Suppressor activity and potency among regulatory T cells is discriminated by functionally active CD44

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CD4CD25+ regulatory T cells are fundamental to the maintenance of peripheral tolerance and have great therapeutic potential. However, efforts in this regard have been hampered by limiting cell numbers in vivo, an anergic phenotype in vitro, and a rudimentary understanding of the molecular basis for the functional state of CD4CD25+ regulatory T cells (Treg cells). Here we show heterogeneity of suppressor activity among activated CD4CD25+ Treg cells and that, within this population, the functionally active, hyaluronan-binding form of CD44 (CD44<act>) is strikingly correlated with superior suppressor activity. Within 16 hours after in vitro activation, CD44<act> can discriminate enhanced suppressive function in in vitro proliferation assays and in an in vivo bone marrow engraftment model. The expression of other surface markers and that of Foxp3 are similar irrespective of hyaluronan binding and associated degree of suppressor potency. Furthermore, CD44<act> is induced on resting CD4CD25+ cells in vivo by allogeneic stimulation, with similar functional consequences. These results reveal a cell-surface marker that delineates functional activity within a population of activated CD4CD25+ regulatory T cells, thereby providing a potential tool for identifying regulatory activity and enriching for maximal suppressor potency. (Blood. 2006;107:619-627) © 2006 by The American Society of Hematology

Introduction

Among regulatory T cells with immunomodulatory properties, emphasis has recently focused on a population of CD4 T cells expressing the IL-2 receptor α chain (CD25).1-3 These cells have been shown to prevent autoimmunity disease through their ability to maintain tolerance to self-antigens and to suppress proliferation and cytokine secretion by other cell types. Because CD4CD25+ regulatory T cells (Treg cells) develop early in the neonatal thymus and are exported to the periphery competent to suppress the activation of other self-reactive T cells, they are referred to as naturally occurring Treg cells.4,5

Activation and expansion of CD4CD25+ cells prolonged over one week in vitro or in vivo results in enhancement of their suppressor function.6,7 However, heterogeneity of function within a population of activated CD4CD25+ Treg cells has not been described, in part due to the absence of a marker that selectively discriminates such differential suppressor activity. The integrin αEβ7 has recently been described to distinguish potent suppressor activity independent of CD25 expression.8,9 Similarly, α4β1 and α4β7 distinguish differential suppressor populations within CD4CD25+ Treg cells in humans.10 The ability to enrich for potent activated Treg cells has been hampered by the ubiquitous expression of most known activation markers across T-cell populations. While many of these markers, such as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and lymphocyte activation gene-3 (LAG-3), do distinguish Treg cells from unactivated nonregulatory T cells,11-15 they do not distinguish activated Treg cells from activated T effectors (Teff cells) nor, pertinent to the studies presented here, do they distinguish the degree of suppressor potency within a population of activated Treg cells.

Our laboratory has characterized a primary adhesion (rolling) interaction mediated by the activated form of CD44 (CD44<act>) on peripheral T cells interacting with its ligand hyaluronan (HA) on microvascular endothelium.16 We have established that the CD44-initiated adhesion pathway mediates specific T-cell egress during an immune response into an inflamed site,17 that microvascular endothelial cells respond to proinflammatory stimuli with the induction of elevated luminal surface HA permitting capture,18 and that CD44<act> in conjunction with the integrin VLA-419,20 mediates the extravasation of activated T cells in vivo.17,18,20,21 CD44 has been implicated in a number of murine models of autoimmune disease,22-24 as well as in humans.25 These data support the hypothesis that CD44<act> is a significant participant in the well-described enhanced homing of activated T cells to inflamed tissues and suggest relevance of the CD44/HA interaction at target sites of autoimmunity.

While highly expressed, CD44 on resting lymphocytes is inactive and binds to HA only when conformationally activated.16,26-28 While the biochemical basis for CD44 activation in T cells is not clear, it does not require variant exon splicing.20,29 We...
have shown that in both mouse and human T cells, the activation of CD44 is achieved via physiologic triggering through the T-cell receptor (TCR) in vitro and in vivo.\textsuperscript{25,28} While CD44 is expressed at high levels and essentially uniformly on T cells with strong TCR stimuli, conversion to its activated form occurs only on a subset of activated T cells. These observations led us to examine T cells that are similar by other phenotypic criteria but that differ with respect to this unique marker.

Here we explore the significance of CD44\textsuperscript{ex} expression in naturally occurring Treg cells. We have examined the characteristics of CD4CD25\textsuperscript{+} cells induced to express CD44\textsuperscript{ex} and their behavior in vitro and in vivo. In contrast to the anergic properties of CD4CD25\textsuperscript{+} cells in vitro, including inability to flux calcium, proliferate, or secrete IL-2, we show that they have a striking capacity to up-regulate the ligand-binding form of CD44 compared with similarly treated CD4CD25\textsuperscript{+} cells. Moreover, we establish that this marker is highly correlated with functional suppressor activity activated within CD4CD25\textsuperscript{+} Treg cells in both in vitro and in vivo models. These results suggest that CD44\textsuperscript{ex} delineates a population containing highly potent suppressor activity identifiable with a simple surface stain and that this marker may aid in the tracking of such regulatory function over the course of immune and autoimmune responses. The results further imply utility in identifying and enriching for potent suppressors in strategies to deliver these cells therapeutically for control of dysregulated or pathologic immune responses.

