mAb (green). Arrows indicate the coexpression of LAG-3 and CD4 or CD8 (yellow). C. Lymphocytes isolated from metastatic lymph nodes and PBMCs from five patients with stage IV melanoma, as well as CD4+ and CD8+ T cells purified from both sets of lymphocytes, were analyzed for LAG-3 mRNA expression by quantitative PCR. Results are expressed as mean mRNA ΔCt values (±SD, n = 5). *p < 0.05, **p < 0.01, metastatic lymph node data (dark gray) compared with PBMC data (light gray). D. Expression of LAG-3 at the surfaces of CD8+, CD4+, CD4+CD25highFoxp3+, CD56–CD3+, and CD56+CD3− subpopulations isolated from peripheral blood (n = 14), healthy lymph nodes (n = 3), and metastatic lymph nodes (n = 5), all from patients with stage IV melanoma, as determined by flow cytometry. Results are expressed as mean percentages (±SD). **p < 0.01, ***p < 0.001, metastatic lymph nodes (dark) compared with PBMCs (gray).](http://www.jimmunol.org/)

![FIGURE 2. MHC II molecules on melanoma cells could interact with LAG-3–positive cells. Allogenic lymphocytes from healthy donors were activated by PHA for 48 h then cultured with A375 melanoma cells (ratio 1:5 and 1:1) in the presence of anti–LAG-3 Ab (17B4) or isotype control (IgG1) for 4 d. Lymphocyte proliferation was determined by CFSE staining. Results are expressed as mean values ± SD, n = 3, of the total percentage of proliferating cells (CFSElow) determined as described in Materials and Methods. *p < 0.01, anti–LAG-3–treated (dark gray) compared with IgG1-treated (light gray) cells.](http://www.jimmunol.org/)
M74 in the presence of sLAG-3 was evaluated by sub-G1 analysis (data not shown).

To confirm further that engagement of MHC II on melanoma cells by LAG-3 can provide protection against Fas-mediated apoptosis, we examined whether LAG-3–expressing cells could similarly protect A375 cells from this apoptosis. Given that LAG-3 is expressed on various subsets of tumor-infiltrating leukocytes, we used CHO-LAG-3* cells as a model of LAG-3–expressing cells. A375 cells were cultured alone or with CHO-LAG-3* at a ratio of 1:2 or 1:5 for 1 h. Anti-Fas mAb was then added, and cultures were continued for another 48 h. The interaction of Mel1 cells with CHO-LAG-3* cells significantly decreased etoposide-induced apoptosis in a ratio-dependent manner.

**FIGURE 3.** Soluble and membrane LAG-3 protects MHC II-positive A375 melanoma cells from Fas-mediated apoptosis. A–C, Fas-sensitive MHC II-positive A375 (A, B) and MHC II-negative M74 (C) melanoma cells were cultured in the presence or absence of sLAG-3 (10 μg/ml) for 1 h, then treated or not with anti-Fas mAb 7C11 0.1 μg/ml for 24 h and 48 h. Apoptosis was determined by loss of mitochondrial transmembrane potential (ΔΨm) and flow cytometry analysis (A, C) or by sub-G1 analysis (B). Results in A and C are presented as mean percentages of apoptotic cells (±SD) from three independent experiments. *p < 0.05, comparison of cells incubated with anti-Fas mAb in the presence of sLAG-3 and cells incubated with anti-Fas mAb alone. In B, a representative FACS histogram plot of DNA content analysis at 48 h is shown, and the percentages of cells in sub-G1 phase are indicated. D, MHC II-positive A375 cells were cocultured with LAG-3–transfected CHO cells (CHO-LAG-3*) at a ratio of 1:2 and 1:5 for 1 h and were then treated with anti-Fas mAb 7C11 0.1 μg/ml for 48 h. *p < 0.05 anti-Fas-treated A375 cells cocultured with CHO-LAG-3* cells compared with anti-Fas-treated A375 cells.

