1083-8791/04/1005-0002$30.00/0
doi:10.1016/j.bbmt.2003.12.302

CD25 Expression Distinguishes Functionally Distinct Alloreactive CD4$^+$ CD134$^+$ (OX40$^+$) T-Cell Subsets in Acute Graft-versus-Host Disease

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Received July 15, 2003; accepted December 21, 2003

ABSTRACT

CD134 (OX40) is expressed on activated CD4$^+$ donor T cells in allogeneic stem cell transplant recipients with acute graft-versus-host disease. The data presented here reveal that differential expression of CD25 by CD4$^+$ CD134$^+$ T cells allows separation of these activated cells into 2 phenotypically and functionally distinct alloreactive T-cell subsets. These subsets exhibit distinct tissue associations, with CD4$^+$ CD134$^+$ CD25$^-$ T cells preferentially found in lymphoid tissues and CD4$^+$ CD134$^+$ CD25$^+$ T cells located in lymphoid tissues and inflamed extralymphoid tissues. The CD25$^-$ T-cell subset exhibited potent proliferative responses to both concanavalin A and allogeneic host leukocytes. By contrast, the CD25$^+$ T-cell subset proliferated minimally in response to either treatment and inhibited alloantigen-induced proliferation of the CD25$^-$ subset. Proliferative unresponsiveness associated with the CD25$^+$ T-cell subset did not extend to cytokine secretion. When stimulated with alloantigen, both CD4$^+$ CD134$^+$ T-cell subsets responded by secreting interferon-γ and interleukin (IL)-10, and neither T-cell subset produced detectable levels of IL-2 or IL-4. Three-day treatment of the CD25$^+$ T-cell subset with IL-2 restored the proliferative responsiveness of these cells to host alloantigens, suggesting that the proliferative unresponsiveness associated with this T-cell subset reflected a requirement for IL-2. The preferential tissue associations and distinct functional properties associated with these separable alloreactive CD4$^+$ CD134$^+$ T-cell subsets suggest that they participate differentially in clinical graft-versus-host disease.

KEY WORDS

CD134 • OX40 • Allogeneic stem cell transplantation • Graft-versus-host disease

INTRODUCTION

Acute graft-versus-host disease (GVHD) is a major and frequently lethal complication of allogeneic stem cell transplantation [1-4]. The disease is characterized by damage to epithelial cells and involves multiple organ systems, including the skin, gastrointestinal tract, and liver [2,5]. The disease occurs when mature donor T cells respond immunologically to host-derived major histocompatibility complex (MHC) and/or minor histocompatibility antigens presented by host antigen-presenting cells [6-9], and recent data indicate that disease progression can occur in the absence of MHC class I or II molecule expression by target epithelial cells [10]. Although clearly implicated in disease, the physiologic contributions of CD4$^+$ T cells to disease induction and progression remain poorly understood.

CD134 (OX40) is a member of the tumor necrosis factor receptor superfamily and functions as a costimulatory receptor, providing a complementary signal, or "second signal," to CD4$^+$ T cells after signal-

ing through the T-cell receptor [11]. Naive T cells do not express CD134; however, this molecule is rapidly upregulated after T-cell receptor engagement [12], and CD134 expression is associated with activated antigen-specific $CD4^+$ T cells in autoimmune disease processes and in GVHD [13,14]. The naturally occurring ligand of CD134 (CD134L) is expressed by antigen-presenting cells—including dendritic cells, B cells, and macrophages—and has been shown to deliver a second signal to $CD4^+$ T cells [15-20]. CD134L has also been reported on endothelial and microglial cells [21,22]. Signaling of $CD4^+$ T cells through CD134 ligation has been shown to enhance cell accumulation, increase cell survival, increase the expression of antiapoptotic proteins, and promote the generation of T-helper type 1 and 2 cytokines [23-28]. In addition, cell signaling via CD134 has been implicated in the generation of memory T cells and the reversal of peptide-induced peripheral tolerance or anergy [13,23,26,29-32].

CD134 is expressed on activated $CD4^+$ donor T cells in rats, mice, and humans with GVHD [14,33-36]. In the rat, CD134 was found to be rapidly upregulated on donor $CD4^+$ T cells after transplantation, and cells within this population were shown to be alloreactive [14]. In the mouse, blocking the interaction between CD134 and CD134L by administering a monoclonal anti–CD134L-specific antibody prevented GVHD, suggesting that CD134 ligation is critical for disease progression [33]. Blazar et al. [37] extended these studies by demonstrating that a CD134-specific antibody with receptor agonist activity promoted disease and that the disease was less severe in animals deficient for CD134 or CD134L. Thus, these observations strongly implicate $CD4^+$T cells that coexpress $CD134^+$ in GVHD.

Activated GVHD-associated T cells can also express CD25, the interleukin (IL)-2 receptor (IL-2R) α chain. To evaluate the relationship between GVHD-associated $CD4^+$ $CD134^+$ and $CD4^+$ $CD25^+$ T cells, lymphocytes from rats with GVHD were assessed for expression of all 3 markers. In this study, we report that GVHD-associated $CD4^+$ $CD134^+$ T cells can be divided into 2 T-cell subsets distinguished by expression of CD25. Because both CD25 and CD134 are associated with T-cell activation, we hypothesized that these phenotypically distinguishable $CD4^+$ $CD134^+$ T-cell subsets would exhibit distinct biological properties. The results of the studies described here reveal that the $CD4^+$ $CD134^+$ T-cell subsets exhibit distinct tissue localization patterns and distinct in vitro proliferative responses, that the $CD25^+$ subset is immune suppressive, and that both subsets are composed of alloreactive T cells. These data suggest that the separable $CD4^+$ T-cell subsets introduced here differentially contribute to the complex pathology associated with clinical GVHD.

MATERIALS AND METHODS

Rats

Female or male Buffalo and (Lewis $\times$ Buffalo)$_{F1}$ rats 8 to 16 weeks of age were used in this investigation. These animals were obtained from the breeding colony at the Portland Veterans Affairs Medical Center (Portland, OR), and the breeder rats maintained in this colony were from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All rats were housed at the Veterans Affairs Medical Center veterinary medicine unit (Portland, OR) and cared for according to institutional guidelines, with free access to food and water. Within any experiment, animals were matched for sex and age.

Induction of GVHD

GVHD was induced by injection of parental Buffalo lymphocytes into (Lewis $\times$ Buffalo)$_{F1}$ recipients as described previously [14]. Briefly, bone marrow cells (2×10^7) and lymph node cells (5×10^7) isolated from Buffalo rats were injected intravenously into sublethally irradiated (Lewis $\times$ Buffalo)$_{F1}$ recipients. Animals received 600R gamma irradiation with a Mark 168A Irradiator (J.L. Sheperd and Associates, San Fernando, CA). For 1 week before and throughout the experiments, animals were given water containing neomycin sulfate 1.1 mg/mL and polymyxin B sulfate 0.167 mg/mL to limit the passage of endotoxin across the gut wall as a consequence of host conditioning. Animals were monitored throughout the course of disease and were killed when a near-moribund state was identified, as determined by the onset of diarrhea or a 30% loss of body weight.

