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*J Immunol* 1999; 162:2748-2753; ; http://www.jimmunol.org/content/162/5/2748

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Lymphocyte Activation Gene-3, a MHC Class II Ligand Expressed on Activated T Cells, Stimulates TNF-α and IL-12 Production by Monocytes and Dendritic Cells

Marie-Noëlle Avice,* Marika Sarfati,† Frederic Triebel,‡ Guy Delespesse,* and Christian E. Demeure2*

Lymphocyte activation gene-3 (LAG-3) is an MHC class II ligand structurally and genetically related to CD4. Although its expression is restricted to activated T cells and NK cells, the functions of LAG-3 remain to be elucidated. Here, we report on the expression and function of LAG-3 on proinflammatory bystander T cells that are activated in the absence of TCR engagement. LAG-3 is expressed at high levels on human T cells cocultured with autologous monocytes and IL-2 and synergizes with the low levels of CD40 ligand (CD40L) expressed on these cells to trigger TNF-α and IL-12 production by monocytes. Indeed, anti-LAG-3 mAb inhibits both IL-12 and IFN-γ production in IL-2-stimulated cocultures of T cells and autologous monocytes. Soluble LAG-3Ig fusion protein markedly enhances IL-12 production by monocytes stimulated with infra-optimal concentrations of sCD40L, whereas it directly stimulates monocyte-derived dendritic cells (DC) for the production of TNF-α and IL-12, unravelling an enhanced responsiveness to MHC class II engagement in DC as compared with activated monocytes. Thus similar to CD40L, LAG-3 may be involved in the proinflammatory activity of cytokine-activated bystander T cells and most importantly it may directly activate DC. The Journal of Immunology, 1999, 162: 2748–2753.

Lympocyte activation gene-3 (LAG-3) is an MHC class II ligand that is structurally related to CD4 (1, 2). Like CD4, LAG-3 was recently found to associate with the CD3-TCR complex (3). In spite of these similarities, LAG-3 differs from CD4 in several regards. It has higher affinity than CD4 for MHC class II molecules (4), and whereas CD4 transduces signals via the protein tyrosine kinase lck (5), the intracytoplasmic tail of LAG-3 has no site to interact with lck (6). Unlike CD4, LAG-3 is not expressed on resting T cells but is readily induced on activated CD4+ and CD8+ T cells as well as NK cells (2, 7). The expression of LAG-3 on human T cells correlates with their capacity to produce IFN-γ and is up-regulated by IL-2 and IL-12 (8, 9). In vivo, it has been detected in lymphoid tissues (10) and on T cells infiltrating renal cell carcinoma (11).

The function of LAG-3 has not been investigated thoroughly. LAG-3 seems to have the capacity to exert both effector and receptor activities. It was reported to transduce a positive signal into LAG-3+ NK cells, as indicated by the defective killing of certain tumor targets by NK cells from LAG-3−/− mice (12). However, blocking LAG-3/MHC class II interactions with a neutralizing anti-LAG-3 mAb fails to inhibit Ag-driven T cell cytotoxicity and MLR, suggesting that LAG-3 does not provide a positive signal in TCR-driven interactions (10). Conversely, LAG-3 cross-linking on activated T cells before restimulation via the CD3-TCR complex induces a state of unresponsiveness at both proliferation and cytokine production levels (3). Finally, during T-T cell interaction, MHC class II engagement by LAG-3 was found to provide a negative signal leading to decreased proliferation and cytokine production (6).

