Inhibitory mechanisms of LAG-3-dependent T cell suppression

Atypical motifs in the cytoplasmic region of the inhibitory immune co-receptor LAG-3 inhibit T cell activation

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ABSTRACT

T cell activation is tightly regulated by both stimulatory and inhibitory co-receptors and has been a focus in the development of interventions for managing cancer or autoimmune diseases. Targeting the inhibitory co-receptors programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) has successfully eradicated tumors but induced immune-related adverse-events in human and mice. The beneficial and adverse effects of targeting these co-receptors highlight their importance in cancer immunity and also autoimmunity. Although therapeutic potencies of other inhibitory co-receptors are under extensive investigation, their inhibitory mechanisms and their functional differences are not well understood. Here we analyzed the inhibitory mechanisms of lymphocyte activation gene-3 (LAG-3), another inhibitory co-receptor by using an in vitro T cell activation system and a high-affinity anti–LAG-3 Ab that strongly interferes with the binding of LAG-3 to its ligand. We found that the expression level of LAG-3 strongly correlates with the inhibitory function of LAG-3, suggesting that LAG-3 functions as a rheostat rather than as a breaker of T cell activation. By evaluating the inhibitory capacities of various LAG-3 variants relative to their expression levels, we found that LAG-3 transduces two independent inhibitory signals through an FxxL motif in the membrane-proximal region and the C-terminal EX repeat. These motifs have not been previously reported for inhibitory co-receptors, suggesting that LAG-3 inhibits T cell activation through a non-redundant inhibitory mechanisms along with the other inhibitory co-receptors. Our findings provide a rationale for combinatorial targeting of LAG-3 and the other inhibitory co-receptors to improve cancer immunotherapy.

LAG-3 has four immunoglobulin (Ig)-like domains that are called domain 1 (D1) to D4 in its extracellular (EC) region. LAG-3 has been
reported to associate with major histocompatibility complex class II (MHCII) using its D1 with higher affinity than CD4 and inhibit T cell activation by interfering the engagement of CD4 by MHCII (12,13). However, LAG-3 can inhibit T cell activation independently of CD4 and thus, the molecular mechanism of inhibition by LAG-3 has been enigmatic (8). We have recently found that LAG-3 does not bind to MHCII universally but selectively binds to stable complex of peptides and MHCII (pMHCII) and preferentially inhibits the activation of T cells reactive to stable pMHCII (14). We have also demonstrated that LAG-3 does not interfere CD4-pMHCII and TCR-pMHCII interactions. Instead, the inhibitory function of LAG-3 requires its intracellular (IC) region, suggesting that LAG-3 transduces inhibitory signal via its IC region (14).

The IC region of LAG-3 consisting of about 60 amino acid residues lack a typical signaling motif with a known signaling mechanism such as immuno-receptor tyrosine based inhibitory motif (ITIM) and immuno-receptor tyrosine based switch motif (ITSM). However, amino acid sequences from different species show substantial similarity. Workman et al. reported that the KIEELE sequence in the middle of the IC region was conserved among species and was required for LAG-3 to inhibit the antigen-dependent activation of 3A9 hybridoma T cells. They also demonstrated that the amino acid substitution of the K in the KIEELE sequence to A was enough to abrogate the inhibitory capacity of LAG-3 (15). However, the other regions have not been extensively analyzed and how this K transduces inhibitory signal is currently unknown. Iouzalen et al. identified LAG-3 associated protein (LAP) as a molecule that bound to the C-terminal region of LAG-3 with 10 to 15 repeats of E and favorably but not limited to P (EX repeat) (16). Currently, the function of LAP as well as EX repeat is unknown. Thus, the molecular basis of the inhibitory signal by LAG-3 remains elusive.

In the current study, we used an in vitro T cell activation system and high-affinity anti-LAG-3 Ab that strongly interfered the interaction of LAG-3 and pMHCII to analyze the inhibitory function of LAG-3. We demonstrated that the expression level of LAG-3 strongly correlated with the inhibitory function of LAG-3. Intriguingly, deletion of KIEELE sequence did not abrogate the inhibitory function of LAG-3. Instead, we found that LAG-3 transduces two independent inhibitory signals through an FxxL motif in the membrane-proximal (PR) region and the C-terminal EX repeat. These motifs have not been reported for the other inhibitory co-receptors to date, suggesting that LAG-3 inhibits T cell activation using non-redundant inhibitory mechanisms with the other inhibitory co-receptors.