Cell Preparations

Single-cell leukocyte suspensions from lymph nodes and spleen were prepared by passage of cells through a 250-μm stainless-steel sieve. Liver-associated leukocytes were obtained after portal vein perfusion to remove leukocytes contained with the vasculature. Briefly, portal vein perfusion of the liver was performed serially with phosphate-buffered saline (PBS) and then with PBS containing 1% collagenase D (Roche, Indianapolis, IN) and 2% fetal bovine serum (FBS). Perfused livers were minced and incubated with PBS containing 1% collagenase D and 2% FBS (30 minutes at 37°C and 5% CO_2). The liver cell preparations were passed through a 250-μm stainless-steel sieve to remove undigested tissue, and leukocytes were recovered from the cell suspension by Ficoll gradient centrifugation. Lungs were minced before incubation with 1% collagenase in PBS with 2% FBS and then processed as with the liver.

Antibodies and Flow Cytometric Analyses

Antibodies directed against CD45.1 (RT7.1), CD4 (OX38), CD25, CD134 (OX40), CD62L, CD45RC, and MHC class II (OX6) were purchased from BD PharMingen (La Jolla, CA). Lewis and $(\text{Lewis} \times \text{Buffalo})_{F1}$ lymphocytes express CD45.1, but Buffalo cells do not. Antibody labeling of lymphocyte suspensions was conducted with routine methods, and labeled cells were evaluated by using a FACSCaliber (BD Biosciences, San Jose, CA). Results were analyzed with CellQuest (BD Biosciences).

Cell Sorting

Single-cell suspensions from lymph nodes, spleen, liver, or lungs of transplant recipients were labeled with antibodies directed against CD45.1, CD4, CD134, and/or CD25 in PBS with 2% FBS and sorted with a FACSVantage cell sorter (BD Biosciences). The sort gate was set on $CD45.1^-$ (donor) lymphocytes, and $CD45.1^-$ cells were sorted into $CD4^+$ $CD134^-$, $CD4^+$ $CD134^+$, $CD4^+$ $CD134^+$ $CD25^-$, or $CD4^+$ $CD134^+$ $CD25^+$ subsets. An alternative method to purify these cell subsets was to select $CD4^+$ or $CD134^+$ cells by using microbead technology (Miltenyi Biotec, Auburn, CA) and to subsequently label and FACSVantage-sort the microbead-selected cells. In the setting where microbead selection was used, cells were labeled with a fluorochrome-conjugated antibody directed against CD134 or CD4, and the labeled cells were positively selected by using microbeads coated with anti–mouse immunoglobulin G. Selected cells were then labeled with fluorochrome-conjugated antibody directed against CD4, CD134, and/or CD25 before FACSVantage sorting. Results obtained with the 2 methods were comparable.

IL-2 Rescue

Sorted T cells were cultured in RPMI medium (Invitrogen, Grand Island, NY) containing 10% FBS (Hyclone, Logan, UT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 mmol/L (Fisher Scientific, Fair Lawn, NJ), L-glutamine 2 mmol/L (Invitrogen), minimal essential medium nonessential amino acids (Sigma Chemical Co., St. Louis, MO), sodium pyruvate 1 mmol/L (Sigma), 2-mercaptoethanol 50 μmol/L (Invitrogen), penicillin 100 U/mL, and streptomycin 100 μg/mL (Invitrogen; complete medium). For IL-2 rescue, cells were cultured in complete medium supplemented with human recombinant IL-2 (10 U/mL; Chiron, Emeryville, CA) in 24-well plates.

T-Cell Proliferation

Proliferation assays were used to examine the responses of sorted donor T-cell subsets after their isolation from transplant recipients. The stimulatory agents used in these assays included irradiated (3000R) parental syngeneic or host allogeneic splenocytes, concanavalin A (ConA) plus syngeneic splenocytes, and antibodies targeting CD3 and CD28, or CD3 and CD134. For these assays, sorted donor T cells were cultured in complete medium at 3 to 4×10^4 cells per well in 96-well plates at 37°C in 5% CO_2. Syngeneic and allogeneic stimulator cells were added at concentrations of 1×10^5 cells per well, and ConA was used at a concentration of 2 μg/mL. In the setting of antibody-dependent T-cell stimulation, the anti-CD3 antibody was immobilized onto plates (preincubated for 2 hours with antibody at a concentration of 5 μg/mL in PBS), and anti-CD28 and anti-CD134 antibodies were added to culture medium at a concentration of 5 μg/mL. For assays of immune suppression, the number of $CD4^+$ $CD134^+$ $CD25^-$ T cells was held constant for each condition, and variable numbers of $CD4^+$ $CD134^+$ $CD25^+$ T cells were added to the cultures. The cultures were incubated for 3 to 4 days, and 1 μCi of ^{3}H-thymidine was added to each well for the last 18 hours of incubation. The cells were harvested, and thymidine uptake was assessed with a liquid scintillation counter. The data are reported as the mean counts per minute ± SEM for 3 to 6 wells for each treatment.

Cytokine Detection

Capture enzyme-linked immunosorbent assays (ELISAs) were performed to quantify cytokine secretion by sorted donor T-cell subsets isolated from transplant recipients. Cytokine production was monitored in culture supernatants from cells stimulated with parental syngeneic and host allogeneic splenocytes. Culture conditions were the same as those described for T-cell proliferation, except that culture supernatants were harvested for analysis of cytokine content after 72 hours of culture. OptEIA capture ELISA reagent kits (BD PharMingen) were used to assess supernatants for IL-2, IL-4, IL-10, and interferon (IFN)-γ. The reagents in each cytokine quantitation kit included a capture antibody, a biotinylated detection antibody, and an avidin/horseradish peroxidase conjugate. The horseradish peroxidase was detected by using tetramethyl benzidine as a substrate, and 2N H_2SO_4 was used as a stopping reagent. ELISAs were performed according to the manufacturer's instructions, and optical density was read with a Bio-Tek Instruments, Inc. (Winooski, VT) plate reader.

Statistical Analyses

Data from these studies were analyzed with the SPSS statistical program (SPSS Inc., Chicago, IL).

Comparisons included analysis of variance or the Student *t* test.

