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LAG-3 in Cancer Immunotherapy

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Abstract

LAG-3 (CD223) is a cell surface molecule expressed on activated T cells (Huard et al. Immunogenetics 39:213–217, 1994), NK cells (Triebel et al. J Exp Med 171:1393–1405, 1990), B cells (Kisielow et al. Eur J Immunol 35:2081–2088, 2005), and plasmacytoid dendritic cells (Workman et al. J Immunol 182:1885–1891, 2009) that plays an important but incompletely understood role in the function of these lymphocyte subsets. In addition, the interaction between LAG-3 and its major ligand, Class II MHC, is thought to play a role in modulating dendritic cell function (Andreae et al. J Immunol 168:3874–3880, 2002). Recent preclinical studies have documented a role for LAG-3 in CD8 T cell exhaustion (Blackburn et al. Nat Immunol 10:29–37, 2009), and blockade of the LAG-3/Class II interaction using a LAG-3 Ig fusion protein is being evaluated in a number of clinical trials in cancer patients. In this review, we will first discuss the basic structural and functional biology of LAG-3, followed by a review of preclinical and clinical data pertinent to a role for LAG-3 in cancer immunotherapy.

Keywords

Anergy; CD4 lymphocyte; CD8 lymphocyte; Checkpoint; Tolerance; Treg; Tumor immunology; LAG-3

1 Structural Aspects of LAG-3

LAG-3 was initially discovered in an experiment designed to selectively isolate molecules expressed in an IL-2-dependent NK cell line (Triebel et al. 1990). A unique, 489-amino acid membrane protein was found, and further analyses showed that the coding region for this protein was located on the distal portion of the short arm of human chromosome 12, adjacent to the coding region for CD4. Analysis of the amino acid sequence of LAG-3 revealed an Ig superfamily member, with four IgG loops, similar to that of CD4, and subsequent studies have been, to a large part, guided by this homology.

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1.1 Basic Structure

The structure of LAG-3 is shown in Fig. 1. As above, both LAG-3 and CD4 molecules include four IgG domains. Although this structural homology is high, at the amino acid level LAG-3 is less than 20% homologous to CD4, indicating that the two genes likely diverged early in evolution (Dijkstra et al. 2006). The membrane-distal D1 domain of LAG-3 contains a unique "extra loop," to which antibodies have been raised (Baixeras et al. 1992), and which is not present on any CD4 molecules thus sequenced. As will be discussed below, LAG-3 has been demonstrated to bind to Class II MHC primarily through a small set of amino acids localized to the D1 domain (Huard et al. 1997) – this is in sharp contrast to CD4 which interacts with Class II MHC through a fairly large surface involving multiple residues (Fleury et al. 1991; Moebius et al. 1993). In addition, the intracellular portion of LAG-3 is relatively short, containing a unique motif (KIEELE) that is required for LAG-3 modulation of T cell function (Workman and Vignali 2003).

1.2 LAG-3 Expression

LAG-3 is in many ways a T cell activation marker, expressed on both CD4 and CD8 T cells 3–4 days post activation (Huard et al. 1994). It is also expressed on natural killer (NK) cells, although its function on that cell type is of uncertain significance (Huard et al. 1998). One study suggests expression on activated B cells, although those data have not been widely replicated (Kisielow et al. 2005). Finally, LAG-3 mRNA can also be found in the thymic medulla, the splenic red pulp, and the base of the cerebellum (Workman et al. 2002). When T cells are activated, LAG-3 expression is first detectable approximately 24 h post activation, peaking around day 2 and then gradually declining by day 8. Early studies on LAG-3 suggested that its expression might serve to distinguish T_H1 from T_H2 CD4 T cells (Annunziato et al. 1996); i.e., IL-12 potently stimulated LAG-3 expression, and blockade of IFN-γ decreased LAG-3 expression (Annunziato et al. 1997). More recently, these findings have been called into question, with one study showing that LAG-3 expression might not reliably distinguish T_H1 from T_H2 cells, at least in humans (Rogala et al. 2002).

