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## Cutting Edge: Molecular Analysis of the Negative Regulatory Function of Lymphocyte Activation Gene-3<sup>1</sup>

Creg J. Workman, Kari J. Dugger,<sup>2</sup> and Dario A. A. Vignali<sup>3</sup>

**Lymphocyte activation gene (LAG)-3 (CD223) is a CD4-related activation-induced cell surface molecule that binds to MHC class II molecules with high affinity and negatively regulates T cell expansion and homeostasis. In this study, we show that LAG-3 inhibits CD4-dependent, but not CD4-independent, T cell function via its cytoplasmic domain. Although high affinity interaction with MHC class II molecules is essential for LAG-3 function, tailless LAG-3 does not compete with CD4 for ligand binding. A single lysine residue (K468) within a conserved "KIEELE" motif is essential for interaction with downstream signaling molecules. These data provide insight into the mechanism of action of this important T cell regulatory molecule. *The Journal of Immunology*, 2002, 169: 5392–5395.**

Many cell surface molecules participate in the cooperative regulation of effector T cell expansion and homeostasis (1). Although significant progress has been made in identifying positive costimulatory molecules, relatively few cell surface proteins have been described that down-modulate T cell function. Previous studies have suggested that human lymphocyte activation gene (LAG)-3 (CD223)<sup>4</sup> may function as a negative regulator of activated T cells (2–4). LAG-3 is particularly interesting due to its close relationship with CD4. Their genomic organization is very similar and both molecules have four extracellular Ig-like domains, with conserved structural motifs between the D1 and D3 domains as well as the D2 and D4 domains (5, 6). However, they share <20% amino acid sequence homology. A unique distinguishing feature of LAG-3 is a proline-rich 30-aa

loop between the C and C'  $\beta$  strands of the D1 domain (5, 7). Although both human and murine LAG-3 possess this loop, their homology is lowest in this region. Although the structure and function of this loop is unknown, it appears to be solvent-exposed and several residues at its base mediate ligand binding.

LAG-3 is expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and a subset of NK cells (3, 5, 8, 9). Interestingly, LAG-3 also binds to MHC class II molecules but with a much higher affinity than CD4, implying a functional connection between the two molecules (8–10). Ab cross-linking experiments with human T cells have suggested that LAG-3 associates with the TCR:CD3 complex and negatively regulates signal transduction (4). Although the initial analysis of LAG-3<sup>-/-</sup> mice did not reveal a defect in T cell function (11), we have recently shown that LAG-3 regulates the expansion of activated T cells and T cell homeostasis.<sup>5</sup> However, it is unknown what residues and motifs in LAG-3 mediate its function.

The cytoplasmic domain of LAG-3 is quite distinct from CD4 and contains a number of interesting motifs. There are three regions that are conserved between murine and human LAG-3 (see Fig. 3A). The first region is a potential serine phosphorylation site, which may be analogous to the protein kinase C binding site in CD4 (12). The second is a conserved KIEELE motif with no homology to any other known protein. The third is an unusual glutamic acid-proline (EP) repetitive sequence (12). This motif has been found in a wide variety of functionally distinct mammalian, parasitic, and bacterial proteins in diverse cellular locations, raising the possibility that it mediates interaction with a broad range of molecules or a ubiquitous protein that performs a common function (12–15). Other proteins that contain the EP motif include molecules known to mediate signaling, such as the platelet-derived growth factor receptor, LckBP1, and SPY75. Taken together, these features suggest that the LAG-3 cytoplasmic tail mediates intracellular signal transduction and/or molecular aggregation.

In this study, we took advantage of our observation that murine T cell hybridomas do not express LAG-3, even after activation (data not shown), to address the following questions. First, does ectopic expression of LAG-3 reduce T cell function? Second, does LAG-3 interfere with CD4 binding due to its high affinity for MHC class II molecules? Third, is LAG-3 function dependent on CD4? Fourth, is high affinity binding to MHC class II molecules required for LAG-3 function? Fifth, does LAG-3 mediate its function via its cytoplasmic domain and, if so, what motifs/residues mediate downstream signal transduction?

