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A Soluble Lymphocyte Activation Gene-3 Molecule Used as a Vaccine Adjuvant Elicits Greater Humoral and Cellular Immune Responses to Both Particulate and Soluble Antigens

Samir El mir,* and Frédéric Triebel2*†

The lymphocyte activation gene-3 (LAG-3) 3 molecule is related to CD4 at the gene and protein levels (1, 2). LAG-3 is expressed in activated CD4(+) and CD8(+) T lymphocytes where it is associated with the CD3/TCR complex at the cell surface (3, 4). LAG-3, like CD4 (5), may oligomerize at the cell surface to interact more efficiently with MHC class II molecules, as shown by the finding of three dominant negative mutations in the LAG-3 domain 1 that were able to inhibit the binding of wild-type LAG-3 molecules to class II molecules in a cell-cell adhesion assay (6).

As a soluble fusion protein, LAG-3 has been shown to bind MHC class II molecules with a much higher avidity than CD4 (7) and also to increase the capacity of phagocytic cells (MHC class II molecules in a cell-cell adhesion assay (6)). In addition, the potential of LAG-3 as an adjuvant for both humoral and cell-mediated immune responses to a defined protein Ag has not been explored in previous studies.

In this report, we have evaluated the adjuvant effect of mLAG-3g (a fusion protein between murine LAG-3 and the Fc fraction of a murine IgG2a mAb) in two different strains of mice (BALB/c and C57BL/6) for the induction of both CD4 and CD8 T cell responses in vitro (8). The changes brought about in the APCs during such an activation of the MHC class II signaling pathway are not fully understood but probably involve a combination of improved Ag processing, increased expression of costimulatory and adhesion molecules, and an up-regulation in the production of cytokines such as IL-12 and TNF-α (8). In vivo, tumor regression and tumor cell-specific CD8(+) T cell responses have been shown to be induced by LAG-3-transfected tumor cells or by a soluble LAG-3 fusion protein together with irradiated tumor cells (9). However, the potential of LAG-3 as an adjuvant for both humoral and cell-mediated immune responses to a defined protein Ag has not been explored in previous studies.

Materials and Methods

Experimental animals

Female C57BL/6 (H-2b) mice, 6–8 wk old, were purchased from IFFA-CREDO Laboratories (Lyon, France). Female BALB/c (H-2b) mice, 4–8 wk old, were purchased from Janvier Laboratories (Le Genest St. Isle, France; and † Laboratoire d'Immunologie Cellulaire, Institut Gustave-Roussy, Villejuif, France; and † Laboratoire d'Immunologie Cellulaire, Institut Gustave-Roussy, Villejuif, France)

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Abbreviations used in this paper: LAG-3, lymphocyte activation gene-3; DC, dendritic cell; mLAG-3g, fusion protein between murine LAG-3 and the Fc fraction of a murine IgG2a mAb; HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; alum, aluminium hydroxide.
France). All of these mouse strains were raised in specific pathogen-free conditions.

**Antigens**

Purified HBsAg particles (a subtype) were purchased from Fitzgerald (Concord, MA), and purified chicken OVA (grade IV) was from Sigma (St. Louis, MO). Ags were dissolved in PBS and stored at −20°C.

**mLAG-3Ig, peptides, and aluminum hydroxide (alum)**

Recombinant soluble mLAG-3 molecules were generated by fusing the extracellular domain of mLAG-3 to a murine IgG2a Fc portion (9, 10). The resulting recombinant protein, mLAG-3Ig, was produced in Chinese hamster ovary cells and purified on protein-A columns (Dr. M. Subramanyam and M. Tepper, Ares Advanced Technology, Randolph, MA). The total protein purity was >95% by Coomassie blue SDS-PAGE densitometry. Potential contamination of the purified protein with bacterial endotoxin (LPS) was determined using the chromogenic Limulus amoebo- cyte lysate assay (BioWhittaker, Walkerville, MD), which measures the activity of a L. amoeboocyte protease after activation by LPS. A calibration curve based on enzymatic activity vs LPS was constructed to determine endotoxin units in the test sample, and values of less than 1 EU/mg were obtained for mLAG-3Ig.

The hepatitis B virus (HBV) S peptide (aa 28–39, JPIQSLDSWWTSDL-H2d restricted) and the OVA peptide (aa 257–264, SIINFKEL-H2b restricted) were synthesized by solid-phase technique and purified by reverse-phase HPLC (Institut Gustave-Roussy, Villejuif, France). Peptides were dissolved in PBS and stored at −20°C. Alum was purchased from Sigma (St. Louis, MO) and stored at room temperature.