**Materials and methods**

**Reagents**

Rooster comb sodium hyaluronate was purchased from Sigma (St Louis, MO) and fluoresceinated hyaluronan (Fl-HA) was prepared as described.\textsuperscript{16} OVA\textsuperscript{131-139} peptide was synthesized at UTSWMC (University of Texas Southwestern Medical Center, Dallas).

**Mice**

Six- to 8-week-old Balb/c and C57BL/6 mice were obtained from the National Cancer Institute (NCI). C57BL/6-Thy1.1 congenic mice were provided by Dr James Forman (UTSWMCD). DO11.10 TCR transgenic mice maintained under specific pathogen-free conditions and used at 10 to 12 weeks. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee at UTSWMC.

**Antibodies and cytokines**

Purified biotin- and/or fluorochrome-conjugated antimouse antibodies were purchased from the following companies: CD3\textepsilon, CD4, CD8, CD25, CD28, CD44 (IM7), CD45RB, CD62L, CD69, CD90.2, CD152 (BD Pharmingen, San Diego, CA); anti-asialo GM1 (Wako, Richmond, VA); anti–mouse GITR (R&D Systems, Minneapolis, MN); HA-blocking rat anti–mouse CD44 (KM81; ATCC, Manassas, VA). IL-2 was obtained from R&D Systems (Minneapolis, MN) and fluoresceinated hyaluronan (Fl-HA) was prepared as described.\textsuperscript{16} Rooster comb sodium hyaluronate was purchased from Sigma (St Louis, CA). Cytokines: IL-2 was obtained from R&D Systems (Minneapolis, MN); anti–asialo GM1 (Wako, Richmond, VA); anti–mouse CD44 (IM7); CD45RB, CD62L, CD69, CD90.2, CD152 (BD Pharmingen, San Diego, CA). Purified biotin- and/or fluorochrome-conjugated antimouse antibodies were purchased from BD Biosciences (San Jose, CA).

**Flow cytometry**

Cells were stained in PBS/5% FCS on ice with the exception of CTLA-4 surface staining, which was done at 37°C for 30 minutes. Analysis of stained cells was performed on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

**Cytokine analyses**

Enzyme-linked immunosorbent assays for IFN\textgamma were carried out using Quantikine\textregistered Immunoassays (R&D Systems) according to the manufacturer’s instructions.

**PCR analysis**

**Semi-quantitative cytokine mRNA analysis.** Total RNA was isolated from equal numbers of sorted cells using NReasy kits (Qiagen, Valencia, CA). Reverse transcription was done on 0.5 to 1 \mu g of RNA using SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA). Ten percent of resulting cDNA was used in multiplex polymerase chain reactions (PCRs) for T-helper (TH1/TH2) cytokines (Biosource) and inflammatory cytokines (Maxim Biotech, San Francisco, CA). Bands were resolved on 2% agarose gels.


**Cell separations**

To isolate CD4CD25\textsuperscript{+} cells, CD4 or total T cells purified using CD4 or T-cell columns (R&D Systems) were incubated with anti-CD25–biotin in PBS/2% FCS, washed, incubated with anti-biotin microbeads (Miltenyi Biotec, Auburn, CA), and separated according to the suggested protocol. Retained cells were eluted from the column and confirmed as at least 95% CD4CD25\textsuperscript{+} cells by fluorescence-activated cell sorter (FACS) analysis. For some experiments, CD4CD25\textsuperscript{+} cells were purified on a FACDiva Cell Sorter (BD Biosciences) using anti-CD25–PE plus anti–CD4–FITC. The resultant purity of sorted cells was greater than 98%. CD44\textsuperscript{hi}/FIHA-positive (CD44\textsuperscript{hi}) and CD44\textsuperscript{lo}/FIHA-negative (CD44\textsuperscript{lo}) cells were sorted using the FACDiva. Antigen-presenting cells (APCs) were prepared from T-depleted splenocytes, irradiated at 30 Gy, and plated at 5 \times 10^5 well. Bone marrow cells for adoptive transfer were obtained by flushing femurs and tibiae with PBS using a 23-gauge needle.

**In vitro assays**

Cultures were done in RPMI1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 \mu g/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 \mu M 2-mercaptoethanol. T cells were activated by incubating cells at 1 × 10^6 cells/mL in plates previously coated with anti-CD3 (5 \mu g/mL unless otherwise noted) plus anti-CD28 (5 \mu g/mL) or with 25 \mu M OVA\textsuperscript{233-239} peptide plus 1 × 10^6 irradiated APCs. For suppressor assays, 5 × 10^4 CD4CD25\textsuperscript{–} T cells were cultured in triplicate in 96-well plates with 5 × 10^5 irradiated APCs plus 0.5 \mu g/mL anti-CD3 along with the indicated number of CD4CD25\textsuperscript{+} cells. Cultures were incubated for 72 hours, pulsed with 1 \mu Ci/well (0.037 MBq/well) 3H-thymidine for the last 12 hours of culture, and counted.

