Soluble Human LAG-3 Molecule Amplifies the *In vitro* Generation of Type 1 Tumor-Specific Immunity

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Abstract

The adjuvant activities of the human lymphocyte activation gene-3 (*LAG-3*) molecule have been evaluated in a human setting by investigating the ability of a soluble recombinant human LAG-3 protein (hLAG-3Ig) to enhance the *in vitro* induction of viral- and tumor-specific CTLs. We found that soluble human LAG-3 significantly sustained the generation and expansion of influenza matrix protein Melan-A/MART-1 and survivin-specific $CD8^+$ T lymphocytes in peripheral blood mononuclear cells (PBMC) of both cancer patients and healthy donors, showing its ability to boost $CD8^+$ T-cell memory response or to prime naive T cells *in vitro*. The peptide-specific T cells generated in the presence of hLAG-3Ig were endowed with cytotoxic activity and enhanced release of type 1 cytotoxic T (Tc1) cytokines and were able to recognize tumor cells expressing their nominal antigen. Phenotype and cytokine/chemokines produced by antigen-presenting cells (APC) of PBMCs exposed *in vitro* for 2 days to peptide and hLAG-3Ig indicate that the LAG-3–mediated adjuvant effect may depend on a direct activation of circulating APCs. Our data revealed the activity of hLAG-3Ig in inducing tumor-associated, antigen-specific $CD8^+$ T-cell responses in a human setting and strongly support the conclusion that this recombinant protein is a potential candidate adjuvant for cancer vaccines. (Cancer Res 2006; 66(8): 4450-60)

Introduction

The ability to induce a strong immune response that includes the generation and expansion of tumor-specific cytotoxic T cells is indeed the first requirement for a clinically effective cancer vaccine (1). However, the nature of tumor-associated antigens (TAA), which are mainly self-derived proteins to which the immune system could be tolerized, is a major critical issue hampering the success of cancer immunotherapy. To induce sustained systemic immune responses, several strategies have been developed, and besides the variety of vaccine formulations, the efficacy of different immunologic adjuvants has been investigated (2). Although vaccine adjuvants have become of general use, and some of them have been extensively exploited in preclinical and clinical approaches, their mechanisms of action are not yet completely understood. Nevertheless, it is now clear that adjuvants can exert their effects at different stages of vaccine-induced immunologic responses; most of them work mainly by activating and licensing dendritic cells to present antigens in a correct activating milieu (3). Vaccine strategy should also be effective in sustaining the final effector phase of the T-cell response. Several mechanisms arising both from tumor and from immune system itself have been recently described, which can impair the activity of antitumor T cells (4, 5), and one of the most relevant suppressor mechanisms is operated by regulatory T cells (6–8). The overcome of this negative immune-related feedback is indeed a challenging issue that should be taken into consideration in designing new therapeutic cancer strategies. Given the complexity in the generation of a strong and effective response against tumors, the identification and the development of new immunologic adjuvants is, therefore, mandatory.

Lymphocyte activation gene-3 (*LAG-3*) is a member of immunoglobulin superfamily (9) that binds MHC class II molecules (10). It is expressed on activated natural killer and T cells but not on resting T lymphocytes (9, 11). A complex role of LAG-3 in controlling T-cell functions has been recently reported in murine and in human setting (12). Like CD4 and CD8, LAG-3 is associated with the CD3/TCR complex and shown to be involved in down-regulating antigen-induced TCR signaling (13, 14). Recent studies in mice (15, 16) and in humans (17) reported that LAG-3 negatively regulates T-cell function and homeostasis. However, as a soluble molecule, LAG-3 activates antigen-presenting cells (APC) through MHC class II signaling (18, 19), leading to increased antigen-specific T-cell responses *in vivo* (20), and its presence as free molecule in the sera of subgroups of breast cancer patients at diagnosis has been correlated with a better survival (21). Given these critical roles of the protein in the immune system, its physiologic expression in human tumor-infiltrating lymphocytes (22) and its ability to activate APCs *in vitro*, soluble LAG-3 molecule has been exploited as a vaccine adjuvant in *in vivo* mouse models, by using either viral-associated antigens or TAAs given as irradiated LAG-3-transfected syngeneic tumor cells, soluble proteins, or as gene-encoding plasmids (20, 23, 24). Vaccination in the presence of LAG-3 induced a strong adjuvant activity and the generation of a protective long-lasting immunity. However, immunologic mechanisms by which LAG-3 molecule exerts its adjuvant actions have not been studied using complex, physiologically relevant cell populations, such as peripheral blood mononuclear cell (PBMC) and tumor antigens. In the present study, we evaluated the adjuvant effect of human soluble LAG-3 protein in a human setting by investigating its ability to enhance the induction of viral- and tumor-specific CTLs in PBMCs of cancer patients and healthy donors.

Both viral and tumor antigen models were used: the highly immunogenic influenza matrix protein–derived peptide Flu_{58-66}, the melanoma-associated peptide $MART_{27-35}$, its $MART_{26-35}A27L$ analogue, and the survivin-derived $SVV\text{-}1_{95-104}$ peptide. For the first

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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time, we showed that soluble human LAG-3 significantly sustained the generation and the expansion of influenza matrix protein Melan-A/MART-1 and survivin-specific $CD8^+$ T lymphocytes both in melanoma or colorectal cancer patients and in healthy donors, showing a double ability to boost $CD8^+$ T-cell memory response and to prime naive T cells. The generated T cells were endowed with cytotoxic activity and a Tc1 cytokine secretion profile. The LAG-3-mediated adjuvant effect was shown also in $CD4^+CD25^+$-depleted PBMCs, and it might partially rely on direct activation of APCs residing in human peripheral blood that include monocytes, B cells, and dendritic cells. Thus, we were able to show for the first time that the human LAG-3 molecule supported the generation and increased the frequency of tumor-specific $CD8^+$ T-cell responses in the human immune system, therefore validating the use of this protein as a potential adjuvant in immunotherapeutic approaches against cancer.

Materials and Methods

Reagents. The recombinant soluble human LAG-3 fusion protein (hLAG-3Ig) identified as the product IMP321 was provided by Immutep (Orsay, France) from a pre-GMP batch. Briefly, Chinese hamster ovary $DHFR^-$ cells were transfected with a plasmid coding for the D1-D4 extracellular domains of hLAG-3 fused to the Fc tail of a hIgG1 (25). A productive clone was selected after amplification in methothrexate. The purified bulk preclinical batch has a concentration of 1.89 mg/mL hLAG-3Ig in the form of a 200-kDa dimeric protein and 0.75 EU/mg endotoxin (LAL assay, Bio Whittaker, Walkersville, MD), 0.42 ng/mL DNA, and 6 ng/mL host cell protein contents. An aliquot of the hLAG-3Ig preparation used throughout this study has been analyzed by SDS-PAGE, and gels were stained with Coomassie blue or blotted on polyvinyl difluoride membrane (Bio-Rad Laboratories, Richmond, CA) and hybridized with anti human-LAG-3 mouse monoclonal antibody (mAb) 17B4 (see Supplementary Fig. S1) followed by goat anti-mouse horseradish peroxidase IgG (Calbiochem, Darmstadt, Germany). Boiling hLAG-3Ig for 5 minutes abrogated its biological activity, evaluated as the ability to up-regulate CD83 expression on monocytes purified from PBMCs of healthy donors.

Peptide synthesis. Peptides were purchased from Immatics (Tübingen, Germany); their degree of purification was analyzed by high-performance liquid chromatography. All the peptides showed >95% purity. The following peptides were used in this study: survivin-derived SVV-$1_{95\text{-}104}$ (ELTLGEFLKL), MelanA/MART-1–derived $MART_{27\text{-}35}$ (AAGIGILTV) and $MART_{26\text{-}35}$A27L (ELAGIGILTV), influenza matrix protein–derived $Flu_{58\text{-}66}$ (GILGFVFTL), and HIV envelope–derived $HIVenv_{117\text{-}125}$ (KLTPLCVTL).

Antigen stimulation of peripheral blood lymphocytes. Upon informed consent, blood samples were collected from melanoma and colorectal cancer patients or healthy donors. PBMCs were isolated and analyzed for HLA-A*0201 expression as previously described (26).

