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# Role of LAG-3 in Regulatory T Cells

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## Summary

**Regulatory T cells (Tregs) limit autoimmunity but also attenuate the magnitude of antipathogen and antitumor immunity. Understanding the mechanism of Treg function and therapeutic manipulation of Tregs in vivo requires identification of Treg-selective receptors. A comparative analysis of gene expression arrays from antigen-specific $CD4^+$ T cells differentiating to either an effector/memory or a regulatory phenotype revealed Treg-selective expression of LAG-3, a CD4-related molecule that binds MHC class II. Antibodies to LAG-3 inhibit suppression by induced Tregs both in vitro and in vivo. Natural $CD4^+CD25^+$ Tregs express LAG-3 upon activation, which is significantly enhanced in the presence of effector cells, whereas $CD4^+CD25^+$ Tregs from LAG-$3^{-/-}$ mice exhibit reduced regulatory activity. Lastly, ectopic expression of LAG-3 on $CD4^+$ T cells significantly reduces their proliferative capacity and confers on them suppressor activity toward effector T cells. We propose that LAG-3 marks regulatory T cell populations and contributes to their suppressor activity.**

## Introduction

Regulatory T cells (Tregs) have been recognized as an important mechanism of immunologic tolerance (reviewed in Curotto de Lafaille and Lafaille [2002], Jonuleit and Schmitt [2003], Maloy and Powrie [2001], Sakaguchi et al. [2001], and Shevach [2002]). Either emerging directly from the thymus or being induced in the periphery, Tregs are typically reported to be $CD4^+CD25^+$ and function by inhibiting effector T cells. They are important in maintaining immune tolerance to limit autoimmunity and in regulating homeostatic lymphocyte expansion (Almeida et al., 2002; Annacker et al., 2000, 2001). However, they also suppress natural immune responses to parasites (Belkaid et al., 2002) and viruses (Suvas et al., 2003) as well as antitumor immunity induced by therapeutic vaccines (Sutmuller et al., 2001). Manipulation of Treg function is therefore an important goal of immunotherapy since this T cell subset appears central to the balance between immune tolerance and immune responsiveness for multiple disease processes.

Elucidation of Treg mechanisms and manipulation of this subset would be greatly facilitated by the identification of Treg-selective cell surface receptors that modulate their function. While the transcription factor Foxp3 appears to be selectively expressed by thymically derived Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), to date no Treg-specific cell surface receptors have been described. CD25, GITR (McHugh et al., 2002; Shimizu et al., 2002), and CTLA-4 (Read et al., 2000), the cell surface receptors commonly used to mark Tregs, are highly upregulated on effector/memory cells upon antigen-driven activation and are therefore not Treg specific. In order to identify Treg-specific molecules, we performed a differential gene expression analysis of antigen-specific T cells, differentiating to either effector/memory cells in response to viral infection or Tregs upon encounter of cognate antigen as a self-antigen. This analysis revealed that the LAG-3 gene was selectively upregulated in Tregs. The physiologic role of LAG-3, an MHC class II binding CD4 homolog, has not been clearly elucidated. Several studies have suggested that LAG-3 may have a negative regulatory function (Hannier et al., 1998; Huard et al., 1994; Workman et al., 2002a, 2002b, 2004; Workman and Vignali, 2003). Here, we show that membrane expression of LAG-3 selectively marks induced Tregs, that LAG-3 modulates both the in vitro and in vivo suppressive function of Tregs, and that ectopic expression of LAG-3 is sufficient to confer regulatory activity.

## Results

### Induced Tregs with Potent Regulatory Activity

In order to study differences between T cell effector/memory and tolerance induction, we have utilized adoptive transfer of T cell receptor (TCR) transgenic $CD4^+$ T cells (clone 6.5) specific for a model antigen hemagglutinin (HA). In wild-type mice infected with recombinant HA-expressing vaccinia virus (Vac-HA), adoptively transferred HA-specific 6.5 $CD4^+$ T cells differentiate into effector/memory cells upon encounter with HA. The effector/memory response is characterized by a typical expansion/contraction phase and the development of memory markers. When removed from the adoptively transferred animal, these effector/memory cells are hyperresponsive to HA in vitro relative to naive 6.5 $CD4^+$ T cells as assayed by antigen-specific proliferative response and $\gamma$-interferon production. This memory response persists for months after adoptive transfer. In contrast, adoptive transfer of 6.5 $CD4^+$ T cells into C3-HA transgenic mice that ex-

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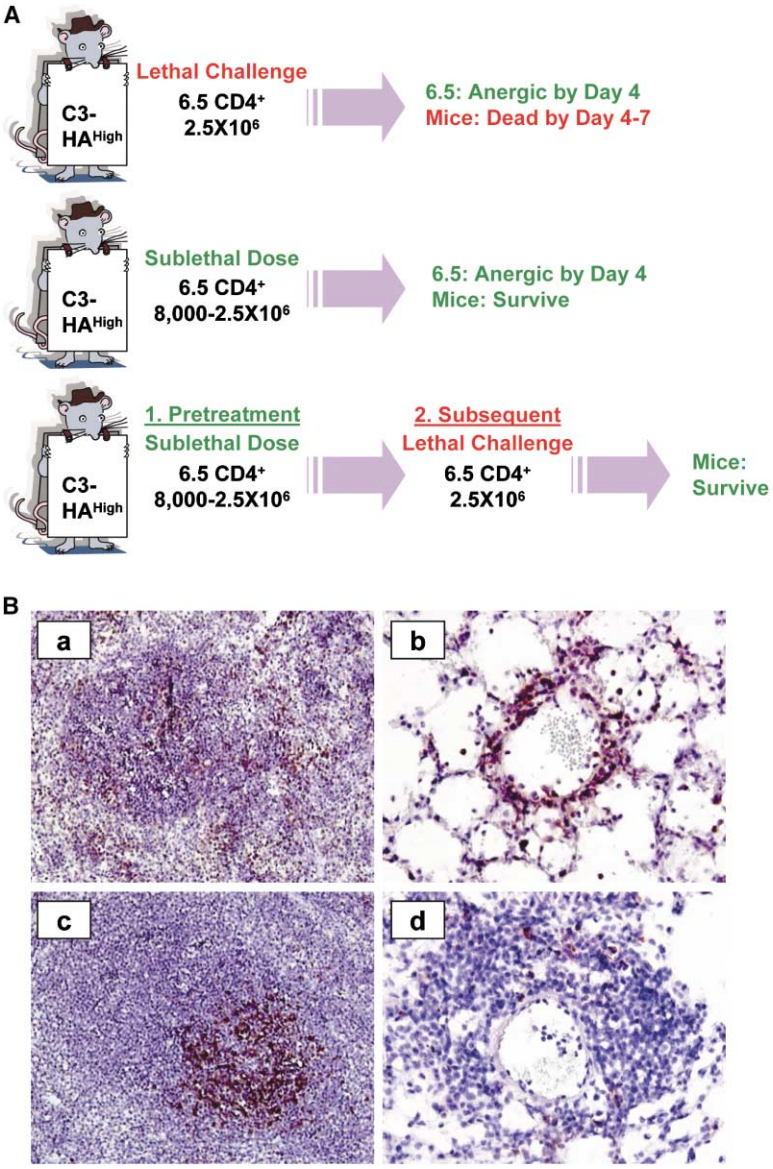



Figure 1. HA-Specific CD4$^+$ T Cells Become Tolerant and Develop Regulatory T Cell Activity upon Adoptive Transfer into C3-HA$^{high}$ Transgenic Mice