**Immunization protocols**

Immunization with HBsAg was conducted on BALB/c mice (five mice per group). Each mouse received two injections s.c. on the ventral surface on days 0 and 21 of 0.1 µg of HBsAg in 200 µl PBS with or without 1 µg of isotype-matched murine control IgG2a (Southern Biotechnology Associates, Birmingham, AL) or 1 µg of mLAG-3Ig.

In other experiments, C57BL/6 mice (five mice per group) received three injections on days 0, 21, and 28 of 50 µg native OVA alone (or adsorbed onto alum) in 200 µl PBS or with 1 µg mL-3Ig or a control IgG2a molecule. No side effects were ever observed with mL-3Ig (even at 10 µg/site) using these immunization protocols.

**Evaluation of CTL response**

Splens were recovered under sterile conditions 1 wk after the last immunization. Cell suspensions were prepared (10³ cells/well) individually from each spleen and suspended in 2 ml of α-MEM tissue culture medium supplemented with 10 mM HEPES buffer, 1 mM Na pyruvate, nonessential amino acids, 5 × 10⁻⁵ M 2-ME, antibiotics, and 10% FCS (Life Technologies, Cergy-Pontoise, France) in 24-well plates. Responder splenocytes or BALB/c spleen cells in the presence of the HBV S peptide (10⁻⁶ M HBV S peptide or 10⁻⁵ M OVA peptide, respectively. After 5 days in culture, half of the medium was replaced with fresh medium and the cells were then used as effectors in a standard 4-h chromium release assay performed 2 or 3 days later. Targets were P815 or EL4 cells labeled with ⁵¹Cr (3.7 MBq/3 l of supernatant was removed from each well and counted on a

**Cytokine assay**

In vivo primed spleen cells were cocultured with Ags in parallel to the proliferation assays. Culture supernatants were collected at 48 and 72 h, and the concentrations of IL-4, IL-10, IFN-γ, and TNF-α were determined by ELISA using commercial kits (R&D Systems, Abingdon, U.K.). In blocking assays, splenocytes were incubated for 48 h with CD4-, CD8-, MHC class I-, or MHC class II-specific mAb (10 µg/ml).

**Serology**

Blood was collected by retrobulbar puncture using heparinized glass pipettes, and mouse anti-HBs or anti-OVA Abs were detected using an ELISA on HBsAg- or OVA-coated wells (1 µg/ml). Bound Abs were detected by anti-mouse biotinylated Abs and then Streptavidin-HRP (Amersham Life Sciences) staining.

**Statistics**

Data were analyzed by the nonparametric Mann-Whitney U rank test, and differences with p < 0.01 were considered statistically significant.

**Results**

**Induction of HBsAg or OVA-specific CTLs by administration of Ag plus mLAG-3Ig**

We first examined spleen cells from BALB/c mice primed with various HBsAg formulations for the development of HBsAg-specific CTL responses. The surface HBV protein (S-Ag) self-assembles into 22-nm particles and is an efficient immunogen for inducing CTL responses in its native, particulate form. Spleen cells from immunized mice were cultured with the HBV S (aa 28–39) peptide (without IL-2) for 7 days and then assayed for cytolytic activity (11, 12). Spleen cells from mice immunized with 1 µg HBsAg developed CTLs that lysed syngeneic H-2d mastocytoma P815 cells in the presence of the HBV S peptide (18% lysis vs 6% without peptide at a 100/1 E:T ratio; Fig. 1A). Spleen cells from mice immunized with a lower dose (0.01 µg) of HBsAg also recognized the HBV S peptide-loaded P815 cells (16% lysis vs 3% without peptide at a 100/1 E:T ratio; data not shown).

The soluble mLAG-3Ig molecule was then evaluated as an adjuvant for boosting CTL responses to these two different doses of HBsAg preparations. When mice were injected with either 1 µg (Fig. 1A) or 0.01 µg (not shown) HBsAg plus 1 µg mL-3Ig, a strong CTL response was obtained. Mice injected with the same dose of HBsAg plus 1 µg of the isotype-matched murine IgG2a control molecule did not show any evidence of increased cytolytic activity. Similar results were obtained when testing these peptide-induced CTL lines on HBsAg-transfected P815 cells (P815/S) or untransfected P815 cells (11, 12) (data not shown). These latter results suggest that the vaccination Ag is capable in the presence of LAG-3 of priming a population of CTLs that can be restimulated during a natural infection, i.e., HBsAg was used as the exogenous Ag that primed BALB/c mice to recognize target cells that had processed the Ag endogenously.