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<sup>4</sup> Abbreviations used in this paper: LAG, lymphocyte activation gene; HEL, hen egg lysozyme; GFP, green fluorescent protein; IRES, internal ribosomal entry site; WT, wild type.

## Materials and Methods

### LAG-3 constructs and retroviral transduction

LAG-3 constructs were produced using rPCR as described (16). Details of primers and strategy can be obtained from C. J. Workman. (creg.workman@stjude.org). The constructs were cloned into a murine stem cell virus-based retroviral vector, murine stem cell virus-internal ribosomal entry site (IRES)-green fluorescent protein (GFP) and retrovirus produced as described (17, 18). The CD4<sup>+</sup>3A9 T cell hybridoma (hen egg lysozyme (HEL) 48–63-specific; H-2A<sup>k</sup>-restricted) (19) and a CD4<sup>-</sup> variant (3A9.N49) T cell hybridoma (20) were transduced as described (9). Cells were sorted on a MoFlow (Cytomation, Ft. Collins, CO) for uniform GFP expression.

### Flow cytometry and the H-2E<sup>k</sup>.γ2aFc multimer-binding assay

LAG-3 expression was assessed with a rat anti-LAG-3 mAb (C9B7W, IgG1 κ) (9). Binding assays were performed using an MCC.96–108:H-2E<sup>k</sup>.γ2aFc.NL multimer (21) directly labeled with Alexa 633 (Molecular Probes, Eugene, OR). Hybridomas (1.5 × 10<sup>5</sup>/well) were incubated with the H-2E<sup>k</sup>.γ2aFc multimer (10 μg/well) in PBS plus 0.1% BSA and 0.02% NaN<sub>3</sub> for 1.5 hr at room temperature in the dark. The cells were washed twice with 100 μl PBS and analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

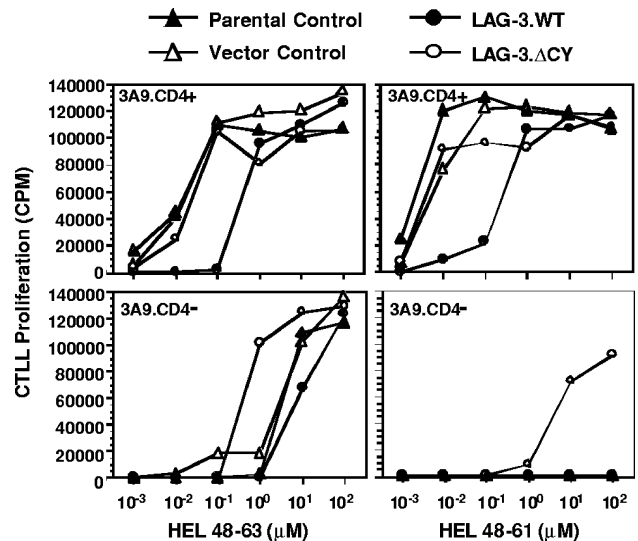
### T cell hybridoma assays

Assays were performed as previously described (20, 22). Briefly, hybridomas were stimulated with LK35.2 B cells as APC and pulsed with either HEL<sub>48–63</sub> or HEL<sub>48–61</sub> peptides. Supernatants were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2-dependent T cell line CTLL-2.