**Results**

**Evaluation of anti–mouse LAG-3 monoclonal Abs**

Monoclonal Abs are useful and essential to analyze the physical and functional properties of molecules. Although the C9B7W clone of anti–mouse LAG-3 Ab is widely used as a blocking Ab of mouse LAG-3, C9B7W has been reported not to interfere the binding of LAG-3 to MHCII but postulated to induce conformational changes of LAG-3 to attenuate its function (13). We have recently developed the TKB58 clone of anti–mouse LAG-3 Ab that can strongly block the binding of LAG-3 to pMHCII (14). We first characterized TKB58 and C9B7W as well as TKB27 clone of anti–mouse LAG-3 Ab, which we developed by immunizing LAG-3 deficient mice with the recombinant protein of mouse LAG-3 EC region. We generated chimeric molecules of mouse and human LAG-3 in which one of the four Ig-like domains of mouse LAG-3 is swapped with the corresponding Ig-like domain of human LAG-3 and tested their reactivity to TKB58, TKB27, and C9B7W. In accordance with the former report, C9B7W recognized D2 of mouse LAG-3 because it failed to recognize the chimeric molecule with human D2 (Fig. 1 A). TKB58 and TKB27 recognized D1 and D4 of mouse LAG-3, respectively (Fig. 1 A). Next, we tested the capacities of TKB58, TKB27, and C9B7W to block the binding of soluble LAG-3 protein (LAG-3-EC) to stable pMHCIIs on IIA1.6 B lymphoma cells (14). As shown in Fig. 1 B, TKB58 but not TKB27 blocked the LAG-3-EC binding to IIA1.6 cells. On the other hand, C9B7W slightly reduced the staining intensity of LAG-3-EC to IIA1.6 cells, suggesting that the conformational change induced by C9B7W might result in the weak attenuation of the interaction between LAG-3 and pMHCII.

To examine the capacities of TKB58, TKB27, and C9B7W to block the inhibitory function of LAG-3, we used DO11.10 hybridoma T cells that recognize 323-339 segment of chicken ovalbumin (pOVA323-339) in the context of I-Ad. As we have reported before, LAG-3 efficiently inhibited the activation and the secretion of IL-2 from DO11.10
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T cells upon co-culturing with pOVA23-33-pulsed IIA1.6 cells (Fig. 1 C) (8). TKB58 completely blocked the inhibitory effects of LAG-3 at as low as 0.5 µg/ml, whereas TKB27 did not block the inhibitory effects of LAG-3 even at 25 µg/ml (Fig. 1 D and E). In agreement with the former report, we could observe the blocking effect by C9B7W albeit to a lesser extent than TKB58 (13). However, C9B7W did not completely block the inhibitory effect of LAG-3 even at 25 µg/ml, probably because C9B7W attenuates LAG-3 function by inducing the conformational change of LAG-3 but not by masking the binding surface of LAG-3 to pMHCII (Fig. 1 E).

Then we measured binding affinities of TKB58, TKB27, and C9B7W to LAG-3 using bio-layer interferometry (Fig. 2). The association rate of TKB58 was 26 and 23 times faster than C9B7W and TKB27, respectively, whereas the dissociation rate of TKB58 was 2.1 and 3.6 times slower than TKB27 and C9B7W, respectively. Accordingly, Kd value of TKB58 was 48 and 94 times smaller than TKB27 and C9B7W, respectively, indicating that TKB58 binds to mouse LAG-3 much more strongly than TKB27 and C9B7W (Fig. 2).

Correlation of cell surface amount of LAG-3 and its inhibitory effect

LAG-3 is not expressed on naive T cells but inducibly expressed on T cells upon activation (17). Although the stronger TCR signal induces higher LAG-3 expression, the correlation of cell surface amount of LAG-3 and its inhibitory effect has not been elucidated. Therefore, we overexpressed LAG-3 on DO11.10 T cells to a various level using seven different promoters (pLTR, pEF1α, pCAG, β-actin, pCMV, pSV40, and pMC1) with or without polyadenylation (polyA) signal, which generally prolongs the longevity of transcripts (Fig. 3 A and B). The amount of cell surface LAG-3 was calculated relative to that of DO11.10 T cells expressing LAG-3 under pLTR. LAG-3-dependent inhibitory effects were calculated by comparing the amount of IL-2 in the presence or absence of anti-LAG-3 blocking Ab (TKB58). As shown in Fig. 3 C and D, the inhibitory effect of LAG-3 showed strong positive correlation to the cell surface amount of LAG-3 (R² = 0.9287). Intriguingly, the inhibitory effect of LAG-3 was not saturated even when LAG-3 was expressed with the strongest promoter tested (pLTR). These results indicate that LAG-3 functions as a rheostat rather than a breaker of T cell activation.

