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Expression Profiling of Murine Double-Negative Regulatory T Cells Suggest Mechanisms for Prolonged Cardiac Allograft Survival

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Recent studies have demonstrated that both mouse and human αβTCR+CD3+NK1.1−CD4−CD8− double-negative regulatory T (DN Treg) cells can suppress Ag-specific immune responses mediated by CD8+ and CD4+ T cells. To identify molecules involved in DN Treg cell function, we generated a panel of murine DN Treg clones, which specifically kill activated syngeneic CD8+ T cells. Through serial cultivation of DN Treg clones, mutant clones arose that lost regulatory capacity in vitro and in vivo. Although all allogeneic cardiac grafts in animals preinfused with tolerant CD4/CD8 negative 12 DN Treg clones survived over 100 days, allograft survival was unchanged following infusion of mutant clones (19.5 ± 11.1 days) compared with untreated controls (22.8 ± 10.5 days; p < 0.001). Global gene expression differences between functional DN Treg cells and nonfunctional mutants were compared. We found 1099 differentially expressed genes (q < 0.025%), suggesting increased cell proliferation and survival, immune regulation, and chemotaxis, together with decreased expression of genes for Ag presentation, apoptosis, and protein phosphatases involved in signal transduction. Expression of 33 overexpressed and 24 underexpressed genes were confirmed using quantitative real-time PCR. Protein expression of several genes, including FcεRIγ subunit and CXCR5, which are >50-fold higher, was also confirmed using FACS. These findings shed light on the mechanisms by which DN Treg cells down-regulate immune responses and prolong cardiac allograft survival. The Journal of Immunology, 2005, 174: 4535–4544.

Regulatory T (Treg) cells play an important role in modulating various diseases, including autoimmune diseases (1, 2), transplantation (3), allergy (4), infections (5), and cancer (6). Many types of Treg cells are known to be present in various animal and human disease models. The most extensively studied Treg cell populations are CD4+, which include CD4+CD25+ cells (7), Tr1 cells (8), CD4+CD103+ T cells (9), and CD4+CD25− T cells (10). In addition to CD4+ T cells, CD8+CD28− cells (11), γδ T cells (12), NK T cells (13), and αβTCR+CD3+NK1.1−CD8− double-negative (DN) T cells (14–18) have also been shown to have potent immunoregulatory function. Currently, identification of Treg cells is based primarily on nonspecific cell surface markers such as CD25 and CD62L. However, these markers are present only on certain subsets of Treg cells, as well as on nonregulatory cells.

Global expression analysis using cDNA microarray technology is a very powerful tool for high-throughput comparison of gene expression profiling. This technique allows for the identification of genes that are expressed cooperatively or are coregulated by a given treatment or stimulus. Microarray analysis has been successfully used to study many aspects of transplantation, including graft rejection (19, 20) and early transplant rejection prognosis (21). Recently, several groups have used this approach in an attempt to identify specific markers for CD4+CD25+ Treg cells. By comparing CD4+CD25+ Tr cells to CD4+CD25− T cells (9, 10, 22, 23), it was discovered that CD4+CD25+ Treg cells have a unique expression profile of various genes, including suppressors of cytokine signaling and glucocorticoid-induced TNFR family-related receptor, a member of the TNFR superfamily (22). Furthermore, differences in the regulation of apoptosis, cell cycle, cytokine receptor, cell-cell interaction, and stress pathway genes were also observed (23). In addition, levels of αβ integrin appear to correlate with presence of highly suppressive CD4+CD25+ Treg cells (9). Expression of the transcription factor scurfin, encoded by the Foxp3 gene, has been correlated to CD4+CD25+ and CD8+CD25+ Treg cells (24, 25). Foxp3 and neuropilin-1 are currently accepted as the best candidate markers for these Treg cells (26). However, it is not known whether the genes that are preferentially expressed in CD4+CD25+ T cells have a unique expression profile of various genes, including suppressors of cytokine signaling and glucocorticoid-induced TNFR family-related receptor, a member of the TNFR superfamily (22). Furthermore, differences in the regulation of apoptosis, cell cycle, cytokine receptor, cell-cell interaction, and stress pathway genes were also observed (23). In addition, levels of αβ integrin appear to correlate with presence of highly suppressive CD4+CD25+ Treg cells (9). Expression of the transcription factor scurfin, encoded by the Foxp3 gene, has been correlated to CD4+CD25+ and CD8+CD25+ Treg cells (24, 25). Foxp3 and neuropilin-1 are currently accepted as the best candidate markers for these Treg cells (26). However, it is not known whether the genes that are preferentially expressed in CD4+CD25+ T cells have a unique expression profile of various genes, including suppressors of cytokine signaling and glucocorticoid-induced TNFR family-related receptor, a member of the TNFR superfamily (22). Furthermore, differences in the regulation of apoptosis, cell cycle, cytokine receptor, cell-cell interaction, and stress pathway genes were also observed (23). In addition, levels of αβ integrin appear to correlate with presence of highly suppressive CD4+CD25+ Treg cells (9). Expression of the transcription factor scurfin, encoded by the Foxp3 gene, has been correlated to CD4+CD25+ and CD8+CD25+ Treg cells (24, 25). Foxp3 and neuropilin-1 are currently accepted as the best candidate markers for these Treg cells (26).
expressed in CD4^+CD25^+ Treg cells are also expressed in other Treg cells. Furthermore, the molecular mechanisms by which Treg cells control immune responses remain elusive.