**In vivo transfer assays**

All C57BL/6 or C57BL/6-Thy1.1 recipient mice received myeloablative doses of total body irradiation from a 137Cesium source (900-950 cGy).
followed by intraperitoneal injection of 20 µg anti–asialo GM1. For suppression of reactivity in a graft-versus-host (GVH) model, CD4CD25− cells from Balb/c mice were labeled in 5 µM CFSE (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR). Effectors (1 × 10^6) mixed with indicated numbers of unlabeled fresh unactivated CD4CD25+ cells or activated CD4CD25+ cells sorted on CD44+ expression were injected intraperitoneally into C57BL/6 hosts. Three days later, recipient mice were killed and cells in the peritoneal cavity of each mouse were harvested by lavage with 5 mL RPMI 2 mM EDTA, 10 ng/mL murine IL-3, 10 ng/mL murine IL-6, and 50 ng/mL murine SCF, Methocult M3234; Stem Cell Technologies, Vancouver, BC, Canada) with 3 hours after irradiation C57BL/6 animals were reconstituted intravenously (SD/H11006 CD45RB. However, the naturally occurring CD4CD25+ subset constitutively expresses many of these activation markers shared with effector/memory cells.6,32 Therefore, we monitored temporal changes in activation markers on total naive CD4 T cells in conjunction with that of activated CD44 as assessed by staining with FITC-HA (Figure 1A). Using plate-bound anti-CD3/anti-CD28 stimulation, CD25 and CD69 are rapidly induced to maximal and uniform expression by 6 to 8 hours. At 24 hours, while T cells generally conform to a typical activated T-cell–staining profile of CD25+, CD69+, CD62L+, TCR+, only a relatively minor fraction of activated cells express CD44+ (Figure 1A-B). We additionally used OVA32-39 TCR transgenic (DO11.10) naive CD4 T cells, thereby eliminating the diversity and variable affinity of the TCR. Again, only a similar limited fraction of cells respond with the expression of CD44+ after 24 hours in vitro with high-dose OVA peptide–pulsed APCs (Figure 1C). Thus, the population of stimulated CD4 T cells distinguished by the functionally active form of CD44 represents only a fraction of cells otherwise fully activated by standard phenotypic criteria.

**CD44CD25+ regulatory T cells have a pronounced capacity to up-regulate CD44act**

To examine the regulation of CD44 activation on CD4CD25+ compared with CD4CD25− T cells, peripheral Balb/c CD4 cells were fractionated on the basis of CD25 expression. As anticipated, as an effector/memory marker, overall CD44 expression was slightly higher in the freshly isolated CD4CD25+ population than in corresponding CD4CD25− cells (Figure 2A). However, endogenous CD44+ expression, as measured by anti-CD44 (KM81) blockable Fl-HA binding, was not detectable. Fractionated cells

**Results**

**CD44act as an activation marker that discriminates a subset within a larger population of activated T cells**

The classic T-cell surface markers after activation include the acquisition of CD69 and CD25 and decreases of CD62L, TCR, and CD45RB. However, the naturally occurring CD4CD25+ subset constitutively expresses many of these activation markers shared with effector/memory cells.6,32 Therefore, we monitored temporal changes in activation markers on total naive CD4 T cells in conjunction with that of activated CD44 as assessed by staining with FITC-HA (Figure 1A). Using plate-bound anti-CD3/anti-CD28 stimulation, CD25 and CD69 are rapidly induced to maximal and uniform expression by 6 to 8 hours. At 24 hours, while T cells generally conform to a typical activated T-cell–staining profile of CD25+, CD69+, CD62L+, TCR+, only a relatively minor fraction of activated cells express CD44+ (Figure 1A-B). We additionally used OVA32-39 TCR transgenic (DO11.10) naive CD4 T cells, thereby eliminating the diversity and variable affinity of the TCR. Again, only a similar limited fraction of cells respond with the expression of CD44+ after 24 hours in vitro with high-dose OVA peptide–pulsed APCs (Figure 1C). Thus, the population of stimulated CD4 T cells distinguished by the functionally active form of CD44 represents only a fraction of cells otherwise fully activated by standard phenotypic criteria.

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**Statistical analysis**

Comparisons were done by Student t test, except where otherwise noted.