Requirement of the PR region for the inhibitory function of LAG-3

Although the KIEELE sequence in the middle of IC region of LAG-3 has been reported to be essential for LAG-3 function (15), we could not reproduce the result in our experimental system using DO11.10 T cells (Fig. 4 A–C). Therefore, we examined the requirements of PR region (amino acid 467-492) and EX repeat (amino acid 493-521) of LAG-3 for its inhibitory function. We generated deletion mutants of LAG-3 and introduced them into DO11.10 T cells (Fig. 4 A and B). Because deletion mutations affected the cell surface expression level of LAG-3 to variable degrees and we observed a strong correlation between the cell surface amount of LAG-3 and its inhibitory effect (Fig. 3 D), we evaluated the inhibitory capacity of each LAG-3 mutant by comparing its expression level and inhibitory effect with the reference values obtained from the panel of DO11.10 T cells expressing LAG-3 to variable degrees (Fig. 3 D). In accordance with the former observation, LAG-3ΔIC failed to inhibit the IL-2 secretion from DO11.10 T cells upon antigen stimulation, suggesting that LAG-3 inhibits T cell activation by transducing an inhibitory signal via its IC region (Fig. 4 D and E) (14,15). Intriguingly, LAG-3ΔPR but not LAG-3ΔEX and LAG-3ΔKIEELE showed substantially reduced inhibitory capacity, indicating that PR region but not KIEELE sequence is required for the inhibitory function of LAG-3 (Fig. 4 D and E).

Requirement of the FxxL motif in the PR region for the inhibitory function of LAG-3

To further characterize the PR region, a series of mutants with alanine substitutions in 5 consecutive amino acid residues in PR region were generated and tested for their expression levels on DO11.10 T cells (Fig. 5 A). LAG-3 mutants with 473-477A and 476-480A substitutions showed reduced inhibitory capacities to the similar level as LAG-3 ΔPR, suggesting that the responsible amino acid residue(s) locates between R473 to H480 (Fig. 5 B–D). Then, we tested single alanine substitution mutants and found that LAG-3ΔF475A and LAG-3ΔL478A showed reduced inhibitory capacities to the similar level as LAG-3ΔPR, suggesting that F475 and L478 play pivotal roles in the PR.
region-dependent inhibitory function of LAG-3 (Fig. 5 E–H). LAG-3 with both F475A and L478A mutations also showed reduced inhibitory capacities to the similar level as LAG-3ΔPR, LAG-3-473-477A and LAG-3-476-480A (Fig. 5 F–H). Therefore, F475 and L478 likely function in the same inhibitory process and we named this motif as FxxL motif.

**Weak EX repeat-dependent inhibition of T cell activation by LAG-3**

Although the inhibitory capacities of LAG-3ΔPR and F475A/L478A are substantially attenuated, they still weakly inhibited IL-2 production from DO11.10 T cells upon activation. Because LAG-3ΔIC completely lacked the inhibitory function, this residual inhibitory effect is likely mediated by the EX repeat. As expected, by deleting the EX repeat of LAG-3-F475A/L478A, the residual inhibitory effect was completely abrogated (Fig. 6 A and B). Therefore, LAG-3 likely inhibits T cell activation by using two distinct mechanisms that are dependent on the FxxL motif and the EX repeat.

LAG-3 mutant that is defective in both FxxL motif- and EX repeat-dependent inhibitory mechanisms (LAG-3ΔEX/F475A/L478A) rather increased the IL-2 production, indicating that LAG-3ΔEX/F475A/L478A functioned as a co-stimulator (Fig. 6 A–B). The co-stimulatory effect was dependent on the interaction of LAG-3ΔEX/F475A/L478A to pMHCII because anti–LAG-3 blocking Ab (TKB58) completely canceled the additive effect. As mentioned above, LAG-3 has a structural similarity with CD4 in the EC but not IC region. CD4 is known to associate with Lck that is essential for the initiation of the TCR signal. In agreement with former reports (18,19), the overexpression of CD4 not only augmented the production of IL-2 upon activation but also rendered DO11.10 T cells dependent on CD4 for activation as anti–CD4 blocking Ab (GK1.5) completely abrogated the IL-2 production rather than merely canceling the additive effect. These results suggest that the PR region of LAG-3 may associate with a signaling molecule(s) that additively augment the strength of the TCR signal (Fig. 6 C).

