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LAG-3 Expression Defines a Subset of CD4^+CD25^{high}Foxp3^{+} Regulatory T Cells That Are Expanded at Tumor Sites

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Human natural regulatory CD4^+ T cells comprise 5–10% of peripheral CD4^+ T cells. They constitutively express the IL-2 receptor-α chain (CD25) and the nuclear transcription Foxp3. These cells are heterogeneous and contain discrete subsets with distinct phenotypes and functions. Studies in mice report that LAG-3 has a complex role in T cell homeostasis and is expressed in CD4^+CD25^+ T regulatory cells. In this study, we explored the expression of LAG-3 in human CD4^+ T cells and found that LAG-3 identifies a discrete subset of CD4^+CD25^{high}Foxp3^{+} T cells. This CD4^+CD25^{high}Foxp3^{+}LAG-3^{+} population is preferentially found in the thymus, but differentiate in the periphery from naive CD4^+ T cells or induced Treg cells. Induced Treg cells are not present in the immunosuppressive cytokines IL-10 and TGF-β. Immunology sorted from in vitro expanded CD4^+CD25^{high}Foxp3^{+}LAG-3^{+} T cells showed that this subset of cells is endowed with potent suppressor activity that requires cell-to-cell contact. Our data show that LAG-3 defines an active CD4^+CD25^{high}Foxp3^{+} regulatory T cell subset whose frequency is enhanced in the PBMCs of patients with cancer and is expanded at tumor sites. *The Journal of Immunology, 2010, 184: 000–000.

In the immune system, several mechanisms are in operation to finely tune the immune response. An active role in preventing autoimmune diseases and maintaining immune homeostasis is played by the so-called regulatory T cells (Treg). Human Treg cells comprise two main groups of CD4^+ T cells with different origins: natural occurring Treg cells generated in the thymus, and adaptive or induced Treg cells. Induced Treg cells are not present in the thymus, but differentiate in the periphery from naive CD4^+ T cells upon polyclonal or Ag-specific activation in the presence of specialized immunoregulatory cytokines. Two subsets of induced Treg cells have been described and shown to exist in humans: Tr1 cells, which secrete high levels of IL-10 and arise from CD4^+ T cells upon encountering Ags in a tolerogenic environment (1), and Th3 cells, which are induced by oral Ag administration and secrete high levels of TGF-β (2). Moreover, recent experimental evidence demonstrated that in humans, adaptive Treg cells may also emerge from the memory CD4^+CD25^{−}CD45RO^{+} T cell pool in the presence of antigenic stimulation. These converted Treg cells, once generated, are highly susceptible to apoptotic death, thus representing a highly dynamic T cell pool that mirrors its responsive counterpart (3–5).

Naturally occurring Treg cells in mice are unequivocally defined as CD4^+CD25^{+}Foxp3^{+} T cells. This same set of markers, together with a low expression level of CD127 (6, 7), also identifies human natural Treg cells. However, unlike in mice, human Foxp3^{+} T cells are heterogeneous. For example, studies have reported that TCR-mediated activation of CD4^+CD25^{-} T cells induces the transient expression of Foxp3 without conferring suppressive activity (8, 9). Similarly, it was shown that CD4^+CD25^{low}Foxp3^{low} T cells, which display limited to no suppressive capacity, are present in human PBMCs (10).

Further phenotypic studies revealed that other subclasses of human natural Treg cells could be identified based on the expression of molecules such as MHC-II, CD45RO/RA, CCR7, and costimulatory molecules such as ICOS (11–14). The expression of CD45RO/RA and CCR7 identifies populations that are at different in vivo differentiation stages, whereas their combined expression defines the presence of the natural naive, effector memory, or central memory Treg cells.

Natural Treg cells, upon TCR engagement (15, 16), exert their immunosuppressive functions in a contact-dependent fashion. However, it has been shown that the expression of MHC-II and ICOS characterizes natural Treg cells that are functionally different (13, 14). These latest data suggest that Treg cells can be further differentiated into discrete subsets that differently rely on cell-to-cell contact and on IL-10 or TGF-β production for their functional activities.