## Results

### The negative regulatory function of LAG-3 requires the cytoplasmic domain and is dependent on CD4

We took advantage of our observation that the HEL<sub>48–63</sub>-specific, H-2A<sup>k</sup>-restricted murine T cell hybridoma 3A9 (19, 20) does not express LAG-3, even after activation (data not shown). Wild-type LAG-3 (LAG-3.WT) and cytoplasmic tailless LAG-3 (LAG-3.ΔCY) were ectopically expressed on CD4<sup>+</sup> and CD4<sup>-</sup> variants of 3A9. Retrovirus was produced using a murine stem cell virus-based retroviral vector that contained an IRES and GFP (17). The IRES allows for the translation of GFP and the test protein from the same mRNA, thus facilitating the verification of coexpression. Uniform LAG-3 expression was obtained with both constructs and was comparable to normal activated T cells (data not shown). Transductants expressing a uniform level of GFP/LAG-3 were analyzed in an Ag presentation assay with the CD4-dependent peptide HEL<sub>48–61</sub>, and the largely CD4-independent peptide HEL<sub>48–63</sub> (20). Expression of LAG-3.WT significantly reduced the IL-2 response of the CD4<sup>+</sup> T cell hybridoma to both peptides (Fig. 1). This effect was not absolute, but was very reproducible. Further analysis provided four interesting observations. First, LAG-3.WT reduced the response of the CD4<sup>+</sup> T cell hybridoma to HEL<sub>48–63</sub>, but had no effect in the absence of CD4, suggesting that it specifically blocked CD4-mediated T cell function. Second, the inhibitory effect of LAG-3 was lost following deletion of the cytoplasmic tail, suggesting that it is required to mediate LAG-3 function. Third, LAG-3.ΔCY had no effect on CD4-dependent T cell function, raising the possibility that LAG-3 may not disrupt CD4:MHC class II interaction. Fourth, LAG-3.ΔCY partially restored function in the CD4<sup>-</sup> T cell hybridoma, suggesting that the LAG-3 extracellular domains can provide some positive “coreceptor” function as a consequence of its interaction with MHC class II molecules. However, it is likely that this is a noncognate interaction that is independent of the TCR. This latter observation is interesting because it cannot simply be due to the absence of the inhibitory cytoplasmic domain as LAG-3 does not inhibit T cell function in the absence of CD4 (Fig. 1, bottom left panel). Rather,



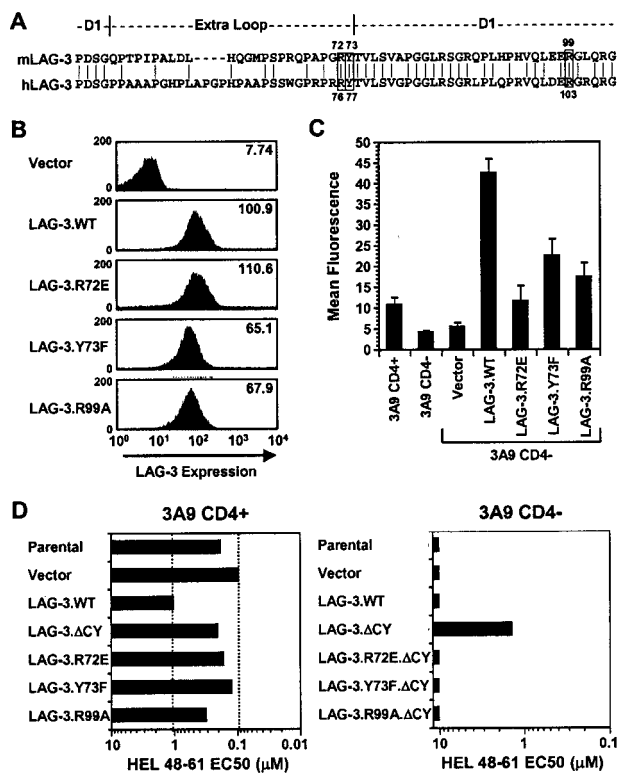
**FIGURE 1.** Ectopic expression of wild-type, but not tailless, LAG-3 reduces CD4-dependent T cell function. LAG-3.WT and LAG-3.ΔCY were expressed on CD4<sup>+</sup> (top panels) and CD4<sup>-</sup> (bottom panels) variants of the 3A9 T cell hybridoma by retroviral transduction. Controls include the unmanipulated hybridoma (Parental Control) and cells transduced with retrovirus carrying the empty vector (Vector Control). Data represent IL-2 production, measured by proliferation of the IL-2-dependent T cell line CTLL-2, in response to HEL<sub>48–63</sub> (left) or HEL<sub>48–61</sub> (right). Data are representative of three experiments.

it is possible that the LAG-3 tail is tethered to a molecule or structure that prevents it from functioning as a positive regulator. Taken together, these data demonstrate that the negative regulatory function of LAG-3 is mediated by its cytoplasmic domain rather than competing with CD4 for MHC class II binding, and may interfere with CD4 coreceptor function.