LAG-3 protects MHC II-positive Mel1 melanoma cells from drug-induced apoptosis

Melanoma tumors are known to be highly resistant to drugs and chemotherapy. This resistance can be attributed, at least in part, to tumor cell interactions with elements from the microenvironment, being it soluble factors or stromal or immune cells. This prompted us to test whether LAG-3–MHC II interactions could also protect melanoma cells from drug-induced apoptosis. Etoposide is one of the drugs currently used in melanoma chemotherapy. Thus, we tested whether the interaction of MHC II-positive, etoposide-sensitive melanoma cell line Mel1 with CHO-LAG-3* cells may affect Mel1 apoptosis induced by etoposide. Mel1 and CHO-LAG-3* cells were cocultured at ratios of 1:0.5, 1:1, 1:2, and 1:5 for 1 h. Etoposide was added, and cultures were then continued for another 48 h. The interaction of Mel1 cells with CHO-LAG-3* cells significantly decreased etoposide-induced apoptosis in a ratio-dependent manner.
dependent manner, reaching 59% at a ratio of 1:5 \((p < 0.01)\), compared with apoptosis due to etoposide alone (Fig. 4A).

Similar to CHO-LAG-3⁺ cells, sLAG-3 also protected Mel1 melanoma cells from etoposide-induced apoptosis as evaluated by measurement of \(\Delta \Psi_m\) modifications (Fig. 4A) and by sub-G₁ analysis (Fig. 4B). However, sLAG-3 did not have any protective effect on the MHC II-negative, etoposide-sensitive melanoma cell line 42/95 (Fig. 4C). Thus, LAG-3 MHC II signaling protects MHC II-positive Mel1 melanoma cells from etoposide-induced death.

**Protection from Fas- and etoposide-induced apoptosis by LAG-3 occurs in various MHC II-positive melanoma cells but not in MHC II-negative melanoma cells**

We then confirmed the capacity of LAG-3 MHC II signaling to prevent Fas-mediated and etoposide-induced apoptosis of melanoma cells using a panel of MHC II-positive and MHC II-negative melanoma cell lines. Similar to the A375 melanoma cell line, Meljuso, WM983A, and WM983B melanoma cell lines as well as HM11 and WAC melanoma cells express MHC II molecules (Table I) and are sensitive for Fas-mediated apoptosis. Skmel5 melanoma

![Figure 4](image_url)
cell line is similar to M74 being MHC II-negative (Table I) and sensitive for Fas-mediated apoptosis. The relative apoptosis mediated by Fas in MHC II-positive melanoma cells in the presence of sLAG-3 ranged between 70 and 80% of the apoptosis mediated by Fas in the absence of sLAG-3 (Fig. 5A), with a mean percentage of 75.7 ± 4.8% (±SD, n = 6). In contrast, the presence of sLAG-3 did not affect Fas-mediated apoptosis of the MHC II-negative Skmel5 melanoma cell line (data not shown).

Similar to Mel1, Meljuso, WM983A, and WM983B melanoma cell lines as well as SL8 and WAC melanoma cells were concurrently positive for MHC II and susceptible to etoposide-induced apoptosis. The relative apoptosis induced by etoposide in MHC II-positive melanoma cells in the presence of sLAG-3 ranged between 60 and 75% of the apoptosis induced by etoposide in the absence of sLAG-3 (Fig. 5B), with a mean percentage of 67.8 ± 6.5% (±SD, n = 6). In contrast, the presence of sLAG-3 did not affect etoposide-induced apoptosis of MHC II-negative Skmel5 and M74 melanoma cell lines (data not shown).

These results indicate that LAG-3 provides significant protection to MHC II-positive melanoma cells against Fas-mediated apoptosis (p < 0.001), ranging between 20 and 30%, and it also significantly protects them from etoposide-induced apoptosis (p < 0.001) with a range of 25 to 40%. In contrast, LAG-3 does not protect MHC II-negative melanoma cells from either apoptosis.