(A) C3-HA$^{high}$ transgenic mice express high levels of HA in various epithelial compartments, with the highest level expressed in pulmonary epithelia. C3-HA$^{high}$ recipients die 4–7 days after adoptive transfer of 2.5 $\times$ 10$^6$ HA-specific TCR transgenic (6.5) CD4$^+$ T cells due to pneumonitis associated with a transient effector phase of activation occurring prior to development of an anergic phenotype. Transfer of smaller numbers of 6.5 CD4$^+$ T cells results in less severe pulmonary pathology, and the C3-HA$^{high}$ recipients survive the transfer. Residual 6.5 T cells become anergic as defined by their inability to produce $\gamma$-interferon or proliferate to HA antigen in vitro. Mice receiving a sublethal dose of 6.5 T cells are protected from subsequent infusion of 2.5 $\times$ 10$^6$ naive 6.5 T cells. Thus, the initial tolerized T cells develop Treg activity that suppresses lethal pneumonitis induced by the second high dose of 6.5 T cells. (B) Localization of effector/memory versus suppressed T cells in C3-HA$^{high}$ mice. Naive 6.5 T cells (Thy1.1$^+$/1.2$^-$) were adoptively transferred into C3-HA$^{high}$ recipients (Thy1.1$^-$/1.2$^+$), either in the absence or in the presence of 6.5 anergic cells/Tregs (Thy1.1$^-$/1.2$^+$). Spleens and lungs were harvested 3 days after adoptive transfer, and Thy 1.1$^+$ cells were stained by immunohistochemistry (Thy1.1$^+$ effector T cells are brown; sections are counterstained with hematoxylin). In the absence of Tregs, T effector cells (brown) are scattered in the splenic follicles (a) and infiltrate the pulmonary vessels (b). In the presence of Tregs, suppressed HA-specific 6.5 T cells (brown) become sequestered in the splenic periarteriolar lymphatic sheath (c) and fail to infiltrate the pulmonary vessels (d).

press HA in multiple epithelial tissues results in tolerance (Adler et al., 1998, 2000). Similar to the effector/memory response, there is a rapid expansion/activation phase characterized by proliferation and expression of effector cytokines, such as $\gamma$-interferon. However, after the activation phase, the total HA-specific T cell pool contracts and residual 6.5 cells fail to produce $\gamma$-interferon or proliferate in vitro upon antigen stimulation 4–7 days after adoptive transfer (Adler et al., 2000; Huang et al., 2003). The extinction of the capacity to produce lymphokines such as IL-2 and $\gamma$-interferon and to proliferate in response to antigen represents the standard operational definition of the anergic phenotype.

The intensity of the initial in vivo effector phase in C3-HA mice that precedes tolerance induction is proportional to the number of 6.5 CD4$^+$ T cells adoptively transferred as well as the expression level of HA antigen in the recipient mice. Thus, C3-HA$^{low}$ mice tolerate the transfer of 2.5 $\times$ 10$^6$ 6.5 CD4$^+$ T cells quite well, but C3-HA$^{high}$ mice, which have 1000-fold higher HA expression than C3-HA$^{low}$ mice, die within 4–7 days after transfer of 2.5 $\times$ 10$^6$ 6.5 CD4$^+$ T cells (Figure 1A). The cause of death is lethal pulmonary vasculitis due to infiltration of transgenic 6.5 CD4$^+$ T cells in the lung where HA expression is highest. Adoptive transfer of less than 2.5 $\times$ 10$^5$ 6.5 CD4$^+$ T cells into C3-HA$^{high}$ mice causes pulmonary vasculitis of less severity, and the recipients survive (Figure 1A) (Huang et al., 2003). Interestingly, 6.5 CD4$^+$ T cells transferred at a sublethal dose acquire a regulatory phenotype as they are capable of protecting mice from death upon subsequent infusion of what would be a lethal dose of 6.5 CD4$^+$ T cells in unprotected C3-HA$^{high}$ mice. This in vivo regulatory function is extremely potent, since transfer of as few as 8000 cells (0.3% of the lethal dose) will completely protect animals from death upon subsequent infusion of 2.5 $\times$ 10$^6$ naive 6.5 CD4$^+$ T cells. Protection is observed as early as 4 days after the initial transfer and remains active up to

6 months (Figure 1A). Depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, before adoptive transfer totally eliminates the protective effect, thereby defining the Treg phenotype of anergized clonotypic 6.5 $CD4^+$ T cells.

In unprotected mice, the infiltrating T effector cells that cause the pulmonary vasculitis are scattered in the spleen and accumulate around the vessels in the lung (Figures 1Ba and 1Bb). Suppression of lethal pneumonitis is accompanied by an accumulation of the initial input (Treg) 6.5 T cells in the lungs and a drastic reduction in the number of infiltrating T effector cells from the second infusion (Figure 1Bd). Paradoxically, this results in a greater cellular infiltration but no death. Instead of accumulating in the lungs, as occurs in the absence of Tregs, the T effector cells accumulate in the splenic periarteriolar lymphatic sheath (Figure 1Bc). Further evidence that the anergic cells demonstrate Treg function comes from the finding that they inhibit the activation of cytotoxic HA-specific $CD8^+$ T cells in vivo (data not shown). Elimination of $CD25^+$ T cells prior to the first (protective) adoptive transfer did not affect the development of Tregs capable of protecting animals from a subsequent lethal challenge of 6.5 T cells. Therefore, it is likely that the Treg phenotype of the initial input T cells was acquired after adoptive transfer as opposed to being a consequence of naturally occurring Tregs among the adoptively transferred population. These findings are highly compatible with the observations of Von Boehmer and colleagues, who demonstrated that 6.5 $CD4^+$ T cells rendered tolerant after transfer into transgenic mice expressing HA in the B cell compartment in fact exhibit Treg function (Jooss et al., 2001).

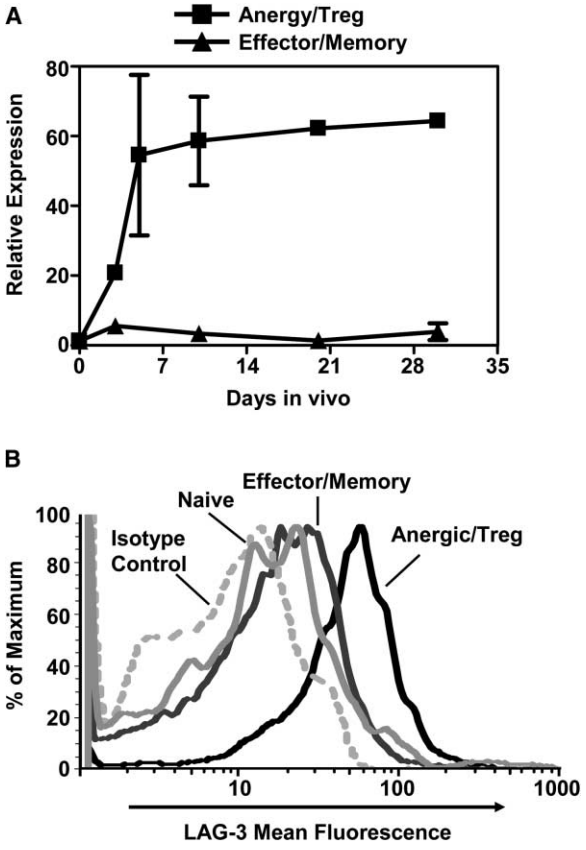



Figure 2. LAG-3 Is Differentially Expressed between Anergic/Treg and Effector/Memory $CD4^+$ T Cells

(A) The differential expression revealed by gene chip analysis was confirmed by quantitative real-time RT-PCR. The differential expression of LAG-3 at early time points (day 2 to day 4) extends to 30 days after adoptive transfer.

(B) Cell surface LAG-3 protein levels were assessed by antibody staining. Splenocytes were harvested from C3-$HA^{high}$, wild-type B10.D2 mice immunized with Vac-HA or wt B10.D2 mice 5 days after i.v. injection with $6.5^+$ $Thy1.1^+$ splenocytes. All samples were first incubated with whole rat IgG to block Fc receptors and then stained with an anticlonotypic 6.5 TCR mAb plus streptavidin-APC and LAG-3-PE or an irrelevant rat IgG1-PE control. Cells were gated on $6.5^+$ T lymphocytes. Isotype control, dashed light gray line; naive cells, solid light-gray line; effector/memory cells, solid dark-gray line; anergic cells/Tregs, solid black line.