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Analyses comparing CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells. CD4 cells were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> fractions and activated at 1 x 10<sup>6</sup> cells/well with increasing concentrations of plate-bound anti-CD3 with (open) or without (closed) 5 μg/mL anti-CD28. The percentage of CD69<sup>+</sup> cells (top) and blast-positive cells (by scatter, middle) are nearly indistinguishable between populations, whereas the percentage of Fl-HA–binding cells differs significantly between CD4CD25<sup>+</sup> and CD4CD25<sup>-</sup> for both treatments (bottom; P ≤ .01, Wilcoxon test). (A) Abundance of CD44<sub>act</sub> expression on resting CD4CD25<sup>+</sup> and CD4CD25<sup>-</sup> T cells, CD4 cells were separated into CD4CD25<sup>+</sup> and CD4CD25<sup>-</sup> fractions and activated at 1 x 10<sup>6</sup> cells/well with increasing concentrations of plate-bound anti-CD3 with (open) or without (closed) 5 μg/mL anti-CD28. The percentage of CD69<sup>+</sup> cells (top) and blast-positive cells (by scatter, middle) are nearly indistinguishable between populations, whereas the percentage of Fl-HA–binding cells differs significantly between CD4CD25<sup>+</sup> and CD4CD25<sup>-</sup> for both treatments (bottom; P ≤ .01, Wilcoxon test). (B) Kinetics of activation marker expression on CD25<sup>+</sup> and CD25<sup>-</sup> CD4 T cells. CD4 cells were separated into CD4CD25<sup>+</sup> and CD4CD25<sup>-</sup> fractions and activated at 1 x 10<sup>6</sup> cells/well with increasing concentrations of plate-bound anti-CD3 with (open) or without (closed) 5 μg/mL anti-CD28. Data shown are the mean ± SD of 3 independent experiments (*P ≤ .001; **P ≤ .01). FACS analyses from a representative experiment are shown below for the 18-hour time point with and without the HA-blocking anti-CD44 antibody, KMB1.

Figure 2. CD44<sub>act</sub> is preferentially expressed on CD4CD25<sup>+</sup> Treg cells after activation.

Activated CD44 is associated with elevated expression of transcripts for relevant cytokines in CD4CD25<sup>+</sup> Treg cells

It has been demonstrated that prior stimulation of CD4CD25<sup>+</sup> regulatory cells improves their suppressor activity. We therefore asked whether this marker discriminates cells with differential cytokine expression profiles. CD4CD25<sup>+</sup> cells were stimulated for 16 to 18 hours with plate-bound anti-CD3/anti-CD28 to induce expression of CD44<sub>act</sub>, as in Figure 2. At this time the population is fully activated by the criteria of maximal CD69 expression. Cells were sorted into CD44<sup>+</sup> HA-binding (CD44<sup>act</sup>) and CD44<sup>-</sup> non–HA-binding fractions (Figure 3A) and directly lysed for comparison of cytokine mRNA expression by reverse transcriptase (RT)–PCR. The HA-binding fraction expressed markedly elevated mRNA levels for a number of cytokines, including

Figure 3. CD44<sub>act</sub> expression on CD4CD25<sup>+</sup> Treg cells is associated with elevated expression of relevant cytokine mRNA.

(A) CD4CD25<sup>+</sup> T cells were activated for 16 to 18 hours with plate-bound anti-CD3/anti-CD28 and then sorted immediately on the basis of CD44<sub>act</sub> expression (gates indicated by boxes). (B) Sorted activated CD4CD25<sup>+</sup> cells and freshly isolated, unactivated (Unact) CD4CD25<sup>+</sup> cells were immediately lysed for multiplex RT-PCR to assess cytokine mRNA levels. Results shown are representative of 4 independent experiments. Cycles for each cytokine: GMCSF, TNF<sub>α</sub>, 25 cycles; IL-10, IFN<sub>γ</sub>, TGF<sub>β</sub>, 30 cycles; IL-4, -5, -10, 40 cycles. Internal control GAPDH was amplified for 25 cycles. (C) Relative cytokine mRNA levels as measured by real-time quantitative PCR analysis in CD44<sup>act</sup> and CD44<sup>-</sup> fractions of CD4CD25<sup>+</sup> cells activated for 24 hours and separated as in panel A. Cytokine levels in unactivated CD4CD25<sup>+</sup> T cells are shown for comparison. Data shown are the mean ± SD of 3 experiments. *P ≤ .001 and **P ≤ .005 for comparisons of CD44<sub>act</sub> to CD44<sup>-</sup> mRNAs.
those associated with suppression: IL-10, TGFβ, and IL-4 (Figure 3B). That mRNA for some proinflammatory cytokines is also expressed in these cells is consistent with previous literature, particularly given the strong TCR stimulus used, and was confirmed by quantitative real-time RT-PCR (Figure 3C). These results clearly indicate a skewing in transcript levels of many cytokines in the CD44 act-bearing fraction of CD4CD25 Treg cells, suggesting that this marker may in turn be associated with greater functional capacity.