Recent studies have also shown that CD4^+ Treg cells are implicated in the suppression of the immune response against tumors (17–19). There is accumulating evidence that demonstrates a significant increase in the number of Treg cells in the peripheral blood and tumor microenvironment of patients with various types...
of cancer (20, 21). Moreover, although data remain controversial and depend on the type of tumor studied (21), a higher accumulation of Treg cells is often associated with advanced disease stages and is inversely correlated with favorable prognosis and overall survival (22, 23). It has been clearly demonstrated that in most human solid tumors as well as hematologic malignancies, Ag-specific Treg cells actively suppress the proliferation of CD4+CD25+ and CD8+ effector T cells, thereby limiting the immune response against cancer and contributing to tumor growth (24).

LAG-3 has been recently described in mice as a novel Treg-associated marker that is directly involved in the control of T cell expansion and homeostasis (25, 26). There is increasing evidence on its involvement in tumor-infiltrated T function in Hodgkin’s lymphoma (28). Similarly, an Ag-specific LAG-3+CD25+Foxp3+ induced regulatory CD8+ T cell subset has been identified in a series of patients with tuberculosis (29).

In the current study, we show that inside the suppressor CD4+CD25highFoxp3+ T cell population, LAG-3 expression identifies a discrete subset of cells that displays an effector-memory/terminal-effector phenotype. This subset of T cells is expanded in peripheral blood and tumor sites of cancer patients, suggesting that discrete compartments of Treg cells are selectively modulated in cancer patients.

Materials and Methods

Blood and tissue samples

Upon obtaining informed written consent, blood samples were collected from healthy donors or patients with melanoma or colorectal cancer at different stages of disease. PBMCs were isolated by Ficoll/PaqueTM PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation as previously described (30). Lymphocytes were also obtained from tumor-free or tumor-invaded lymph nodes, whereas tumor-infiltrated lymphocytes were collected from visceral and subcutaneous metastases of advanced cancer patients who underwent curative resection at the Melanoma and Sarcoma Unit or at the Colorectal Surgery Unit of our institute.

Abs and flow cytometry

The following Abs were used: allophycocyanin-conjugated anti-CD4, PE-Cy7–conjugated anti-CD25, FITC- or PE-conjugated anti-CD45RA and –CCR7, FITC-conjugated Ki67, and as an isotype control, fluorochrome-conjugated mouse IgG (BD Biosciences, San Jose, CA); FITC-conjugated or biotin-conjugated anti–LAG-3 (clone 17B4; Aotech; Epalung, Switzerland); and PE- or allophycocyanin-conjugated anti–Foxp3 (eBioscience, San Diego, CA). Intracellular staining for human Foxp3 was performed according to the manufacturer’s protocol (eBioscience). Ki67 FITC staining was performed using eBioscience fixation and permeabilization buffers.

Intracellular staining for IL-10, IL-2, and TGF-β1 was performed as follows: lymphocytes that were freshly isolated or activated overnight with anti-CD3/CD28 beads (Dynal beads; CD3/CD28 T cell Expander; Invitrogen Dynal AS, Oslo, Norway) or with 1 μg/ml Golgi Plug (BD Biosciences), were stained for cell surface markers CD4, CD25, and LAG-3, washed, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), and stained with PE-labeled anti–IL-10, anti–IL-2 (BD Biosciences) or PE-labeled anti–TGF-β1 (IQ Products, Groningen, The Netherlands) mAb. The fluorescence intensity was evaluated using a BD FACSScalibur flow cytometer and analyzed using BD CellQuest software (BD Biosciences) or FlowJo Cytometry Analysis software (Tree Star, Ashland, OR).

Isolation of CD4+ and CD4+CD25+ T cells

CD4+ T cells were purified from freshly isolated PBMCs or from lymph nodes (LNs) by immunomagnetic depletion of non-CD4+ T cells using the human CD4+ T Cell Isolation Kit II following the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated cells was checked by surface staining with anti-CD4 mAb. CD4+CD25+ T cells were purified from freshly isolated PBMCs by immunomagnetic sorting using human the CD4+CD25+ Regulatory T Cell Isolation Kit and following the manufacturer’s instructions (Miltenyi Biotec). The purity of the isolated cells was checked by surface staining with anti-CD4 and anti-CD25 mAb.