### LAG-3 function is dependent on ligation with MHC class II molecules

Previous studies examining the interaction between human LAG-3 and MHC class II had identified several residues in the D1 and D2 domains responsible for this high affinity interaction (7). We reasoned that mutation of residues that were conserved between murine and human LAG-3 might have a comparable effect on binding to MHC class II molecules. Initially, we made three mutations in the D1 domain of murine LAG-3 (R72E (compared with R76E in human LAG-3), Y73F (Y77F), and R99A (R103A)) that were previously shown to either increase 3-fold, abolish, or reduce by half, respectively, the binding affinity of human LAG-3 for MHC class II (Fig. 2A; Ref. 7). 3A9 CD4<sup>+</sup> and CD4<sup>-</sup> T cell hybridomas were transduced with retrovirus carrying these LAG-3 mutants and sorted for uniform levels of GFP expression, as described above. Expression of the R72E mutant was comparable to LAG-3.WT, while the other two were ~35% lower (Fig. 2B).

To examine the ability of the three LAG-3 point mutations to bind to MHC class II, binding studies were performed with the 3A9 CD4<sup>-</sup> LAG-3 transductants using an H-2E<sup>k</sup>.γ2aFc multimer (21). By using an H-2E<sup>k</sup> reagent, we were able to examine LAG-3 binding to MHC class II without concerns about TCR-specific interaction with the 3A9 TCR, which is H-2A<sup>k</sup>-restricted. Significant binding of H-2E<sup>k</sup>.γ2aFc multimer to the LAG-3.WT transductants was observed, with minimal binding seen with the unmanipulated CD4<sup>-</sup> cells or vector alone transductants (Fig. 2C). Weak but reproducible binding was seen with the CD4<sup>+</sup> hybridomas, consistent with the weaker affinity between MHC class II molecules and



**FIGURE 2.** LAG-3 function is dependent on its high affinity interaction with MHC class II molecules. *A*, Sequence comparison between murine LAG-3 (mLAG-3) and human LAG-3 (hLAG-3) in the region around the extra loop in the D1 domain. The three mutated residues are boxed with residue numbers shown. *B*, Ectopic expression of LAG-3 and the three LAG-3 point mutations on 3A9 CD4<sup>-</sup> T cell hybridomas. Mean fluorescence is shown (*top right* of each histogram). Data are comparable to CD4<sup>+</sup> 3A9 transductants. *C*, Binding of H-2E<sup>k</sup>.γ2aFc.NL multimer to LAG-3.WT and the three point mutants. Data represent mean  $\pm$  SE of three experiments. *D*, Functional analysis of 3A9 CD4<sup>+</sup> (*left panel*) and 3A9 CD4<sup>-</sup> (*right panel*) hybridoma expression in wild-type and mutant LAG-3. Data are expressed as the concentration of HEL<sub>48-61</sub> that gave 50% maximal IL-2 production (EC<sub>50</sub>). Dashed lines in the *left panel* highlight the LAG-3.WT and vector controls.