RESULTS

CD25 Expression Distinguishes 2 GVHD-Associated CD4+ CD134+ T-Cell Subsets That Exhibit Distinct Tissue Associations

Our previous work revealed that the activation antigen CD134 is upregulated on T cells in GVHD and that expression is associated with cells of the CD4 lineage [14]. Cells from lymph nodes and inflamed liver at day 10 of disease illustrate this association (Figure 1A). Because expression of CD25 is also observed on activated T cells in GVHD, we sought to determine the relationship between $CD4^+$ $CD134^+$ and $CD4^+$ $CD25^+$ GVHD-associated T cells. Donor ($CD45.1^-$) T cells from rats with GVHD, posttransplantation day 10, were evaluated for expression of both CD134 and CD25. The assessment revealed that GVHD-associated $CD4^+$ $CD134^+$ T cells could be subdivided into $CD25^+$ and $CD25^-$ subsets (Figure 1B). Further, these T-cell subsets exhibited distinct tissue associations (Figure 1B; Table 1). In organized lymphoid tissues, such as lymph nodes and spleen, the $CD25^+$ and $CD25^-$ T-cell subsets were present at similar frequencies. By contrast, in extralymphoid sites such as inflamed liver and lung, the $CD25^+$ T-cell subset predominated, and these cells represented 65% to 85% of the total $CD4^+$ $CD134^+$ T cells. Similar results have been observed between days 5 and 18 of disease (data not shown). Thus, these data reveal the existence of 2 GVHD-associated $CD4^+$ $CD134^+$ T-cell subsets that exhibit distinct tissue associations.

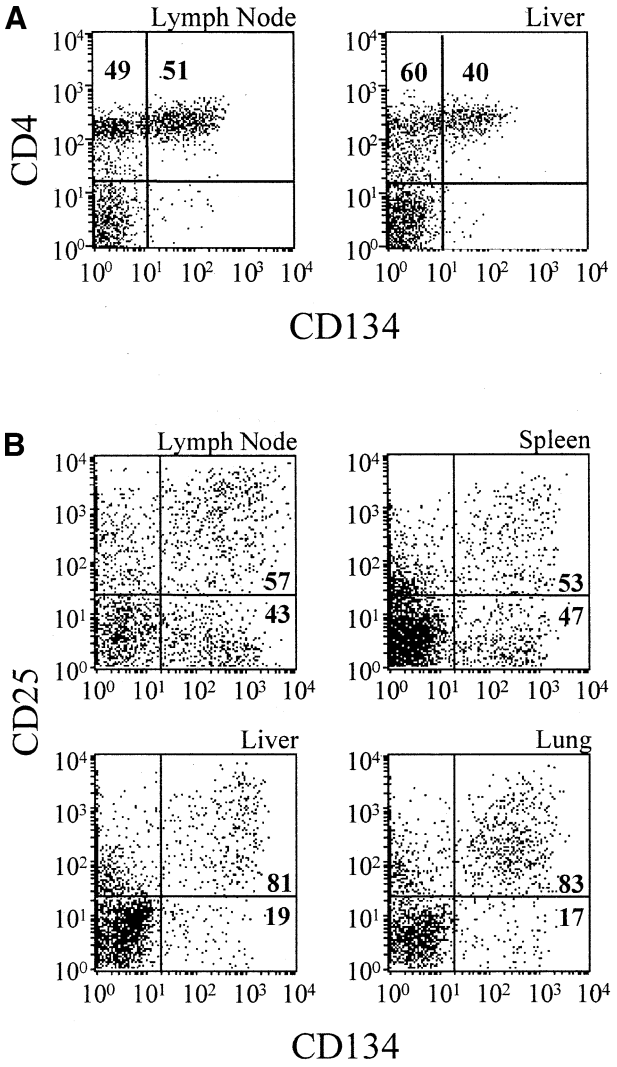

Figure 1. Acute GVHD-associated $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T-cell subsets are nonrandomly distributed in lymphoid versus extralymphoid tissues. Lymphocytes were isolated from the tissues of animals with GVHD on day 10 after bone marrow transplantation. Lymph nodes and spleen were used as representative lymphoid organs, and liver and lung were used as the source of lymphocytes from extralymphoid tissues. Isolated lymphocytes from lymph node and liver were labeled with antibodies to CD4 and CD134. Analysis by flow cytometry (A) revealed that the vast majority of $CD134^+$ cells were $CD4^+$. Isolated lymphocytes were also labeled with antibodies to CD45.1, CD134, and CD25 (B). The plots shown are gated on donor $CD45.1^-$ cells, which represent 90% to 95% of total leukocytes in day 10 transplant recipients. The percentage of total $CD4^+$ $CD134^+$ T cells that were $CD25^+$ or $CD25^-$ is indicated. Similar results were observed in 4 independent experiments, and the results shown are from a representative animal.

Expression of CD134 or CD25 by GVHD-Associated CD4+ T Cells Suggested That These Cells Had Been Recently Activated

To further define the activation state of these cells, lymph node–derived cells were sorted into $CD4^+$ $CD134^+$ $CD25^+$ and $CD4^+$ $CD134^+$ $CD25^-$ T-cell fractions by using a fluorescence-activated cell sorter (FACS; Figure 2A), and sorted cells were stained and evaluated for expression of CD45RC, CD62L, and MHC class II (Figure 2B). In the rat, these molecules are differentially expressed on naive versus activated T cells; CD62L and CD45RC were downmodulated and MHC class II was increased with activation. Lymph node cells in the $CD25^+$ subset were predominantly negative for CD45RC, expressed low levels of CD62L, and expressed high levels of MHC class II, a phenotype consistent with T-cell activation. Cells contained within the $CD25^-$ T-cell subset were negative for CD45RC and expressed low levels of CD62L, as anticipated for activated T cells. However, most of the cells within the $CD25^-$ T-cell subset expressed low to intermediate levels of MHC class II. In contrasting MHC class II expression levels on $CD25^+$ versus $CD25^-$ cells, the mean fluorescence

Table 1. *Acute GVHD-Associated CD4$^+$ CD134$^+$ CD25$^-$ and CD4$^+$ CD134$^+$ CD25$^+$ T-Cell Subsets Are Nonrandomly Distributed in Lymphoid versus Extralymphoid Tissues**

Variable	Lymph Nodes	Spleen	Liver	Lungs
Total cells ($\times 10^5$)	434.3 ± 57.6	200 ± 65.9	302 ± 83.1	34 ± 22.4
CD4$^+$ CD134$^+$ CD25$^+$ cells as a percentage of total CD4$^+$ CD134$^+$ T cells	54.8 ± 1.2	46.3 ± 4.1	70.3 ± 2.5	73.3 ± 4.0
CD4$^+$ CD134$^+$ CD25$^-$ cells as a percentage of total CD4$^+$ CD134$^+$ T cells	45.2 ± 1.2	53.7 ± 4.1	29.7 ± 2.5	26.7 ± 4.0

*The data shown are pooled from 10 independent experiments. One to 3 animals were used in each experiment, and cells were obtained on days 10 to 18 after bone marrow transplantation. The distinct tissue associations (lymphoid versus extralymphoid) of the CD4$^+$ CD134$^+$ CD25$^+$ and CD4$^+$ CD134$^+$ CD25$^-$ subsets were observed throughout the course of disease; $P < .05$.

intensities were 494 and 144 units, respectively (Figure 2B; Table 2). These data suggest that the 2 CD4$^+$ CD134$^+$ T-cell subsets are differentially activated.