Expansion of peripheral Treg cells was performed following a previously published protocol (31). CD4+CD25+ T cells (2 × 10^5 per milliliter) were stimulated with 1 μg/ml OKT3 in the presence of 10% per milliliter allogeneic gamma-irradiated (10,000 cGy) feeder PBMCs and 10^5 per milliliter allogeneic gamma-irradiated lymphoblastoid cell line. Cell cultures were maintained in X-VIVO-15 medium (Lonza, Basel, Switzerland) supplemented with 10% FCS (Lonza), 1% heat-inactivated human serum, 2 mM glutamine (Lonza), and antibiotics. Recombinant IL-2 (Proleukin-Chiron, Amsterdam, The Netherlands) was added 3 d after activation at 40 U Cetus/ml. Isolated CD4+CD25+ T cells, activated in vitro as described above, were stained with FITC-conjugated anti–LAG-3 mAb and sorted using a FACS-Vantage DiVa (BD Biosciences).

Suppression assay

In vitro suppression assays were performed in 96-well round-bottom plates or HST Transwell-96 plates (Corning Glass, Corning, NY). The responder CD4+CD25+ T cells were stimulated using anti-CD3/CD28 beads and incubated alone or with increasing numbers of freshly isolated autologous CD4+CD25+ LAG-3+ T cells. The proliferation of the responder T cells was evaluated 72 h after the incubation of T suppressor cells with [3H]Thymidine (Amersham Biosciences, Piscataway, NJ). [3H]Thymidine was then added at 1 μCi per well for an additional 18 h. For some experiments, the proliferation of responder cells was evaluated by CFSE labeling (see Materials and Methods, CFSE proliferation assay). For certain experiments performed in transwell...
plates, anti–IL-10 (10 μg/ml) or anti–TGF-β1 (10 μg/ml; R&D, Minneapolis, MN) was added to the samples. The whole human IgG molecule (10 μg/ml; Chemicon International, Temecula, CA) was used as a control. All experiments were performed in triplicate. The percentage of inhibition was calculated as follows: % of inhibition = 100 × [1 – (mean proliferation [responder and suppressor] / mean proliferation [responder only with beads])]%

CSFE proliferation assay

For in vitro CFSE proliferation assays, purified CD4+CD25– T cells were labeled by incubation with 5 μM CFSE (Invitrogen, Carlsbad, CA) in RPMI 1640 medium containing 10% FCS for 15 min at 37˚C. This reaction was blocked by the addition of cold FCS, followed by washing with RPMI 1640 medium supplemented with 10% FCS. The cells were subsequently prepared for a suppression assay (see Materials and Methods, Suppression assay).

Statistical analysis

The differences between groups were assessed using the two-tailed Student t test. Differences were regarded as significant at p ≤ 0.05 (∗p ≤ 0.05; ∗∗p ≤ 0.02; ∗∗∗p ≤ 0.005).

Results

LAG-3 is preferentially expressed by CD4+CD25high T cells and defines a discrete subset of CD4+CD25highFoxp3+ T cells

Studies in mice indicated that LAG-3 has a complex role in T cell homeostasis and is expressed by CD4+CD25+ Treg cells. To explore the distribution of LAG-3 in human CD4+ T cells, the expression of the LAG-3 molecule was evaluated inside the CD4+CD25neg, CD4+CD25low and CD4+CD25high gated populations of PBMCs from healthy donors. We consistently observed the LAG-3 molecule within the CD4+CD25high compartment (Figure 1 A–C).

Human natural Treg cells constitutively express a high level of CD25 (15, 31–34) and the nuclear transcription factor Foxp3 (10, 35, 36). Thus, the expression of Foxp3 and LAG-3 inside the CD4+CD25high gated cells was evaluated by multiparametric flow cytometry. As expected, Foxp3 uniformly stained all the CD4+CD25high T cells. Conversely, LAG-3 expression defined a discrete subset of T cells inside the CD4+CD25highFoxp3+ compartment (Figure 1D–G).