**Discussion**

In the present study, by using an in vitro T cell activation system and high-affinity anti–LAG-3 Ab that interferes the interaction of LAG-3 and stable pMHCII, we analyzed the inhibitory function of LAG-3 against T cell activation. First we demonstrated that the cell surface amount of LAG-3 strongly correlated with its inhibitory function, which indicates that LAG-3 functions as a rheostat rather than a breaker of T cell activation. LAG-3 is not expressed on naive T cells but inducibly expressed on T cells upon activation (17). Protein kinase C signaling pathway has been reported to regulate the expression level of LAG-3 by controlling its trafficking from the lysosomal compartment to the cell surface (20). The cell surface expression level of LAG-3 is also regulated by the shedding of the EC region by two transmembrane metalloproteases, ADAM10 and ADAM17 (21). The current findings clearly indicate that the inhibitory effect of LAG-3 was not saturated even when LAG-3 was expressed with the strongest promoter tested. In addition, changes in the amount of LAG-3 on cell surface directly affected the inhibitory effect of LAG-3, indicating that the regulation of its cell surface amount is effective for the regulation of T cell activation.

The expression level of LAG-3 has been reported to differ largely depending on the cell types. CD4+ T cells with the exhausted-like phenotypes are reported to express high amount of LAG-3, while the small subset of intestinal CD4+ T cells with regulatory function are reported to express LAG-3 weakly (7,22-25). The impact of LAG-3 on cell-autonomous function may thus differ among different types of cells. In addition, the stronger TCR signal induces higher LAG-3 expression, suggesting that the induction of LAG-3 likely serves as a negative feedback to prevent excess activation (8).

By dissecting the IC region of LAG-3, we found that LAG-3 inhibits T cell activation by using two distinct mechanisms that are dependent on the FxxL motif in the PR region and the EX repeat at the C-terminus. Because LAG-3ΔEX showed comparable inhibitory capacity with intact LAG-3, the FxxL motif-dependent inhibition predominates over EX repeat-dependent inhibition at least in the inhibition of IL-2 production by DO11.10 T cells upon activation. On the other hand, EX repeat-dependent inhibition functions complementarily in the absence of FxxL motif-dependent inhibition. Intriguingly, LAG-3 mutant that is defective in both FxxL motif- and EX repeat-dependent inhibitory mechanisms provided co-stimulation, suggesting that the PR region of LAG-3 may contain a co-stimulatory motif(s) in addition to co-inhibitory motifs.
Further analyses are required to reveal the physiological meaning of the co-stimulatory potential of LAG-3.

Because the FxxL motif resembles YxxL sequence in ITIM, phospho-tyrosine independent signaling adaptor proteins may be involved in the inhibitory signal via the FxxL motif in LAG-3. For example, μ-adaptin that is involved in the endocytosis of membrane molecules has been reported to recognize the FxxL motif in β-arrestin2 (26). The C-terminal tail of platelet-derived growth factor β-receptor is also rich in E and P. Intriguingly, this C-terminal tail has been reported to inhibit its tyrosine kinase activity autonomously (27). Although LAG-3 itself does not have a tyrosine kinase activity, EX repeat of LAG-3 may also inhibit another tyrosine kinase(s) that is activated by antigen stimulation. Currently, how the FxxL motif and EX-repeat regulate TCR proximal and downstream molecules how these motifs independently or cooperatively regulate signaling pathways in T cell activation.

LAG-3 inhibits the proliferation of T cells and production of cytokines such as IL-2 and IFN-γ from T cells (8,28,29). In addition, LAG-3 has been reported to play critical roles in regulatory T cells (24). In the current study, we clearly demonstrated that LAG-3 inhibits IL-2 production from T cells upon antigen stimulation by using the FxxL motif and EX-repeat. The requirement of both or either of these motifs in the LAG-3-dependent regulation of other T cell functions remains to be verified.