### LAG-3 Is Differentially Expressed on Induced Tregs

In order to identify genes associated with the anergic/Treg phenotype in our in vivo system, we performed Affymetrix chip analysis on purified 6.5 $CD4^+$ T cells either after adoptive transfer into nontransgenic recipients followed by Vac-HA immunization to generate effector/memory T cells or after transfer into C3-$HA^{high}$ mice to generate anergic cells/Tregs. $Thy1.1^+/Thy1.2^-$ congenic 6.5 T cells were purified from $Thy1.1^-/Thy1.2^+$ Vac-HA-infected wild-type (effector/memory) or C3-$HA^{high}$ (anergic/Treg) recipients with a sequential isolation procedure involving MACS column depletion of $CD8^+$ T cells, B cells, and Thy $1.2^+$ T cells followed by flow cytometric sorting to $>95\%$ purity. This protocol avoids the use of TCR-specific or CD4 coreceptor-specific antibodies that could potentially alter TCR- or CD4-dependent gene expression patterns.

RNA was isolated from naive 6.5 $CD4^+$ T cells as the day 0 sample. RNA was also isolated from 6.5 $CD4^+$ T cells at days 2, 3, and 4 postadoptive transfer for chip analysis. HA-specific T cells show increasing unresponsiveness to in vitro stimulation from days 2–4. This development of anergy correlates with the acquisition of suppressive activity in in vitro suppression assays (data not shown). Genes that were differentially expressed between anergic/Treg populations and effector/memory populations were ranked according to an algorithm that summed their differential expression from days 0–4. A surprisingly large number of genes were selectively activated in anergic/Treg populations even at these early time points postadoptive transfer. Many of these genes represented ESTs with no known function. Among the genes that had been previously identified, LAG-3 was among the most differentially expressed in anergic/Treg populations relative to effector/memory populations. This result was subsequently validated by quantitative RT-PCR analysis with a LAG-3 primer-probe pair for various time points extended to 1 month postadoptive transfer. After a minimal initial increase in the effector/memory cells, LAG-3 expression returns to baseline by 20 days postadoptive transfer. In striking contrast, LAG-3 expression increases 20- to 50-fold over the first 5 days in anergic cells/Tregs and remains high over the subsequent 4 week analysis (Figure 2A). In contrast, levels of Foxp3, GITR, and CTLA-4 showed modest increases (1.5- to 4-fold) that were similar in both effector/

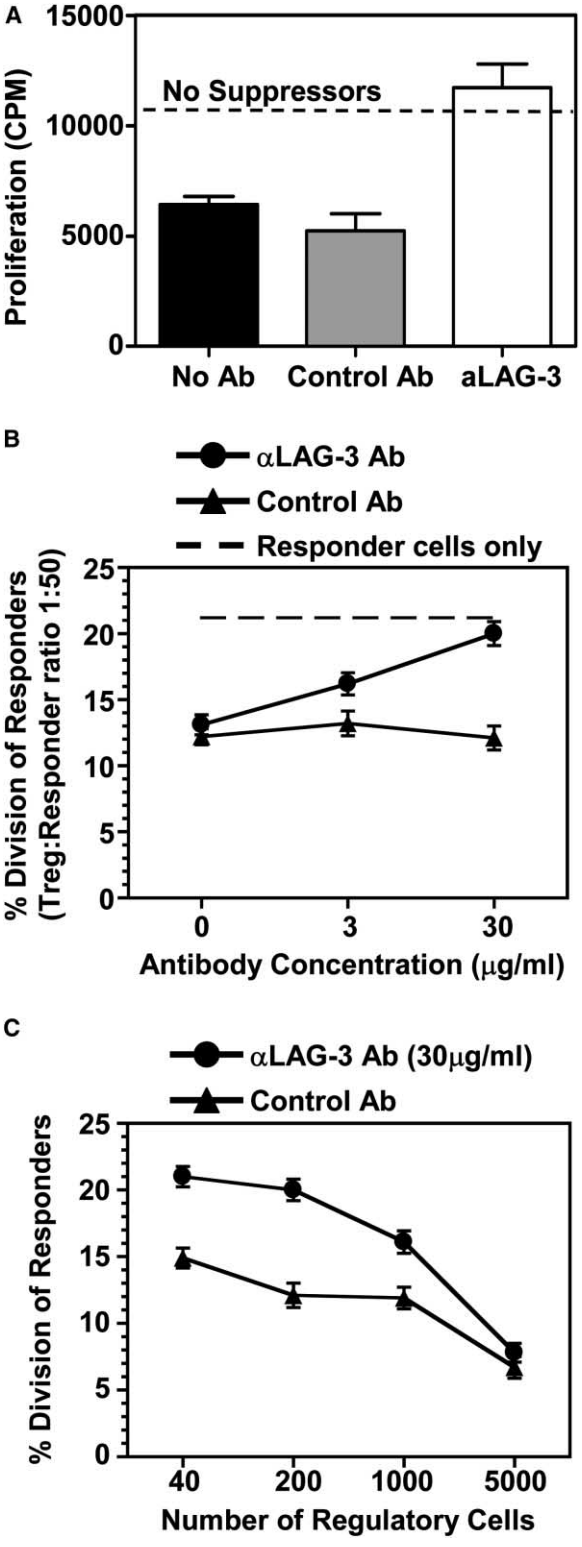



Figure 3. Anti-LAG-3 Antibodies Block In Vitro Treg Activity

(A) Monoclonal anti-LAG-3 antibody added to the in vitro suppression assay at a concentration of 2 μg/ml totally reverses the suppression of naive 6.5 $CD4^+$ T cell proliferation in vitro by 6.5 $CD4^+$ suppressors at a suppressor:responder ratio of 1:25. Dashed line, no Tregs (suppressors).

(B) T cells with regulatory function (Treg) were induced by adoptive transfer of $2.5 \times 10^6$ HA-specific $Thy1.1^+$ CD4 T cells to $Thy1.2^+$ C3-HA recipients. On day 4 after adoptive transfer, $CD4^+$ $Thy1.1^+$ T cells (Treg) were purified by FACS sorting. Tregs were mixed at a ratio of 1:50 with naive CFSE-labeled HA-specific $Thy1.2^+$ $CD4^+$ responder T cells in the presence of increasing concentrations of anti-LAG-3 antibody (circles) or isotype control (triangles). After 60 hr, cells were harvested and analyzed. Division of responder cells was determined with the method of Lyons (Lyons, 2000) and the software package FlowJo (Tree Star Inc, Ashland, OR). Dashed line, no Tregs (responder cells only). Mean ± RMS (Root Mean Square) error for triplicate wells is shown.

(C) Tregs were isolated as above and cocultured at increasing numbers with naive CFSE-labeled HA-specific $Thy1.2^+$ $CD4^+$ responder T cells in the presence of anti-LAG-3 or isotype control antibody (30 μg/ml). Mean ± RMS error for triplicate wells is shown.

memory cells and the induced anergic cells/Tregs over the first 4–5 days (data not shown).

Cell surface expression of LAG-3 on populations of anergic/Treg 6.5 $CD4^+$ T cells relative to effector/memory 6.5 $CD4^+$ T cells was then analyzed with an anti-LAG-3 monoclonal antibody (Workman et al., 2002b). While there are very low levels of LAG-3 staining on effector/memory cells, the majority of anergic cells/Tregs from C3-$HA^{high}$ transgenic mice display moderate to high levels of LAG-3 staining, correlating with the gene expression results (Figure 2B).