CD44 act on CD4CD25 Treg cells distinguishes a population with highly enhanced suppressor activity in vitro

To address whether differential CD44 act expression is associated with differential functional activity, CD4CD25 cells were fully activated and fractionated as described in Figure 3 into HA-binding and non–HA-binding populations and placed in a standard anti-CD3 plus APC in vitro suppressor assay with CD4C25 T cells as responders. Activated CD44 act+ CD4CD25 cells suppressed proliferation more robustly than either activated CD44 act– CD4CD25 cells or freshly isolated unactivated CD4CD25 cells (Figure 4A). We have observed an approximate 6-fold difference in the number of CD44 act+ cells required to achieve 50% suppression compared with the CD44 act–, non–HA-binding population and consistently a more than 10-fold difference compared with the direct ex vivo–isolated Treg cells (Figure 4B). The enhanced suppressor function of the CD44 act–expressing population is particularly notable in view of the fact that when the non–HA-binding cells are returned to anti-CD3/anti-APC stimulation in the suppressor assay, they continue to give rise to a significant percentage of new CD44 act+ cells, albeit not as bright as in the original positive population (Figure 4C). Thus, at least some of the suppression observed in the CD44 act– population potentially derives from the de novo generation of an HA-binding population within it.

While unactivated Treg cells do not express CD44 act initially (Figure 2A), a distinct portion of cells up-regulates this form after stimulation during a suppressor assay (Figure 4D). In addition, total activated Treg cells containing about half as many Fl-HA–binding cells as the sorted CD44 act+ population (98% pure) require approximately twice as many cells to give 90% suppression compared with CD44 act+ Treg cells (Figure 4E). The correspondence between the degree of suppression and the fraction of CD44 actcells in the unmanipulated total population further indicates that the heightened suppressor function observed in the CD44 act–enriched population is not due to direct signaling through HA during separation, as has been suggested by some studies for low–molecular-weight HA. These observations together substantiate a strong correlation between expression of this marker and functionally active Treg cells.

Activated CD4CD25 Treg cells differing distinctly with respect to expression of CD44 act do not show parallel differences for other activation markers

Since the CD4CD25 population is known to bear increased effector/memory markers, it was of interest to determine whether differences in such surface markers correlated with activated Treg cells differing in expression of CD44 act. CD4CD25 cells were therefore stimulated with anti-CD3/anti-CD28 for 24 hours, then stained for CD4, CD25, CD69, CD45RB, CD62L, CTLA-4, and GITR in combination with Fl-HA. Cells were gated on CD44 act+, Fl-HA–positive, and Fl-HA–negative populations as indicated and assessed for the other markers. As expected, activation resulted in increases in CD25, CD69, CTLA-4, and GITR, as well as decreases in CD27 and CD62L and increases in CD69, CD45RB, and GITR, as well as decreases in CD27 and CD62L and increases in CD69, CD45RB, and GITR.
in CD62L and CD45RB compared with unactivated Treg cells (Figure 5A). However, except for slight shifts in CTLA-4 and GITR in the HA-binding population, all other markers were nearly indistinguishable between the HA-binding and HA-nonbinding subsets, providing additional evidence for the natural CD4CD25 \(^+\) Treg cell phenotype of the entire population and the otherwise similar phenotypic state of activation of cells that differ with respect to CD44\(^{act}\) expression. The magnitude difference seen in FI-HA binding (Figure 5A, top) together with the functional data presented in Figure 4 suggests that among cell-surface activation markers, CD44\(^{act}\) most clearly delineates functional activity among activated CD4CD25 \(^+\) Treg cells.

**Foxp3 transcript levels do not correlate with CD44\(^{act}\) expression or degree of suppressor activity in activated Treg cells**

As a highly characterized marker for this subset, Foxp3 has been shown to be expressed after in vitro activation of CD4CD25 \(^+\) cells. However, prior studies have not addressed the extent to which the level of Foxp3 expression correlates with suppressor function. When mRNA from the activated CD4CD25 \(^+\) populations fractionated on the basis of CD44\(^{act}\) was examined by quantitative real-time PCR for Foxp3, levels were essentially equivalent (P > .5; Figure 5B). Moreover, these results are consistent with previous reports of decreases in Foxp3 mRNA after stimulation of CD4CD25 \(^+\) cells, although still much higher than the CD44CD25 \(^+\) cells (P > .1 for both activated CD4CD25 \(^+\) populations). These data serve to further confirm the suppressor phenotype of the CD44\(^{act}\) population and, together with cell-surface staining data, suggest that CD44\(^{act}\) is a unique marker within the CD4CD25 \(^+\) population that discriminates the degree of regulatory function.