Donor CD4$^+$ CD134$^+$ T Cells, a Mixture of CD25$^+$ and CD25$^-$ Cells, Proliferate Minimally when Stimulated with Host Alloantigen

The abundance of CD4$^+$ CD134$^+$ T cells in GVHD suggests that these cells play an active role in disease. To assess the functional properties of these cells, T cells from the lymph nodes of animals with GVHD were sorted into CD4$^+$ CD134$^+$ and CD4$^+$ CD134$^-$ populations. Liver-derived CD4$^+$ CD134$^+$ T cells were also sorted. These populations were then cultured with syngeneic Buffalo stimulator cells, host allogeneic (Lewis $\times$ Buffalo)$_{F1}$ stimulator cells, or syngeneic Buffalo stimulator cells plus ConA. ^{3}H-Thymidine incorporation was used as a measure of cell proliferation. In contrasting proliferation of these cells in response to syngeneic versus allogeneic stimulator cells, proliferation was minimal for all cell populations (Figure 3A). The CD4$^+$ CD134$^+$ populations yielded small but statistically significant allospecific responses, whereas CD4$^+$ CD134$^-$ cells did not. These low-level proliferative responses were unexpected in that our previous data had shown substantial allospecific proliferative responses by CD4$^+$ CD134$^+$ T cells after short-term in vitro culture in medium containing IL-2 [14]. ConA stimulation yielded a more robust response from all populations (Figure 3B). However, the response of lymph node–derived CD4$^+$ CD134$^-$ T cells to ConA was notably stronger than the responses of CD4$^+$ CD134$^+$ T-cell populations from either lymph nodes or liver. Thus, these data indicate that at the time of isolation, alloreactive T cells contained within the CD4$^+$ CD134$^+$ T-cell population proliferate poorly in response to stimulation with host alloantigen. Further, the ConA responses of the CD4$^+$ CD134$^+$ T-cell subsets were weak relative to CD4$^+$ CD134$^-$ T cells.

CD4$^+$ CD134$^+$ CD25$^-$ and CD4$^+$ CD134$^+$ CD25$^+$ T Cells Exhibit Differences in Their Proliferative Responses after Stimulation with ConA or Alloantigen

The presence of 2 CD4$^+$ CD134$^+$ T-cell subsets with distinct tissue associations in GVHD suggested that the minimal proliferative responses observed for the mixed (CD25$^-$ and CD25$^+$) CD4$^+$ CD134$^+$ population might not have accurately reflected the activities of each T-cell subset. To test this concept, CD4$^+$ CD134$^+$ cells were sorted into CD4$^+$ CD134$^+$ CD25$^+$ and CD4$^+$ CD134$^+$ CD25$^-$ T-cell subsets and analyzed functionally in proliferation assays. Sorts were performed as in Figure 2, and the GVHD-associated cells used in this series of experiments were lymph node derived. CD4$^+$ CD134$^+$ T cells, a mixture of both CD25$^+$ and CD25$^-$ cells, were also sorted from these lymph node cell preparations. The proliferative capacities of the CD4$^+$ CD134$^+$ CD25$^+$, the CD4$^+$ CD134$^+$ CD25$^-$, and the total CD4$^+$ CD134$^+$ cell population (containing both the CD25$^+$ and the CD25$^-$ subsets) were assessed after stimulation with parental syngeneic stimulator cells, host allogeneic stimulator cells, or syngeneic stimulator cells plus Con A (Figure 4). The CD25$^-$ subset exhibited a strong proliferative response to both allogeneic stimulator cells and to ConA. By contrast, the CD25$^+$ subset failed to proliferate after stimulation with allogeneic stimulator cells and responded very poorly to ConA. The total CD4$^+$ CD134$^+$ fraction proliferated poorly after stimulation with allogeneic stimulator cells and responded modestly to ConA.

CD4$^+$ CD134$^+$ CD25$^+$ T Cells Are Immune Suppressive

Because the lymph node CD4$^+$ CD134$^+$ T-cell population contains roughly equal numbers of CD25$^-$ and CD25$^+$ cells, proliferation of this mixed population after stimulation with allogeneic stimulator cells would have been predicted to be roughly 50% of that observed for the CD25$^-$ subset. However, the ob-

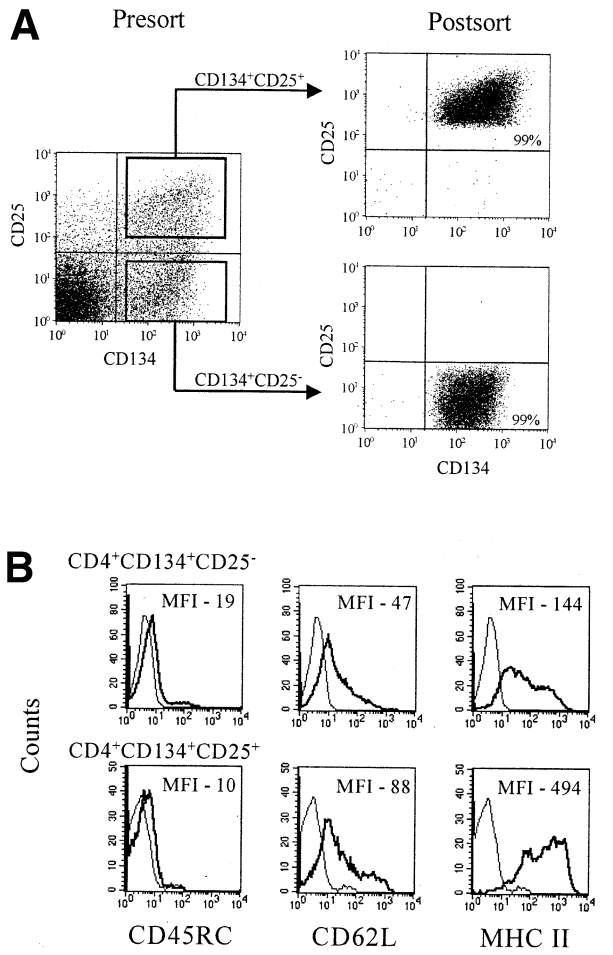

Figure 2. Phenotypic analysis of $CD4^+$ $CD134^+$ T cells suggests recent activation. Lymphocytes were isolated from the lymph nodes of animals with GVHD on day 10 after bone marrow transplantation. Isolated cells were labeled with antibodies directed against CD45.1, CD134, and CD25 and FACS-sorted into donor $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ cell subsets. In this representative cell sort (A), the section on the left illustrates the CD134 and CD25 sort gates and the presort profile of donor $CD45.1^-$ cells. The sections on the right illustrate reanalyzed sorted donor-derived $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T-cell subsets. After sorting, these cells were labeled with CD45RC, CD62L, and MHC class II and analyzed by flow cytometry. Results are shown as histograms (B), with specific staining shown with a bold line and isotype control staining shown with a fine line. Values presented are the mean fluorescence intensity (MFI) of specifically stained cells. Similar results were observed in 4 independent cell sorts, and the results shown are from a representative animal.

served response was <15% of the predicted value (Figure 4), suggesting that the $CD25^+$ subset suppressed proliferation of the $CD25^-$ subset.