DN Treg cells comprise 1–3% of peripheral T lymphocytes in rodents (15). These cells can down-regulate syngeneic CD8^+ T cell-mediated immune responses to self-Ags, suggesting a role for DN Treg cells in regulation of autoimmunity (27). We have previously shown that DN Treg cells isolated from mice that have permanently accepted allogeneic xenogeneic T cells and dose-dependent manner (15, 18). Interestingly, both mouse and human DN Treg donor-specific allogeneic skin graft survival when infused into syngeneic naive mice (15, 17). Furthermore, injection of DN Treg clones can attenuate graft vs host disease caused by infusion of allogeneic CD8^+ T cells, suggesting a role in vivo suppression of anti-host CD8^+ T cells (14). Recently, human DN Treg cells have been isolated and characterized. These cells compose 0.8–1% of total human peripheral blood CD3^+ T cells and 2.5% of lymph node T cells (18). Similar to what has been found in murine models, human DN Treg cells can also suppress immune responses mediated by syngeneic CD8^+ T cells in an Ag-specific and dose-dependent manner (15, 18). Interestingly, both mouse and human DN Treg cells are cytotoxic to syngeneic CD8^+ T cells that express the same TCR specificity as DN Treg cells (15, 18). Although these findings suggest that DN Treg cells are important in regulating immune responses both in vitro and in vivo, the molecular mechanisms behind their effects are poorly understood.

The goal of this study was to analyze global gene expression patterns that differentiate DN Treg clones that have potent immunoregulatory functions in vitro and in vivo from nonfunctional mutant cell lines derived from these clones to gain insight into the molecular mechanisms that contribute to DN Treg cell function. By comparing the expression profiles of DN Treg clones with those from the nonregulatory mutant strains, we identified 1099 highly differentially expressed genes revealing increased expression of genes involved in cell proliferation, immune regulation, and chemotaxis and an associated decrease in expression of genes involved in Ag presentation, apoptosis, and protein phosphatases involved in signal transduction pathways. A subset of overexpressed and underexpressed genes were validated using quantitative real-time PCR (QRT-PCR) or FACS analysis. The potential involvement of these molecules in controlling DN Treg cell function is discussed.

Materials and Methods

Mice

C57BL/6 × BALB/c (CB6F_1) and BALB/c-H-2-dm2 (dm2) were purchased from The Jackson Laboratory. A breeding stock of 2C-transgenic mice (on C57BL/6 background) was provided by Dr. D. Y. Loh (Nippon Research Center, Kanagawa, Japan). The 2C mice were bred with dm2 mice to obtain anti-L^d^ transgenic TCR (2C^+^dm2F_1, H^2^dm2) mice. The anti-L^d^ transgenic TCR can be detected by a specific mAb IB2 (29). All of the animals were kept in the animal facility at the University Health Network. All protocols were approved by the University Health Network Animal Care Committee, which meets the guidelines of the Canadian Council on Animal Care.

Generating and maintaining DN Treg and mutant clones

Generation of DN Treg clones was performed using previously described methods (15). Briefly, splenocytes were collected from either naive or tolerant (2C^+^dm2F_1) mice that permanently accepted CB6F_1 skin allografts following donor lymphocyte infusion and stimulated with irradiated L^d^ CB6F_1 splenocytes in the presence of 30 U/ml recombinant human IL-2 and 30 U/ml rIL-4 for 10 days. Activated IB2^+ T cells were subse-

quentely cultured in 96-well tissue culture plates at limiting dilutions of 0.5 cells/well. Additionally, cells arising from these wells were subcloned at dilutions of 0.5 cells/well to ensure clonality. To maintain the T cell clones, 5 × 10^5 cells were cultured in a 24-well plate containing 5 × 10^5 irradiated L^d^ CB6F_1 splenocytes as stimulators in α-ΜΕΜ supplemented with 10% FBS, 0.1% 2-ME, and 30 U/ml recombinant human IL-2 and 30 U/ml rIL-4. The cells were incubated at 37°C with 5% CO_2. Cells were restimulated in the same way every 3–4 days.