1.3 Binding of Class II MHC

Based on the structural (but not amino acid) homology between LAG-3 and CD4, early studies were performed to determine whether LAG-3 might interact with Class II MHC. COS-7 cells were transfected with human LAG-3 and shown to rosette human B cell tumors (Baixeras et al. 1992). This interaction could be blocked via antibodies to either LAG-3 or HLA-DR, indicating the specificity of binding. Later studies characterized this interaction with a soluble LAG-3–Ig fusion protein (Huard et al. 1995) and determined that the K_d for this association was a remarkable 60 nM, several orders of magnitude higher affinity than that of CD4 for class II MHC (10^{-4} M). Mutagenesis studies localized the LAG-3 residues involved in MHC Class II binding to the D1 loop. Surprisingly, only a handful of residues appeared to be involved in this interaction, again in sharp contrast to the extensive Class II binding surface of CD4 (Fleury et al. 1991; Moebius et al. 1993). The implications of these findings are not clear; it seems surprising that a high affinity, nanomolar, interaction could be mediated by such a limited contact region.

1.4 Localization of LAG-3 in T Cells

Because LAG-3 bears structural homology to CD4, it seemed logical to hypothesize that LAG-3 might co-segregate with CD4 in T cell activation. Instead, initial studies showed that LAG-3 does not co-segregate with CD4 but rather localizes with CD8 and CD3/TCR complexes (Hannier and Triebel 1999). These results have been recently readdressed using a new murine anti-LAG-3 antibody, and once again it was found that LAG-3 was not co-localized with CD4 either at the cell surface or intracellularly (Woo et al. 2010). Interestingly, a significant fraction of the LAG-3 molecules in a CD4 T cell was stored in intracellular compartments in close association with the microtubule organizing center, potentially facilitating rapid transit to the T cell surface during activation.

2 LAG-3 Function

2.1 Role in CD4 T Cell Function and Expansion

Early studies using a monoclonal antibody to LAG-3 showed that human CD4 T cell clones exhibited more persistent proliferation when LAG-3 was blocked in vitro (Huard et al. 1995). This proliferation was accompanied by enhanced cytokine production with a mixed pattern (IL-2, IL-4, IFN-γ). These pro-inflammatory effects were limited to antigen-dependent stimuli and were not noted in CD8 T cells. These data were the first to suggest a negative regulatory effect of LAG-3 on T cell function, a role confirmed by later studies using human cells (Macon-Lemaitre and Triebel 2005). However, the development of LAG-3 knockout (Miyazaki et al. 1996) animals allowed a more precise inquiry into the role of LAG-3 on T cells in murine models. These experiments showed a role for LAG-3 in regulating the in vitro and in vivo expansion of both CD4 and CD8 T cells, thus confirming its role as a negative regulator (Workman et al. 2002). Further studies with a LAG-3 molecule lacking the KIEELE domain demonstrated a critical role for this motif in the negative regulatory function of LAG-3; i.e., LAG-3 molecules lacking this domain could not negatively modulate T cell function in vitro or in vivo (Workman and Vignali 2003).

A negative regulatory role for the LAG-3/Class II MHC interaction, however, is not without controversy. Using a series of mixed lymphocyte reactions, one group showed that soluble LAG-3 clearly down-modulated human CD4 T cell function in vitro, suggesting that the interaction between LAG-3 and Class II MHC in these culture conditions was a stimulatory one (Subramanyam et al. 1998). Interestingly, this down-modulation of the MLR response was not noted in human CD8 T cells, suggesting that the interaction between Class II and LAG-3 on CD8 T cells might be functionally distinct from that on CD4 cells. These results are seemingly contradictory to a subsequent series of experiments (see below), in which soluble LAG-3–Ig was shown to function in vitro and in vivo as an activator of dendritic cells.

2.2 Role of LAG-3 on Regulatory T Cells

Using microarray analyses, our group found that LAG-3 was relatively upregulated on CD4 T cells that encounter self-antigen in vivo and adopt a regulatory phenotype (Huang et al. 2004). In this model of self-tolerance, we found that a LAG-3− blocking antibody appeared to mitigate regulatory T cell (Treg) function in vivo, and transfection of antigen-specific