**LAG-3 activates MAPK/Erk and PI3K/Akt pathways in MHC II-positive melanoma cells**

The MAPK/Erk and PI3K/Akt are recognized as survival signaling pathways that are highly involved in melanoma development and progression (39). Therefore, we examined the capacity of LAG-3 to activate these pathways in MHC II-positive melanoma cells.

Stimulation of A375 cells with sLAG-3 induced a substantial increase in the phosphorylation levels of Erk1/2 at 2 h that was sustained up to 4 h and decreased to baseline after 8 h of stimulation (Fig. 6a, upper panel). Treatment with sLAG-3 also induced a potent increase in Akt phosphorylation at 4 h that was sustained up to 8 h at least (Fig. 6b, upper panel). Similar results were obtained with Mel1 melanoma cells (data not shown). However, stimulation of the MHC II-negative M74 melanoma cell line enhanced the phosphorylation of neither Erk1/2 nor AKT (Fig. 6, lower panels). These data provide evidence of the capacity of LAG-3 to activate both MAPK/Erk and PI3K/Akt pathways in MHC II-positive melanoma cells, further suggesting the possible involvement of LAG-3 in melanoma progression.

**LAG-3–induced protection from apoptosis involves MAPK/Erk and PI3K/Akt pathways**

We then conducted inhibition studies to determine whether LAG-3–induced MAPK/Erk and/or PI3K/Akt activation could be involved in LAG-3–induced protection against apoptosis. Fas-sensitive A375 cells were cultured in the presence or absence of the MEK-1 inhibitor PD98059 or PI3K/Akt inhibitor LY294002 for 1 h. Then, sLAG-3 was added for 1 h before cells were triggered with anti-Fas mAb 7C11 0.1 μg/ml for 48 h (A) or treated (or not) with etoposide 20 μg/ml for 48 h (B). Apoptosis was measured by sub-G1 analysis. The percentage of Fas- or etoposide-induced apoptotic cells detected in the absence of sLAG-3 was reported to 100 to allow comparison between melanoma cell lines. Results are presented as mean percentages (±SD, gray bars) of Fas-mediated or etoposide-induced apoptosis obtained in the presence of sLAG-3 relative to respective Fas-mediated or etoposide-induced apoptosis detected in the absence of sLAG-3 (white bars) from three independent experiments.
To test the involvement of these pathways in LAG-3–induced protection against etoposide-induced cell death, etoposide-sensitive Mel1 cells were similarly treated with the MEK-1 and PI3K/Akt inhibitors, and etoposide-induced apoptosis was evaluated. A significant reduction in the ability of sLAG-3 to protect Mel1 cells from etoposide-induced apoptosis (p < 0.01) was observed compared with apoptosis induced by etoposide alone (Fig. 7B). The PI3K/Akt inhibitor Ly294002 only partially (by 30%) inhibited the sLAG-3–induced protection from etoposide-induced apoptosis (p < 0.05), whereas the MEK-1 inhibitor PD98059 completely abrogated this effect (p < 0.01) compared with etoposide-induced apoptosis in the presence sLAG-3 (Fig. 7B). Together, these results indicate that both MAPK/Erk and PI3K/Akt pathways are involved in LAG-3–induced protection against Fas- and drug-induced apoptosis of melanoma cells, although at different levels.