The frequency of CD4+CD25highFoxp3+LAG-3+ T lymphocytes is increased in PBMCs of tumor-bearing patients and at tumor sites

To investigate whether CD4+CD25highFoxp3+LAG-3+ T cells were expanded in cancer patients and could thus possibly represent
a subset of tumor-induced Treg cells, LAG-3 expression was analyzed in PBMCs of patients with advanced tumor-bearing stage III and IV melanoma and colorectal cancer. Similar to what we found in healthy donors, LAG-3Foxp3+ cells were mainly confined to the CD4+CD25high compartment (Fig. 1F, 1G). However, the frequency of LAG-3Foxp3+ cells among CD4+CD25high T cells was significantly increased in the PBMCs of patients with cancer, compared with those of healthy donors (Fig. 2A).

The effect of a tumor on the percentage of CD4+CD25high Foxp3+LAG-3+ T cells became more evident in the analysis of T lymphocytes derived from tumor-positive or tumor-negative lymph nodes of stage III melanoma and colorectal cancer patients as well as lymphocytes infiltrating s.c. or visceral stage IV melanoma metastasis. Results reported in Fig. 2B show that lymphocytes of tumor-invaded lymph nodes and tumor-infiltrated lymphocytes were significantly enriched in CD4+CD25highFoxp3+LAG-3+ T cells.

CD4+CD25highFoxp3+LAG-3+ T cells display phenotypic features of effector-memory T cells

CD4+CD25highFoxp3+LAG-3+ T cells of cancer patients were then characterized for expression of CCR7, CD45RA and Ki67. CD45RA and CCR7 expression analysis indicated that both LAG-3− and LAG-3+ CD4+CD25highFoxp3+ T cells mainly displayed an effector-memory phenotype. However, a small percentage of cells that reacquired CD45RA and displayed a terminal effector phenotype was detectable in the LAG-3− compartment (Fig. 3).

As previously reported (5), we found that a high percentage of CD4+CD25highFoxp3+ T cells express the proliferation marker Ki67 (Fig. 4B). However, analysis in regard to the expression of LAG-3 revealed that among CD4+CD25highFoxp3+ T cells, the proliferating compartment was mainly represented by LAG-3− negative cells (Fig. 4B), and only a small percentage of Ki67+ LAG-3− cells was found.

CD4+CD25highFoxp3+LAG-3+ T cells from patients with cancer produce immunosuppressive cytokines

To assess whether LAG-3+ Treg cells from cancer patients were endowed with functional activities, we evaluated the production of the immunosuppressive cytokines IL-10 and TGF-β1, which are known to play a key role in the generation and function of natural Treg cells and Tr1 cells (14, 37–39), ex vivo via intracellular staining, and FACS. IL-10 and TGF-β1 were exclusively found in the CD4+CD25high Treg cells. Moreover, these cytokines were mainly produced by the LAG-3− subset (Fig. 5A). Conversely, IL-2 was detectable only in CD4+CD25low gated cells, while the CD4+CD25high compartment remained negative for IL-2 (Fig. 5A). These results exclude the possibility that CD4+CD25highFoxp3+LAG-3+ cells were recently activated conventional effector T cells. Cumulative data reporting on the percentage of cytokine-secreting cells inside the CD4+CD25highFoxp3+LAG-3− and CD4+CD25highFoxp3+LAG-3− gated populations of PBMCs from patients with cancer are reported in Fig. 5B. As previously reported (5), we found that a high percentage of CD4+CD25highFoxp3+ T cells express the proliferation marker Ki67 (Fig. 4B). However, analysis in regard to the expression of LAG-3 revealed that among CD4+CD25highFoxp3+ T cells, the proliferating compartment was mainly represented by LAG-3− negative cells (Fig. 4B), and only a small percentage of Ki67+ LAG-3− cells was found.
cancer and lymphocytes of tumor-invaded LNs confirmed the preferential association between LAG-3+ Treg cells and the production of suppressive cytokines. However, this broad analysis indicated that TGF-β1 displays a less restricted distribution; therefore, a high proportion of LAG-3+ Treg cells also produces this immunosuppressive cytokine (Fig. 5C).