Here, we first report on the role of LAG-3 in the cytokine-driven activation of T cells cocultured with autologous monocytes in the absence of TCR/CD3-mediated stimulation. We show that T cells expressed LAG-3 and that LAG-3/MHC class II interaction, together with CD40-CD40 ligand (CD40L) interaction, is involved in both IL-12 and IFN-γ production. Blocking LAG-3/MHC contact with anti-LAG-3 mAb not only suppresses the positive signal given to monocytes via MHC class II but also inhibits T cell response to IL-12. In addition, we show that recombinant soluble LAG-3 (LAG-3Ig) dose-dependently induces TNF-α production and costimulates sCD40L-induced IL-12 production by monocytes. Most interestingly, LAG-3Ig also directly stimulates dendritic cells (DC) to produce both IL-12 and TNF-α without additional stimulatory or costimulatory signal.

Materials and Methods

Reagents and cell lines

sLAG-3Ig molecules produced in Chinese hamster ovary cells as described (6) were kindly provided by M. Subramanyam and M. Tepper (Ares Advanced Technology, Randolph, MA). Preparations contained no detectable endotoxin (<15 pg/ml) as determined by the Limulus amebocyte lysate assay (QCL-100; BioWhittaker, Walkerville, MD). The anti-LAG-3 mAb 17B4 was previously described (2), and the anti-CD3 UCHT-1 was kindly provided by Dr. P. Beverley (University College and Middlesex School of Medicine, London, U.K.). PHA was from Sigma (St. Louis, MO), and the tuberculin purified protein derivative was from BCG Seizo (Tokyo, Japan). The blocking goat anti-human IL-12 Ab and rIL-2 were kind gifts from Dr.
Monocytes were obtained by aggregation of adult PBMCs in the cold (13) and were depleted of T cells by rosetting on 2-aminoethylisothiouronium bromide (AET)-treated SRBC as previously described (14). The resulting preparations were 95% CD14+, as determined by FACS (Becton Dickinson, Mountain View, CA) using phycoerythrin-(PE-) conjugated anti-CD14 mAb (Ancell, London, Canada). Autologous T cells were purified by rosetting the monocyte-depleted PBMC with AET-SRBC followed by Lympho-Kwik T treatment (One Lambda, Canoga Park, CA).

Monocytes were incubated in 6-well culture plates (5 x 10^6 cells/3 ml per well) in serum-free RPMI 1640. After 1 h, nonadherent cells were removed and adherent cells were cultured in 3 ml of complete RPMI 1640 medium supplemented with 25 ng/ml GM-CSF and 25 ng/ml IL-4. On day 4, one-half of the culture medium was replaced with fresh medium containing GM-CSF and IL-4, and nonadherent cells were harvested on day 7. Upon microscopical analysis, >98% nonadherent cells presented cellular projections. Analysis by FACS revealed that preparations consisted of a homogenous (>96%) population of CD2+, CD14+CD16+, CD16+, CD40+, CD80+, CD83+, CD86+, CD11c+, and HLA-DR+ large cells, in agreement with previous reports (15-17). Less than 1% of CD3+, CD19+, or CD56+ cells could be detected.

Culture conditions

All cultures were performed in complete HB101 medium (Irvine, Santa Ana, CA) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 IU penicillin, 100 μg/ml streptomycin (BioWhitaker), and 10 μg/ml Polymyxin B (Sigma).

For coculture experiments, T cells (10^6/ml) were incubated with autologous monocytes (0.2 x 10^6/ml) and IL-2 (50 U/ml). Cell supernatant was collected at day 3 and DNA synthesis at that time was assessed by the [3H]thymidine (1 μCi/well; Amersharm, Arlington Heights, IL) for 6 h before harvesting the cells and counting incorporated radioactivity by liquid scintillation. T cell cultures in the presence of IL-1 and IL-12 were performed exactly as described (19). The anti-LAG-3 mAb 17B4 and its isotype-matched control (anti-CD5, clone OKT1; American Type Culture Collection, Manassas, VA) were used in culture at 5 μg/ml. Neither anti-CD5 nor a control mAb of the same isotype not binding to T cells (anti-Rye, prepared in our laboratory) affected proliferation or cytokine production. When cultured alone, monocytes (2 x 10^6/ml) were preincubated overnight in the presence of GM-CSF (25 ng/ml) and IFN-γ (500 IU/ml) as previously reported (18) before stimulation at 10^6/ml. DC were also stimulated at 10^6/ml.