**LAG-3 signaling promotes the survival of melanoma cells**

If LAG-3 can contribute to the resistance of melanoma cells to immune system attacks as well as to elimination by drugs, could sLAG-3 or the interactions of melanoma cells with LAG-3–positive cells be among the resistance strategies of these tumors? To provide insights to this possibility, we evaluated the effect of LAG-3 on spontaneous apoptosis of the MHC II-positive A375 melanoma cell line and on MHC II-positive SLM8 melanoma cells. Tumor cells were cultured in the presence or absence of sLAG-3 for 48 h and were analyzed for their spontaneous apoptosis by measurement of ΔΨm modifications. A significant 41.5% decrease in A375 spontaneous apoptosis was observed in the presence of sLAG-3 compared with A375 baseline apoptosis (p < 0.05) (Fig. 8A). The protection was even more pronounced when A375 melanoma cells were cocultured with CHO-LAG-3+ cells: a 58% decrease in spontaneous apoptosis was observed at a ratio of 1:2 (p < 0.01) (Fig. 8A). Spontaneous apoptosis of SLM-8 melanoma cells was also significantly decreased by both sLAG-3 and by their interaction with CHO-LAG-3+ cells in comparison with baseline apoptosis (p < 0.05) (Fig. 8B). Analyzing our panel of MHC II-positive melanoma cells (n = 6), we found that the presence of sLAG-3 in the cultures reduced the relative spontaneous cell death of MHC II-positive melanoma cells to 67 ± 7.1% (mean ± SD) of the spontaneous apoptosis observed in the absence of sLAG-3 (taken as 100%). However, sLAG-3 did not prevent the spontaneous apoptosis of MHC II-negative melanoma cell lines (Fig. 8C). Thus, the presence of LAG-3 within the melanoma microenvironment might be among the elements that provide death resistance and persistence of these tumors.

**Discussion**

The poor efficacy of current immunotherapeutic approaches used to treat distant metastatic melanomas underscores an urgent need for novel therapies based on a better understanding of the elements regulating the resistance of these tumors to therapeutic protocols. The MHC II ligand LAG-3 molecule plays a key role in the regulation of T cell functioning, and several subsets of LAG-3–expressing T cells are expanded in PBMCs of advanced tumor-bearing patients and at tumor sites (12, 26, 40). Based on data concerning the signal transduction function of MHC II molecules (8) and the expression of MHC II in melanoma (1, 2, 4, 5, 7), we analyzed the effect of LAG-3 on apoptosis of melanoma cells. In this study, we show that LAG-3 MHC II signaling in melanoma cells hinders their apoptosis, whether it is induced via Fas, by drugs, or spontaneous. Our study proposes LAG-3–MHC II molecular interaction as a novel pathway enabling melanoma cells not only to escape immune attack but also to counter drug effects and to survive.

We showed that LAG-3 can activate MAPK/Erk and PI3K/Akt survival pathways, both being highly implicated in melanoma resistance to apoptosis and progression (41). We have previously shown that anti–HLA-DR L243 activates MAPK/Erk but not PI3K/Akt pathway in melanoma cells (10). Discrepancies between pathways activated by L243 mAb and sLAG-3 have also been reported in dendritic cells (42). The L243 mAb is known to recognize HLA-DR in a manner mimicking that of TCR recognition, whereas the interaction of LAG-3 with MHC II may rather mimic that of CD4 (15, 27). Therefore, different epitopes recognized by each MHC II ligand might underlie the observed discrepancies between the effects of LAG-3 and anti–HLA-DR. LAG-3 and L243 also activate MAPK/Erk with different kinetics; engagement of MHC II in melanoma cells by L243 induces a sustained MAPK/Erk activation for up to 24 h (10), whereas in this study we show that LAG-3 rather induces a transient activation from 2 to 4 h.