We have recently reported that LAG-3 does not bind to MHCII universally but selectively binds to stable pMHCII and preferentially inhibits the activation of T cells reactive to stable pMHCII (14). The identification of FxxL motif and EX-repeat in the current study further supports the idea that LAG-3 accumulates at the immunological synapse and transduces inhibitory signal via its IC region to attenuate TCR proximal signal.

As mentioned above, we could not reproduce the former observation by Vignali and colleagues that the deletion of KIEELE sequence abrogated the inhibitory function of LAG-3. Although the reason for this discrepancy is currently unknown, it should be noted that the PR region was not analyzed in the study and thus the relative effects of PR region and KIEELE sequence have not been compared (15). Based on our current observation, the deletion of PR region might have shown greater effect compared with the deletion of KIEELE sequence in the former experimental condition as well. In addition, the expression levels of LAG-3 mutants substantially differed among different mutants. As we observed the strong positive correlation between the cell surface amount and the inhibitory effect of LAG-3, we need to take the expression level of mutants into consideration. Otherwise, we may under- or over-estimate the inhibitory capacities of mutants. By comparing the expression level and the inhibitory effect of mutants with the reference values obtained from the panel of DO11.10 T cells expressing LAG-3 to variable degrees, we could obtain unambiguous results.

While we are preparing this manuscript, Chen and colleagues reported that LAG-3 inhibited T cell activation by associating with fibrinogen-like protein 1 (FGL1)(30). Although the exact contribution of FGL1 and stable pMHCII to the inhibitory function of LAG-3 needs future verification, the complete blockade of LAG-3 function by TKB58 that interferes the binding between D1 of LAG-3 and pMHCII strongly suggests that stable pMHCII is primarily involved in the inhibition by LAG-3 (14).

Because of the success of tumor immunotherapy targeting CTLA-4 and PD-1, therapeutic potencies of the other inhibitory co-receptors are being extensively explored. However, their actual inhibitory mechanisms, functional differences, and collaboration are not well understood. Here, we demonstrated that LAG-3 transduced two independent inhibitory signals through the FxxL motif in the PR region and the C-terminal EX repeat. These motifs have not been reported for inhibitory co-receptors before, suggesting that LAG-3 inhibits T cell activation using non-redundant inhibitory mechanisms with the other inhibitory co-receptors. Thus, combinatorial therapy of LAG-3 with the other inhibitory co-receptors is expected to provide synergistic effects.

Experimental procedures

Cell culture

DO11.10 T and IIA1.6 cells, which were kindly provided by Tasuku Honjo (Kyoto University) and Tomohiro Kurosaki (Osaka University), respectively were cultured in RPMI 1640 medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest), 0.5 mM Monothioglycerol (Wako), 2 mM...
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L-alanyl-L-glutamine dipeptide (Gibco), 100 U/ml penicillin (Nacalai Tesque), and 100 µg/ml streptomycin (Nacalai Tesque). Plat-E cells, which were kindly provided by Toshio Kitamura (University of Tokyo) were maintained in Dulbecco’s Modified Eagle Medium (D’MEM, Invitrogen), supplemented with 10% (v/v) FBS, 100 U/ml penicillin (Nacalai Tesque), and 100 µg/ml streptomycin (Nacalai Tesque).

Plasmids and retroviral gene transduction

Fragments of cDNA were amplified by PCR and cloned into retroviral expression plasmid vectors modified from pFB-ires-Neo (Agilent) with which cDNAs are transcribed under pLTR. Mutant cDNAs with deletions and amino acid substitutions and chimeric cDNAs of mouse and human LAG-3 were generated by overlap extension PCR. For controlling the expression levels, fragments of cDNA were cloned into retroviral expression plasmid vectors modified from pSUPER.retro.puro (OligoEngine), the promoter region of which was replaced with promoters of EF-1α (human elongation factor-1 alpha), CAG (cytomegalovirus enhancer/chicken beta-actin), β-actin, pCMV (cytomegalovirus), SV40 (simian virus 40), and MC1 (polyoma virus enhancer/herpes simplex virus thymidine kinase) coupled with or without polyadenylation (polyA) signal. Plasmids were transfected using the FuGENE HD (Promega) into Plat-E cells cultured in D’MEM, high glucose (Gibco) supplemented with 20% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and supernatants containing viruses were used to transduce genes into target cells. Infected cells were selected with G418 (Wako), puromycin (Sigma-Aldrich), or by cell sorting.