On day 0, PBMCs were seeded in 24-well plates at a concentration of 10^6/mL in RPMI 1640, supplemented with 10% heat-inactivated human serum, 2 mmol/L L-glutamine (Bio Whittaker Europe, Verviers, Belgium), and antibiotics. To induce the generation of peptide-specific $CD8^+$ T lymphocytes, cells were stimulated for 10 days with the indicated peptide at a concentration of 2 μg/mL ($Flu_{58\text{-}66}$) or 3 μg/mL ($MART_{26\text{-}35}$A27L, $MART_{27\text{-}35}$, or SVV-1), with or without 16 μg/mL hLAG-3Ig. As control, induction of peptide-specific T cells was also done in the presence of 16 μg/mL of human IgG, Fc fragment–purified protein, or human IgG, whole molecule purified protein (Chemicon International, Temecula, CA). On day 2, interleukin 2 (IL-2; EuroCetus, Amsterdam, the Netherlands) was added at a final concentration of 60 IU/mL; then cells were maintained under the same conditions, replacing half of the medium and IL-2 every 2 days. For experiments aimed at evaluating the effect of hLAG-3Ig on APCs, supernatants were collected and stored after 48 hours of cell culture, and cells were harvested and analyzed for the surface expression of activation markers. If required, restimulation was done using autologous PBMCs at a stimulator/responder ratio of 4:1, pulsed with peptide either alone or in the presence of 16 μg/mL hLAG-3Ig for 2 hours, and then γ-irradiated (3,000 rad).

Depletion of $CD4^+CD25^+$ T cells from freshly isolated PBMCs was done by immunomagnetic sorting with a human $CD4^+CD25^+$ Isolation kit following the manufactory instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Depleted cells were stimulated at the same experimental conditions set for the whole PBMC population (see above). When needed, restimulation was conducted using $CD4^+CD25^+$-depleted, peptide-pulsed, γ-irradiated autologous PBMCs at a stimulator/responder ratio of 4:1.

T-cell stimulation assay. The presence of an antigen-specific $CD8^+$ T-cell response was first detected by evaluating IFN-γ release in a T-cell stimulation assay. HLA-A*0201^+ T2 cells were pulsed for 2 hours at 37°C with 2 μg/mL of the stimulating peptide and of a control HIV-derived epitope (HIV $env_{117\text{-}125}$, HLA-A*0201 restricted). For experiments aimed at evaluating the presence of a $CD8^+$ T cell response against TAAs (Melan-A/MART-1 and survivin), HLA-A*0201^+ tumor cells were also used as a target. In particular, Melan-A/MART-1-specific T-cell response was analyzed by using the HLA-A*0201^+ Melan-A/MART-1^+ Me501 melanoma cell line (generated in our laboratory from tissue obtained from a metastatic melanoma patient); the survivin$^+$, HLA-A*0201^+ SW480 colon cancer cell line (American Type Culture Collection, Rockville, MD) was used as a target for survivin-specific T cells. Inhibition experiments were done by adding to tumor cells 1 μg/mL of anti-class I HLA A6-136 mAb.

IFN-γ and perforin enzyme-linked immunospot. Evaluation of IFN-γ and Perforin release by stimulated T cells was done by using the enzyme-linked immunospot (ELISPOT) method, as previously described (25). A detailed description of the protocol used in this study for the detection of both proteins is provided in the Supplementary Data.

Cytometric bead array assay. To simultaneously detect and measure multiple soluble analytes released in the supernatants of cell cultures, the Becton Dickinson Cytometric Bead Array (CBA) was used in this study. Each assay was conducted according to the manufactory instructions. See Supplementary Data for details.

Tetramer staining. Staining of Melan-A/MART-1- or Flu-specific T cells was done using commercially available phycoerythrin-conjugated HLA-A*0201/$MART_{26\text{-}35}$A27L and HLA-A*0201/$Flu_{58\text{-}66}$ tetramers (Immunomics, Beckman Coulter, Marseilles, France). Briefly, 5×10^5 stimulated T cells were incubated for 45 minutes at 4°C with 10 μL of tetramer, then washed twice in PBS, and incubated for 30 minutes at 4°C with anti-CD8 mAb (Becton Dickinson, Heidelberg, Germany). Fluorescence intensity was evaluated using a FACSCalibur flow cytometer and analyzed using CellQuest software (Becton Dickinson). A phycoerythrin-conjugated HLA-A*0201-negative tetramer (Immunomics, Beckman Coulter) was used as a control (data not shown).

Flow cytometric immunofluorescence analysis. Evaluation of APC activation in whole PBMC populations was done by flow cytometry. To specifically identify and enumerate peripheral blood monocytes and B cells, alternative staining with FITC-conjugated CD14 or CD19 mAb was done: cells resulting positive for either marker were analyzed for the surface expression of CD80, CD83, and CD86 proteins by using phycoerythrin-conjugated mAb (all purchased from Becton Dickinson). Peripheral blood dendritic cells (PBDC) were identified as lineage (CD3, CD14, CD16, CD19, CD20, CD56) negative and $CD123^{low/high}$ and then analyzed for the surface expression of CD80, CD83, and CD86 proteins by using phycoerythrin-conjugated mAb. Fluorescence intensity was evaluated using a FACSCalibur flow cytometer and analyzed using CellQuest software (Becton Dickinson).

Results

Soluble human LAG-3 enhances the expansion of tumor- or viral-specific $CD8^+$ T lymphocytes. To evaluate the ability of LAG-3-soluble molecule to enhance the priming of naive $CD8^+$ T cells or to boost a $CD8^+$ memory T-cell response, four different antigen models have been used that include the influenza matrix protein–derived $Flu_{58\text{-}66}$ peptide, the TAA peptide $MART_{27\text{-}35}$, its

$MART_{26-35}$A27L analogue, and the survivin-derived $SVV\text{-}1_{95-104}$ peptide. PBMCs from healthy donors were used as responder cells, being known that whereas influenza-specific $CD8^+$ T cells carry features of antigen-experienced T cells, anti-Melan-A/MART-1 $CD8^+$ T cells in healthy individuals all belong to the naive T-cell subset. Moreover, activities of LAG-3 molecule in association with tumor-derived peptides were also tested in cancer patient PBMCs. Circulating $CD8^+$ T cells specific for tumor antigens in cancer patients include T cells belonging to both memory and naive compartments, as shown for Melan-A/MART-1 (27). In addition, in view of a possible usage of LAG-3-soluble molecule in cancer vaccine, it was crucial to test its biological functions using TAAs and patients' derived PBMCs as responder cells.

PBMCs from HLA-A*0201$^+$ donors were stimulated *in vitro* with the Flu_{58-66} peptide (GILGFVFTL), with the wild-type $MART_{27-35}$ nonapeptide (AAGIGILTV), or with the highly immunogenic $MART_{26-35}$A27L peptide analogue (ELAGIGILTV), whereas melanoma or colon cancer patients' PBMCs were stimulated with the two mentioned Melan-A/MART-1 peptides and with the survivin-derived $SVV\text{-}1_{95-104}$ peptide (ELTLGEFLKL), respectively. *In vitro* stimulation was done in the presence or absence of 16 μg/mL of LAG-3 recombinant protein (hLAG-3Ig), and functional and phenotypic analysis of the peptide-sensitized T cells was conducted after 1 or 2 weeks of *in vitro* culture.

Proliferation of each cell culture was monitored by counting the total number of lymphocytes in the single culture conditions at different time points.

We found that the addition of hLAG-3Ig induced an expansion of the cultured lymphocytes for all the peptides used (Fig. 1), whereas the total number of cells remained unchanged when the *in vitro* stimulation was done with peptide alone. This LAG-3-dependent proliferation started early during the *in vitro* culture, being detectable at day 10 of culture. Moreover, the difference in the yield of the peptide-stimulated T cells was amplified after the second week of culture, and all the T cells grown in the presence of hLAG-3Ig showed a fold increase ranging from 2.5 (Fig. 1*A* and *E*) to 6 (Fig. 1*C* and *F*).