### LAG-3 Is Required for Maximal Treg Function In Vitro

To determine whether LAG-3 has a direct role in regulating suppression by induced Tregs, we first determined whether anti-LAG-3 antibodies could block the ability of LAG-3-expressing cells to suppress the in vitro proliferative responses of naive HA-specific $CD4^+$ 6.5 TCR transgenic T cells. Anti-LAG-3 antibodies at concentrations as low as 2 μg/ml inhibit suppression by Treg 6.5 $CD4^+$ T cells in this in vitro assay system (Figure 3A). Over the 2 day assay period, anti-LAG-3 antibodies did not affect proliferative responses of 6.5 T cells stimulated in the absence of Tregs, confirming that the effect of anti-LAG-3 antibodies was indeed on the Tregs and not the effector cells (data not shown). In order to directly assess the effect of the induced Tregs on responder T cell proliferation, a similar assay was performed with CFSE-labeled naive 6.5 T cells. Flow cytometry was used to determine the percentage of cells with reduced CFSE fluorescence ($CFSE^{lo}$), which would be indicative of proliferation. Our experiments show that titration of the anti-LAG-3 antibody results in a progressive reduction in Treg activity as indicated by a reciprocal increase in the percentage of $CFSE^{lo}$ cells, whereas the isotype control had no effect (Figure 3B). Although the anti-LAG-3 antibody was clearly able to block a significant proportion of the suppressor activity at low Treg numbers, the effect was more limited at high Treg numbers (Figure 3C). This is not surprising as it is likely that Tregs utilize multiple molecules to mediate suppression, and these cells appear to have strong regulatory potential. The ability of anti-LAG-3 antibodies to block in vitro suppression by Tregs demonstrates that LAG-3 is not simply a Treg-selective marker but is a molecule that is required for maximal Treg activity.

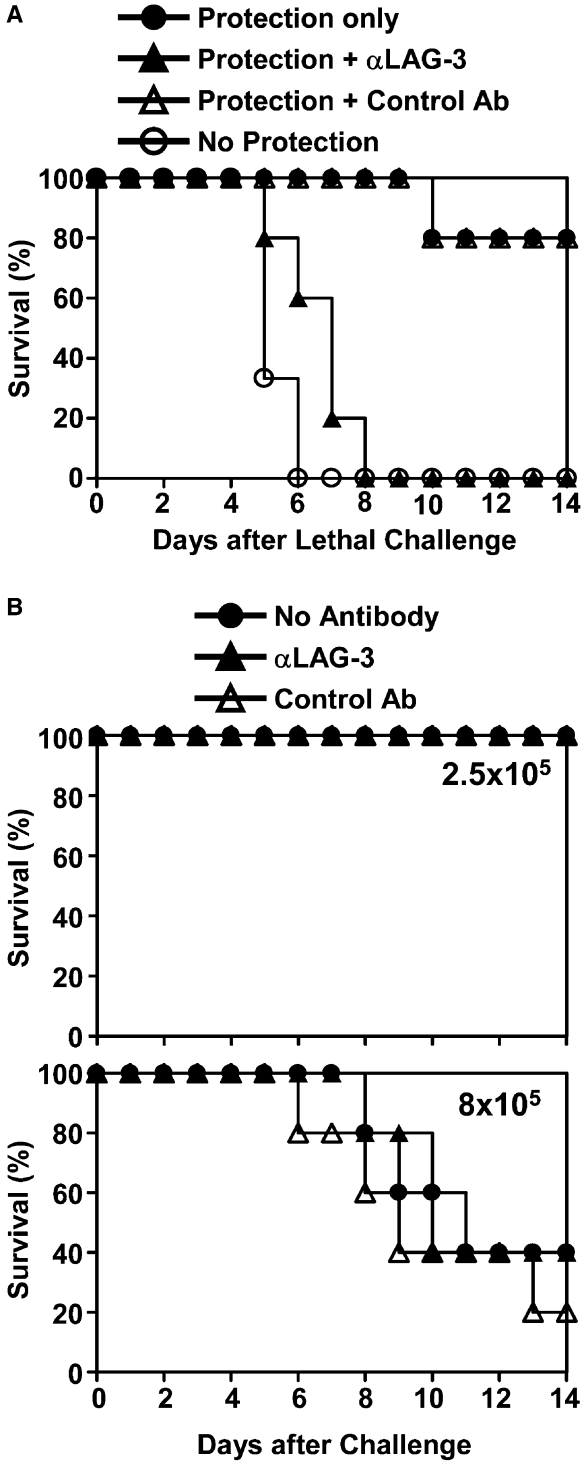



Figure 4. In Vivo Anti-LAG-3 Antibody Treatment Blocks 6.5 $CD4^+$ Treg-Mediated Protection from Lethal Pulmonary Vasculitis

(A) C3-$HA^{high}$ mice pretreated with 8000 6.5 $CD4^+$ T cells survived subsequent challenge with $2.5 \times 10^6$ 6.5 $CD4^+$ T cells given 4 days after the initial transfer establishment of Treg population (protection only). Without the sublethal pretreatment, the C3-$HA^{high}$ recipients died 4–6 days after lethal challenge (no protection). Monoclonal anti-LAG-3 antibody (200 μg) was given i.v. to the C3-$HA^{high}$ mice with the lethal dose of 6.5 T cells 4 days after they were pretreated with 8000 6.5 $CD4^+$ T cells, and another dose of 200 μg was given 2 days later. Anti-LAG-3 antibody-treated mice could no longer tolerate the subsequent lethal challenge (protection + αLAG-3). In contrast, treatment with isotype control antibody rat IgG1 could not eliminate the in vivo suppression (protection + control Ab). A representative experiment (three to five animals per group) of five is shown.

(B) Anti-LAG-3 mAb does not hyperactivate naive 6.5 $CD4^+$ T cells in the absence of Tregs. C3-$HA^{high}$ mice received either $2.5 \times 10^5$ (sublethal dose) or $8 \times 10^5$ (partial lethality between 7 and 14 days after transfer) naive 6.5 $CD4^+$ T cells in combination with anti-LAG-3 antibody, control rat IgG1, or no antibody. No lethality was observed with the anti-LAG-3 antibody infusions at the $2.5 \times 10^5$ dose, whereas lethality at the $8 \times 10^5$ dose was not affected by anti-LAG-3 antibody.

**LAG-3 Is Required for Induced Treg Activity In Vivo**

We next evaluated the role of LAG-3 in modulating in vivo Treg function by determining whether administration of anti-LAG-3 antibodies could block suppression of lethal pneumonitis by Tregs in C3-$HA^{high}$ mice. C3-$HA^{high}$ mice were pretreated with 8000 (sublethal dose) 6.5 $CD4^+$ T cells followed by a subsequent dose of $2.5 \times 10^6$ naive 6.5 $CD4^+$ T cells 4 days after the first transfer. As described above, Tregs have already developed at this point. Anti-LAG-3 antibody (200 μg) was administered i.v. together with the subsequent challenge of $2.5 \times 10^6$ 6.5 cells, and another 200 μg was given 2 days later. This antibody treatment totally eliminated the in vivo suppressive activity of the Tregs, and the mice died in a time frame comparable to the C3-$HA^{high}$ mice lethally challenged without protective sublethal 6.5 pretreatment (Figure 4A). On the contrary, mice with established Tregs treated with isotype control antibody (Rat IgG1) or no antibody survived subsequent challenge with $2.5 \times 10^6$ naive 6.5 T cells. Importantly, parallel control experiments clearly show that the anti-LAG-3 antibody does not deplete the protective Thy1.1$^+$ 6.5$^+$ T cells in vivo. These cells are readily detected ex vivo by staining with an anti-rat IgG1 antibody, which binds to the in vivo anti-LAG-3 antibody-coated Tregs (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/4/503/DC1/). Furthermore, we saw no reduction in the percentage of Thy1.1$^+$ cells in the anti-LAG-3-treated mice compared with the untreated controls (data not shown).

An alternate possibility was that the anti-LAG-3 antibodies were hyperactivating the T cells in the challenge population such that they overcame the inhibitory effects of the Tregs. To rule out this possibility, we asked whether in vivo administration of anti-LAG-3 antibodies together with a dose of 6.5 T cells just below the lethality threshold would cause lethality in the absence of a preestablished Treg population. We therefore administered $2.5 \times 10^5$ 6.5 $CD4^+$ T cells (the maximal dose that will not cause lethality) or $8.0 \times 10^5$ 6.5 $CD4^+$ T cells (roughly 50% lethality between 7 and 14 days after transfer) into C3-$HA^{high}$ mice together with anti-LAG-3 antibodies or isotype control. Our data clearly show that the anti-LAG-3 treatment did not render the $2.5 \times 10^5$ 6.5 $CD4^+$ T cell dose lethal nor did it enhance the partial lethality of the $8.0 \times 10^5$ 6.5 $CD4^+$ T cell dose (Figure 4B). In summary, these data confirm that the anti-LAG-3 antibody prevents protection by blocking LAG-3-mediated Treg function rather than mediating their deletion or promoting lethality mediated by the effector population.