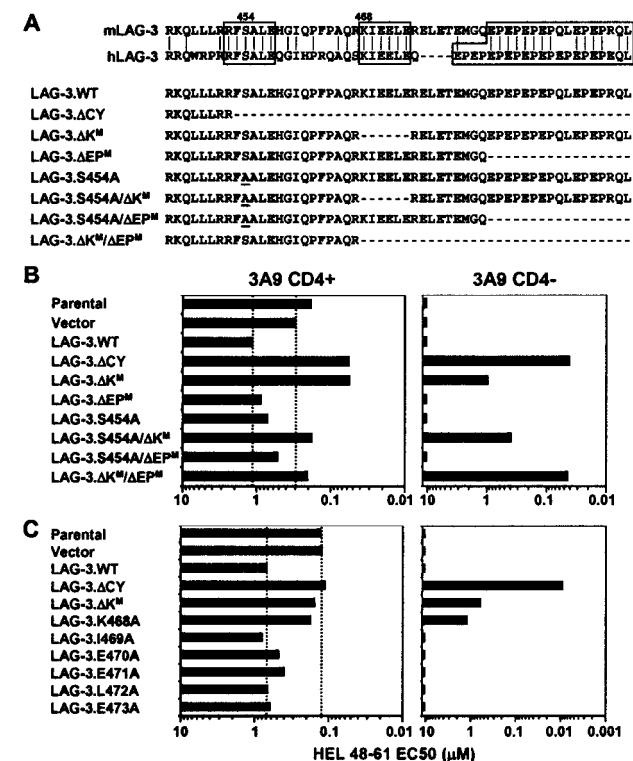
CD4 compared with LAG-3 (10, 23). Substantially reduced, but detectable, binding of the H-2E<sup>k</sup>.γ2aFc multimer to the three LAG-3 point mutants was observed. This was in contrast to observations made with comparable mutations in human LAG-3 (7). This could be due to differences in methodology as the latter study used a cell-cell adhesion assay compared with our flow cytometry-based assay. Alternatively, differences may exist in the use and structure of these residues in murine vs human LAG-3. The R72E mutation appears to reduce LAG-3 affinity to that of CD4, as far as can be determined using this assay. Although reduced binding of the H-2E<sup>k</sup>.γ2aFc multimer to the Y73F and R99A mutants was also observed, it is likely that it is partially due to their reduced expression.

Functional analysis was performed with CD4<sup>+</sup> and CD4<sup>-</sup> 3A9 transductants as described above. First, the effect of these mutations on the negative regulatory function of LAG-3 was determined by expressing LAG-3.WT, LAG-3.ΔCY, and the three LAG-3 point mutations (LAG-3.R72E, LAG-3.Y73F, and LAG-3.R99A) in the CD4<sup>+</sup> T cell hybridoma (Fig. 2*D*). All three LAG-3 point mutations substantially reduced LAG-3 function to a level comparable to the LAG-3.ΔCY mutant. Second, the effect of these mutations on the ability of LAG-3.ΔCY to act as a coreceptor

in the CD4<sup>-</sup> 3A9 T cell hybridoma was assessed using tailless versions of these LAG-3 point mutants. In all three cases, the coreceptor activity exhibited by LAG-3.ΔCY was lost. Take together, these data demonstrate that the high affinity LAG-3:MHC class II interaction is essential for LAG-3 function.

#### LAG-3 function is mediated through a conserved KIEELE motif

Comparison of the murine and human LAG-3 cytoplasmic tail sequences reveals three conserved regions: a potential serine phosphorylation site (S454), a unique KIEELE motif and multiple EP repeats (Fig. 3*A*). To determine which residues mediate LAG-3 function, we generated a series of mutants that lacked one or two of these motifs (Fig. 3*A*). Removal of the EP motif or mutation of S454 had little effect on LAG-3 function in either CD4<sup>+</sup> or CD4<sup>-</sup> T cells (Fig. 3*B*). However, deletion of the conserved KIEELE motif completely abrogated LAG-3 function in CD4<sup>+</sup> T cells. Interestingly, the negative regulatory capacity of LAG-3 was only completely abrogated (up to the level of LAG-3.ΔCY) in CD4<sup>-</sup> T cells if both the KIEELE and EP motifs were removed. Thus, it is possible that the EP motif may play a role in preventing LAG-3 from acting as a coreceptor, but may not cooperate with the KIEELE motif in mediating the negative regulatory activity of LAG-3.