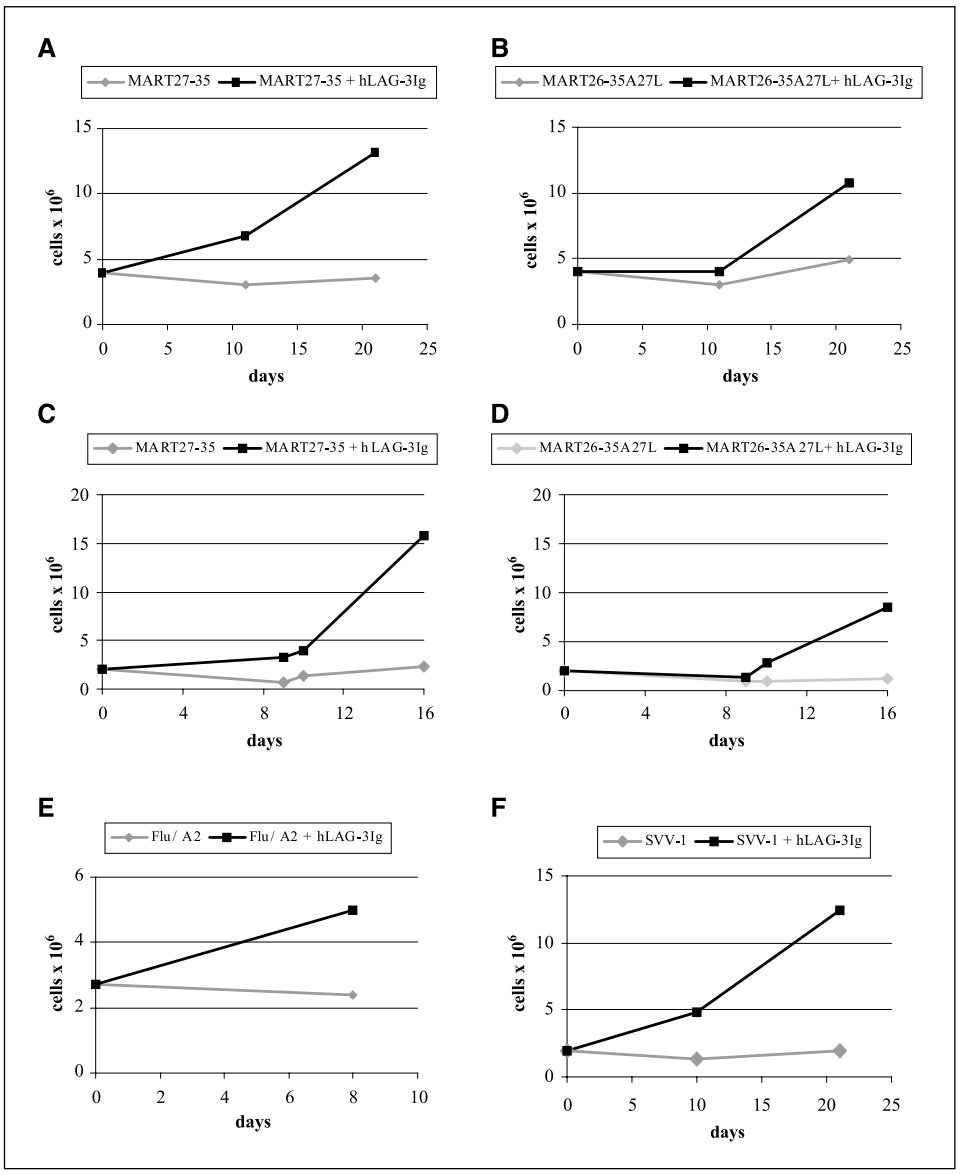

Figure 1. hLAG-3Ig increased the proliferation of antigen-stimulated PBMCs. PBMCs from healthy donors (*A, B*, and *E*), melanoma patients (*C* and *D*), and colorectal cancer patients (*F*) were stimulated once or twice with the indicated peptide, either alone (*gray lines*) or in the presence of 16 μg/mL hLAG-3Ig (*black lines*), and their growth was monitored at various time points. Data report the overall number of T lymphocytes present in each cell culture at the indicated time points. Six independent T-cell cultures representative of a total of 20 have been reported.

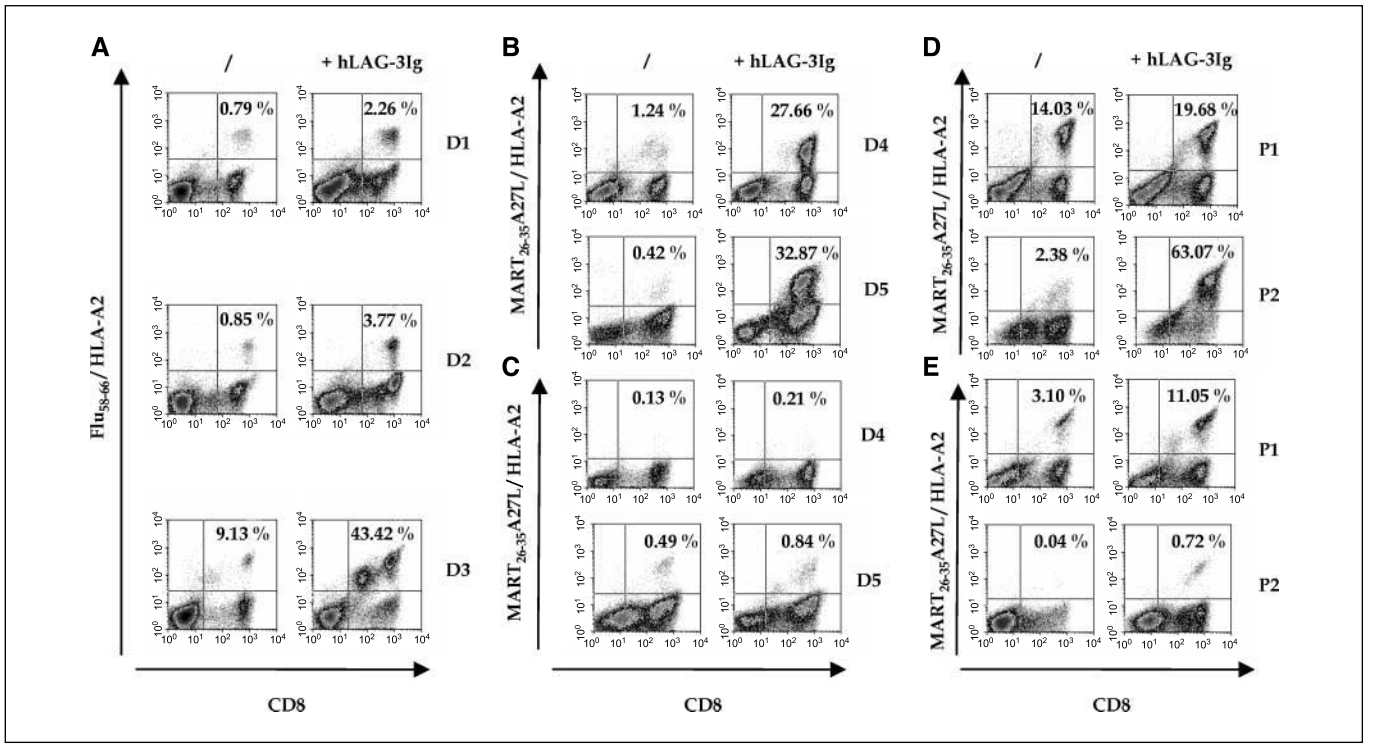

Figure 2. Tetramer-guided analysis of antigen-specific CD8$^+$ T cells. PBMCs from healthy donors were stimulated with Flu_{58-66} peptide (*A*: D1, D2, one round of stimulation; D3, two rounds of stimulation), with $MART_{26-35}A27L$ analogue peptide (*B*: D4 and D5, two rounds of stimulation), and with $MART_{27-35}$ wild-type nonapeptide (*C*: D4 and D5, two rounds of stimulation) either alone (*left*) or in the presence of 16 μg/mL hLAG-3Ig (*right*). After staining with FITC-conjugated anti-CD8 mAb and phycoerythrin-conjugated Flu_{58-66}/HLA-A*0201 tetramers (*A*) or PE-conjugated $MART_{26-35}A27L$/HLA-A*0201 tetramers (*B* and *C*), the percentage of CD8$^+$, peptide-specific T cells was determined by flow cytometry (see top right quadrant of each dot plot). PBMCs from melanoma patients P1 and P2 were subjected to two rounds of *in vitro* stimulation with $MART_{26-35}A27L$ analogue peptide (*D*) or with the $MART_{27-35}$ wild-type nonapeptide (*E*) either alone (*left)* or with the addition of 16 μg/mL hLAG-3Ig (*right)*. After staining with phycoerythrin-conjugated $MART_{26-35}A27L$/HLA-A*0201 tetramers and FITC-conjugated anti-CD8 mAb (*D* and *E*), the percentage of CD8$^+$, antigen-specific T lymphocytes was determined by flow cytometry (see top right quadrant of each dot plot).

We next asked whether this strong proliferative response achieved in the presence of hLAG-3Ig was also associated with an increase in the number of peptide-specific T cells. Therefore, we evaluated the presence and frequency of antigen-specific CD8$^+$ T lymphocytes after one or two rounds of peptide stimulation by using antigen-specific tetramer staining. We found that the addition of hLAG-3Ig induced a significant increase of the frequency of antigen-specific CD8$^+$ T cells in all the *in vitro* cultures.

PBMCs of HLA-A*0201$^+$ healthy donors, stimulated with the influenza matrix protein–derived Flu_{58-66} peptide, showed an increased frequency of peptide-specific, CD8$^+$ T lymphocytes when cultured in the presence of hLAG-3Ig as detected by staining with the HLA-A*0201/Flu_{58-66} tetramer (Fig. 2*A*, donors D1 and D2), and this difference was amplified after a second round of stimulation (Fig. 2*A*, donor D3).