Cytofluorometric analysis

Binding of LAG-3Ig to monocytes and DC was assayed by indirect immunofluorescence. Briefly, preactivated monocytes or DC were incubated with LAG-3Ig (5 μg/ml) in the presence of normal human IgG (300 μg/ml) for 1 h at 4°C. Cells were then stained with biotinylated Goat anti-human IgG (Tago, Burltime, CA) for 1 h at 4°C followed by PE-labeled streptavidin (Ancell). Expression of LAG-3 and CD40L was monitored daily by flow cytometric analysis using anti-CD3 mAb (Becton Dickinson). To assess the purity of cellular preparations, fluoro-labeled overnight in the presence of GM-CSF and IL-12 (50 U/ml) interaction and IL-12 production (13). To examine the role of LAG-3 in this TCR-independent T cell activation, cultures were supplemented with neutralizing anti-LAG-3 mAb. As seen in Fig. 2, anti-LAG-3 inhibits T cell proliferation (42% suppression) and the production of both IFN-γ (83% suppression) and IL-12 (56% significantly reduces its expression (Fig. 1D).

T cells cocultured with autologous monocytes and IL-2 proliferate and secrete IFN-γ by a mechanism involving CD40-CD40L interaction and IL-12 production (13). To examine the role of LAG-3 in this TCR-independent T cell activation, cultures were supplemented with neutralizing anti-LAG-3 mAb. As seen in Fig. 2, anti-LAG-3 inhibits T cell proliferation (42% suppression) and the production of both IFN-γ (83% suppression) and IL-12 (56% suppression).
inhibition). Anti-CD5 mAb, used as an IgG1 isotype control that also binds to T cells, has no effect. Anti-LAG-3 mAb does not inhibit IL-12 production by down-regulating CD40L expression or by inducing the release of IL-10, which remained undetectable (data not shown). Therefore, it was possible that anti-LAG-3 suppressed IL-12 production by inhibiting the interaction between LAG-3 on T cells and MHC class II molecules on monocytes. Indeed, ligation of MHC class II on murine DC by means of Abs expressed IL-12 production, anti-LAG-3 mAb also inhibits the release of IL-12 as well as TNF-α by monocyte-derived DC (Fig. 4). LAG-3Ig markedly costimulates IL-12, as well as TNF-α, production in response to suboptimal but not optimal sCD40L stimulation (Fig. 5B). The enhancing effect of LAG-3Ig on sCD40L-induced IL-12 production is observed only when sCD40L is used at an infraoptimal concentration (Fig. 6). The ability of LAG-3Ig to markedly enhance IL-12 production in response to suboptimal but not optimal sCD40L stimulation may explain the suppressive effect of anti-LAG-3 mAb on IL-12 production in IL-2-stimulated cultures of CD4+ T cells and autologous monocytes. Indeed, in these cultures T cells express very low, albeit functionally significant, levels of CD40L (13). These observations may also account for the finding that the same anti-LAG-3 mAb fails to inhibit IFN-γ production when T cells are activated with Ag or mitogen (Table I), stimuli known to induce high levels of CD40L expression (24). These effects of LAG-3Ig on monocytes are specific inasmuch as they are prevented (75%) by preincubation of LAG-3Ig with anti-LAG-3 mAb (Table II).