To further assess the immune-suppressive nature of $CD4^+$ $CD134^+$ $CD25^+$ T cells, FACS-sorted $CD4^+$ $CD134^+$ $CD25^+$ and $CD4^+$ $CD134^+$ $CD25^-$ T cells were evaluated independently or when mixed for responsiveness to syngeneic versus allogeneic stimulator cells. For these mixing studies, the number of $CD4^+$ $CD134^+$ $CD25^-$ T cells was held constant for each condition, and variable numbers of $CD4^+$ $CD134^+$ $CD25^+$ T cells were added to the cultures. Figure 5 illustrates that lymph node–derived $CD4^+$ $CD134^+$ $CD25^-$ T cells respond well to host alloantigen and that the $CD4^+$ $CD134^+$ $CD25^+$ T-cell population was unresponsive. The results also show that the response of $CD4^+$ $CD134^+$ $CD25^-$ T cells was reduced in a dose-dependent fashion by the addition of increasing numbers of $CD4^+$ $CD134^+$ $CD25^+$ T cells, confirming that cells contained in the $CD25^+$ subset are able to inhibit proliferation of cells in the $CD25^-$ subset.

Co-Stimulation Partially Overcomes $CD4^+$ $CD134^+$ $CD25^+$ T-Cell Unresponsiveness

The lack of a proliferative response by the $CD4^+$ $CD134^+$ $CD25^+$ T-cell subset to ConA (Figure 4B) was unique in these studies. To characterize further the response potential of these cells, the stimulatory effects of antibodies targeting CD3 and CD28 (anti-CD3/CD28) or CD3 and CD134 (anti-CD3/CD134) were compared with the stimulatory effects of ConA. The proliferative responses of sorted lymph node $CD4^+$ $CD134^-$ T cells were contrasted with the proliferative responses of lymph node $CD4^+$ $CD134^+$ $CD25^+$ T cells. As anticipated, the $CD4^+$ $CD134^-$ T-cell subset responded well to stimulation with anti-CD3/CD28, anti-CD3/CD134, and ConA (Figure 6). By contrast, the $CD4^+$ $CD134^+$ $CD25^+$ T cells responded poorly to ConA, but these cells did respond to anti-CD3/CD28 and anti-CD3/CD134. The increased responsiveness of the $CD25^+$ subset to stimulation with anti-CD3/CD28 or anti-CD3/CD134 supports the concept that proliferative unresponsiveness within the $CD25^+$ T-cell subset can be partially overcome by the delivery of potent signals through the T-cell receptor and the co-stimulatory receptors CD28 and CD134.

Alloreactive Cells Are Contained within the $CD4^+$ $CD134^+$ $CD25^+$ T-Cell Subset

The unresponsive properties of the $CD25^+$ T-cell subset precluded effective assessment of host specific

Table 2. *Expression of Activation Markers by $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T Cells**

	Marker		
Cell Subset	**CD45RC**	**CD62L**	**MHC Class II†**
$CD4^+$ $CD134^+$ $CD25^-$	**16.5 ± 3.7**	**50.5 ± 12.9**	**121.2 ± 15.2**
$CD4^+$ $CD134^+$ $CD25^+$	**46.0 ± 13.8**	**82.7 ± 22.6**	**395.4 ± 42.8**

*The data shown are pooled from 4 independent experiments.
†Significant difference between the $CD4^+$ $CD134^+$ $CD25^+$ and $CD4^+$ $CD134^+$ $CD25^-$ T-cell subsets; $P < .05$.

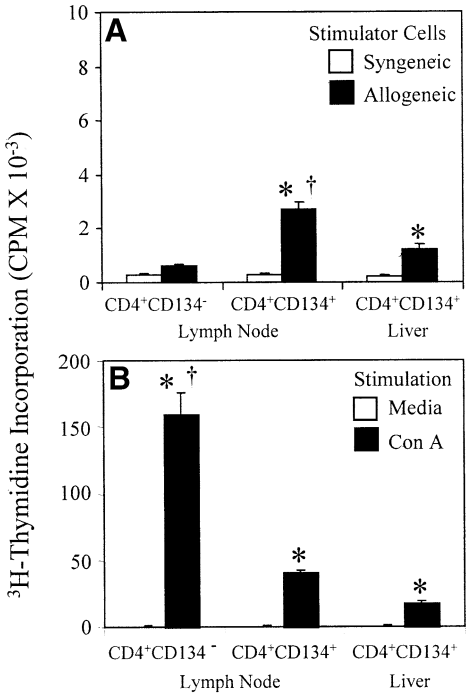

Figure 3. Proliferative responses of CD4$^+$ CD134$^-$ and CD4$^+$ CD134$^+$ T-cell subsets when stimulated with alloantigen or ConA. FACS-sorted donor-derived lymph node and liver CD4$^+$ CD134$^+$ cells and lymph node CD4$^+$ CD134$^-$ cells from animals with day 10 GVHD were cultured with syngeneic or allogeneic stimulator cells or with syngeneic stimulator cells plus ConA. Proliferative responses to syngeneic and allogeneic stimulator cells are shown in (A), and responses of these cells to ConA are illustrated in (B). Note the difference in scale between (A) and (B). The proliferation rate is presented as ^{3}H-thymidine incorporation. Similar results were observed in at least 4 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. *Significant differences between stimulation conditions; †significant differences between the CD4$^+$ CD134$^+$ and CD4$^+$ CD134$^-$ cell populations; $P < .05$.

alloreactivity in the studies illustrated in Figures 4 and 5. However, because exposure of GVHD-derived CD4$^+$ CD134$^+$ T cells to IL-2 had previously revealed alloreactive cells within this mixed population [14], we hypothesized that IL-2 would reverse the unresponsive properties associated with the CD25$^+$ T-cell subset and that subsequent allostimulation of IL-2 rescued cells would reveal host-specific alloreactivity. To test this hypothesis, FACS-sorted GVHD-associated CD4$^+$ CD134$^+$ CD25$^+$ T cells were cultured for 3 days in medium containing IL-2, and cells recovered from these cultures were evaluated for reactivity to parental syngeneic and host allogeneic splenocytes. Results are shown in Figure 7 and reveal that when unresponsiveness in the CD25$^+$ T-cell subset is overcome by exposure to IL-2, treated cells proliferate in response to stimulation with host allogeneic stimulator cells. Thus, the CD25$^+$ T-cell subset is composed of alloreactive T cells.