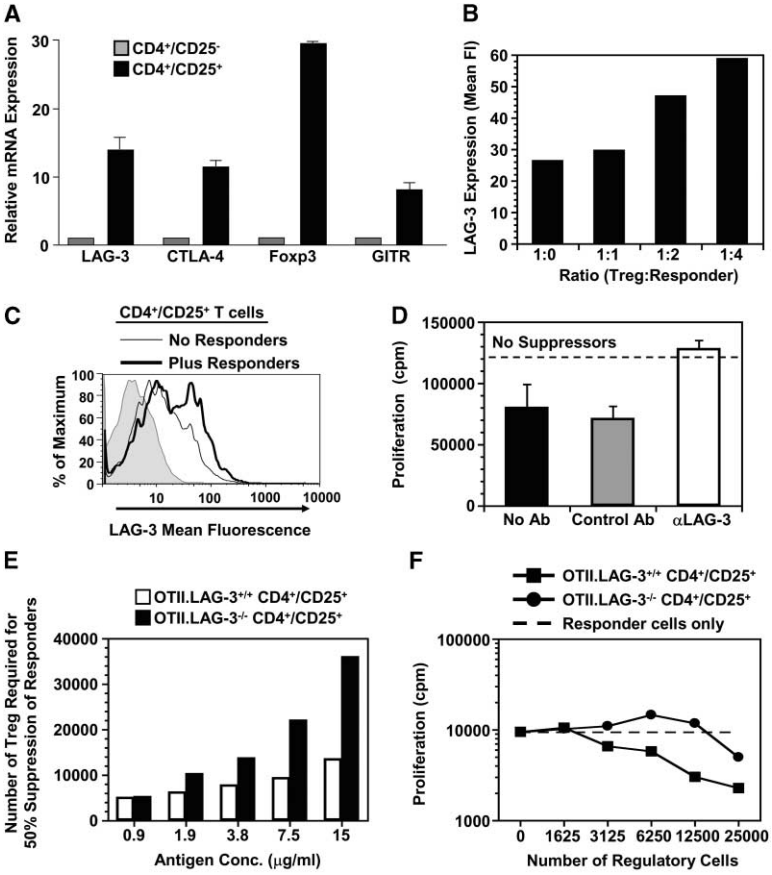



Figure 5. LAG-3 Is Expressed on Natural $CD4^+CD25^+$ Tregs and Is Required for Maximal Natural $CD4^+CD25^+$ Treg Function

(A) Natural $CD4^+CD25^+$ Tregs have higher levels of LAG-3 mRNA expression compared to their $CD4^+CD25^-$ counterparts. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from wild-type BALB/c lymph nodes. $CD4^+CD25^+$ T cells, the population documented to contain natural regulatory T cells, have significantly higher mRNA levels for CD25 and LAG-3, as well as for CTLA-4, GITR, and Foxp3, as compared to the $CD4^+CD25^-$ cells (expression of each mRNA in the $CD4^+CD25^-$ subset was normalized to a value of one).

(B) LAG-3 expression (Mean FI) was determined on $CD4^+CD25^+$ Tregs that were sorted from BALB/c spleens and activated with anti-CD3 antibody (5 μg/ml) and APCs in the absence or presence of increasing numbers of $CD4^+CD25^-$ responder T cells.

(C) Expression of LAG-3 on $CD4^+CD25^+$ T cells after activation with (bold line) or without (thin line) $CD4^+CD25^-$ T cells present at a ratio of 1:2 (Treg:responder). The shaded histogram represents the negative staining control. The data are representative of four experiments.

(D) Sorted $CD4^+CD25^+$ T cells from BALB/c mouse lymph nodes were used as suppressors and $CD4^+CD25^-$ T cells as responders in an in vitro suppression assay (suppressor:effector ratio of 1:25) with anti-CD3 antibodies (0.5 μg/ml) as the T cell stimulus. Anti-LAG-3 antibodies at the concentration of 50 μg/ml reverse the in vitro suppression of natural $CD4^+CD25^+$ regulatory T cells, whereas isotype control antibody does not. Dashed line, no Treg suppressors. Irradiated (3000 rads) BALB/c splenocytes were used as APC.

(E) $CD4^+CD25^+$ T cells were positively sorted from spleens of OT-II.LAG-$3^{+/+}$ and OT-II.LAG-$3^{-/-}$ mice. The sorted T cells were mixed at different ratios with irradiated (3000 rads) C57BL/6 APC, naive $CD4^+CD25^-$ OT-II.LAG-$3^{-/-}$ T cells ($2.5 \times 10^4$ cells/well), and various concentrations of OVA 326–339 peptide. The number of Tregs required to achieve 50% suppression of the responders was determined at each antigen concentration. The data represent the mean of three individual experiments. The percentage of $CD25^+CD4^+$ Tregs that are OT-II$^+$ ($V\alpha2^+V\beta5^+$) is 52.3% ± 8.1% in wild-type mice and 56.7% ± 3.8% in LAG-3.KO mice (mean of four mice per genotype).

(F) A standard Treg proliferation assay was performed with a titration of $CD4^+CD25^+$ T cells from OT-II.LAG-$3^{+/+}$ and OT-II.LAG-$3^{-/-}$ mice in the presence of APCs, $CD4^+CD25^-$ OT-II.LAG-$3^{-/-}$ T cells, and a high peptide concentration (7.5 μg/ml).

### LAG-3 Is Expressed by Natural Tregs and Is Required for Maximal Regulatory Activity

Taken together, these data confirm an important role for LAG-3 in mediating suppressor function of induced Tregs. Given that the relationship between induced Tregs and natural Tregs remains unclear, it was of interest to see whether the LAG-3 message was expressed selectively in $CD4^+CD25^+$ T cells from wild-type mice. LAG-3 mRNA (along with CTLA-4, Foxp3, and GITR mRNA) is indeed selectively expressed in $CD4^+CD25^+$ cells compared with $CD4^+CD25^-$ cells (Figure 5A). Paradoxically, we could not detect any LAG-3 protein on the cell surface of $CD4^+CD25^+$ T cells directly ex vivo. This observation was consistent with our previous finding that significant amounts of LAG-3 mRNA are detectable in the splenic red pulp, but the percentage of LAG-$3^+$ splenocytes is less than 2% (Workman et al., 2002b). Interestingly, LAG-3 possesses a rather long 5′ untranslated sequence that is rich in CT and GT repeats, which could play a role in posttranscriptional regulation of gene expression (Mastrangeli et al., 1996). However, stimulation of $CD4^+CD25^+$ Tregs with anti-CD3 and APCs induced significant levels of LAG-3, which also occurred in the presence of MHC class $II^{-/-}$ APCs (Figures 5B and 5C, and data not shown). Interestingly, coculture of Tregs with anti-CD3-stimulated naive $CD4^+CD25^-$ T cells induced a 2-fold increase in LAG-3 expression, suggesting that the presence of effector T cells may enhance their LAG-3-mediated regulatory activity. It is noteworthy that this increase occurred in a dose-dependent manner (Figure 5B).

To directly evaluate the role of LAG-3 in the regulatory function of natural Tregs, we asked whether anti-LAG-3 antibodies could inhibit in vitro suppression mediated by purified $CD4^+CD25^+$ cells. As shown in Figure 5D, $CD4^+CD25^+$ Treg activity is blocked by anti-LAG-3 antibodies. Finally, we assessed whether natural $CD4^+CD25^+$ Tregs from LAG-$3^{-/-}$ OT-II TCR transgenic mice have comparable regulatory activity to those derived from wild-type OT-II transgenic mice. In these experiments, we assessed the influence of both Treg number and antigen dose. The data are expressed as the number of Tregs required to give 50% suppression. At low peptide concentrations, where Tregs can com-

fortably control $CD4^+CD25^-$ T cell proliferation, LAG-$3^{-/-}$ and wild-type Tregs have equivalent suppressive capacity. However at high peptide concentrations, where maximal Treg activity is obligatory, 2.6 times as many LAG-$3^{-/-}$ versus wild-type $CD4^+CD25^+$ Tregs are required to control $CD4^+CD25^-$ T cell proliferation (Figures 5E and 5F). Taken together, these data suggest that LAG-3 plays a significant role in suppression mediated by natural, as well as induced, Tregs.