A similar effect in healthy donors' PBMCs was seen also when considering the induction of Melan-A/MART-1–specific T-cell responses. HLA-A*0201$^+$ T lymphocytes stimulated with the highly immunogenic decapeptide $MART_{26-35}A27L$ exhibited a significant increase of epitope-specific CD8$^+$ T cells when cultured with hLAG-3Ig. In fact, the frequency of CD8$^+$, HLA-A*0201/$MART_{26-35}A27L$ tetramer-positive cells reached the 27.66% or 32.87% for hLAG-3Ig/peptide–stimulated cells compared with a 1.24% or 0.42% frequency when the same PBMCs were stimulated in the absence of hLAG-3Ig (Fig. 2*B*, donors D4 and D5). Although the total number of specific T cells was lower, the same trend was also observed for the less immunogenic wild-type $MART_{27-35}$ nonapeptide, with frequency values obtained with hLAG-3Ig showing almost 2-fold increase in respect to that achieved after stimulation with the peptide alone (Fig. 2*C*). Thus, the adjuvant effect of hLAG-3Ig on peptide-stimulated T cells was observed for PBMCs of healthy donors both with viral- and melanoma-associated antigen, suggesting a role for this molecule in a general mechanism of activation, regardless of the naive or effector status of the responding CD8$^+$ T cells.

Increased frequencies of Melan-A/MART-1–specific T cells were consistently observed also in patients' PBMCs stimulated with $MART_{26-35}A27L$ or $MART_{27-35}$ in the presence of hLAG-3Ig (Fig. 2*D* and *E*, respectively). For patient P2, two rounds of peptide stimulation in the presence of hLAG-3Ig led to the generation of a T-cell culture in which the 63.07% of cells was represented by CD8$^+$ Melan-A/MART-1–specific T cells. It is important to note that the hLAG-3Ig–mediated effect is presumably independent of the frequency of circulating antigen-specific T cells. In fact, hLAG-3Ig treatment increased the frequency of antigen-specific T cells also in individuals whose PBMCs reached a significant number of CD8$^+$ T-cell precursors by peptide stimulation alone (Fig. 2*A*, donor D3; Fig. 2*D* and *E*, patient P1) as well as in those where peptide stimulation alone led to a low, barely detectable frequency of antigen-specific T cells (Fig. 2*B*, donor D5; Fig. 2*E*, patient P2).

Antigen-specific T lymphocytes generated in the presence of hLAG-3Ig release high levels of IFN-γ. We next analyzed the functional activity of the cultured T cells known to contain antigen-specific CD8$^+$ T cells by tetramer staining analysis. Because a significant difference, in terms of frequency, was observed between cells stimulated in the presence or absence of hLAG-3Ig, we asked

whether the CD8$^+$ T cells generated with hLAG-3Ig were also endowed with functional activity to exclude that they could indeed represent anergic CD8$^+$ T cells or T lymphocytes with low antigen affinity and unable to be functionally triggered by their nominal antigen. To explore the specificity of the *in vitro* activated T cells, IFN-γ ELISPOT assays were first done. The function of influenza matrix–specific CD8$^+$ T cells was evaluated by analyzing IFN-γ release in response to Flu_{58-66}-loaded T2 cells (Fig. 3*A*). This HLA-A*0201–restricted viral antigen is highly immunogenic, and specific IFN-γ release could be detected after a single round of sensitization with the peptide alone (see Fig. 3*A, left*, donors D1 and D2). However, the addition of hLAG-3Ig to the lymphocyte culture strongly amplified the antigen-specific IFN-γ release, and in all the tested cultures, the presence of hLAG-3Ig led to a number of specific T cells that exceeded the range limit of the ELISPOT assay (Fig. 3*A, right*).

The role of hLAG-3Ig in the induction of tumor-specific reactivity was then evaluated. For two of four CD8$^+$ T-cell cultures generated *in vitro* by stimulating HLA-A*0201$^+$ melanoma patients' or donors' PBMCs with the highly immunogenic $MART_{26-35}$A27L analogue peptide, no reactivity was detectable (Fig. 3*B, left*, D4 and P2), whereas the two additional cultures tested displayed significant release of cytokine in response to specific peptides (Fig. 3*B, left*, D5 and P1). When PBMCs of the same individuals were cultured with $MART_{26-35}$A27L analogue peptide together with hLAG-3Ig, their specific reactivity was strongly enhanced (Fig. 3*B, right*, D5 and P1), and in the presence of hLAG-3Ig, specific IFN-γ release was detected also in those PBMCs for which the culture with peptide alone failed to achieve functional specificity (Fig. 3*B, right*, D4 and P2). Moreover, all the Melan-A/MART-1–specific CD8$^+$ T cells generated in the presence of hLAG-3Ig recognized their antigen when naturally processed by melanoma, as shown by