CD4 T cells with full-length, but not truncated, LAG-3 could confer in vitro regulatory properties. This finding is supported by studies in patients with Hodgkins lymphoma, showing elevated Treg levels when patients' disease was active. In vitro studies showed that depletion of LAG-3+ CD4 T cells enhanced tumor-specific CD8 T cell reactivity, consistent with a role for LAG-3 in suppressing antitumor immunity (Gandhi et al. 2006). This finding is supported by more recent studies showing an enhanced suppressive capacity of LAG-3+ CD4+ CD25+ cells versus LAG-3− cells from the tumor sites of cancer patients (Camisaschi et al. 2010). A recent study also reports a role for LAG-3 in a FoxP3+ subset of CD8 T cells with regulatory function (Joosten et al. 2007), a novel finding that is especially interesting as regulatory CD8 T cells enjoy a resurgent interest (Kapp and Bucy 2008).

2.3 Role of LAG-3 on CD8 T Cells

Although early studies questioned a role for LAG-3 in CD8 T cells, such findings were curious given the five- to eightfold increased expression of LAG-3 in activated CD8 versus CD4 cells, as well as the relative co-localization of LAG-3 and CD8 in activated T cells. Studies using LAG-3 knockout animals confirmed a role for LAG-3 in regulating CD8 T cell homeostatic proliferation, as well as in the in vivo response to a superantigen (Workman et al. 2004). We confirmed this role by adoptively transferring antigen-specific CD8 T cells to mice bearing their cognate antigen as either a self or a tumor antigen (Grosso et al. 2007). In this setting, LAG-3 knockout CD8 T cells showed enhanced proliferation and cytokine production. Interestingly, administration of a LAG-3-blocking antibody around the time of adoptive T cell transfer showed a similar enhancement of immune function, suggesting a potential direct role for the blocking antibody on CD8 T cells. This was verified by administering the blocking antibody to mice receiving an adoptive transfer of LAG-3 knockout T cells, although here no additional effects were noted. Recent studies using antibodies that block CTLA-4 found evidence for a similar direct role on effector T cells, confirming that immune checkpoint blockade may, in some circumstances, function via a cell-intrinsic mechanism (Peggs et al. 2009). In terms of the intersection of LAG-3 with other immune checkpoints, it is important to note a recent seminal study involving exhausted CD8 T cells in a model of chronic viral infection. Here, it was found that nonfunctional CD8 T cells could express multiple checkpoint molecules, and that some cells co-expressed both LAG-3 and the well-described immune checkpoint molecule PD-1 (Blackburn et al. 2009). In this model, blockade of both PD-1 and LAG-3 resulted in an improved antiviral immune response as compared to either molecule alone. We observed a similar phenotype in our model of self-antigen tolerance (Grosso et al. 2009), demonstrating a population of nonfunctional CD8 T cells that express both LAG-3 and PD-1. These studies have recently been extended to human ovarian carcinoma samples, where a significant fraction of tumor antigen-specific CD8 T cells co-express LAG-3 and PD-1 (Matsuzaki et al. 2010). Taken together, these important studies suggest that immunotherapy of chronic infections and cancer may require the blockade of multiple immune checkpoints.

2.4 LAG-3 Mechanism of Action

The precise mechanisms by which LAG-3 negatively modulates T cell function are not completely understood. As above, it is clear that the unique intracellular KIELLE domain is required for these effects. However, early studies on LAG-3 were able to demonstrate a

soluble form of the molecule in the sera of certain patients, suggesting that cleavage of LAG-3 might play some physiological role (Triebel et al. 2006). In an elegant series of studies, the Vignali group expanded on these findings, showing that LAG-3 is cleaved near the cell surface by two members of the TNF alpha converting enzyme (TACE) family of metalloproteases known as ADAM 10 and ADAM 17 (Li et al. 2007). Expression of a non-cleavable form of LAG-3 mediated an irreversible defect in T cell function, showing that LAG-3 cleavage was a major mechanism by which its negative regulatory function was mitigated. Interestingly, these studies revealed no role for the cleaved form of LAG-3, in sharp contrast to the studies below involving a LAG-3–Ig fusion protein.