### Ectopic Expression of LAG-3 Confers Regulatory Activity

The data presented thus far suggest that LAG-3 is required for maximal Treg function. However, is it sufficient? We performed a series of transduction experiments to determine if ectopic expression of LAG-3 on T cells confers regulatory activity. For these experiments, 6.5 $CD4^+$ T cells were first depleted of any $CD25^+$ natural Tregs and then transduced with MSCV-based retrovirus encoding either GFP alone, GFP + wild-type LAG-3, or GFP + a mutant LAG-3.Y73FΔCY that has substantially reduced affinity for MHC class II and cannot mediate downstream signaling (Workman et al., 2002a). After a 10 day rest period, essentially no endogenous LAG-3 staining was observed on $GFP^+$ $CD4^+$ 6.5 T cells transduced with the MSCV-GFP retrovirus, whereas high levels of LAG-3 staining were observed on $GFP^+$ 6.5 cells transduced with the MSCV-LAG-3/GFP and MSCV-LAG-3.Y73FΔCY/GFP retrovirus.

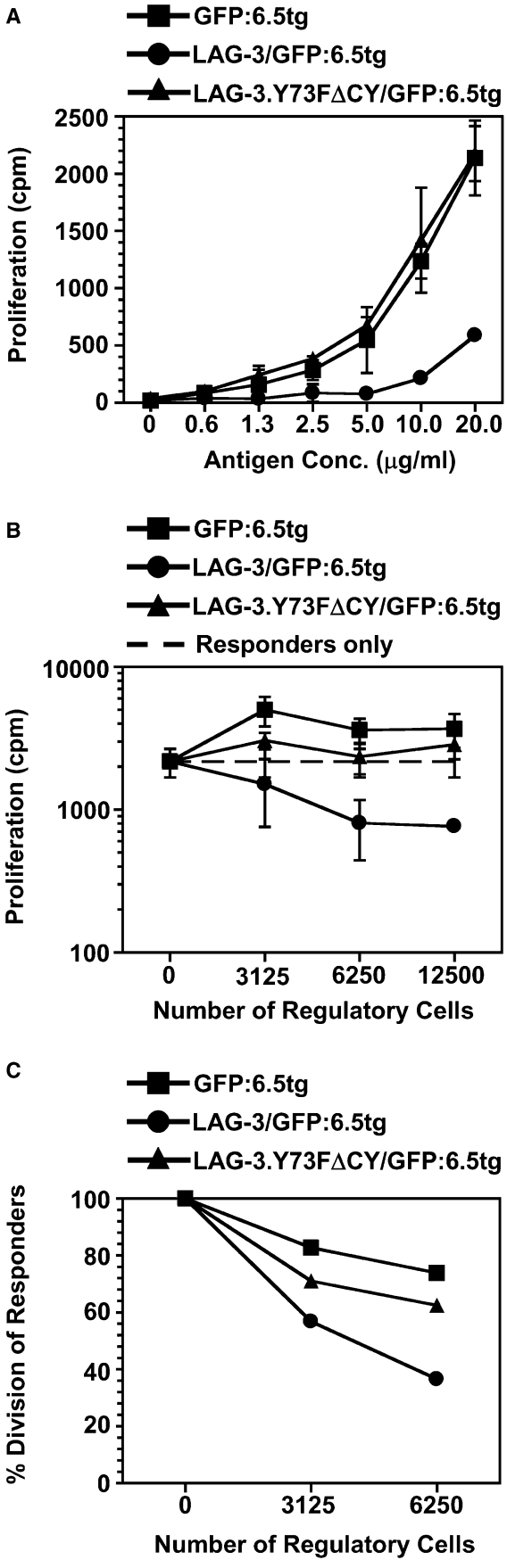
We first assessed the effect of LAG-3 expression on T cell proliferation. $GFP^+$ cells from each group were sorted and stimulated with $HA^{110–120}$ peptide plus APC. T cells expressing the nonfunctional LAG-3 mutant or GFP alone responded comparably. In contrast, substantially reduced proliferation was seen with LAG-3-transduced 6.5 T cells after peptide stimulation (Figure 6A). We next asked if ectopic expression of LAG-3 was sufficient to confer regulatory activity toward naive T cells. $GFP^+$ cells were mixed at different ratios with APC, $HA^{110–120}$ peptide, and naive 6.5 $CD4^+CD25^-$ T cells in a standard Treg proliferation assay. No suppression was observed with control 6.5 cells expressing GFP alone or the nonfunctional LAG-3.Y73FΔCY mutant (Figure 6B). In fact, total proliferation was somewhat increased as these cells contribute to $[^3H]$thymidine uptake. In striking contrast, 6.5 cells expressing wild-type LAG-3 potently suppressed proliferation of the naive 6.5 cells. This was a robust and reproducible finding, which was also duplicated with nontransgenic T cells stimulated with anti-CD3 and OT-II TCR transgenic T cells stimulated with cognate peptide (data not shown). Furthermore, this suppression was contact- or proximity-dependent as determined in transwell assays (data not shown). Surprisingly, the suppressive capacity of LAG-3 was comparable to that seen in parallel experiments with ectopically expressed Foxp3.

As these experiments measure the combined proliferation of the transduced and naive 6.5 T cells, it is formally possible that much of the reduced $[^3H]$thymidine uptake is a consequence of the reduced proliferation of the LAG-3-transduced cells. To assess the proliferation of the effector population directly, naive $Thy1.2^+$ $CD25^-$ 6.5 T cells were labeled with CFSE and cocultured with $Thy1.1^+$ $GFP^+$-transduced cells in a standard Treg assay as described above. The percentage of $Thy1.2^+$ T cells with reduced CFSE expression (two or more divisions, $CFSE^{lo}$) was determined by flow cytometry. Some reduction in the percentage of $CFSE^{lo}$ cells was seen with increasing numbers of $GFP^+$ or LAG-3.Y73FΔCY/$GFP^+$-transduced T cells, which is likely due to competition for peptide-pulsed APC as the control cells in this instance are also responding (Figure 6C). However, the percentage of $CFSE^{lo}$ cells seen in the presence of LAG-3-transduced T cells was considerably lower, suggesting that these cells do have a direct effect on the proliferative capacity of naive $CD25^-$ 6.5 T cells. It should be noted that as the LAG-3-transduced cells proliferate poorly (Figure 6A), they are less likely to compete with the responders for antigen and APC, and thus the reduction seen is a direct consequence of suppression by the LAG-3-transduced cells. Taken together, these data provide direct evidence that confirms the functional role of LAG-3 in suppression. Interestingly, LAG-3 transduction did not induce other genes associated with Tregs, including Foxp3, CD25, CD103, and GITR (data not shown). Likewise, we have not seen any induction of LAG-3 after Foxp3 transduction. This result, together with the lack of significant differential expression of Foxp3, CD25, CD103, and GITR between 6.5 T cells differentiating to effector/memory versus anergic/Treg phenotypes suggests that LAG-3 may mediate a distinct pathway of regulatory T cell function independent of the Foxp3 pathway.

## Discussion

These findings identify LAG-3 as a cell surface molecule selectively upregulated on Tregs that may be directly involved in mediating Treg function. Given the many systems in which both natural and induced Treg activity has been defined, it remains to be determined whether LAG-3 is a universal Treg marker or selectively marks only certain Treg subsets. Our results suggest that in addition to the induced $CD4^+$ Tregs studied here, LAG-3 plays at least some role in mediating suppression by natural $CD4^+CD25^+$ Tregs. Furthermore, other experimental data demonstrate a role for LAG-3 in the regulation of homeostatic lymphocyte expansion by natural Tregs (C.J.W. and D.A.A.V., unpublished data). At this stage we do not propose that LAG-3 is a "lineage marker" for Tregs, and it remains to be determined, as is the case for Foxp3, whether LAG-3 is expressed in all cells with regulatory activity. In fact, it is not clear that Tregs represent a stable lineage or differentiation state capable of promoting tolerance in a noncell-autonomous fashion (von Boehmer, 2003). However, our data do suggest that LAG-3 may mark cells with regulatory activity.