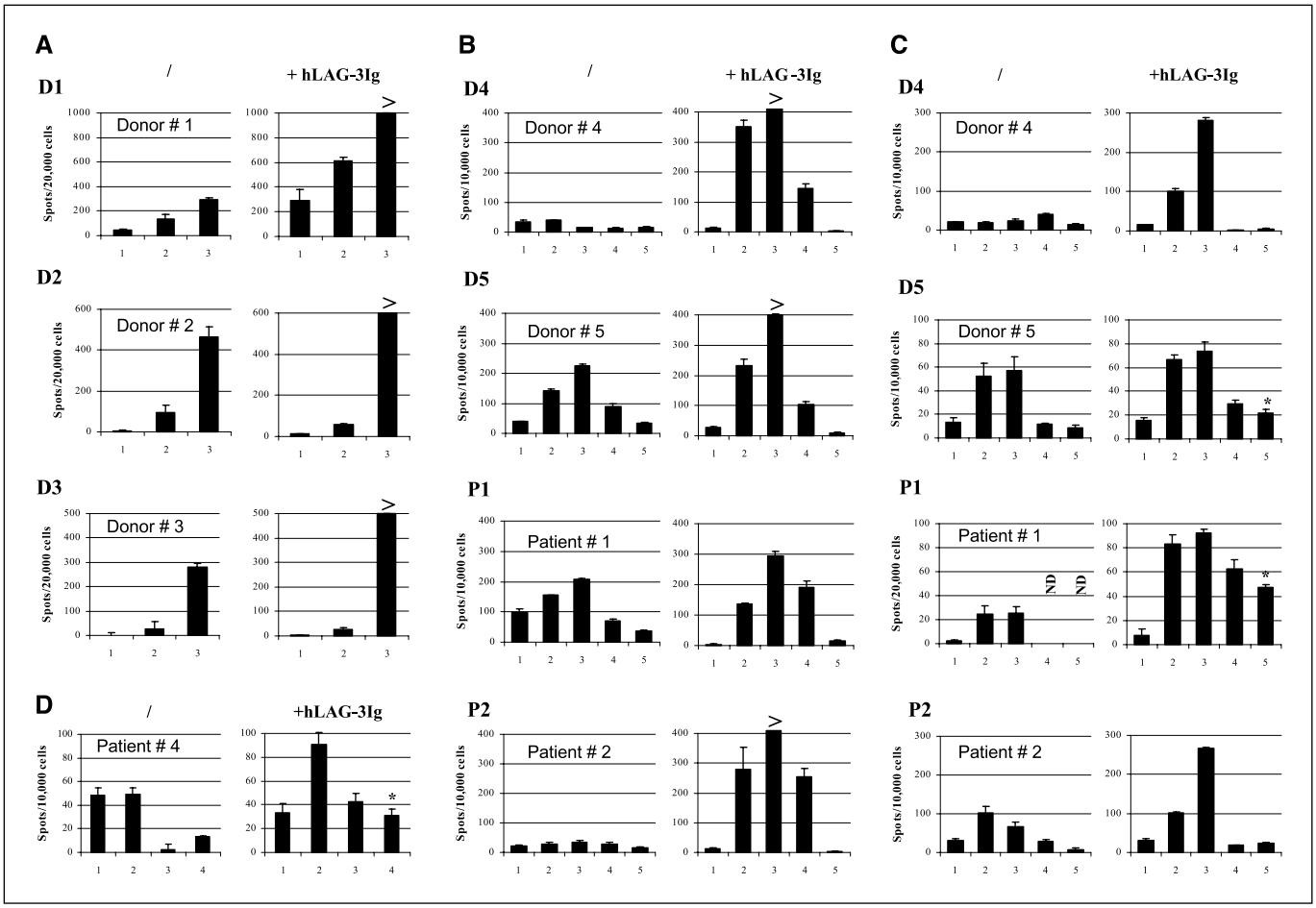

Figure 3. Addition of soluble hLAG-3Ig to PBMC cultures of healthy donors or melanoma and colorectal cancer patients leads to the generation of a high number of functionally active antigen-specific T cells. *A,* PBMCs of healthy donors D1, D2, and D3 were subjected to one (D1) or two (D2 and D3) rounds of *in vitro* stimulation with HLA-A*0201–restricted Flu_{58-66} peptide and then analyzed in ELISPOT assay for the presence of IFN-γ–secreting cells. In the T-cell stimulation assay, lymphocytes were incubated alone (*column 1*) or with T2 cells loaded with 2 μg/mL HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *column 2*) or with Flu_{58-66} peptide (*column 3*). Cell cultures were done with or without the addition of 16 μg/mL hLAG-3Ig (*top*). *B* and *C,* PBMCs of healthy donors D4 and D5 and of melanoma patients P1 and P2 were cultured *in vitro* for 10 days with $MART_{26-35}$A27L (*B*) or $MART_{27-35}$ (*C*) peptide either alone or in the presence of 16 μg/mL hLAG-3Ig and then restimulated with peptide-pulsed, γ-irradiated autologous PBMCs. The frequency of IFN-γ–secreting cells was evaluated by IFN-γ ELISPOT. For the T-cell stimulation assay, antigen-stimulated T cells were cultured with T2 cells loaded with 2 μg/mL HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *column 1*), 2 μg/mL $MART_{27-35}$ peptide (*column 2*), or 2 μg/mL $MART_{26-35}$A27L peptide (*column 3*); HLA-A*0201$^+$ Melan-A/MART-1$^+$ Me501 melanoma cell line, alone (*column 4*) or in the presence of blocking A6-136 anti-HLA class I mAb (*column 5*). *ND,* not detected. *D,* PBMCs of colorectal cancer patient P4 were subjected to two rounds of *in vitro* stimulation with SVV-1 peptide and then analyzed in the ELISPOT assay for the presence of IFN-γ–secreting cells. Cell culture was done with or without the addition of 16 μg/mL hLAG-3Ig. For the assay, lymphocytes were stimulated with T2 cells loaded with 2 μg/mL HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *column 1*) or of SVV-1 peptide (*column 2*), HLA-A*0201$^+$ survivin$^+$ SW480 colon cancer cell line, alone (*column 3*) or in the presence of blocking anti-HLA class I A6-136 mAb (*column 4*). Data are expressed as the number of spots per well, depending on each experiment. >, number of spots in the wells exceeds counting limits. *, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), compared with the recognition of the same target in the absence of anti-HLA class I A6-136 mAb.

Table 1. Different cytokine profile of peptide-stimulated T cells as evaluated by Th1/Th2 CBA assay

hLAG-3Ig	Cytokine release (pg/mL)					
	TNF-α		IL-2		IL-4	
	−	+	−	+	−	+
P1						
Lymphocytes only	0	0	0	0	0	0
Peptide-stimulated lymphocytes	94.7	449.3	168.8	384.0	19.2	55.4
Tumor-stimulated lymphocytes	10.2	38.0	0	0	0	0
P2						
Lymphocytes only	0	0	0	0		
Peptide-stimulated lymphocytes	0	124.1	0	56.2		
Tumor-stimulated lymphocytes	0	10.9	0	0		

NOTE: PBMCs of melanoma patients P1 and P2 were subjected to two rounds of *in vitro* stimulation with $MART_{26-35}$A27L peptide and analyzed for their production of Th1 or Th2 cytokines, alone and after stimulation with the specific peptide or with the Me501 melanoma cell line. Data indicate the quantitative measurement of cytokines expressed as pg/mL. IFN-γ release was not included in this analysis, whereas IL-6 and IL-10 levels were under the limits of sensitivity of the assay in all the conditions tested. Of note, T cells from patient P1 did also release IL-4. Assay sensitivity = 10 pg/mL. Values below the sensitivity threshold were reported to 0. SDs calculated on three replicates were <10% of the indicated value.

the class I HLA–restricted recognition of a Melan-A/MART-1$^+$, HLA-A*0201$^+$ melanoma cell line (Fig. 3*B, right, columns 4* and *5*).

Similar results were also obtained by stimulating T cells from melanoma patients and healthy donors with the wild-type $MART_{27-35}$ nonapeptide (Fig. 3*C*). In fact, when PBMCs from melanoma patients and healthy donors were stimulated with the wild-type epitope, the addition of hLAG-3Ig to the cell cultures induced a significant amplification of IFN-γ release in response to antigen. All the T-cell cultures recognized their nominal antigen when loaded on T2 cells (Fig. 3*C, right, columns 2* and *3*). Partially because of its low binding affinity to HLA-A2.1 molecule, the immunogenicity of the wild-type $MART_{27-35}$ peptide is known to be weaker than that of the modified $MART_{26-35}$ A27L decapeptide. For donor 5 and patient 1, the frequency of specific T cells obtained by the stimulation with the wild-type peptide was low, not sufficient to lead to a strong tumor recognition, and T cells generated from these PBMCs showed only borderline tumor recognition (Fig. 3, D5 and P1). Conversely, for donor 4 and patient 2 in front of a quite high number of MART-1-specific T cells, no tumor recognition occurred, suggesting that LAG-3 molecules might have expanded preexiting T cells bearing TCR with low affinity/avidity for their nominal antigen not sufficient to be activated by melanoma cells (Fig. 3, D4 and P2).

Therefore, the presence of hLAG-3Ig led to the generation of Melan-A/MART-1–specific T cells in all the cultures tested, independently from the source of lymphocytes (patients' or healthy donors' PBMCs) and from the type of Melan-A/MART-1 peptide used (the $MART_{26-35}$A27L analogue decapeptide or the wild-type $MART_{27-35}$ nonapeptide).

Considering other TAA models, T cells generated *in vitro* from PBMCs of a HLA-A*0201$^+$ colorectal cancer patient by peptide stimulation in the presence of hLAG-3Ig showed an increased release of IFN-γ in response to peptide-loaded T2 cells and also to survivin$^+$, HLA-A*0201$^+$ SW480 colon cancer cell line (Fig. 3*D, left* and *right*, see legend).

In conclusion, all data obtained with IFN-γ ELISPOT assays confirmed that hLAG-3Ig enhanced the generation of peptide-specific T cells that were endowed with functional activity and that, in the case of tumor-derived peptides, had the ability to recognize antigen-positive tumor cells. Such a functional activity of the hLAG-3Ig recombinant protein in enhancing the generation of peptide-specific T cells *in vitro* was due to the LAG-3 molecule itself and not dependent by the IgG portion of the recombinant protein. In fact, neither human purified IgG Fc fragments nor human purified whole IgG proteins when added during the T-cell culture conditions were able to significantly increase the induction of antigen-specific T cells *in vitro* (for details, see Supplementary Information).

hLAG-3Ig favors the generation of anti-Melan-A/MART-1 T cells with a type 1 pattern of cytokine release that includes IL-2. To better characterize the type of anti-Melan-A/MART-1 CD8$^+$ T cell responses, antigen-stimulated T lymphocytes obtained from melanoma patients P1 and P2, cultured alone or in the presence of peptide-loaded T2 cells or Me501 melanoma cell line, were studied for the release of Th1/Th2 cytokines by CBA assay (Table 1). T cells generated in the presence of hLAG-3Ig in both patients produced more cytokines than the corresponding T cells grown with peptide alone, although with some differences in their total levels due to individual variations. Moreover, a type 1 cytokine pattern that included tumor necrosis factor-α (TNF-α) and IL-2 production could be identified for both patients upon T-cell stimulation with nominal peptide. Because melanoma cells probably express limited amount of antigen, and because they did not bear other relevant costimulatory molecules, they failed to fully activate T cells, and these Mart-1-specific T cells did not release IL-2 upon tumor stimulation.

CD8$^+$ T cells generated in the presence of hLAG-3-Ig are cytotoxic and express terminally differentiated effector phenotype. To exert their antitumor reactivity, tumor-specific CD8$^+$ T cells should be able to potentially home into tumor tissues and be cytotoxic. We therefore tested whether the CD8$^+$ T cells generated by peptide stimulation and hLAG-3Ig displayed cytotoxic functions and were characterized by a final effector phenotype. Perforin is a pore-forming protein produced and exocytosed in

cytoplasmic granules by effector T lymphocytes after encountering target cells. Thus, to determine cell-mediated cytotoxicity, we investigated the release of perforin by our activated T cells: its production by antigen-stimulated T cells was evaluated by a specific ELISPOT assay. As shown in Fig. 4*A* and *B* for melanoma patient P1, PBMCs stimulated either with $MART_{26-35}$A27L (Fig. 4*A*) or with wild-type $MART_{27-35}$ epitope (Fig. 4*B*) in the presence of hLAG-3Ig were able to release perforin in response to T2 cells loaded with either peptide (*column 2* and *3*, respectively), but not with a HLA-A*0201–restricted, HIV-derived control peptide $HIVenv_{117-125}$ (*column 1*). The frequency of CTLs in $MART_{26-35}$A27L-stimulated population was higher than in the $MART_{27-35}$-stimulated one. Moreover, both populations released perforin in response to Me501 melanoma cell line (*column 4*), and this CTL activity was inhibited by the anti-class I HLA mAb A6-136 (*column 5*).