Suppressor activity of LAG-3+ Treg cells

Our analysis showed that inside the CD4+CD25+Foxp3+ T cell compartment, LAG-3 positive cells display a regulatory phenotype and produce suppressive cytokines that are involved in mediating Treg cell function. Thus, we investigated the suppressive ability of these cells. To this end, Treg cells from PBMCs of healthy donors were magnetically isolated (purity >98%) and shortly expanded in vitro according to a published protocol (31). Cells were collected and analyzed for CD25 and LAG-3 surface expression. All cells were CD25-positive and displayed varying percentages of LAG-3 positivity (Fig. 6A, B). Cells were sorted into LAG-3+ (Fig. 6A, 6B, indicated as suppressor 1) and LAG-3+/low fractions (Fig. 6A, 6B, indicated as suppressor 2); their ability to inhibit the proliferation of autologous freshly isolated CD4+CD25- T cells was evaluated in a [3H]thymidine incorporation assay. As shown in Fig. 6A and 6B, LAG-3+ T cells displayed enhanced suppressor activity as compared with their LAG-3+/low counterparts.

Suppression experiments were also performed in transwell plates in which sorted LAG-3+ Treg cells and responder T cells were separated by a membrane permeable to soluble molecules, but that prevented direct cell-to-cell contact. As shown in Fig. 6C, LAG-3+ Treg cells exerted their suppressor activity mainly via a direct cell-to-cell interaction, because the prevention of a physical interaction between LAG-3+ Treg and responder T cells permitted responder T cells to completely reacquire their proliferation activity. The addition of anti–IL-10 or anti–TGF-β1 mAb did not affect the inhibitory activity of LAG-3+ Treg cells. These data indicate that LAG-3+ Treg cells exert their suppressor activities in a contact-dependent fashion, in line with what is known for CD4+CD25+Foxp3+ Treg cells. The possible in vivo contribution of IL-10 and TGF-β1 still remains to be investigated.

Discussion

The LAG-3 molecule has been defined as an activation marker that is expressed by human and mouse CD4+ T cells. LAG-3 has been shown to play a key role in T cell homeostasis and has been associated with murine CD4+ and CD8+ Treg cells (25, 40). Moreover, recent data in humans identified a new subset of CD8+ Treg cells expressing LAG-3 that mediate immunosuppression via the secretion of CCL4 (29). Based on these observations, we analyzed the expression of this marker in human CD4+ T cells to...
explore its distribution in relation to CD25 and its involvement in human Treg cells.

In this study, we found that the expression of LAG-3 identifies a discrete population of CD4+CD25\textsuperscript{high}Foxp3\textsuperscript{+} Treg cells in human peripheral blood. Treg cells are no longer considered a homogeneous group of cells, but are instead known to contain subclasses of T cells that can be differentiated by their expression of molecules, such as CD45RO/RA,CCR7,MHC-II, and the costimulatory molecule ICOS (13, 14). Our data on LAG-3 add to the complexity to the world of human Treg cells.

Furthermore, we show that this subset of LAG-3\textsuperscript{+} Treg cells is expanded in PBMCs of advanced tumor-bearing patients, as well as in CD4\textsuperscript{+} T cells found at tumor sites. Importantly, in patients with melanoma or colorectal cancer, LAG-3 expression defines a subpopulation of T cells that produce the suppressive cytokines IL-10 and TGF-\textbeta1. These findings, together with the observation that CD4+CD25\textsuperscript{high}LAG-3\textsuperscript{-} cells display an effector-memory/terminal-effector phenotype and lack a significant proliferation capacity, suggest that CD4+CD25\textsuperscript{high}LAG-3\textsuperscript{-} cells could be considered as activated or differentiated Treg cells.