**FIGURE 3.** The conserved KIEELE motif is essential for LAG-3 function. *A*, Sequence comparison between murine LAG-3 (mLAG-3) and human LAG-3 (hLAG-3) cytoplasmic domains (entire sequence shown). Three conserved regions are boxed: a possible serine phosphorylation site at S454, a KIEELE motif (designated K<sup>M</sup>), and a long EP repeat (designated EP<sup>M</sup>). Position of the murine S454 and K468 residues are shown. Mutants generated by rPCR are detailed. *B*, LAG-3.WT and various LAG-3 cytoplasmic tail mutants were ectopically expressed on 3A9.CD4<sup>+</sup> (*left panels*) and 3A9.CD4<sup>-</sup> (*right panels*) T cell hybridomas by retroviral transduction. The data represent the concentration of HEL<sub>48-61</sub> that gave 50% maximal IL-2 production (EC<sub>50</sub>). Dashed lines highlight the LAG-3.WT and vector controls. *C*, Point mutations of the KIEELE motif were made by alanine substitution. Hybridomas expressing these mutants were generated and analyzed as in *B*.

Subsequent alanine scanning mutagenesis of individual residues in the KIEELE motif clearly showed that substitution of lysine 468 (K468) abrogated LAG-3 function (Fig. 3C). A small but reproducible increase in CD4<sup>+</sup> T cell function was also observed following mutation of the two glutamic acid residues at 470 and 471 (E470/E471). Taken together, these data show that a single lysine at position 468 in the cytoplasmic domain was essential for the inhibitory function of LAG-3.

## Discussion

In this study, we have shown that murine LAG-3 inhibits CD4-dependent T cell function, a process that is entirely dependent on the cytoplasmic domain. Interestingly, tailless LAG-3 did not inhibit CD4-dependent function. This suggests that LAG-3 does not function by disrupting CD4:MHC class II interaction, which is surprising given that LAG-3 has a considerably higher affinity for MHC class II molecules than CD4 (9, 10, 23). This could be due to spatial separation of CD4 and LAG-3 within the immunological synapse or nonoverlapping binding sites on MHC class II molecules. An alternative possibility is that deletion of the LAG-3 cytoplasmic domain alters its location in the membrane. For instance, LAG-3 had been suggested to localize in lipid rafts (24). Curiously, it has been shown that human LAG-3:Ig fusion proteins can disrupt CD4:MHC class II interaction, but not CD4/MHC class II-dependent cytotoxicity (10). Colocalization or similar studies could resolve this issue.

Our data also showed that ectopically expressed LAG-3 blocked IL-2 production in CD4<sup>+</sup> but not CD4<sup>-</sup> hybridomas. Our favored interpretation is that LAG-3 function is dependent on the presence of CD4. However, we cannot rule out the possibility that the effect of LAG-3 is not evident at the higher peptide concentrations required to stimulate the CD4<sup>-</sup> T cell hybridomas. It has been suggested that LAG-3 associates with the TCR:CD3 complex and interferes with TCR signaling (4). Thus, it is conceivable that LAG-3 achieves this by disrupting coreceptor function. Given that LAG-3 is expressed on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and that the absence of LAG-3 affected the expansion of both T cell populations,<sup>5</sup> it is possible that LAG-3 may also interfere with CD8 function. Indeed, LAG-3 has been found to associate with CD8 following TCR ligation (25). Thus, it is probable that LAG-3 interferes with a molecule/pathway common to both coreceptors.

Our study clearly showed that a single lysine residue 468 within a conserved KIEELE motif in the LAG-3 cytoplasmic domain was indispensable. This motif is conserved between murine and human LAG-3 and has not been previously described, suggesting that it may recruit a unique molecule. The LAG-3 cytoplasmic domain also contains an unusual EP (glutamic acid/proline) repeat that may recruit a novel protein LAG-3-associated protein, which was identified in a yeast two-hybrid screen (24). However, LAG-3-associated protein:LAG-3 association in T cells has not yet been confirmed and thus the importance of this protein has yet to be determined. Our studies showed that deletion of the EP motif had no effect on LAG-3 function, although it may function cooperatively with the KIEELE motif in preventing coreceptor activity. It will clearly be important to identify the molecule(s) that associate with LAG-3, particularly the KIEELE motif, and mediate downstream signaling and function.

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