Soluble protein

The fragment of cDNA encoding the D1 to D4 of mouse LAG-3 (LAG-3-EC) was amplified by PCR. The five-stranded coiled-coil domain of cartilage oligomeric matrix protein (COMP) (31) with DYKDDDDK-tag, TEV cleavage site, and PA-tag was added to the C-terminus of LAG-3-EC for pentamer. The Fc region of human IgG1 (hIgG1Fc) was added to the C-terminus of LAG-3-EC for dimer. The streptag was added to the C-terminus of LAG-3-EC for monomer. Chimeric cDNAs were cloned into expression vectors modified from pEBMulti-Neo (Wako). Plasmids were transfected into Plat-E cells using Avalanche-Omni Transfection Reagent (EZ Biosystems) and the culture supernatants were collected after 48 hours. Pentameric LAG-3-EC was purified using anti–PA-tag Ab beads (Wako) followed by the cleavage by TurboTEV Protease (Accelagen). Dimeric LAG-3-EC was purified using protein G (GE Healthcare).

Mice

BALB/cCrSlc-Lag3 litter (LAG-3–/–) mice (8) were housed under specific pathogen-free conditions in environmentally controlled clean rooms. All mouse protocols were approved by the Animal Experimentation Committee of Tokushima University. All experimental procedures complied with institutional regulations complying with the Act on Welfare and Management of Animals and the related guidelines in Japan.

Generation of anti–mouse LAG-3 monoclonal Abs

BALB/cCrSlc-Lag3 litter mice were immunized with dimeric mouse LAG-3-EC and lymph node cells of immunized mice were fused with SP2/o cells using the envelope of Hemagglutinating Virus of Japan (GenomONE-CF, Ishihara Sangyo Kaisha). Culture supernatants of hybridoma clones were tested for their reactivity to DO11.10 T cells by flow cytometry.

Antibody and Flowcytometric analysis

Cells were stained with the indicated Abs. Data were obtained with Gallios (Beckman Coulter) and analyzed using FlowJo (Tree Star). Anti–mouse LAG-3 Ab (C9B7W) and its isotype control (rat IgG1, eBRG1) were purchased from Thermo Fisher Scientific. Anti–DYKDDDDK-tag (L5) Ab and isotype control for TKB58 (mouse IgG1, MOPC-21) were purchased from Biolegend. FITC-labeled F(ab′)2 fragment of goat anti–mouse IgG (H+L) Ab was purchased from Jackson ImmunoResearch. FITC-labeled goat anti–rat IgG (H+L) Ab was purchased from SouthernBiotech. TKB58 and TKB27 were purified from culture supernatants of hybridomas using protein G sepharose. Purified TKB58 was labeled with CF®633-Dye (Biotium).

Bio-layer interferometry

Monomeric mouse LAG-3-EC with streptag was immobilized to the streptavidin coated biosensor chips (Pall ForteBio) and the association of anti–mouse LAG-3 Abs at different concentration was monitored using BLItz (Pall ForteBio). Chips were washed with PBS to
analyze the dissociation kinetics. The constants of association rate (ka), dissociation rate (kd), and dissociation (kD) were calculated using BLItz Pro software.

**Stimulation of DO11.10 T cells**

DO11.10 T cells infected with or without retroviral vectors containing indicated cDNAs (5 x 10^5 cells/well) were co-cultured with IIA1.6 cells (1 x 10^4 cells/well) with cognate OVA_{323-339} peptide (ISQAVHAAHAEINEAGR, >95% purity) for 24 hours. The concentration of IL-2 in the culture supernatant was quantified by ELISA (BioLegend). To block the mouse LAG-3-mediated inhibition, anti–mouse LAG3 Ab or isotype matched control IgG was added throughout the co-culture assay. The percent LAG-3-dependent inhibition of IL-2 production was calculated as the ratio of IL-2 concentration in the presence to absence of anti-LAG-3 blocking Ab (unless specified, TKB58). To block CD4, anti–mouse CD4 Ab (5 µg/ml, GK1.5, Thermo Fisher Scientific) was added throughout the co-culture assay. The estimated inhibitory effect by the intact LAG-3 at the same expression level of each LAG-3 mutant was deduced from the regression line obtained from the panel of DO11.10 T cells expressing LAG-3 to variable degrees. The expression-adjusted inhibitory function of each LAG-3 mutant was calculated by dividing the raw percent inhibition by each LAG-3 mutant by the estimated percent inhibition by the intact LAG-3 at the same expression level.