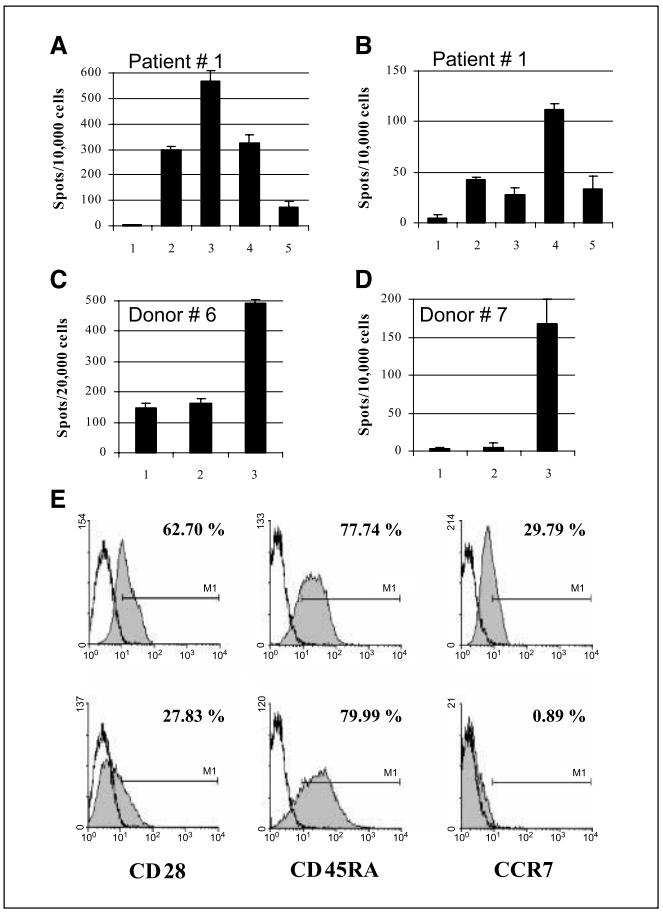

Figure 4. Antigen-specific $CD8^+$ T cells, generated in the presence of hLAG-3Ig, maintain CTL activity and the features of final effectors. *A* and *B,* PBMCs of melanoma patient P1, subjected to two rounds of stimulation with $MART_{26-35}$A27L (5A) or $MART_{27-35}$ (5B) peptide in the presence of hLAG-3Ig, were analyzed for their CTL activity by perforin ELISPOT. In the T-cell activation assay, lymphocytes were cultured with: T2 cells loaded with 2 μg/mL HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *column 1*), 2 μg/mL $MART_{27-35}$ peptide (*column 2*), or 2 μg/mL $MART_{26-35}$A27L peptide (*column 3*); HLA-A*0201$^+$ Melan-A/MART-1$^+$ Me501 melanoma cell line, alone (*column 4*) or in the presence of blocking A6-136 anti-HLA class I mAb (*column 5*). *C* and *D,* frequency of antigen-specific CTLs in PBMCs of healthy donors D6 and D7, stimulated once (D7) or twice (D6) with Flu_{58-66} peptide in the presence of hLAG-3Ig. For the T-cell activation assay, lymphocytes were incubated alone (1) or in the presence of T2 cells loaded with HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *2*) or with Flu_{58-66} peptide (3). Data are expressed as number of spots per well, depending on each experiment. *E,* phenotypic analysis of *in vitro* generated $MART_{26-35}$A27L–specific $CD8^+$ T cells after two rounds of *in vitro* stimulation. Activated T lymphocytes of melanoma patient P1 were stained with phycoerythrin-conjugated $MART_{26-35}$A27L/HLA-A*0201 tetramers, PerCP-conjugated anti-CD8 mAb, and with FITC-conjugated anti-CD28, anti-CD45RA, or anti-CCR7 mAb and analyzed on a flow cytometer. After gating on tetramer$^+$ $CD8^+$ cells, the expression of the indicated surface molecules on this subpopulation was determined. The percentage of positive cells for each marker is indicated in each panel. *Top,* phenotype of cells cultured with the peptide alone; *bottom,* phenotype of cells cultured with the peptide in the presence of hLAG-3Ig.

Perforin ELISPOT was done also on influenza matrix–specific $CD8^+$ T lymphocytes cultured in the presence of hLAG-3Ig. As an example, healthy donors' PBMCs stimulated with Flu_{58-66} peptide (Fig. 4*C* and *D*, donors D7 and D4, respectively) released perforin in response to T2 cells loaded with the specific peptide (*C* and *D, column 3*) but not with a HLA-A*0201–restricted, HIV-derived control peptide $HIVenv_{117-125}$ (*column 2*). Basal release of perforin was evaluated by incubating stimulated T lymphocytes only in the presence of medium (*column 1*).

The phenotype of antigen-specific T cells generated in the presence of hLAG-3Ig was also analyzed. As shown in Fig. 4*E*, T cells stimulated with $MART_{26-35}$A27L did show positive staining for CD45RA, and the addition of the recombinant protein to the cell culture induced a significant reduction of CCR7 and CD28 surface expression in the gated $CD8^+$ tetramer$^+$ T-cell subpopulation (Fig. 4*E, bottom and top*, respectively), indicating the acquisition of a phenotype of terminally differentiated effector cells.

Adjuvant effect of hLAG-3Ig in the generation of antigen-specific $CD8^+$ T cell is preserved in the absence of $CD4^+CD25^+$ regulatory T cells. Given the recent evidence suggesting a functional role of LAG-3 in T regulatory cells at least in the mouse system (15), we asked whether $CD4^+CD25^+$ T regulatory cells could be the main target of the mechanisms by which hLAG-3Ig recombinant protein exerts its adjuvant activities in the induction of antigen-specific T lymphocytes. Indeed, at least in mice, LAG-3 engagement seems to be necessary for suppressive activity of T regulatory cells (15). Soluble LAG-3 added during PBMC stimulation might potentially compete for MHC class II binding with membrane-bound LAG-3 molecule expressed on the surface of T regulatory cells, thereby inhibiting T regulatory cells activity and thus promoting effector T-cell expansion. Therefore, we investigated whether the T-cell promoting effect of hLAG-3Ig was dependent on the presence of $CD4^+CD25^+$ T regulatory cells. Following depletion of $CD4^+CD25^+$ T cells, freshly isolated PBMCs of different healthy donors were subjected to two rounds of *in vitro* stimulation with $MART_{26-35}$A27L (Fig. 5*A*) and $MART_{27-35}$ (Fig. 5*B*) peptides in the presence or in the absence of hLAG-3Ig (Fig. 5, *left* and *right*, respectively). hLAG-3Ig treatment strongly increased the frequency of IFN-γ-releasing antigen-specific T cells even in $CD4^+CD25^+$-depleted PBMCs. As shown in Fig. 5, $CD4^+CD25^+$-depleted PBMCs stimulated twice with $MART_{26-35}$A27L (Fig. 5*A*) and $MART_{27-35}$ (Fig. 5*B*) peptides in the presence of hLAG-3Ig and released high levels of IFN-γ in response to T2 cells loaded with either peptide (*column 2* and *3*, respectively) but not with a HLA-A*0201–restricted, HIV-derived control peptide $HIVenv_{117-125}$ (*column 1*). Conversely, the same stimulations done in the absence of hLAG-3Ig did not show a significant and specific release of IFN-γ in response to both antigens. Tetramer analysis confirmed ELISPOT results (data not shown). The addition of LAG-3 induced the expansion of MART-1-specific T cells; however, in this case, as previously showed also for donor D4 and patient P2 (Fig. 3), the induced T cells did not recognized the HLA-A2-positive melanoma (Fig. 5*A* and *B, right*), confirming the possibility that LAG-3 molecules might have expanded