Our results demonstrate that the LAG-3 molecule, whether soluble or on the cell surface, provides protection to melanoma cells against apoptosis induced either through Fas or by etoposide. We showed that this protection involves MAPK/Erk and PI3K/Akt activation by LAG-3, although at different levels. LAG-3–induced protection from Fas-mediated death seems to involve mainly the PI3K/Akt pathway, whereas LAG-3–induced protection against...
the DNA-damaging drug etoposide seems to rather involve the MAPK/Erk pathway. The kinetics of MAPK/Erk and PI3K/Akt activation by LAG-3 suggests that the partial involvement of MAPK/Erk pathway in LAG-3–induced protection of melanoma cells from Fas-mediated apoptosis could be at early stages of the apoptotic cascade. In support, L243-induced activation of MAPK/Erk in melanoma cells reduces the Fas-mediated processing and activation of caspase-8 and that of Bid (10); both are early events of Fas-mediated melanoma apoptosis before the implication of the mitochondria pathway (32). Importantly, specific inhibition of PI3K/Akt pathway abolishes completely LAG-3–induced protection of A375 melanoma cells from Fas-induced apoptosis. Thus, PI3K/Akt in this model seems to have a predominant role in such protection. It is well established that PI3K/Akt is a foremost regulator of the mitochondrial death pathway (43). In melanoma, even though Fas-induced death is initiated by activation of the classical receptor death cascade, its execution depends on the subsequent activation of the mitochondrial death cascade (33).

In contrast to LAG-3–induced protection from Fas-mediated cell death, protection from apoptosis induced by DNA-damaging etoposide mainly relied on the activation of the MAPK/Erk pathway. Our results are in line with previous studies using another DNA-damaging drug, cisplatin, and showing that the high constitutive activation of the MAPK/Erk pathway in melanoma underlies its resistance to this drug (44). Accordingly, it seems that in addition to the PI3K/Akt pathway, known to regulate drug-induced apoptosis (45, 46), melanoma resistance to DNA-damaging drugs also involves the MAPK/Erk pathway. This does not rule out the implication of PI3K/Akt pathway in LAG-3–

FIGURE 7. LAG-3 protects melanoma cells from Fas- and etoposide-induced apoptosis, and this protection involves MAPK/Erk and PI3K/Akt pathways. A, A375 melanoma cells were treated with specific PI3K/Akt (Ly294002) or MEK-1 (PD98059) inhibitors or not, then were treated with anti-Fas mAb 7C11 0.1 µg/ml or not in the presence or absence of sLAG-3 for 48 h. Apoptosis was determined by loss of mitochondrial transmembrane potential (ΔΨm) and flow cytometry analysis. Results presented are mean percentages of apoptotic cells (±SD) from three independent experiments. *p < 0.05, **p < 0.01. B, Mel1 melanoma cells were treated with specific MEK-1 (PD98059) or PI3K/Akt (Ly294002) inhibitors or not, then were treated with etoposide 20 µg/ml or not in the presence or absence of sLAG-3 for 48 h. Cell death was determined by ΔΨm measurement and presented as in A. *p < 0.05, **p < 0.01.

FIGURE 8. LAG-3 protects MHC II-positive but not MHC II-negative melanoma cells from spontaneous apoptosis. A and B, A375 (A) and SLM-8 (B) MHC II-positive melanoma cells were cultured in the absence (medium) or in the presence of sLAG-3 (10 µg/ml) or CHO-LAG-3+ cells (ratio 1:2) for 48 h. Cell death was determined by ΔΨm measurement and flow cytometry analysis. Results are presented as the mean percentages of spontaneous apoptosis (±SD) observed in the presence of LAG-3 relative to spontaneous apoptosis detected in the absence of sLAG-3 from two independent experiments. *p < 0.05, **p < 0.01. C, Three MHC II-negative melanoma cell lines (M74, Skmel5, 42/95) were cultured in the presence or absence of sLAG-3 (10 µg/ml) for 48 h, and apoptosis was measured by ΔΨm modifications and presented as in A and B.
induced rescue against etoposide because specific inhibitor of the pathway also partially decreased the LAG-3 protective effect. Indeed, growing evidence today supports the notion that DNA-damaging drug-induced apoptosis and the resistance of tumors to these drugs is the result of the interplay between PI3K/Akt and MAPK signaling pathways (47).