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3.1 Preclinical Studies

Shortly after the generation of a LAG-3–Ig fusion protein for use in biochemical and functional studies, this reagent was studied in vivo in a murine tumor model. In contrast to the findings in a human mixed lymphocyte reaction, as well as to those involving the cleaved portion of the molecule, soluble LAG-3–Ig mediated tumor control and regression in mice bearing RENCA (kidney), MCA 205 (sarcoma), or TS/A (mammary) tumors (Prigent et al. 1999). These findings could be replicated by transduction of tumor cells with LAG-3, suggesting that LAG-3 might mediate an antitumor effect by binding to Class II MHC on antigen-presenting cells and potentially mediating their maturation or function. Indeed, in vitro studies using human monocyte-derived dendritic cells confirmed this hypothesis, showing that LAG-3–Ig upregulated the expression of co-stimulatory molecules and increased IL-12 expression in dendritic cells (Andreae et al. 2002). These phenotypic changes resulted in an enhanced ability of LAG-3–Ig-matured dendritic cells to mediate T_H1 response, as documented by an increased production of IFN-γ by responding T cells. These results also suggested that LAG-3–Ig could potentially function as an adjuvant, potentiating a vaccine response. This was indeed the case, as LAG-3–Ig was shown to markedly enhance the CD8 T cell response to a soluble antigen vaccine (Ovalbumin), as well as the humoral response to a particulate antigen (hepatitis B surface antigen) in mice (El and Triebel 2000). This adjuvant effect was extended to a cancer vaccine setting; here LAG-3–Ig was able to prevent mammary carcinogenesis when administered along with a weak DNA vaccine in HER-2/neu transgenic mice (Cappello et al. 2003). It should be appreciated that these results, though exiting, seem to be somewhat contradictory to the fairly well-documented negative role of LAG-3 on T cell proliferation and function. Indeed, it seems counterintuitive that the interaction between LAG-3 and Class II could on one hand mediate T cell downregulation, yet on the other hand send a pro-inflammatory maturation signal to the class II expressing dendritic cells. In this context, one recent study appears to contradict a pro-immune effect of LAG-3/Class II engagement on dendritic cells, suggesting that LAG-3 on regulatory CD4 T cells in fact inhibits dendritic cell function (Liang et al. 2008).

3.2 Clinical Studies

Shortly after the discovery of LAG-3, it was noted that some renal cell cancer (RCC) patients have a dramatic expansion of LAG-3+ CD4+ tumor infiltrating lymphocytes (Angevin et al. 1997). In follow-up work, it was found that LAG-3 expression in RCC TIL

varied from 11% to 48%, whereas significant levels of CTLA-4 or the checkpoint molecule 4-1BB could not be detected (Demeure et al. 2001). However, LAG-3 blockade did not seem to augment CD8 T cell lysis in these studies, suggesting that LAG-3 blockade might perhaps be more important in the early, i.e., priming phase of T cell activation, or perhaps reflecting the technical limitations inherent in using expanded human TIL as a reagent.

Based on the interesting murine studies using LAG-3–Ig, this reagent is being commercially developed (IMP321, Immutep, Paris) and tested in several clinical trials. In the first Phase I trial, IMP321 was administered in increasing doses with a standard influenza vaccine (Brignone et al. 2007a). No dose-limiting toxicity was observed, and adverse effects were minimal. No augmentation of the humoral vaccine response was noted, but a TH1 CD4 T cell response could be detected in several participants. A second, similar trial combined IMP321 with a commercial hepatitis B vaccine (Brignone et al. 2007b). Interestingly, at higher dose levels, CD4 and CD8 T cell responses could be detected after a single IMP321 treatment. These results were subsequently extended to patients with renal cell carcinoma in a single-agent, dose-escalation trial (Brignone et al. 2009). The agent was once again well tolerated, and treatment appeared to correlate with the development of an effector phenotype in CD8 but not CD4 T cells in the periphery. As is typical of Phase I trials in cancer immunotherapy, no objective responses were noted, but several patients showed stable disease. A more innovative trial combined IMP321 with taxane-based chemotherapy in women with breast cancer (Brignone et al. 2010). This single-armed trial demonstrated an objective response rate of 50%, as compared with a historical response rate of approximately 25%. Although single-armed studies in cancer immunotherapy must be interpreted with caution, a number of additional phase II trials are either underway or in the planning phase (www.clinicaltrials.gov; www.immutep.com).