**Statistical analysis**

Two-tailed unpaired Student’s t-test was used to evaluate statistical significance.
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Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: T.K.M., D.S., and T.O. designed the experiments. T.K.M., D.S., I.O., and T.M. established experimental systems, generated experimental materials, performed the staining and functional experiments using cultured cells. D.S. and T.O. wrote the manuscript with all authors contributing to writing. T.O. oversaw the entire project.
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FOOTNOTES
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The abbreviations used are: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; D, domain; IC, intracellular; Ig, immunoglobulin; ITIM, immuno-receptor tyrosine based inhibitory motif; ITSM, immuno-receptor tyrosine based switch motif; LAG-3, Lymphocyte activation gene-3; PR, membrane-proximal; MHC, major histocompatibility complex class II; NOD, non-obese diabetic; PD-1, programmed cell death 1; pMHCII, complex of peptide and MHCII
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Figure 1. Characterization of anti-mouse LAG-3 Abs. A, Recognition of mouse LAG-3 D1, D4, and D2 domains by TKB58, TKB27, C9B7W clones of anti-mouse LAG-3 Abs. DO11.10 T cells expressing chimeric LAG-3 with the indicated human-derived Ig-like domain were stained with indicated Abs. Mean fluorescent intensities (MFI) are indicated. B, Capacities of anti-mouse LAG-3 Abs to block the binding of LAG-3-EC to stable pMHCII on IIA1.6 cells. IIA1.6 cells were stained with LAG-3-EC (5 µg/ml) in the presence or absence of TKB58, TKB27, or C9B7W (5 µg/ml) and the bound LAG-3-EC was detected with the secondary Ab (anti-DYKDDDDK-tag Ab). MFIs are indicated. C, Capacities of anti-mouse LAG-3 Abs to block LAG-3 function. DO11.10 T cells with or without LAG-3 were stimulated with pOVA-pulsed (1 µM) IIA1.6 cells in the presence or absence of TKB58, TKB27, and C9B7W (1 µg/ml). The concentration of IL-2 in the culture supernatant was determined by ELISA. Numbers denote the percent LAG-3-dependent inhibition of IL-2 production in the presence of the indicated Ab. D, Titration of the capacity of TKB58 to block LAG-3 function. DO11.10 T cells with or without LAG-3 were stimulated as above (C) in the presence of the indicated amount of TKB58 or its isotype control (ctrl Ig). The concentration of IL-2 in the culture supernatant was determined by ELISA. E, Comparison of the capacities of anti-mouse LAG-3 Abs to block LAG-3 function. The percent LAG-3-dependent inhibition of IL-2 production in the presence of indicated amount of each Ab is shown. Data are the mean ± SD of technical duplicates in one experiment. Data are representative of more than three independent experiments.
Figure 2. Binding affinities of TKB58, TKB27, and C9B7W to mouse LAG-3. A–C, Binding affinities of anti–mouse LAG-3 Abs to mouse LAG-3 protein. LAG-3-EC was immobilized to the biosensor chip and the association of TKB58 (A), TKB27 (B), and C9B7W (C) at the indicated concentration was monitored by bio-layer interferometry. Chips were washed with PBS to analyze the dissociation kinetics. The constants of association rate (ka), dissociation rate (kd), and dissociation (kD) are shown. Data are representative of more than two independent experiments.
Figure 3. Correlation between the amount of LAG-3 and its inhibitory effect. A, Schematics of retroviral expression vectors. Mouse LAG-3 cDNA was overexpressed in DO11.10 T cells using indicated promoters with or without polyA signal. B, DO11.10 T cells with various expression levels of LAG-3. The expression levels of LAG-3 were evaluated by flowcytometry using anti–LAG-3 Ab (TKB58). MFI's are indicated. C, LAG-3-dependent inhibition of IL-2 production in DO11.10 T cells with various LAG-3 expression levels. DO11.10 T cells overexpressing LAG-3 under the indicated promoter with or without polyA signal were stimulated with pOVA-pulsed (1 µM) IIA1.6 cells in the presence of anti–LAG-3 blocking Ab (TKB58) or its isotype control (ctrl Ig). The concentration of IL-2 in the culture supernatant was determined by ELISA. D, The correlation between the percent LAG-3 dependent inhibition and the expression level of LAG-3. The amount of LAG-3 was calculated relative to that of DO11.10 T cells expressing LAG-3 under the LTR promoter. The solid line represents the regression line and dashed lines represent 95% prediction intervals. The correlation coefficient is shown. Data are the mean ± SD of technical duplicates in one experiment. Data are representative of more than three independent experiments.
Inhibitory mechanisms of LAG-3-dependent T cell suppression