In APCs, MHC II signaling modulates both cell activation and death (8). The MHC II-mediated death occurs also in some types of lymphoid malignancies (48). In A375 melanoma cells, we previously showed that anti–HLA-DR signaling does not induce death but only provides protection against apoptosis (10). LAG-3 as a natural ligand of MHC II also failed to induce cell death in MHC II–positive melanoma cell lines as well as in freshly isolated melanoma cells, and instead provided protection from spontaneous apoptosis. These results confirm the absence of MHC II–mediated death in melanoma cells, in contrast to APCs and certain lymphoid malignancies. HLA-DR signaling mediates activation and death through distinct signaling pathways (49). In melanoma, it seems that MHC II signaling is coupled to signaling pathways that control activation and growth rather than cell death. This may be due to the compartmentalization of MHC II into different microdomains of the plasma membrane and/or their association with other cell surface molecules, which are critical in determining the outcome of HLA-DR signaling toward cell death versus activation and growth in APCs (8, 49).

The mechanisms by which melanoma tumors escape immune surveillance are complex and not fully understood. A study with a skin tumor model showed that a squamous cell carcinoma that expresses high levels of MHC II, in the absence of costimulatory CD80 and CD86 molecules, had a growth advantage compared with MHC II–negative squamous cell carcinoma, which tends to regress spontaneously (50). In this study, we demonstrate the capacity of a physiological MHC II ligand, LAG-3, either soluble or expressed on the cell surface, to protect from apoptosis induced via Fas, known as an important pathway of immune-mediated killing of melanoma. On one hand, our results provide evidence that our previously reported MHC II–mediated hampering of the killing by immune cells is physiologically valid. On the other hand, they may provide one possible explanation into the growth advantage of MHC II–positive skin tumors (50) and into the long-standing correlation between MHC II expression and degree of melanoma malignancy (2, 5–7). However LAG-3 also protects from drug-induced apoptosis. Thus, during interaction between melanoma cells and effector immune cells, the engagement of MHC II by LAG-3 expressed on tumor-infiltrating leukocytes would elevate not only MAPK/Erk but also PI3K/Akt activity in melanoma cells and contribute, along with growth factors (51, 52), to their resistance to immune system attacks as well as to chemotherapy.

All these results suggest LAG-3 and its MHC II signaling as a critical resistance element within the microenvironment of melanoma tumors. Our results showed that LAG-3 is expressed by various subsets of tumor-infiltrating leukocytes including NKT and NK cells in addition to CD4+, CD8+, and CD25^{high}FOXP3+ T cells. Previous studies have shown that LAG-3 engagement inhibits T cell activation likely through regulating homeostatic responses and inducing regulatory activity (18, 26, 40). This is strongly supported by studies in LAG-3 knockout mice, which clearly demonstrated this LAG-3 activity in vivo (21). Furthermore, in chronic viral infection, simultaneous blockade of LAG-3 and PD-L1 induced a greater T cell CD8\(^+\) cytotoxic response (53). The importance of LAG-3–mediated regulatory effect on T cells with regard to antitumor immune response has also been elegantly demonstrated by Grosso et al. (54). The authors showed a direct role for LAG-3 in inhibiting the activity of CD8\(^+\) T cells in a murine model of tumor tolerance and suggested that LAG-3 blockade may be a potential cancer treatment (54). The role of LAG-3 in downregulating NK and NKT cell activation is not yet fully addressed. Nevertheless, LAG-3 signaling on activated NKT cells was suggested to downregulate their proliferation (55), although LAG-3 does not seem to have a transducing activity involved in NK cytotoxicity (40, 56). Although further studies are warranted, the current state of knowledge and our data presented in this article propose the LAG-3–MHC II interaction as a bi-directional immune escape pathway in melanoma tumors with direct consequence shared by both melanoma and immune cells. Then again, our studies resume the interest of MHC II phenotyping for more efficient therapeutic strategies.

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Disclosures
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