To confirm that the lysis of peptide-pulsed P815 target cells could be attributed to MHC class I-restricted CTLs, we performed blocking assays with anti-CD4-, CD8-, MHC class I and -MHC class II mAbs (Fig. 1B). Lysis was inhibited in the presence of 10 µg/ml CD8- or MHC class I-specific and not CD4- (not shown) or MHC class II-specific mAbs, showing that the amplified response of splenocytes to the HBV S epitope in mice immunized with mL-3Ig as an adjuvant was mediated by MHC class I-restricted CD8⁺ T cells.

mLAG-3Ig was also evaluated as an adjuvant for inducing cytotoxic T lymphocyte responses to a soluble Ag. The well-described OVA system was chosen as a model to determine whether soluble exogenous proteins could prime CTLs when delivered with
A low dose (1 μg) of mLAG-3Ig. The EL4 mouse T lymphoma cells were used as target cells in the presence or absence of the OVA 257–264 peptide. Fig. 2A shows that splenocytes from C57BL/6 mice immunized three times with OVA (50 μg) and mLAG-3Ig (1 μg) had Ag-specific cytolytic activity (87% and 70% lysis vs 8% and 7% without peptide at a 100/1 and 30/1 E:T ratios, respectively), whereas mice injected with OVA alone or OVA plus 1 μg of an isotype-matched control Ab (IgG2a) had little detectable cytolytic activity. In a second experiment (Fig. 2B), OVA was adsorbed onto alum, and a significant CTL response was observed (36% lysis vs 11% without peptide at a 100/1 E:T ratio). This was probably due to the alum providing a depot that could enhance the uptake of OVA by macrophages leading to some CTL priming in vivo. Under these conditions, which are close to the vaccination formulations used in humans, a low dose of mLAG-3Ig (1 μg) further increased the priming of CTLs (60% and 34% lysis vs 10% and 7% without peptide at a 100/1 and 30/1 E:T ratio, respectively; Fig. 2B).

Induction of Ag-specific T cell proliferative responses by addition of mLAG-3Ig with either a particulate (HBsAg) or a soluble (OVA) Ag

We examined spleen cells from mice primed with the various HBsAg (Fig. 3) or OVA (Fig. 4) formulations for the development of proliferative HBsAg- or OVA-specific T cell responses. Spleen cells from primed mice were tested for their capacity to proliferate at days 4 and 5 in response to various doses of either HBsAg or OVA. Mice immunized with Ag plus 1 μg mLAG-3Ig had stronger Ag-specific proliferative responses than the mice immunized with either the particulate or soluble Ag alone. Fig. 3 describes the proliferative response obtained at day 4 in mice vaccinated with 1 μg HBsAg plus 1 μg mLAG-3Ig, with mice injected with HBsAg plus 1 μg IgG2a as a control group. Similar results were obtained with a low HBsAg dose (0.01 μg/mouse). Fig. 4A shows that splenocytes of mice vaccinated with OVA (50 μg/mouse) together with 1 μg mLAG-3Ig had a stronger proliferative response at day 4 as well as at day 5 (not shown) than mice injected with Ag alone or with Ag plus an isotype-matched control Ab (IgG2a). This increased response was also observed in groups of mice receiving OVA adsorbed onto alum (Fig. 4B).
midine incorporation is expressed as the mean ± SEM for the values for individual animals in each group. *p < 0.01 differences between the LAG-3-treated mice and each control group.

Induction of Th1- but not Th2-type cytokine responses in mice injected with mLAG-3Ig as an adjuvant

Spleen cells were also tested 1 wk after the last injection for their capacity to produce cytokines at 48 or 72 h in response to various doses of HBsAg or OVA. Splenocytes of mice vaccinated with either 1 μg (Fig. 5) or 0.01 μg (data not shown) HBsAg plus mLAG-3Ig exhibited significantly greater IFN-γ and TNF-α production in response to 3 μg/ml HBsAg than did the splenocytes of mice immunized with Ag alone or with Ag plus the control IgG2a molecule. A similar increase in Th1-type cytokine response was also observed with a low dose (0.3 μg/ml) of HBsAg (data not shown). In contrast, no significant increase of IL-4 or IL-10 (Fig. 5C and D) Th2-type cytokine production was observed in mice immunized with either of the two doses of HBsAg.