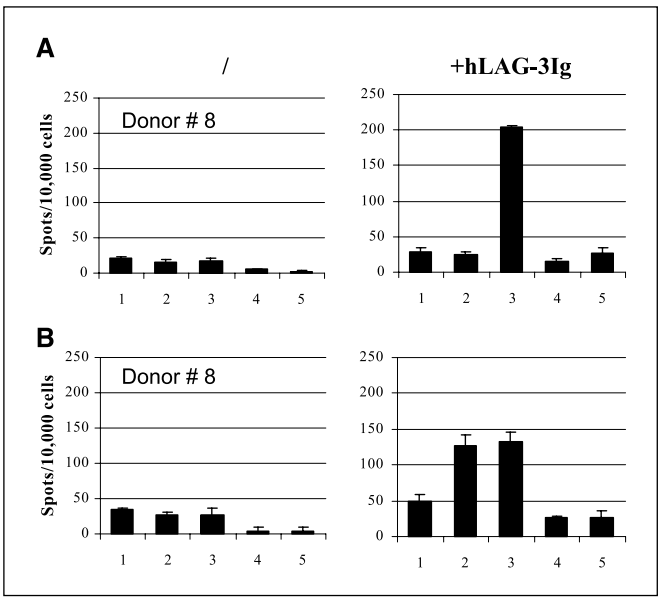

Figure 5. Depletion of $CD4^+CD25^+$ T cells does not affect the adjuvant function of hLAG-3Ig in the generation of an antigen-specific T-cell response. After depletion of $CD4^+CD25^+$ T cells, freshly isolated PBMCs of healthy donors were stimulated twice with $MART_{26-35}A27L$ (*A*) and $MART_{27-35}$ (*B*) peptides in the presence or in the absence of hLAG-3Ig (*left* and *right*, respectively) and then analyzed in ELISPOT assay for the presence of IFN-γ-secreting cells. In the T-cell stimulation assay, lymphocytes were incubated with T2 cells loaded with 2 μg/mL HLA-A*0201–restricted control peptide (HIV $env_{117-125}$, *column 1*), 2 μg/mL $MART_{27-35}$ peptide (*column 2*), or 2 μg/ml $MART_{26-35}A27L$ peptide (*column 3*); HLA-A*0201$^+$ Melan-A/MART-1$^+$ Me501 melanoma cell lines, alone (*column 4*) or in the presence of blocking anti-HLA class I mAb (*column 5*).

preexiting T bearing a TCR with low affinity/avidity for their nominal antigen.

Based on these findings, it is likely that hLAG-3Ig exerts its activity, targeting cells other than T regulatory, although we can not formally rule out the possibility that LAG-3 molecule may also indirectly affect $CD4^+CD25^+$ functions.

Soluble LAG-3 (hLAG-3Ig) activates peripheral blood APCs, inducing the release of chemokines and proinflammatory cytokines. It has been extensively shown that the hLAG-3Ig induced dendritic cell activation through a direct interaction with the HLA class II molecules expressed by the APC, whereas no major involvement of the Fc potion of the hLAG-3Ig molecules was found in mediating such a functional activity of hLAG-3Ig molecules (18, 19). To define the major subset of cells directly interacting with the recombinant protein and to identify the molecular and cellular network leading to the amplification of the immune response, the effect of hLAG-3Ig treatment was evaluated on the phenotype and functional activity of monocytes, dendritic cells, and B cells included in such analysis because they have been recently shown to be efficient APCs, able to stimulate strong antigen-specific T-cell responses (28).

PBMCs of healthy donors were cultured for 48 hours in the presence of 3 μg/mL $MART_{26-35}A27L$ peptide and 16 μg/mL hLAG-3Ig, as previously done for antigen stimulation. Cells were harvested, and the activation status of the different subpopulations of APCs present in the whole culture was analyzed by flow cytometry for the surface expression of CD80, CD83, and CD86 molecules; supernatants of the same cell cultures were evaluated for the presence of cytokines and chemokines.

Exploiting the expression of their distinctive cell surface markers, we identified $CD14^+$ monocytes, $CD19^+$ B lymphocytes, and $Lin^-/CD123^{low/high}$ PBDCs. As shown in Table 2, treatment with hLAG-3Ig induced significant up-regulation of CD83 and CD86 on all APC populations. In particular, CD83 expression levels ranged from 1.3% to 20.4% on PBDCs and from 0.3% to 18.6% on monocytes, whereas CD86 ranged from 45.4% to 70.4% on PBDCs and from 65.3% to 81.7% on monocytes. No increase in CD80 expression was detectable in any of the cell subsets. These phenotypic changes in the profile of costimulatory molecules expressed by each APC subset were confirmed on isolated APCs purified from PBMCs and exposed *in vitro* to hLAG-3Ig for 48 hours (data not shown).

To characterize the immune modulation induced by hLAG-3Ig treatment, the extent of cytokine and chemokine release in the same PBMC cultures was evaluated by CBA assays (Table 3). Antigen stimulation in the presence of hLAG-3Ig strongly increased the production of RANTES, TNF-α, IL-4, IL-6, and IL-1β and, to a lesser extent, of IFN-γ. IP-10 and MIG, ligands for the same

Table 2. Antigen stimulation of human PBMCs in the presence of hLAG-3Ig induces expression of maturation markers on the surface of different APC subpopulations

	% Positive cells in gated populations					
	Monocytes		B lymphocytes		PBDCs	
	/	+hLAG-3Ig	/	+hLAG-3Ig	/	+hLAG-3Ig
CD80	10.8	3.4	3.6	3.0	0.2	0.7
CD83	0.3	18.6	6.3	12.2	1.3	20.4
CD86	65.3	81.7	12.7	24.9	45.4	70.4

NOTE: Healthy donors' PBMCs, resuspended in RPMI supplemented with 10% human serum, were seeded in six-well plates and cultured with 3 μg/mL $MART_{26-35}A27L$ peptide either alone or in the presence of 16 μg/mL hLAG-3Ig. After a culture period of 48 hours, cells were harvested and analyzed for the surface expression of CD80, CD83, and CD86 molecules in $CD14^+$ (monocytes), $CD19^+$ (B lymphocytes), and $Lin^-/CD123^{low/high}$ (PBDCs) populations. Data presented here are representative of three different experiments. Numbers indicate the percentage of marker-positive cells in $CD14^+$ (monocytes), $CD19^+$ (B cells), or $Lin^-/CD123^{low/high}$ (PBDCs) gated populations.

Table 3. Stimulation of human PBMCs by hLAG-3Ig induces the release of an activating pattern of proinflammatory cytokines and chemokines

	Cytokine/chemokine release (pg/mL)							
	IFN-γ	TNF-α	IL-1β	IL-4	IL-6	RANTES (CCL5)	MIG (CXCL9)	IP-10 (CXCL10)
/	27	55	118	82	1,254	798	>10,000	>10,000
+hLAG-3Ig	46	5,624	2,871	654	>10,000	3,963	1,051	2,506

NOTE: Results of Th1/Th2, inflammation, and chemokine CBA assays of supernatants from antigen-stimulated healthy donors' PBMCs cultured for 48 hours either alone or in the presence of 16 μg/mL hLAG-3Ig. Data reported are representative of experiments done on three healthy donors and indicate the quantitative measurement of cytokines or chemokines expressed as pg/mL. Assay sensitivity = 10 pg/mL. SDs calculated on three replicates were <10% of the indicated value.

chemokine receptor CXCR3, were still detectable at high levels although down-regulated compared with those found in cultures of PBMCs with peptide alone.

Discussion

In the present study, we show that human soluble LAG-3 protein (hLAG-3Ig) can act as an adjuvant in the induction of an antiviral and antitumor immune response, enhancing the expansion of antigen-specific $CD8^+$ T lymphocytes characterized by CTL activity, release of Tc1 cytokines, and final effector memory phenotype. Our data, by extending to a human setting previous findings obtained in animal tumor models (20, 23, 24), indicate that LAG-3 molecule, physiologically produced by human cells (11) and characterized by *in vitro* strong immunostimulatory properties (18), when used as a soluble recombinant protein, could indeed satisfy the major requirements for a safe and effective adjuvant for vaccine treatment in cancer patients.

In our study, four different antigens have been used to test the *in vitro* adjuvant features of hLAG-3Ig. These antigens included the influenza matrix protein–derived Flu_{58-66} peptide, the TAA peptide $MART_{27-35}$, its $MART_{26-35}$A27L analogue, and the survivin-derived $SVV\text{-}1_{95-104}$ peptide. The *in vitro* and *in vivo* immunologic behavior of these antigens has been already extensively described and discussed (26, 27, 29, 30). We were able to show that hLAG-3Ig added *in vitro* to the T-cell cultures consistently induced a rapid and better proliferation of T cells. This enhanced proliferation was not due to a non-specific mechanism of activation but indeed represented the selective expansion of antigen-reactive T cells. Furthermore, we showed that these peptide-specific T lymphocytes, generated in the presence of hLAG-3Ig, were characterized by a strong CTL activity and by enhanced functional properties.