LAG-3Ig stimulates IL-12 production by monocyte-derived DC

Because DC are an important source of IL-12 and express high levels of MHC class II, we next examined the ability of LAG-3Ig to stimulate IL-12 production by monocyte-derived DC. Whereas LAG-3Ig binds similarly to monocyte-derived DC and to activated monocytes (Fig. 4), LAG-3Ig directly and dose-dependently induces the production of IL-12 as well as TNF-α by monocyte-derived DC (Fig. 7, A and B) and costimulates the production of these cytokines, induced by a low dose of sCD40L. These effects of LAG-3Ig on DC are specific inasmuch as they are prevented...
constitute the majority of the lesional T lymphocyte infiltrate. Our previous in vitro studies have suggested a mechanism accounting for the activation of bystander T cells. Resting peripheral blood T cells were shown to be stimulated for proliferation and IFN-γ production during coculture with autologous monocytes in the presence of either IL-2 or IL-15, a non-T cell–derived cytokine that is produced at inflammatory sites. Such TCR-independent T cell activation is dependent upon endogenous IL-12 and TNF-α production by monocytes that in turn requires the engagement of monocyte-associated CD40 with T cell-associated CD40L. The latter was shown to be expressed at very low but functionally sufficient levels on freshly isolated primary T cells in the absence of TCR/CD3 signal (13). The present results show that LAG-3 is expressed on cytokine-activated “bystander” T cells and that it regulates their proinflammatory activity. Addition of neutralizing anti-LAG-3 mAb suppresses both endogenous production of IL-12 and T cell response to IL-12. The ability to block IL-12 production by either anti-LAG-3 or anti-CD40L mAbs (13) suggests that the LAG-3/MHC class II and CD40/CD40L signaling pathways act in synergy to induce IL-12 production in this culture system. This view is supported by the finding that LAG-3Ig markedly costimulates IL-12 production in response to suboptimal concentrations of sCD40L trimer and allows the production of IL-12 in response to suboptimal sCD40L stimulation. In contrast to IL-12, the production of TNF-α is directly triggered by LAG-3Ig without CD40L stimulation, underlining the differential regulation of these cytokines. In addition to their well-defined functions as peptide-presenting structures and response restriction elements, MHC class II molecules can also transduce signals in the APCs on which they are expressed (27, 28). Cross-linking of MHC class II molecules has been reported to up-regulate TNF-α expression (29–32) as well as IL-1, IL-6, and nitric oxide production by monocytic cells (30, 33, 34) and to induce IL-12 production by murine DC (22). Most interestingly, the enhancing effect of LAG-3Ig on IL-12 production is observed only with suboptimal, and not with optimal, CD40L stimulation. This is consistent with the observations (Ref. 10 and Table I) that anti-LAG-3 mAb does not suppress IFN-γ production by Ag- or mitogen-stimulated T cells, which express much more CD40L than cytokine-activated cells (24). Addition of exogenous IL-12 only partly overcomes the suppressive effect of anti-LAG-3 mAb on IFN-γ production in the T cell/monocyte coculture system, indicating that ligation of T cell–associated LAG-3 inhibits the response to IL-12. This inhibition might result from two mechanisms. First, the blockade of LAG-3/MHC class II interaction may inhibit response to IL-12; this view implies that

TABLE I. Anti-LAG-3 Ab inhibits IFN-γ production by T cells induced without TCR-CD3 engagementa

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Conditions</th>
<th>Control Ab</th>
<th>Anti-LAG-3 Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T cells + Mo + IL-2</td>
<td>2,815</td>
<td>654</td>
</tr>
<tr>
<td>2</td>
<td>T cells + Mo + IL-2 + anti-CD3</td>
<td>43,450</td>
<td>52,910</td>
</tr>
<tr>
<td>3</td>
<td>T cells + Mo + IL-2 + anti-CD3</td>
<td>37,557</td>
<td>13,020</td>
</tr>
<tr>
<td>4</td>
<td>PBMC + PPD</td>
<td>3,613</td>
<td>3,899</td>
</tr>
<tr>
<td>5</td>
<td>PBMC + PPD</td>
<td>7,211</td>
<td>4,647</td>
</tr>
</tbody>
</table>