**Transfer of CD4CD25\(^+\) Treg cells expressing activated CD44 results in enhanced regulatory activity in vivo**

It has been previously shown that effector-cell activation and function, but often not proliferation, are suppressed by CD4CD25 \(^+\) Treg cells in vivo. To assess the effect of suppressors on the activation of effector T cells in vivo, Balb/c CD4CD25 \(^+\) effector T cells were CFSE labeled and injected into C57BL/6-Thy1.1 congenic recipients together with various populations of unlabeled Balb/c CD4CD25 \(^+\) Treg cells. Treg cells were first activated for 24 hours and separated into HA-binding and non–HA-binding populations, as described in Figure 3. These, or control freshly isolated unactivated Treg cells, were injected intraperitoneally along with CFSE-labeled effector cells. After 3 days, peritoneal cells were harvested and assessed for CD25 expression on dividing alloreactive cells as a measure of effector-cell activation in the CFSE/Thy1.2 compartment. Transfer of control autologous Balb/c CD4CD25 \(^+\) T cells showed no proliferation or CD25 up-regulation (not shown). Cotransfer of suppressor cells bearing CD44\(^{act}\) did not affect proliferation of effectors but did significantly reduce their CD25 expression from 69% of CFSE-labeled cells in the presence of no suppressors to 15% in the presence of suppressors expressing CD44\(^{act}\), even at suppressor-effector (S/E) ratios of 1:10 (Figure 6A-B). In contrast, activated CD44\(^{hi}\) non–HA-binding Treg cells exerted only quite modest effects on responder-cell activation. Freshly isolated suppressors likewise have little effect even at higher S/E ratios. Thus, Treg cells expressing CD44\(^{act}\) exhibit pronounced suppressive activity in vivo, and this property is sustained over 72 hours.

An early and major target in GVH is the hematopoietic stem cell, and the CD4CD25 \(^+\) Treg cell population can effectively impede GVH disease (GVHD) in murine models. Therefore we investigated HSC rescue by Treg cells in a GVH model to obtain an early readout and thereby minimize CD44\(^{act}\) populations potentially recovering CD44\(^{act}\) expression over time with further stimulation. Lethally irradiated C57Bl/6 mice were reconstituted with syngeneic bone marrow and coinjected with Balb/c CD4CD25 \(^+\) effector T cells with and without CD4CD25 \(^+\) suppressor populations generated as described in Figure 3. After 5 days, splenic colony-forming units (CFUs) were measured in vitro as a reflection of HSC engraftment. Simultaneously, serum levels of IFNγ, typically elevated early in a GVH reaction, were measured. Syngeneic bone marrow engraftment alone gives rise to baseline numbers of CFUs (110 per 2 x 10\(^4\) plated cells) and negligible levels of IFNγ (Figure 6C). CFUs are dramatically diminished with the addition of allogeneic CD4CD25 \(^+\) T effector cells. While the addition of freshly isolated Balb/c CD4CD25 \(^+\) suppressors restores CFUs to about 20% of baseline, the suppressor population enriched for CD44\(^{act}\) demonstrates the greatest rescue, with CFUs approaching BM reconstitution only, as well as the greatest normalization of

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**Figure 5. Expression of markers associated with CD4CD25\(^+\) Treg cells is similar irrespective of CD44\(^{act}\) levels after activation.** (A) After 24-hour activation in vitro, cells were stained and analyzed by flow cytometry. Gating for histograms was done on the basis of Fl-HA binding, as indicated in the top panel. The HA-negative gate (left) is established based on the limits of KM81 blocking of the HA-positive population. Marker expression for total resting CD4CD25 \(^+\) cells (gray line) is also shown for comparison.

(B) Relative Foxp3 mRNA levels as measured by real-time quantitative PCR analysis in CD44\(^{act}\) and CD44\(^{nonact}\) fractions of CD4CD25 \(^+\) cells activated for 24 hours and separated as in Figure 3. Foxp3 levels in unactivated CD4CD25 \(^+\) and CD4CD25 \(^+\) T cells are shown for comparison. Data shown are the mean ± SD of 3 experiments. P = .003, comparing CD44\(^{act}\) Treg cells to unactivated CD4CD25 \(^+\) cells; P = .007, comparing CD44\(^{act}\) Treg cells to unactivated CD4CD25 \(^+\) cells.
C57BL/6 mice were reconstituted with C57Bl/6 bone marrow cells (3 mice/group. Statistical comparison between relevant groups is shown. *P\leq.001; **P\leq.002. Data are the mean ± SD of 4 experiments. (B) Average in vivo suppression of CD25 expression on T effectors in the presence of Treg cells. Transfers were conducted as in panel A. Experiments were done with 4 mice/group. Statistical comparison between relevant groups is shown. *P<.001; **P≤.002. Data are the mean ± SD of 4 experiments. (C) Lethally irradiated C57BL/6 mice were reconstituted with C57Bl/6 bone marrow cells (3 × 10⁶) and simultaneously given 2 × 10⁶ Balb/c CD4CD25⁺ effector T cells together with 1.5 × 10⁶ freshly isolated CD44⁺/CD4⁺ fractionated CD4CD25⁻ regulatory T cells as in panel A. On day 5, serum was collected for IFNγ measurement and 2 × 10⁴ splenocytes were plated in Methocult M3334 plus stem-cell growth factors. Five to 7 days later, colonies were counted. The number of CFUs per plate is shown as a percentage of BM alone and is an average for at least 5 mice per group (bottom, 5). The □ reflects the values when CFUs for fractionated cells are indexed for homing efficiency relative to freshly isolated, unactivated CD4CD25⁻ cells. IFNγ serum levels for each group are shown in the top panel. Statistical comparison between indicated groups is shown. *P≤.002; **P≤.01. Data shown are the mean ± SD of 5 experiments.