Stimulation of CD4$^+$ CD134$^+$ CD25$^-$ and CD4$^+$ CD134$^+$ CD25$^+$ T Cells with Alloantigen Promotes Secretion of IFN-γ and IL-10

As an alternate means of assessing T-cell function in this system, sorted CD4$^+$ CD134$^+$ CD25$^-$ and CD4$^+$ CD134$^+$ CD25$^+$ donor T cells from animals with GVHD were cultured in the presence of parental syngeneic or host allogeneic stimulator cells. After 3 days in culture, supernatants were harvested and evaluated for the presence of cytokines indicative of T-helper type 1 (IL-2 and IFN-γ), T-helper type 2 (IL-4), or regulatory T-cell (IL-10) responses. Super-

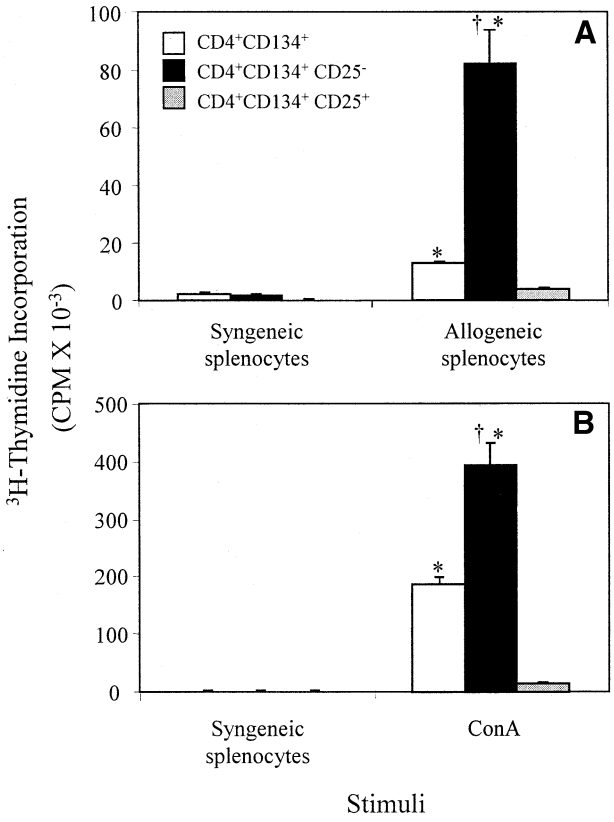

Figure 4. CD4$^+$ CD134$^+$ CD25$^-$ cells are alloreactive, whereas the CD4$^+$ CD134$^+$ CD25$^+$ cells are anergic. Microbead/FACS-sorted donor-derived lymph node T cells from animals with day 10 GVHD were sorted into 3 subsets: CD4$^+$ CD134$^+$, CD4$^+$ CD134$^+$ CD25$^-$, and CD4$^+$ CD134$^+$ CD25$^+$. The 3 subsets were cultured with syngeneic stimulator cells, allogeneic stimulator cells, or syngeneic stimulator cells plus ConA. Proliferative responses to syngeneic and allogeneic stimulator cells are shown in (A), and the responses of these cells to ConA are illustrated in (B). Note the difference in scale between (A) and (B). The proliferation rate is presented as ^{3}H-thymidine incorporation. Similar results were observed in at least 4 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. *Significant differences between the CD4$^+$ CD134$^+$ or CD4$^+$ CD134$^+$ CD25$^-$ subset and the CD4$^+$ CD134$^+$ CD25$^+$ cell subset (within a given stimulation condition); †significant differences between the CD4$^+$ CD134$^+$ CD25$^-$ cell subset and CD4$^+$ CD134$^+$ cells; $P < .05$.

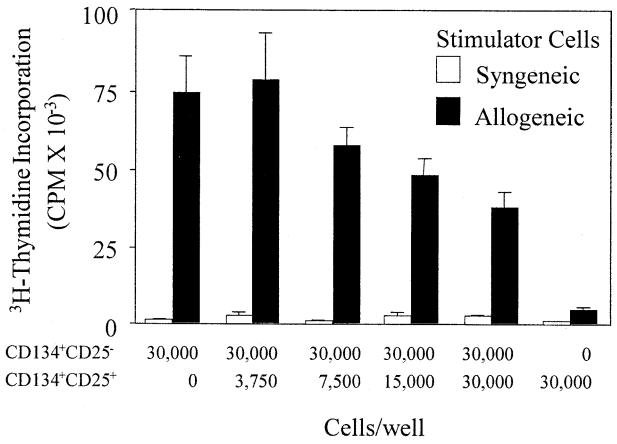

Figure 5. $CD4^+$ $CD134^+$ $CD25^+$ cells inhibit alloantigen induced proliferation of $CD4^+$ $CD134^+$ $CD25^-$ cells. FACS-sorted donor-derived lymph node $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T-cell subsets from animals with day 10 GVHD were tested independently and after being mixed for proliferative responses to allogeneic (closed bars) and syngeneic (open bars) splenic stimulator cells. When the $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T cells were mixed, the number of alloantigen-responsive $CD4^+$ $CD134^+$ $CD25^-$ T cells was held constant for each condition, and variable numbers of the candidate immune-suppressive $CD4^+$ $CD134^+$ $CD25^+$ T cells were added to the cultures. ^{3}H-Thymidine incorporation was used to quantitate proliferation. Similar results were observed in 3 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. Differences in ^{3}H-thymidine incorporation of the $CD4^+$ $CD134^+$ $CD25^-$ T-cell subset as a consequence of adding $CD4^+$ $CD134^+$ $CD25^+$ were significant; $P = .012$.

natants were screened by using capture ELISAs. The results of these analyses revealed that alloantigen stimulation promoted secretion of IFN-γ (Figure 8A) and IL-10 (Figure 8B) by both $CD4^+$ $CD134^+$ T-cell subsets. IL-2 and IL-4 were not detected in these cultures (data not shown). This observation indicates that in the system described here, proliferative unresponsiveness is a poor predictor of responses associated with alternate biological activities. Finally, alloantigen-induced secretion of cytokines by the $CD25^+$ and $CD25^-$ T-cell subsets provides further evidence that both $CD4^+$ $CD134^+$ T-cell subsets are alloresponsive.