A number of studies have suggested a cell-autonomous inhibitory role for LAG-3 (Huard et al., 1994; Workman et al., 2002b). Given our proposed role for LAG-3 in Treg function, it might be expected that LAG-3 knockout mice would display multisystem autoimmunity similar to that seen in Foxp3 knockout or scurfy mice. However, initial studies with LAG-3 knockout mice failed to uncover any evidence for overt autoimmunity or hyperim-




munity (Miyazaki et al., 1996). Nonetheless, there are clearly regulatory T cell defects displayed by LAG-3 knockout mice, such as a defect in regulating cellular homeostasis (C.J.W. and D.A.A.V., unpublished data) and those detailed in the present study. We are in the process of reexamining older LAG-3 knockout mice for more subtle evidence of late-onset autoimmunity as was observed in PD-1 knockout mice (Nishimura et al., 1999, 2001). It is also conceivable that other regulatory mechanisms might have been enhanced in these mice to compensate for the loss of LAG-3 expression.

As LAG-3 is expressed on Tregs, it provides an excellent potential target for selective manipulation of Treg activity to treat both cancer and autoimmune disease. Indeed, our in vivo treatment of mice with anti-LAG-3 completely blocked Treg-mediated protection from lethal pulmonary vasculitis. CD25, the gold standard Treg marker, is induced at high levels in activated cells, as it is a critical component of the IL-2 receptor complex. The apparent reason that $CD4^+CD25^+$ cells are enriched in Treg activity is not because CD25 is specific to Treg function, but rather, because Tregs are chronically stimulated by continuous encounter with self-antigen in the periphery. Recently, the TNF receptor superfamily member 18 molecule (also called GITR) was demonstrated to be upregulated on Tregs. Antibodies to GITR have been reported to inhibit Treg activity both in vivo and in vitro. However, GITR is equivalently upregulated on activated T cells and, therefore, is apparently no more selective as a marker for Tregs than is CD25 (McHugh et al., 2002; Shimizu et al., 2002). Moreover, there are numerous reports that $CD4^+CD25^-$ cell populations can suppress certain immune functions (Annacker et al., 2001; Apostolou et al., 2002; Curotto de Lafaille et al.,

Figure 6. Ectopic Expression of LAG-3 in 6.5 $CD4^+$ T Cells Reduces Proliferative Potential and Confers Potent Regulatory Activity

6.5 $CD4^+$ T cells (6.5tg) were first depleted of any $CD25^+$ natural Tregs and then transduced with MSCV-based retroviral vectors encoding either GFP alone, GFP + wild-type LAG-3, or GFP + a mutant LAG-3.Y73FΔCY that has diminished binding to MHC class II and cannot mediate downstream signaling. After a 10 day rest period, essentially no endogenous LAG-3 staining was observed on $GFP^+$ 6.5 $CD4^+$ T cells transduced with the MSCV-GFP vector, whereas high levels of LAG-3 staining were observed on $GFP^+$ 6.5 cells transduced with the MSCV-LAG-3/GFP and MSCV-LAG-3.Y73FΔCY/GFP vectors. $GFP^+$ cells from each group were sorted for subsequent experiments.

(A) $GFP^+$ 6.5 T cells were stimulated with $HA^{110–120}$ peptide at the concentrations indicated, pulsed with $[^3H]$thymidine on day 2, and harvested on day 3. The standard deviation of duplicate wells is shown. Differences observed were very reproducible and data representative of four experiments.

(B) Different numbers of $GFP^+$ 6.5 T cells were mixed with APC, 5 μg/ml $HA^{110–120}$ peptide, and naive 6.5 $CD4^+CD25^-$ cells in a standard Treg proliferation assay. Data represent the mean ± SEM of three experiments (two for the LAG-3 mutant) performed on separate occasions. In total, over ten experiments were performed at different antigen concentrations with comparable results.

(C) Treg assays were set up as in (B) with the following difference: $GFP^+$ 6.5 T cells were $Thy1.1^+$, and the naive 6.5 $CD4^+CD25^-$ cells used as responders were $Thy1.2^+$ and labeled with CFSE. The percent division of the responders was determined as a percentage of cells that had undergone at least two divisions compared to the total percent of responders that had divided in the absence of $GFP^+$ $Thy1.1^+$ cells. Data are representative of three experiments.

2001; Graca et al., 2002; Shimizu and Moriizumi, 2003; Stephens and Mason, 2000). More recently, neuropilin-1 has been suggested as a specific marker for $CD4^+CD25^+$ Tregs (Bruder et al., 2004). However, our comparative analysis of gene expression array data showed higher levels of neuropilin-1 message on the vac-HA activated/memory cells than in our anergic/regulatory cells (data not presented). Lastly, while Foxp3 has been clearly shown to be expressed on $CD4^+CD25^+$ Tregs, there is still significant debate over its expression on in vivo-induced Tregs (Apostolou and von Boehmer, 2004; Chen et al., 2004; Cobbold et al., 2004). It is noteworthy that the level of LAG-3 expression on in vivo-activated T cells is significantly lower than on our in vivo-generated Tregs, suggesting that LAG-3 may be a more reliable marker of cells with regulatory potential. Perhaps even more importantly, it appears that LAG-3 expression remains high on 6.5 $CD4^+$ Tregs, while LAG-3 expression decreases shortly after activation in effector/memory T cells, indicating that LAG-3 is not simply an activation marker. However, a detailed side-by-side comparison of marker expression and regulatory potential is clearly needed.

Our data show that LAG-3 is required for maximal suppressive activity of both natural and induced Tregs. However, is it sufficient? Thus far, the only molecule shown to confer regulatory activity on activated T cells is Foxp3 (Fontenot et al., 2003; Hori et al., 2003). Importantly, we have shown here that ectopic expression of LAG-3, but not a functionally defective mutant, on $CD4^+$ T cells can also confer regulatory activity. We have also shown that ectopic expression of LAG-3 significantly reduces proliferative potential. Thus, LAG-3 appears to have both intrinsic and extrinsic regulatory activity.

Different mechanisms have been identified for Treg function in different systems (reviewed in Shevach [2002]). We saw a clear correlation between the expression of LAG-3 and IL-10 mRNA in our induced T cells. However, it remains to be determined if IL-10 contributes to the suppressive activity in our system.

How might LAG-3 be involved in the function of Tregs? Our retroviral transduction data and previous studies infer that signaling through the unique KIEELE motif transduces an antiproliferative signal that controls cell growth. Identifying molecules that interact with this motif is clearly a priority. How LAG-3 might mediate extrinsic T cell regulation is more elusive. LAG-3 signaling is required, suggesting that it may induce the expression of regulatory cytokines or cell surface molecules. LAG-3 may also interfere with TCR signaling, although our data suggest that it does not interfere with MHC:CD4 interaction despite its superior affinity. Like Foxp3, the regulatory mechanism of LAG-3 remains to be fully elucidated. However, our data do suggest that LAG-3 may be an important contributor to both induced and natural Treg function.

Lastly, another key question is whether Tregs suppress the reactivity of $CD4^+$ and $CD8^+$ effector cells through direct T-T interactions or through DC intermediaries. The identification of Treg-selective and functional expression of LAG-3, an MHC class II binding molecule, should provide a new handle on dissecting mechanisms and manipulating Treg function for diseases in which these cells play an important role.

## Experimental Procedures

### Mice

The C3-HA transgenic mice have been previously described (Adler et al., 1998, 2000). Further details can be found in the Supplemental Experimental Procedures.

The TCR transgenic mouse line 6.5, which expresses a TCR recognizing an I-$E^d$-restricted HA epitope ($^{110}$SFERFEIFPKE$^{120}$) (generously provided by Dr. Harald von Boehmer, Harvard University, Boston, MA), was backcrossed nine generations onto the B10.D2 genetic background. Transgenic mice used for experiments were 8–24 weeks old.