4 Conclusions

On a basic level, LAG-3 is a remarkably interesting cell surface molecule. Phylogenetic studies show that it arose early, most likely sharing a common ancestor with CD4. LAG-3 plays an important role in modulating T cell expansion and function, and blockade of LAG-3 with monoclonal antibodies can augment T cell function in multiple models. The mechanisms by which LAG-3 exerts its physiological function are relatively poorly understood, but cleavage of LAG-3 by metalloproteases is one way in which LAG-3 function can be attenuated. The intracellular signaling pathways underlying LAG-3 function have been relatively poorly investigated and may yield future insight into its role in T cell phenotype and polarization. Although several key preclinical studies suggest a role for LAG-3 blocking antibodies in cancer immunotherapy, the majority of research in this area revolves around LAG-3–Ig, which modulates dendritic cell function in vitro and in vivo. The ultimate test of LAG-3–Ig as a clinical reagent depends on the completion of several clinical trials, some of which are currently underway, and for which final results are eagerly awaited.

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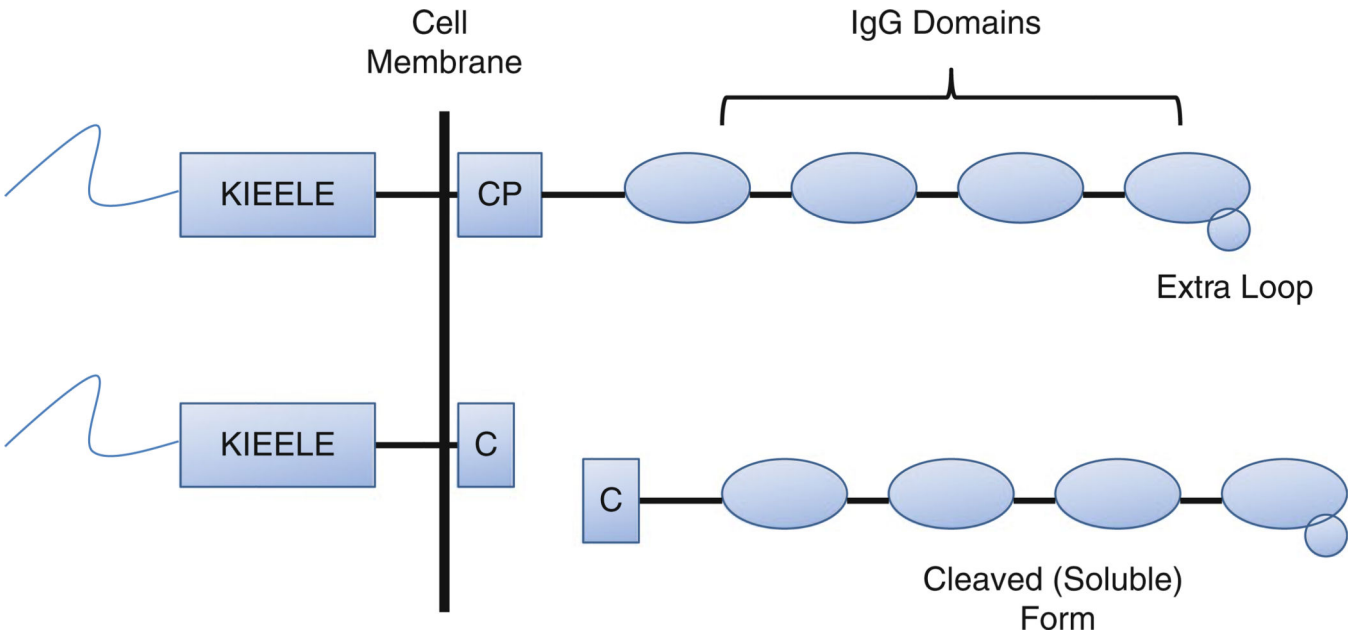

Fig. 1.

LAG-3 structure: LAG-3 is a transmembrane protein with structural homology to CD4, in which it includes four extracellular IgG domains. The membrane-distal IgG domain contains a short amino acid sequence, the so-called extra loop that is not found in other IgG superfamily proteins. The intracellular domain contains a unique amino acid sequence (KIEELE) that is required for LAG-3 to exert a negative effect on T cell function. LAG-3 can be cleaved at the connecting peptide (CP) by metalloproteases to generate a soluble form, which is detectable in serum