DISCUSSION

Increasing evidence indicates that cells expressing the co-stimulatory molecule CD134 play active roles in a variety of immunoinflammatory disease processes. This molecule has been associated with disease-promoting T cells in animals with experimental autoimmune encephalitis [13] and in animals with GVHD [14,33-36]. Studies in rats with GVHD have confirmed that alloreactive $CD4^+$ $CD134^+$ T cells are contained in inflamed livers [14]. In mice, blockade of the CD134/CD134L interaction by administration of a monoclonal anti–CD134L-specific antibody prevented GVHD, suggesting that the biological response associated with CD134 ligation is critical during the induction and/or progression of GVHD [33]. Further support for a role for CD134 ligation in GVHD has been provided by Blazar et. al. [37], who showed that mice deficient for CD134 or CD134L developed less severe disease and that a CD134-specific antibody with receptor agonist activity promoted disease.

In the study described here, we report the tissue distribution, phenotype, and function of $CD4^+$ $CD134^+$ T cells associated with GVHD. The data reveal that $CD4^+$ $CD134^+$ T cells can be separated into 2 functionally distinct alloreactive T-cell subsets on the basis of the expression of CD25. The 2 $CD4^+$ $CD134^+$ T-cell subsets exhibited distinct tissue associations, with $CD25^-$ T cells preferentially found in lymphoid tissues such as lymph nodes and spleen and $CD25^+$ T cells detected in both lymphoid tissues and inflamed extralymphoid tissues. These $CD4^+$ $CD134^+$ T-cell subsets also exhibited distinct biological activities. Lymph node–derived $CD4^+$ $CD134^+$ $CD25^-$ T cells were found to be immediately responsive to alloantigen, proliferating extensively when stimulated with host allogeneic spleen cells. By contrast, lymph node $CD4^+$ $CD134^+$ $CD25^+$ T cells exhibited an unresponsive profile, failing to proliferate

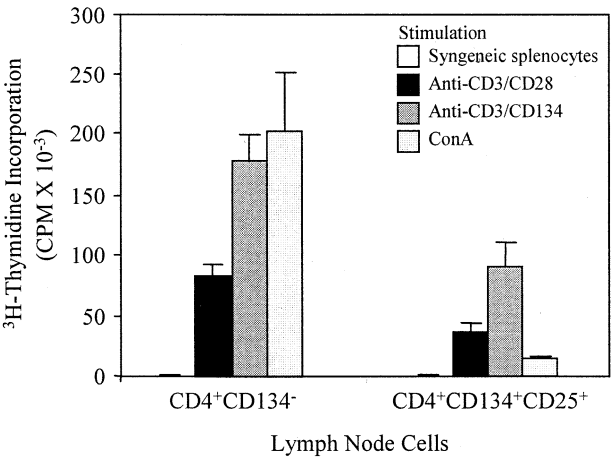

Figure 6. Co-stimulation of $CD4^+$ $CD134^+$ $CD25^+$ cells can be delivered through CD28 or CD134. FACS-sorted donor-derived lymph node $CD4^+$ $CD134^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T cells from day 10 GVHD were incubated with syngeneic stimulator cells, anti-CD3/CD28 antibodies, anti-CD3/CD134 antibodies, or ConA. ^{3}H-Thymidine incorporation was used to quantitate cell proliferation. Similar results were observed in 3 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. Responses of the $CD4^+$ $CD134^-$ and $CD4^+$ $CD134^+$ $CD25^+$ cell subsets to the different treatments (anti-CD3/CD28, anti-CD3/CD134, and ConA) were significantly different; $P < .05$.

when stimulated with host alloantigen and proliferating only minimally when stimulated with ConA. The $CD25^+$ T-cell subset was also found to inhibit alloantigen-induced proliferation of cells contained in the CD25 subset. Proliferative unresponsiveness associated with the $CD25^+$ T-cell subset was overcome by 3 days of in vitro culture in the presence of IL-2. The cells recovered from these IL-2–supplemented cultures maintained expression of CD25 (unpublished data) and were alloreactive, proliferating extensively when stimulated with host alloantigen. The alloresponsiveness of cells within the $CD25^+$ T-cell subset was also demonstrated by using quantitative assays of cytokine secretion, and both cell subsets were found to produce the immune-modulatory cytokines IFN-γ and IL-10. The observation of cytokine secretion and the ability to overcome proliferative unresponsiveness with IL-2 are consistent with the attributes of anergic T cells [38]. Thus, the $CD4^+$ $CD134^+$ T-cell subsets described here exhibited distinct tissue associations and differential responses to alloantigen and ConA, yet both populations are composed of alloreactive cells. These observations suggest that these separable $CD4^+$ T-cell subsets have distinct biological functions and that they may play fundamentally different roles during disease progression.

The predominance of the highly activated $CD4^+$ $CD134^+$ $CD25^+$ T cells in the extralymphoid target tissues of GVHD suggests that cells within this subset are disease-associated effector cells. As effector cells, these cells may directly or indirectly augment the immunoinflammatory process and promote tissue damage by a variety of mechanisms. Potential tissue-damaging mechanisms include the recruitment and activation of host-specific cytotoxic T lymphocytes [39,40], Fas/Fas ligand–mediated target cell killing [39-43], and production of the inflammatory cytokines IL-1 and tumor necrosis factor-α [10,44]. The observation of proliferative unresponsiveness associated with the $CD25^+$ T-cell subset suggested that these cells were exhausted by chronic antigen stimulation and were thus unable to respond when stimulated with alloantigen. However, the finding that these cells are potent secretors of the immune-modulatory cytokines IFN-γ and IL-10 argues against this concept.

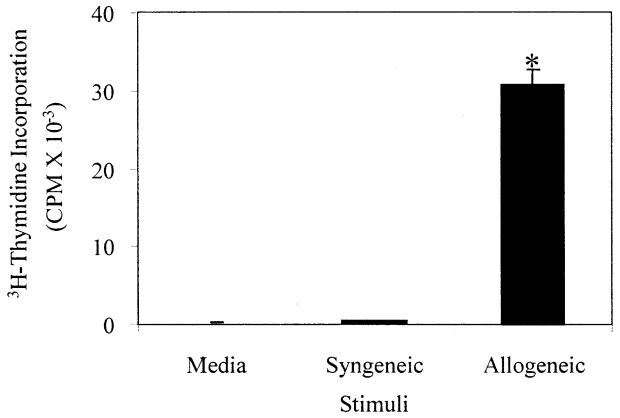

Figure 7. IL-2–treated $CD4^+$ $CD134^+$ $CD25^+$ T cells proliferate when stimulated with allogeneic spleen cells. Sorted donor-derived lymph node $CD4^+$ $CD134^+$ $CD25^+$ T cells from day 10 GVHD were incubated in human recombinant IL-2 (10 U/mL) for 3 days. After IL-2 treatment, cells were harvested, washed, and cultured in medium or medium containing irradiated syngeneic or allogeneic stimulator cells. Similar results were observed in 3 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. *Significant differences between groups; $P < .05$.