LAG-$3^{-/-}$ mice were provided by Yueh-Hsiu Chien (Stanford University, CA) with permission from Christophe Benoist and Diane Mathis (Joslin Diabetes Center, Boston, MA) (Miyazaki et al., 1996). Genome-wide microsatellite analysis demonstrated that 96% of the 109 genetic markers tested were derived from C57BL/6 mice (Charles River Laboratories, Troy, NY). For some experiments, these mice were crossed with OT-II TCR transgenic mice (Ovalbumin 326–339-specific, H-$2A^b$-restricted) (kindly provided by Stephen Schoenberger, La Jolla Institute for Allergy and Immunology, CA, with permission from William Heath, Walter and Eliza Hall Institute, Parkville, Victoria, Australia) (Barnden et al., 1998).

All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committees of the Johns Hopkins University School of Medicine and St. Jude Children's Research Hospital.

### Antibodies, Staining, and Immunohistochemistry

Details can be found in the Supplemental Experimental Procedures.

### Adoptive Transfer

Clonotypic $CD4^+$ or $CD8^+$ T cells were prepared from pooled spleens and lymph nodes of 6.5 transgenic mice. Clonotypic percentage was determined by flow cytometry analysis. The activation marker CD44 was analyzed to ensure that these clonotypic cells were naive in phenotype. After washing three times with Hank's Balanced Salt Solution, an appropriate number of cells were resuspended in 0.2 ml of HBSS for i.v. injection through the tail vein.

### Enrichment and Purification of In Vivo Primed 6.5 $CD4^+$ T Cells

With either effector/memory or tolerance induction in vivo after adoptive transfer, the clonotypic percentage of 6.5 $CD4^+$ T cells in the spleens of recipient mice is only 0.2%–5%. Deliberate enrichment and purification is mandatory to obtain enough clonotypic $CD4^+$ T cells for further studies, such as for Affymetrix gene chip analysis. Donor 6.5 T cells were crossed onto a Thy1.$1^+$/Thy1.$2^-$ background, which allowed for a two-step enrichment and purification procedure after adoptive transfer into Thy1.$1^-$/Thy1.$2^+$ recipients. 6.5 $CD4^+$ T cells were first enriched by using biotinylated anti-CD8 (Ly-2, 53-6.7), anti-B220 (RA3-6B2), anti-Thy1.2 (30-H12) antibodies (antibodies purchased from BD PharMingen, San Diego, CA), MACS streptavidin microbeads, and MACS LS separation columns (Miltenyi Biotech, Auburn, CA) to deplete $CD8^+$ T cells, B cells, and the recipient T cells (Thy1.$2^+$). Since $CD4^+$ T cells and $CD8^+$ T cells are the only populations bearing Thy1.1 and because $CD8^+$ T cells had been depleted during enrichment, sorting for Thy1.$1^+$ cells with FACSVantage SE cell sorter (BD Biosciences) resulted in highly purified 6.5 $CD4^+$ T cells (95%). This technique avoids the use of TCR-specific or CD4 coreceptor-specific antibodies that could potentially alter TCR- or CD4-dependent gene expression patterns.

### Gene Chip Analysis, Statistical Ranking, and Quantitative Real-Time PCR Analysis

Details can be found in the Supplemental Experimental Procedures.

### In Vitro Suppression Assay for Induced 6.5 Regulatory T cells

Purified naive 6.5 $CD4^+$ T cells (responders, $1 \times 10^4$) and 3000-rad-irradiated syngeneic B10.D2 splenocytes (antigen-presenting cells, $6 \times 10^4$ for antibody-blocking studies or $1 \times 10^5$ for all other experiments) were mixed with different numbers of suppressor 6.5 $CD4^+$ T cells and incubated in round bottom 96-well tissue culture plates with 10 μg/ml of HA class II ($^{110}$SFERFEIFPKE$^{120}$) peptide (200 μl

total). Forty-eight to 72 hr later, cultures were pulsed with 1 μCi [$^3$H]thymidine and incubated an additional 16 hr before harvest with a Packard Micromate cell harvester. Determination of the amount of incorporated radioactive counts was performed with a Packard Matrix 96 direct β counter (Packard Biosciences, Meriden, CT). Alternatively, for the CFSE suppression assay, naive 6.5 CD4$^+$ T cells (responders) were labeled with 2.5 μM Vybrant CFDA cell tracer probe "CFSE" (Molecular Probes) and cocultured as above. Proliferation was determined by flow cytometry (FACSCalibur, BD) and analysis with FlowJo software and the Lyons method of calculating the percentage of divided cells (Lyons, 2000).

### In Vitro Suppression Assays for Natural Regulatory T cells

Wild-type BALB/c, OT-II.LAG-3$^{+/+}$, or OT-II.LAG-3$^{-/-}$ mice were used for the natural Treg assays. 5 × 10$^4$ (BALB/c) or 2.5 × 10$^4$ (OT-II.LAG-3$^{-/-}$) FACS-purified CD4$^+$CD25$^-$ T cells (responders) and 5 × 10$^4$ 3000 rad-irradiated BALB/c or C57BL/6 splenocytes (APC) were mixed with different numbers of FACS-purified CD4$^+$CD25$^+$ Tregs and incubated in round bottom 96-well tissue culture plates with 0.5 μg/ml of anti-CD3 antibody or 0.9–15 μg/ml of OVA 326–339 peptide in 200 μl of CTL media. 48 to 72 hr later, cultures were pulsed with 1 μCi [$^3$H]thymidine and incubated an additional 8–16 hr before harvest with a Packard Micromate cell harvester. Determination of the amount of incorporated radioactive counts was performed with a Packard Matrix 96 direct β counter (Packard Biosciences, Meriden, CT).

### LAG-3 Constructs and Retroviral Producer Cell Lines

Production of LAG-3 constructs, retroviral vectors, producers, and transductions have been described (Vignali and Vignali, 1999; Workman et al., 2002a) and are detailed in the Supplemental Experimental Procedures.

### Retroviral Transduction of CD4$^+$/CD25$^-$ T Cells and In Vitro Suppression Assay

Splenocytes from 6.5 Thy1.2$^+$ or 6.5 Thy1.1$^+$ transgenic mice were stained with biotin-labeled anti-B220, anti-Gr1, anti-Mac1, anti-TER119, anti-CD49b, anti-CD8, and anti-CD25 antibody (PharMingen, San Diego, CA). The cells were then incubated with magnetic beads coupled with streptavidin and negatively sorted on an autoMACS (Miltenyi Biotech, Auburn CA) to 90%–95% purity of CD4$^+$/CD25$^-$ T cells. The purified 6.5 CD4$^+$/CD25$^-$ T cells were activated by plate bound anti-CD3 (2C11) and anti-CD28 (35.71). On days 2 and 3 poststimulation, the activated T cells (4 × 10$^5$ cells/ml) were spin transduced (90 min, 3000 rpm) with viral supernatant from vector alone, LAG-3.WT/GFP, or LAG-3.Y73F.ΔCY/GFP retroviral GPE$^+$86 producer cell lines (see Supplemental Experimental Procedures) plus IL-2 (1 ng/ml) and polybrene (6 μg/ml). The cells were allowed to rest for 10 days in the presence of 2 ng/ml IL-2 and then sorted on the top ~30%–35% GFP$^+$/Thy1.2$^+$ T cells.

For the in vitro suppression assays, the purified GFP$^+$ T cells were cultured (2-fold dilutions starting at 2.5 × 10$^4$) with CFSE-labeled or unlabeled 2.5 × 10$^4$ CD4$^+$/CD25$^-$ 6.5 T cells (purified by negative AutoMACS), 5 × 10$^4$ irradiated (3000 rads) splenocytes, and 5 μg/ml or 10 μg/ml HA$^{110–120}$ in a 96-well round bottom plate. The cells were cultured for 72 hr and pulsed with [$^3$H]thymidine 1 μCi/well (Du Pont, Wilmington, DE) in the last 7–8 hr of culture or analyzed for CFSE reduction by flow cytometry.

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