Figure 6. T cells expressing CD44⁺ show an enhanced suppressed effect in alloreactive and GVH models in vivo. (A) C57BL/6-Thy1.1 congenic hosts were injected intraperitoneally with 1 × 10⁶ CFSE-labeled Balb/c CD4CD25⁺ T cells along with 2 × 10⁶ unlabelled unactivated or 1 × 10⁶ activated Balb/c CD4CD25⁺ cells sorted based on CD44⁺ expression. After 3 days, cells in the peritoneum were collected by lavage and analyzed by flow cytometry. Expression of CD25 as a reflection of alloactivation of CFSE-gated CD4CD25⁻ responder T cells is shown. Data are from individual mice and are representative of 4 independent experiments. (B) Average in vivo suppression of CD25 expression on T effectors in the presence of Treg cells. Transfers were conducted as in panel A. Experiments were done with 4 mice/group. Statistical comparison between relevant groups is shown. *P<.001; **P≤.002. Data are the mean ± SD of 4 experiments. (C) Lethally irradiated C57BL/6 mice were reconstituted with C57Bl/6 bone marrow cells (3 × 10⁶) and simultaneously given 2 × 10⁶ Balb/c CD4CD25⁺ effector T cells together with 1.5 × 10⁶ freshly isolated CD44⁺/CD4⁺ fractionated CD4CD25⁻ regulatory T cells as in panel A. On day 5, serum was collected for IFNγ measurement and 2 × 10⁴ splenocytes were plated in Methocult M3334 plus stem-cell growth factors. Five to 7 days later, colonies were counted. The number of CFUs per plate is shown as a percentage of BM alone and is an average for at least 5 mice per group (bottom, 5). The □ reflects the values when CFUs for fractionated cells are indexed for homing efficiency relative to freshly isolated, unactivated CD4CD25⁻ cells. IFNγ serum levels for each group are shown in the top panel. Statistical comparison between indicated groups is shown. *P≤.002; **P≤.01. Data shown are the mean ± SD of 5 experiments.

Figure 7. Conversion of CD44 to its active form occurs on Treg cells in vivo and is associated with increased suppressor function. (A) C57BL/6-Thy1.1 congenic hosts were injected intravenously with 20 × 10⁶ freshly isolated Balb/c CD4CD25⁺ T cells (Thy1.2). After 72 hours, spleen cells were stained with Thy1.2-allophycocyanin, CD44-PE, and Fl-HA. Fl-HA/CD44 staining of cells gated on Thy1.2 is shown with and without KM81 blocking. (B) CD44⁺ and CD44⁻ fractions from the Thy1.2 gate were sorted as shown and placed in an in vitro anti-CD3 suppression assay with 2.5 × 10⁶ CD4CD25⁻ responders. Suppressor activity (mean ± SEM for 2 experiments) is significantly enhanced in the in vivo arising CD44⁺ population. *Statistical comparison of CD44⁺ to CD44⁻ and to unactivated Treg cell groups. P≤.001 for both pairwise comparisons.

Discussion

A variety of cell-surface markers are available that together aid in identifying, isolating, and characterizing the heavily scrutinized CD4CD25⁺ regulatory T-cell subset. However, no surface marker capable of differentiating the degree of suppressor efficacy or potency within this compartment has been described. Like other markers of T-cell activation, CD44⁺ is regulated as the result of physiologic interactions and signaling through the TCR. However, unlike these other markers, conversion to the activated form of CD44 is relatively late and is expressed only on a subpopulation of T cells under conditions of full T-cell activation. Because of their general anergic characteristics and a molecular profile enriched in genes antagonistic to cellular signaling, our expectation was that the CD4CD25⁺ population would be refractory to the induction of CD44⁺. To the contrary, we found these cells highly prone to the expression of this activation marker compared with their CD25⁻ counterparts. Further examination revealed a strong association between the expression of CD44⁺ and degree of suppressor activity. This correlation distinguishes not only suppressor potency between resting and activated CD4CD25⁺ T cells but also among activated Treg cells that appear otherwise phenotypically similar. The results emphasize the physiologic relevance of this marker and its potential for monitoring and isolation of suppressor activity directly ex vivo.

The naturally occurring CD4CD25⁺ subset constitutively expresses several surface markers characteristic of memory T cells. Moreover, on prolonged activation and expansion of these cells there have been reported shifts in expression of several memory/effector activation markers, in agreement with the results shown here (Figure 5A). However, we show that these surface markers are not substantially different when comparing activated Treg cells that differ by nearly a log in CD44-dependent Fl-HA binding, and these Treg cells with proper stimulation (Figure 7A) and further supporting a physiologic relevance for this marker. In addition, when these cells were sorted directly ex vivo on the basis of CD44⁺, then placed in an anti-CD3 suppression assay, superior activity was evident in the CD44⁺ population (Figure 7B).
therefore do not carry the same functional implications. The forkhead/winged helix transcription factor Foxp3 is thought to program the development and function of the CD4CD25+ subset and has proven useful in their identification.3,18,52-54 However, the degree to which suppressor function correlates with Foxp3 mRNA levels among activated Treg cells has not been directly addressed, and reports have generally described down-regulation of Foxp3 mRNA on activation.31,38-40 The use of CD44act to separate activated Treg cells with differential suppressor function allowed us to determine that Foxp3 transcript levels do not differ between the subpopulations defined by this marker (Figure 5B).