The analysis of cytokines released by the peptide-specific T cells upon peptide stimulation did show that in all the Melan-A/MART-1–stimulated cultures hLAG-3Ig strongly promoted a Tc1-like pattern of cytokine release characterized by the production of higher levels of IFN-γ, TNF-α, and IL-2 compared with cultures done with the peptide alone. Indeed, tumor-specific type 1 immunity, which includes tumor antigen-specific Th1 and Tc1 cells, plays a critical role in tumor eradication (31, 32), whereas in cancer patients, a tumor immune response skewed toward a type 2 immunity has been associated to persistence of an active disease (33). Of note, in a melanoma patient, peptide-specific T cells also released IL-4, and this production was further increased upon hLAG-3Ig treatment. This observation, although apparently contradictory, may be explained considering the requirement of IL-4 produced by $CD8^+$ T cells in the generation of tumor-specific Th1 cells (34, 35). In agreement with their complete fully activated status, the antigen-specific T cells generated in the presence of hLAG-3Ig did show a final effector memory phenotype ($CCR7^-CD45RA^+$ and $CD28^{low}$), therefore suggesting that they should be potentially able to home into inflamed tissues where they could exert their antigen-specific cytotoxic activity.

The increased frequency of antigen specificity detected in hLAG-3Ig–stimulated cultures was observed either with viral-associated antigens or TAAs in PBMCs of healthy donors or of cancer patients, suggesting that hLAG-3Ig allowed efficient priming and expansion of naive T cells and complete activation of memory T-cell subset. Treatment with hLAG-3Ig, therefore, may potentially succeed (*a*) in cancer vaccine by expanding preexisting T cells and inducing reacquisition of effector functions and (*b*) in a prophylactic vaccination when the induction of a primary CTL immune response is required.

In the case of melanoma-associated antigen Melan-A/MART-1, a significant increase in antigen-specific T cells was seen either after stimulation with the highly immunogenic $MART_{26-35}$A27L peptide analogue or with the less immunogenic natural counterpart $MART_{27-35}$, therefore highlighting the potential role of hLAG-3Ig in strengthening the response against antigens usually poorly recognized by the immune system.

In general anti-MART-1-specific T cells generated in the presence of hLAG-3Ig were also able to recognize tumor cells, suggesting that the affinity of these T cells for their corresponding nominal antigen was strong enough to ensure the recognition of antigens naturally processed by tumor cells. However, in few cases, this tumor-recognition failed to occur. Indeed, tumor recognition may be dependent by the original TCR repertoire characteristic of each single patient/donor, and hLAG-3Ig mainly worked in expanding all the possible specificities already present in each PBMC. When such specificities included T cells with appropriate TCR avidity/affinity, then the resulting T-cell cultures were also endowed with tumor recognition ability. Although this hypothesis could be proved only by clonal analysis of the affinity/avidity of each T cell generated in the presence or in the absence of hLAG-3Ig; nevertheless, our data still proved that hLAG-3Ig increased the frequency of peptide-specific T cells and expanded T cells endowed with tumor recognition activities if they were already present in the original PBMCs.

Due to unavailability of reliable reagents for tetramer staining, we were unable to document the expansion of T cells expressing

survivin-specific TCR in hLAG-3Ig–treated cultures. Nonetheless, even for this universal tumor antigen, we showed the ability of hLAG-3Ig treatment to cause an overexpansion of T cells releasing INF-γ in response to survivin peptide stimulation, and, more importantly, we were able to show the specific recognition of naturally processed antigen after only two rounds of *in vitro* stimulation, again validating the adjuvant role of hLAG-3Ig for an additional TAA.

Because LAG-3 protein binds HLA class II molecules (10), it is unlikely that the adjuvant action of hLAG-3Ig involved a direct interaction with naive or memory T cells. In agreement with mouse vaccination studies suggesting a possible involvement of APCs in mediating the adjuvant activities of LAG-3 molecules (24, 36), our data confirm that early antigen stimulation of human PBMCs in the presence of hLAG-3Ig induced a more mature phenotype in APCs present in the peripheral blood. Indeed, monocytes, B cells, and PBDCs showed up-regulation of CD83 and CD86, whereas no or very low effect was seen on CD80 expression. These data could contribute to the understanding of the complex mechanism of action of LAG-3 protein. CD83-positive, mature APCs are licensed to efficiently present peptides even in the first phases of stimulation. In addition, through a preferential up-regulation of CD86 compared with CD80, LAG-3 molecule might favor the induction of an activating pathway, which involves CD86-CD28 pairing while limiting the CD80-CD28 contacts described as mainly involved in interaction with T regulatory cells (37, 38).

Our *in vitro* stimulation assays could show that at 48 hours of peptide stimulation, the presence of hLAG-3Ig promoted the release, in the culture medium, of higher levels of endogenous IL-4 and other proinflammatory cytokines, like IL-6, TNF-α, and IL-1β. Although IL-4 is crucial for Th1 cell priming in the early steps during the generation of an antitumor immune response and it is required to sustain dendritic cell differentiation from monocytes (34, 35), cytokines like IL-1β and TNF-α are involved in promoting costimulation between dendritic cells and T cells, therefore enhancing the extent of antigen-specific reactivity (39). Strangely enough, this type 1 immunity did not include any detectable level of IL-12. However, it is still possible that different kinetic points need to be considered, or that all the cytokine produced was immediately used by T cells. In fact, using an intracellular cytokine flow cytometry–based assay, it has been recently shown that IL-12 could be produced by a minority of the activated dendritic cells (40). Huge amounts of IL-6 were induced in stimulations that include hLAG-3Ig protein. Indeed, it has been recently shown that IL-6 favors the shift from neutrophils to monocyte recruitment at site of inflammation and plays a role in directing the transition between innate and acquired immune response (41, 42). Moreover, although with a yet unknown mechanism, IL-6 produced by dendritic cells has been described as a crucial cytokine in hampering the suppressor activity mediated by $CD4^+CD25^+$ T cells (43). In addition to the above described cytokines, the presence of hLAG-3Ig led also to the enrichment of inflammatory chemokines, some of which could be directly produced by the activated dendritic cells (44). RANTES was strongly up-regulated in hLAG-3Ig–treated T-cell cultures. This member of the CC chemokine family of proteins plays an essential role in inflammation by recruiting T cells, macrophages, and eosinophils to inflammatory sites. RANTES coexpressed with other costimulating molecules by APCs has been shown to elicit strong antitumor and recall responses as well as tumor-specific CTL activity in mouse models (45). The milieu of cytokines and inflammatory chemokines induced in the presence of hLAG-3Ig in the early phases of T-cell induction might be responsible of sustaining and supporting the selective expansion of a type 1 tumor-specific immunity. Moreover, the activation pattern of blood APCs and the presence of high amount of IL-6 together with the ability of finally committed T cells to release IL-2 in response to antigen stimulation might be of help in neutralizing the activity of T regulatory cells.

Both basic and translational researches are now aimed at the identification of more potent TRL 1-9 agonists and at defining their role in linking innate and acquired immunity (46–48). Here, we showed that soluble LAG-3, a molecule of human origin, may function as an adjuvant in enhancing tumor-specific responses, and that its activity is likely to be higher than that of type A and type B oligodeoxynucleotides containing unmethylated CG dinucleotides (CpG-A and CpG-B oligodeoxynucleotides) tested under similar conditions (49). In fact, in our experiments, we were able to detect specific anti-Melan-A/Mart-1 or anti-Flu reactivity in PBMCs without the need of enriching the starting PBMC population with $CD8^+$ T cells. Moreover, CpG-A and CpG-B oligodeoxynucleotides have been reported to preferentially target plasmacytoid dendritic cells and B cells, respectively, with CpG-A shown to be superior in expanding preexisting T cells while CpG-B in inducing primary CTL immune response. Our data indicated that the LAG-3 molecule equally activated different APCs in peripheral blood of healthy donors and cancer patients, including PBDCs and B cells, and sustained both primary and recall T-cell responses, combining therefore in a single molecule the adjuvant effects of both CpG-A and CpG-B.

Although the *in vivo* adjuvant activity of hLAG-3Ig remains to be addressed, nevertheless, it has already been shown that LAG-3 does exist as natural soluble molecule in sera of healthy individuals and in patients as well. Moreover, in patients, the level of natural soluble LAG-3 in sera is associated with a cellular immune response, and high levels seemed to be protective in conditions where Th1 responses are an essential component of immune protection, such as tuberculosis or cancer (21, 50).

In conclusion, our data validate the immunostimulatory activity of hLAG-3Ig in inducing TAA-specific $CD8^+$ T-cell responses in a human setting, and they strongly support the use of this recombinant protein as a promising candidate adjuvant for cancer vaccination.

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