By sorting LAG-3\textsuperscript{-} cells from expanded Treg cells, we found that the expression of LAG-3 is associated with strong functional activity. Specifically, we found that LAG-3\textsuperscript{-} Treg cells inhibit the proliferation of CD4+CD25\textsuperscript{-} autologous T cells by contact-dependent mechanisms as expected for CD4+CD25\textsuperscript{high}Foxp3\textsuperscript{+} natural Treg cells. Whether IL-10 and TGF-\textbeta1 contribute to the suppressor activity of this cell subset in vivo still remains to be assessed. These cytokines might play a role in Treg/dendritic cell interactions at tumor sites as reported for the CD4+CD25\textsuperscript{+}ICOS\textsuperscript{+} Treg cells, which suppress T cell proliferation through a contact-dependent mechanism, but also produce IL-10 to counteract dendritic cell function and maturation (14). It is yet to be determined in vivo whether multifunctional Treg cells simultaneously use all of these mechanisms to suppress and restrain the immune response (41). To keep the immune system in check, Treg cells adapt their suppressor mechanisms in response to the local environment. In addition, it is likely that each mechanism plays a specific role in a given inflammatory tissue setting. It has been shown, for example, that Treg-derived IL-10 was mainly necessary for limitation of inflammation in the colon, lung, and skin (42), whereas recent data indicate that, at tumor sites, suppression is also due to the action of a subset of CD4+CD25\textsuperscript{+} Foxp3\textsuperscript{+} T cells that release IL-10 and TGF-\textbeta1 (37, 43).

Our data define phenotypic features that distinguish tumor-related Treg cells. In fact, we showed that CD4+CD25\textsuperscript{high}Foxp3\textsuperscript{+} LAG-3\textsuperscript{-} Treg cells were not only selectively expanded in lymphocytes infiltrating visceral or cutaneous metastasis, but they were also selectively enriched in tumor-invaded LNs as compared to loco-regional tumor-free LNs. Our data are in agreement with previous findings, indicating that melanoma-infiltrating lymphocytes are enriched in a subpopulation of strongly suppressive Treg cells expressing ICOS (44). Like ICOS, LAG-3 becomes expressed by T cells as a consequence of a TCR-mediated activation (45, 46). Interestingly, patients with advanced tumor-bearing cancer display in their peripheral blood an enhanced percentage of CD4+CD25\textsuperscript{high}Foxp3\textsuperscript{+}LAG-3\textsuperscript{-} cells, suggesting the presence of activated Treg cells similar to those found at tumor site.

Our observations raise the question of whether this tumor-associated subset of LAG-3\textsuperscript{-} Treg cells should be considered as tumor-induced/adaptive Treg cells. They certainly differ from classical Treg1 because they express high levels of CD25, are Foxp3\textsuperscript{+}, and exert their suppressive function by direct cell-to-cell contact. In addition, recent data in a human setting seem to stress the functional and numerical relevance of adaptive Treg cells, which are likely constantly produced in the periphery from a differentiation stage that is equipped with suppressor functions (3–5).

Nevertheless, our data are in agreement with previous findings in mice in which the LAG-3 molecule was shown to be expressed both by induced Treg cells and by natural Treg cells upon TCR activation. In mice, evidence is also provided indicating a functional involvement of LAG-3 in mediating suppression (25). In our hands, LAG-3–specific 17B4 Ab, used in vitro in different experimental settings, did not revert the suppressor activity of LAG-3+ Treg cells (data not shown). However, because these results are negative and with no clear evidence of the antagonistic activity of this Ab, the direct contribution of LAG-3 molecule in mediating suppression in humans still remains to be fully addressed, although initial evidence on the role of the Treg–expressed LAG-3 in inhibiting dendritic cell maturation has been recently reported (47).

Our data shed new light on the phenotypic heterogeneity of human Treg cells and provide evidence for a qualitative difference between Treg cells of healthy donors and those found in patients with cancer, showing that LAG-3\textsuperscript{-} Treg cells are selectively expanded in the PBMCs of cancer patients and in the tumor microenvironment.

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Disclosures

The authors have no financial conflicts of interest.

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