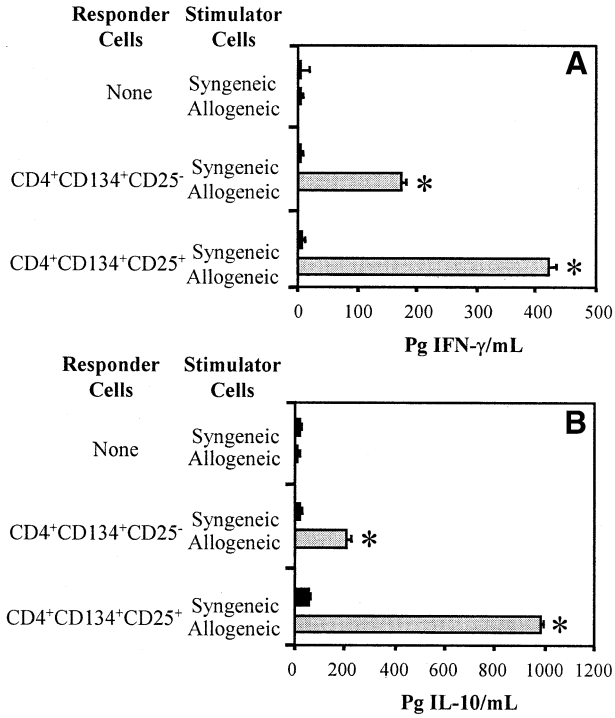

Figure 8. Allostimulation of $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ T cells promotes secretion of IFN-γ and IL-10. Donor-derived lymph node T cells from animals with day 10 GVHD were sorted into $CD4^+$ $CD134^+$ $CD25^-$ and $CD4^+$ $CD134^+$ $CD25^+$ subsets. The subsets were then cultured with parental syngeneic or host allogeneic stimulator cells. After 72 hours, culture supernatants were harvested and evaluated for the presence of IL-10 and IFN-γ. Results shown in (A) indicate IFN-γ concentrations; IL-10 concentrations are illustrated in (B). The limits of sensitivity in these assays were 15.6 pg/mL for IL-10 and 31.2 pg/mL for IFN-γ. Similar results were observed in at least 3 independent experiments, and the results shown are from a representative experiment. Results shown are the mean ± SEM. *Significant differences between groups; $P < .05$.

Regulatory T cells are generally thought to play a protective role in immunoinflammatory processes [45-47], including GVHD [48-50]. In GVHD, this disease-modifying activity was revealed by using 2 experimental strategies: an elimination strategy, in which depletion of naturally occurring regulatory T cells from a transplanted normal T-cell population resulted

in more rapid disease onset and death, and a supplementation strategy, in which supplementation of disease-promoting T cells with regulatory T cells obtained from normal donor animals resulted in protection from disease [48,49]. Supplementation of disease-promoting T cells with ex vivo–generated activation-induced regulatory T cells has also been shown to prevent GVHD [49,50]. Although the GVHD-associated $CD4^+$ $CD134^+$ $CD25^+$ T cells introduced here share phenotypic and functional properties with regulatory T cells, a relationship between the GVHD-associated $CD4^+$ $CD134^+$ $CD25^+$ cells and naturally occurring or activation-induced regulatory cells described by others has not been established. Thus, the data presented here suggest that the $CD4^+$ $CD134^+$ $CD25^+$ T-cell subset is composed of both disease-promoting effector cells and regulatory T cells and that in this model, regulatory T-cell activity is not sufficient to protect from lethal disease.

The selective association of the $CD4^+$ $CD134^+$ $CD25^-$ T-cell subset with organized lymphoid tissues suggests that this cell subset may be central to GVHD. This concept is supported by the potent in vitro proliferative response of this cell population when stimulated directly with alloantigen, a response that suggests the potential for rapid expansion and subsequent migration to extralymphoid inflammatory sites. These lymph node–derived $CD4^+$ $CD134^+$ $CD25^-$ T cells are $CD45RC^-$ $CD134^+$ and express low levels of CD62L, indicating an activated state. However, these cells also express low to intermediate levels of MHC class II and are $CD25^-$, suggesting that they are either not fully differentiated/activated or that they are differentiating toward a memory phenotype. The potential of these cells to further differentiate into $CD4^+$ $CD134^+$ $CD25^+$ effector cells is supported by 2 lines of in vitro evidence: (1) both cell subsets produce the immune-modulatory cytokines IFN-γ and IL-10 when stimulated with alloantigen, and (2) although IL-2 in the absence of stimulatory signals, such as alloantigen, does not support survival of the $CD4^+$ $CD134^+$ $CD25^-$ subset (unpublished data), cells within this T-cell subset are able to upregulate CD25 and MHC class II when stimulated in vitro with alloantigen (unpublished data). Taken together, these data suggest that the 2 $CD4^+$ $CD134^+$ T-cell subsets represent the same T-cell population in different physiologic states.

CD25, the IL-2R α chain, has long been identified as a marker of activated T cells in patients with GVHD. The binding of IL-2 to its cognate receptor promotes T-cell proliferation, and IL-2R–specific antibodies have been generated that block signaling via this receptor [51-54]. IL-2R–specific antibodies are therapeutic in patients with GVHD, and the results of treatment with these IL-2R antagonists are variable but have shown the greatest efficacy in the setting of GVHD involving skin [54]. The limited therapeutic efficacy observed with these agents in other tissues is possibly due to the availability of alternate T-cell growth promoting cytokines such as IL-15, which acts through a distinct cell-surface receptor [55]. An alternate mechanism that may contribute to the limited therapeutic efficacy of IL-2R antagonists could be the presence of a reservoir of disease-producing alloreactive $CD25^-$ T cells that would not be targeted by these antagonists. The identification of an alloreactive $CD4^+$ $CD134^+$ $CD25^-$ T-cell subset in the studies reported here supports this concept.

In summary, the results of this study reveal a high degree of complexity in the $CD4^+$ T-cell compartment in GVHD. Two phenotypically and functionally distinct alloreactive $CD4^+$ $CD134^+$ T-cell subsets were found to increase dramatically in frequency in animals that developed lethal GVHD. The distinct tissue associations and differential expression of activation markers on these cells suggest that cells within the $CD4^+$ $CD134^+$ $CD25^+$ T-cell subset function as disease-associated effector cells and that cells in the $CD4^+$ $CD134^+$ $CD25^-$ T-cell subset may comprise a reservoir of partially activated cells with the capacity to differentiate into effector cells. The data also support the concept that therapeutic agents designed to selectively target $CD25^+$ T cells do not identify all alloreactive $CD4^+$ T cells and suggest that novel therapeutic agents designed to selectively eliminate $CD134^+$ T cells may more effectively limit GVHD.

ACKNOWLEDGMENTS

We thank William H. Fleming and David C. Parker for critically reviewing this manuscript, and John Caldwell and Victor Tam for excellent technical assistance. The work was supported in part by a Veterans Affairs Merit Award to R.T.M.

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