a T cells purified from peripheral blood were stimulated with either syngenic monocytes (Mo) and IL-2 (50 U/ml) with or without anti-CD40 mAb (UCHT-1, 200 ng/ml) or allogenic monocyte-derived DC and IL-2 (50 U/ml). PBMC were stimulated with purified protein derivative (PPD) (12 μg/ml) only. Cultures were performed in the presence of anti-LAG-3 mAb 17B4 or its isotype-matched control (10 μg/ml). b Supernatants were collected after 4 days for IFN-γ measurement.
engagement of LAG-3 by its ligand expressed on monocytes delivers a positive signal to T cells, allowing them to respond to IL-12. Alternatively, LAG-3 ligation may provide direct inhibitory signal to T cells. The second mechanism is supported by the finding that anti-LAG-3 suppresses the response of highly purified T cells to IL-12 plus IL-1. Indeed, this cytokine mixture does not induce the expression of class II molecules on T cells. This view is further supported by the previous findings that ligation of LAG-3 cannot be explained by the differential expression of MHC class II molecules on these two types of APCs. Indeed, the preactivated monocytes employed in our study expressed similar levels of HLA-DR as the DC. The ability of LAG-3 to directly stimulate IL-12 production by DC is consistent with its protective effect on experimental tumor growth. In that study, coinjection of LAG-3Ig together with wild-type tumor cells (or injection of tumor cells transfected with LAG-3) inhibits tumor expansion and confers T cell-dependent protection against rechallenge with wild-type tumor.

In conclusion, the present results indicate that LAG-3, which is typically expressed on TH1-like cells (8), stimulates APC for increased TNF-α and IL-12 production. They further suggest that some anti-LAG3 mAbs have the profile of anti-inflammatory agent capable of blocking the activation of bystander proinflammatory T cells.

Acknowledgments

We thank Dr. M. Gately (Hoffmann La Roche) for his generous gift of anti-IL-12 mAb, and Dr. C. Maliszewski (Immunex) for kindly providing sCD40L.

References


FIGURE 7. LAG-3Ig induces TNF-α and IL-12 production by monocyte-derived DCs. A, DC derived from blood monocytes by culture in GM-CSF and IL-4 were washed and stimulated with LAG-3Ig (5 μg/ml) and/or sCD40L (0.2 μg/ml) as described in Materials and Methods. Supernatants were analyzed after 24 h for TNF-α production and after 72 h for IL-12 production. B, DC were stimulated with graded doses of LAG-3Ig. Shown is mean ± SD of four experiments.

Table II. Specific inhibition of LAG-3Ig-induced cytokine production by anti LAG-3 Ab.a

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Culture</th>
<th>Stimulus</th>
<th>Ab Added</th>
<th>Cytokine Production (pg/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mo.</td>
<td>LAG-3Ig</td>
<td>Control Ab</td>
<td>TNF-α (&lt;100), IL-12 (&lt;50)</td>
</tr>
<tr>
<td>2</td>
<td>Mo.</td>
<td>Anti LAG-3</td>
<td>Control Ab</td>
<td>TNF-α (&lt;100), IL-12 (&lt;50)</td>
</tr>
<tr>
<td>3</td>
<td>DC LAG-3Ig</td>
<td>Control Ab</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC LAG-3Ig</td>
<td>Anti-LAG-3</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

a Monocytes preincubated with GM-CSF and IFN-γ or monocyte-derived DC were stimulated with either LAG-3Ig (5 μg/ml), sCD40L (0.2 μg/ml) or LPS (1 μg/ml) preincubated with either anti LAG-3 mAb 17B4 alone (50 μg/ml; expts. 2 and 3) or a combination of two anti-LAG-3 mAbs (17B4 and 8F5, 25 μg/ml each; expt. 1) or adequate isotype-matched controls or medium for 2 h at 4°C.

b supernatants were collected after 24 h for TNF-α measurement and 72 h for IL-12 measurement. Each experiment shown is representative of at least two experiments.