To characterize the induction of a Th1 cytokine response with mLAG-3Ig used as an adjuvant, we tested the blocking effects of either 1 μg HBsAg or 1 μg of mLAG-3Ig or control IgG2a molecule. Splenocytes were harvested at day 27 and stimulated with various doses (3, 1, or 0.1 μg) HBsAg for 4 days. [3H]Thymidine incorporation is expressed as the mean ± SEM for the values for individual animals in each group. *p < 0.01 differences between the LAG-3-treated mice and each control group.

Influence of mLAG-3Ig on the Ab response

At day 27 after the initial immunization with 1 μg HBsAg alone or with 1 μg mLAG-3Ig or the control IgG2a molecule, Ab levels in BALB/c mice were determined in an ELISA on HBsAg-coated wells. Specific IgG1 levels were about 100-fold higher in those mice immunized with Ag plus mLAG-3Ig as shown in Fig. 8A. Similar results were obtained with mice immunized with a lower dose of HBsAg (0.01 μg; data not shown).

Similarly, mice immunized three times with 50 μg OVA were tested at day 35 using ELISA on OVA-coated plates. Specific IgG levels were also about 100-fold higher in those mice immunized with Ag plus 1 μg mLAG-3Ig compared with mice immunized with Ag alone or Ag plus 1 μg control IgG2a molecule (Fig. 8B).

Because subclasses of Abs may be important in vaccine efficacy, we also examined the Ab subclasses in HBsAg-immunized mice. IgG2a and IgG1 differ in function (for example, they differ in their ability to fix complement), and they have been used as indicators for the induction of Th1 and Th2 responses, respectively. Both HBsAg-specific IgG1 and IgG2a levels were 10- to 100-fold higher in mice immunized with 1 μg HBsAg plus mLAG-3Ig (data not shown).

Discussion

Recently, we reported that a single dose of cell vaccine composed of autologous irradiated cells and 1 μg of recombinant LAG-3Ig...
fusion protein as a form of biological adjuvant elicited an antitumor response (9). The aim of the present study was to assess the humoral and cellular responses (cytotoxicity, proliferative response, and cytokine production) after vaccination with a particulate (HBsAg) or soluble (OVA) protein Ag in the presence of mLAG-3Ig. Our results indicate that the addition of a low dose (1 μg) of mLAG-3Ig to the formulation effectively stimulates the priming of CTLs, activates the proliferative response of spleen T cells along with their Th1-type cytokine production, and induces higher titers of Abs.

Both HBsAg and OVA are model protein Ags that have been widely used due to their immunogenic efficacy having been well established. Immunization with soluble protein Ag preferentially stimulates CD4+ T cells as proteins in the extracellular fluid are processed through the exogenous processing pathway and are degraded into peptides that bind to MHC class II molecules. CD8+ CTLs are stimulated by proteins degraded in an alternative endogenous processing pathway where MHC class I-binding peptides are derived from cytosolic proteins by partial proteolytic degradation. Nonetheless, under certain conditions exogenously acquired Ags, including those from cancer cells, can be cross-primed into the endogenous pathway (13, 14). Cross-priming is particularly efficient when Ags, instead of being obtained free in solution, are engulfed as part of an apoptotic tumor cell or a nanoparticle. In

![Figure 5](https://www.jimmunol.org/)

**FIGURE 5.** Th1- and Th2-type cytokine production of spleen cells from mice immunized with HBsAg plus mLAG-3Ig. BALB/c mice (five per group) were immunized s.c. at days 0 and 21 with 1 μg HBsAg alone or with 1 μg of mLAG-3Ig or control IgG2a molecule. Splenocytes were harvested at day 27, stimulated with 3 μg HBsAg for 48 (open bars) or 72 h (hatched bars), and IFN-γ (A), TNF-α (B), IL-4 (C), and IL-10 (D) production was measured in the supernatants using commercial ELISA kits. Shown are the means ± SEM for the values for individual animals in each group. ∗, Statistically significant (p < 0.01) differences between the LAG-3-treated mice and each control group.