While the association of CD44act with Treg cell function is clear from these studies, the degree to which it is obligatory for function is more difficult to determine. Preliminary antibody-blocking experiments suggest that CD44act ligand binding function is not directly involved in suppression. Since CD44act-expressing Treg cells are highly activated, it is conceivable that elevated CD25 expression and resulting IL-2 consumption could be responsible for increased suppression. However, CD44act low activated Treg cells express similar levels of CD25 (Figure 5B) yet suppress less effectively. Consistent with prior studies,3,33 CD44act Treg cells do not suppress across a semipermeable (Transwell) barrier, making this explanation unlikely (data not shown). Nonetheless, we cannot rule out that local IL-2 depletion occurs with close proximity to effectors. Moreover, increased costimulation and combinations of cytokines do not substantially improve the degree of conversion to CD44act-expressing cells (data not shown). Identifying a stable negative population under conditions of stimulation is made difficult by the continued emergence of CD44act on a portion of activated T cells after depletion of cells binding HA (Figure 4C). Thus, much of the suppressor activity detected in the CD44act- pool may be attributed to this de novo population. To the extent that is the case, functional activity would indeed be closely linked with expression of this marker. Consistent with this interpretation is the observation that freshly isolated Treg cells do up-regulate this marker both in vitro (Figure 4D) and in vivo (Figure 7A). Since our in vivo experiments show relative stability of these functional differences over time (Figures 6-7), by selecting the cells that have the greatest propensity to convert CD44, we may also be selecting the most “triggerable” and thus functionally active subset. The latter interpretation is supported by the fact that while CD44act emerges from a population depleted of such cells on restimulation, the intensity consistently remains lower than on the positive (Figure 4C).

The basis for the predilection of the CD4CD25+ subset to convert CD44 to its functionally activated form is not clear. The fact that this naturally occurring population is thought to have been selected on self-antigens in rigorous fashion and that, as a result, these cells have clear characteristics of memory T cells may be a contributing factor. However, CD4CD25+ cells of memory phenotype isolated directly ex vivo do not show the same degree of CD44 activation as the CD25+ subset (data not shown). CD4CD25+ cells have also been reported to be more sensitive to antigen-induced activation in the presence of IL-2, at least in terms of proliferation,11 and TCR affinity has been shown to play a significant role in the development of the CD25+ subset.55 The inclination to up-regulate CD44act may reflect this and is consistent with a correlation between the expression of CD44act and a heightened state of TCR-mediated cellular activation. When considered in the context of CD44-initiated extravasation, it would be reasonable that a cell armed with a trafficking molecule should also be coordinately poised to execute effector function at the target site—that is, for CD4CD25+ T cells—to exert suppression.

Considerable recent effort has concentrated on the expansion of CD4CD25 Treg cells for controlling pathologic immune responses in the settings of autoimmune disease, transplantation, and GVHD. While anergic and hypoproliferative in vitro, it is clear that this proliferative deficit can be overcome with the addition of exogenous IL-2.53 and increased potency of suppressors is obtained after activation and expansion in vitro or in vivo.6,7,33 However, the 3- to 4-fold improvement in these studies was seen after 7-day activation, whereas in the studies presented here, increased suppression in the populations expressing CD44act was seen with less than 24-hour activation, with a more than 10-fold increase in suppressive function compared with resting cells. In models of GVHD disease, amelioration of disease has been reported after expansion of cells in vitro,47,49,56 and initial attempts to expand alloreactive Treg cells in humans have shown promise.57 Treg cells from nonobese diabetic (NOD) mice can also be expanded in vitro and used therapeutically to protect in autoimmune situations, and autoantigen-specific CD4CD25+ cells were considerably more efficacious than polyclonally expanded counterparts.58 The observations provided by our studies have direct implications for these efforts: (1) the CD44act marker has the potential for preferentially selecting highly potent Treg cells very early after in vitro stimulation for subsequent expansion; (2) since cells bearing this marker can be detected in vivo under autoimmune circumstances (S.D., unpublished observations, July 2003), it may be feasible to directly isolate the Treg cells most relevant to the current autoimmune process (eg, islet-specific, in T1D), and therefore the most efficacious, on the basis of this marker. Importantly, there would be no requirement for prior knowledge of the autoantigen(s) in any autoimmune disease, as the selection itself would be antigen independent.

In conclusion, we have identified a preferentially expressed cell-surface marker demarcating a subset of CD4CD25+ T cells with heightened activation status and functionally primed for enhanced suppression. A significant implication of the CD44act phenotype in the Treg cells compartment is that it may provide a tool to aid in monitoring and isolation of highly potent cells for therapeutic purposes and for furthering the understanding of the signaling pathways leading to suppressor function in this subset.

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Suppressor activity and potency among regulatory T cells is discriminated by functionally active CD44

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