Figure 4. Requirement of the PR region for the inhibitory function of LAG-3. A, Amino acid sequences of the IC region of LAG-3 and its deletion mutants. PR region, KIEELE sequence, and EX repeat are indicated with red, black, and blue lines, respectively. Numbers denote the position of the amino acid from the translation initiation site. B, Expression levels of LAG-3 mutants on DO11.10 T cells. DO11.10 T cells expressing LAG-3 and its indicated mutants were stained with TKB58 and analyzed by flowcytometry. C–E, Defective inhibition of IL-2 production from DO11.10 T cells by LAG-3 lacking its PR and IC region. DO11.10 T cells expressing LAG-3 and its mutants were stimulated with pOVA-pulsed (1 µM) IIA1.6 cells in the presence of anti–LAG-3 blocking Ab (TKB58) or its isotype control (ctrl Ig). The concentration of IL-2 in the culture supernatant was determined by ELISA (C). Relative expression level and the inhibitory effect of LAG-3 mutants are plotted on the standard plot shown in Fig.3D. LAG-3 mutants with weaker function are highlighted in red (D). The inhibitory effect relative to the intact LAG-3 with the same expression level is shown for indicated LAG-3 mutants (E). Data are the mean ± SD of technical duplicates in one representative experiment from three independent experiments (C) or the mean ± SD of three independent experiments (D, E). P values comparing the expression-adjusted relative inhibition of indicated LAG-3 mutants with that of intact LAG-3 are shown (Student's t-test, E).
Figure 5. Requirement of the FxxL motif in the PR region for the inhibitory function of LAG-3. A–D, Defective inhibition of IL-2 production by LAG-3 with alanine substitutions at 473–477 and 476–480. Amino acid sequences of the IC regions of LAG-3 and its mutants are shown. Substituted alanine residues and mutants with weaker function are colored in red (A). DO11.10 T cells expressing LAG-3 and its mutants were stimulated with pOVA-pulsed (1 μM) IIA1.6 cells in the presence of anti–LAG-3 blocking Ab (TKB58) or its isotype control (ctrl Ig). The concentration of IL-2 in the culture supernatant was determined by ELISA (B). Relative expression level and the inhibitory effect of LAG-3 mutants are plotted on the standard plot shown in Fig.3D. LAG-3 mutants with weaker function are highlighted in red (C). The inhibitory effect relative to the intact LAG-3 with the same expression level is shown for indicated LAG-3 mutants (D).

E–H, Defective inhibition of IL-2 production by LAG-3 with alanine substitutions at 475 and 480. Amino acid sequences (E) and inhibitory function (F–H) of indicated LAG-3 mutants are shown as above (A–D). Data are the mean ± SD of technical duplicates in one representative experiment from three independent experiments (B, F) or the mean ± SD of three independent experiments (C, D, G, H). P values comparing the expression-adjusted relative inhibition of indicated LAG-3 mutants with that of intact LAG-3 are shown (Student's t-test, D, H).
Figure 6. EX repeat-dependent inhibition of T cell activation by LAG-3. A and B, EX repeat-dependent partial inhibitory effect of LAG-3 mutant lacking FxxL motif. Amino acid sequences of the IC region of LAG-3 and its mutants are shown (A). DO11.10 cells with LAG-3 and its mutants were simulated with pOVA-pulsed (1 µM) IIA1.6 cells in the presence of anti-LAG-3 blocking Ab (TKB58) or its isotype control (ctrl Ig). The concentration of IL-2 in the culture supernatant was determined by ELISA (B). C, Different manners of co-stimulation by CD4 and LAG-3 mutant lacking both FxxL motif and EX repeat. DO11.10 T cells overexpressing mouse CD4 were stimulated with IIA1.6 cells pulsed with indicated amount of pOVA in the presence or absence of anti-CD4 blocking Ab (GK1.5). The concentration of IL-2 in the culture supernatant was determined by ELISA. Data are the mean ± SD of technical duplicates in one experiment. Data are representative of more than three independent experiments. ND, not detected.
Atypical motifs in the cytoplasmic region of the inhibitory immune co-receptor LAG-3 inhibit T cell activation

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