![Figure 6](https://www.jimmunol.org/)

**FIGURE 6.** Blocking effect of mAb on IFN-γ production of spleen cells from mice immunized with HBsAg plus mLAG-3Ig. BALB/c mice (five per group) were immunized s.c. at days 0 and 21 with 1 μg HBsAg alone or with 1 μg of mLAG-3Ig or control IgG2a molecule. Splenocytes were harvested at day 27 and stimulated with 3 μg HBsAg for 48 h in medium or in the presence of 10 μg/ml of the indicated mAb, and IFN-γ production was measured in the supernatants using commercial ELISA kits. Shown are the means ± SEM for the values for individual animals in each group. ∗, Statistically significant (p < 0.01) differences between the CD4 or MHC class II mAb-treated samples and untreated samples.
particular, an instance in which the entry of such an exogenous protein into the endogenous class I pathway might be of physiological relevance occurs with HBsAg. Indeed, the S protein that self-assembles into 22-nm particles is an efficient immunogen in its particulate form, as shown in the present study in which mice immunized twice with 0.01 mg HBsAg developed CTLs. In contrast, immunization with soluble, nonparticulate proteins such as OVA rarely induces specific CTL responses.

Inefficient CTL priming by protein Ags adjuvanted in alum or mineral oil to induce humoral responses is a general feature of this type of Ag administration (12). Nonetheless, some protein Ags, such as OVA emulsified in CFA (15) or adsorbed onto alum (the present study), may elicit CD8+ MHC class I-restricted CTLs. Innovative adjuvant formulations, such as immune-stimulating complexes (16), liposomes (17, 18), or saponins (19–21), that facilitate in vivo priming of CD8+ CTLs by protein Ags are now being developed. When testing the new formulations, it is of increasing importance to define which compartments of the immune system the different adjuvant preparations selectively enhance and/or suppress. The advantages of our system are that efficient in vivo priming of CTLs was achieved without denaturing the soluble protein Ag (i.e., sparing B cell responses that in fact were increased) and that Th1-type CD4 T cell response was increased.

The development of subunit vaccines requires the use of an adjuvant that acts by stimulating components of the innate immune responses. Our results suggest that signaling APCs via MHC class II molecules is an efficient method of inducing both humoral and cellular responses. These MHC class II molecules may be perceived as “danger signals” to the innate immune system, as are the unmethylated CpG motifs in bacterial DNA (22) and exogenous human hsp60 (23). The precise mechanisms underlying the ability of LAG-3 proteins to promote the induction of CTL activity or to shift toward a Th1 differentiation pattern have yet to be elucidated. DCs, potent APCs located in the skin and lymphoid organs, are likely to be the MHC class II target cells of soluble LAG-3 proteins used as biological adjuvants because these cells play a major role in promoting the immune responses generated by most vaccine formulations. Cross-linking MHC class II molecules expressed on APCs appeared to contribute not only to APC activation but also to the commitment of T cells toward the Th1/Tc1 type in vivo. In accordance with the latter observation, immature human DCs stimulated by hLAG-3Ig have been shown to secrete more IL-12 (a known Th1 inducer) in vitro without the need of a CD40 ligand signal (8). Although no Th2-type cytokine responses were detected in mice injected with mLAG-3Ig, a net increase in serum Ig titers was observed in all mice, including both IgG2 (Th1) and
Levels of the OVA-specific IgG response were determined in five mice for each group at day 27 after immunization at days 0 and 21 with 1 µg HBsAg. OVA indicates the background obtained on OVA-coated plates each group at day 35 after immunization at days 0, 21, and 28 with 50 µg HBsAg. HBsAg indicates the background obtained on HBsAg-coated plates with the serum of an OVA-immunized mouse. B, Levels of the OVA-specific IgG response were determined in five mice for each group at day 35 after immunization at days 0, 21, and 28 with 50 µg OVA. HBsAg indicates the background obtained on OVA-coated plates with the serum of a HBsAg-immunized mouse. The results were obtained in triplicate wells with three dilutions of sera and are shown as the mean ± SEM for the values for individual animals in each group.

IgG1 (Th2) subclasses. The mechanism underlying the adjuvant role of LAG-3 in eliciting both a cell-mediated and a humoral response in vivo is still unclear, but our data in vitro with the blocking mAb indicate that both MHC class I-restricted CD8+ and MHC class II-restricted CD4+ T cells are reacting to the LAG-3 effect.

In any case, the demonstration that a soluble protein Ag (i.e., OVA) conjugated with mLAG-3lg can induce both strong humoral and cellular responses when adsorbed onto alum is certainly of importance for vaccine development using recombinant soluble Ags. Because alum is approved for use in human vaccination and soluble LAG-3 molecules are unlikely to be toxic when given locally in low levels, the combined use of these two agents may be clinically practical. More generally, the ability of soluble LAG-3 proteins to augment both Ab and cell-mediated immune responses suggests that this adjuvant could be a valuable component of subunit vaccines.

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