Cytotoxicity assays

(2C^+^dm2F_1, splenocytes were stimulated with irradiated (20 Gy) allogeneic spleen cells from L^d^ CB6F_1 mice for 3 days. Activated L^d^ specific IB2^+^CD8^+^ T cells then were labeled with [^3]HTdR at a concentration of 10 μCi/ml with 10 μg/ml con A and 50 U/ml IL-2 for 18 h, then washed three times in α-ΜΕΜ and used as targets. DN Treg clones (control naive (CN4) and tolerant CD4^+^CD8^+^ negative (TN12) and mutants (CN4.8 and TN12.8) were used as effector cells at day 3–4 poststimulation and plated in serial dilutions in a round-bottom 96-well tissue culture plate in the presence of 50 U/ml rIL-2, 30 U/ml rIL-4, and irradiated CB6F_1 splenocytes as stimulator cells. After coculture with effector cells at 37°C for 18 h, the cells were harvested and analyzed using a TOP COUNT (Packard Instruments) plate reader. Percent-specific cell killing was calculated using the equation: percentage of specific killing = (S − E)/S × 100, where E (experimental) is cpm of the retained DNA in the presence of effector cells, and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

Mouse cardiac transplantation

Naive 8– to 10-wk-old (2C^+^dm2F_1, mice were preinfused with 10^7/mouse of either the regulatory clone TN12, the mutant clone TN12.8, or left untreated 1 day pretransplantation. Heart grafts from 6– to 8-wk-old CB6F_1 mice were transplanted heterotopically into the abdominal cavity of syngeneic mice as described previously (16). Graft survival was monitored daily by palpation. Grafts were considered rejected when heartbeats could no longer be detected and confirmed by autopsy. Graft survival was expressed as mean survival time ± SD.

cDNA microarray and data analysis

Total RNA was isolated from regulatory and mutant clones using the standard TRIzol reagent protocol (Invitrogen Life Technologies) 3–4 days poststimulation. Before RNA isolation, all clones were tested for their ability to kill activated CD8^+ T cells in vitro as described above. Five of six regulatory clones showed at least 30% cytotoxicity toward allospecific syngeneic CD8^+ T cells and one (TN12 clone) showed reduced but detectable cytotoxicity. None of the mutant clones showed cytotoxicity toward activated syngeneic CD8^+ T cells.

cDNA microarrays were processed using protocols as previously described by our laboratory (19) using 100 μg of total RNA in each channel. Briefly, a 39,000 element cDNA microarray (mouse cDNA array Mouse Microarray Consortium developed at the Stanford Microarray Core Facility (http://microarray. org/sfgf/jsp/home.jsp)) was used for global gene expression profiling. Each cDNA microarray contains ∼50,000 cDNA clones with Unigene cluster identifiers representing 20,182 unique putative genes based on these identifiers (Unigene database build 141). Total RNA (100 μg) was obtained from 12 independent cell cultures of DN Treg cell lines (CN4 and TN12) and noncytotoxic mutant lines (CN4.8 and TN12.8). The DN Treg cell lines (CN4 and TN12) were labeled with Cy3 (green) and mutant cell lines (CN4.8 and TN12.8) labeled with Cy5 (red) and cohybridized in DN Treg: mutant cell pairs to six cDNA microarrays. Hybridized microarrays were scanned using GenePix 4000 (Axon Instruments) and fluorescent images were analyzed with the GenePix Pro software package.

Defective spots were flagged, and data for the six arrays in this study were stored on Stanford Microarray Database (30). Gene lists filtered at retrieval and green:red log normalized ratio collected (positive values represent higher expression in the DN Treg cell lines relative to their respective mutants). Low-stringency retrieval settings (80% representative data, signal:noise ratio of expression measurements > 1.5, signal > 200 in both channels) yields 28,142 cDNA clones. Of these, 1099 clones were found to be differentially expressed (using expression cutoff of 2-fold in one array) and subjected to additional statistical analysis. Cluster analysis (version 3.0) generated hierarchical clusters of the samples measuring similarity of expression of the genes and similarity across arrays, which are visualized with the TreeView program (Ref. 31; www.microarrays.org/ software).

Array data were centered before statistical analysis and significant gene expression differences between the DN Treg cell lines and mutants identified using a false discovery rate threshold of q < 0.025% and one-class response
in Statistical Analysis of Microarray (SAM) (32). Individual genes are ranked by a significance score (the q value) that is the estimate of the percentage of false positives in a set of differentially expressed genes that have the same or larger T statistic. The Predication Analysis of Microarray program (33) was used to identify the minimum gene set that differentiated the two parental cell lines.

Two approaches were used to assess the functional composition of genes assessed as significantly associated with phenotype: first, Expression Analysis Systematic Explorer (EASE; [http://apps1.niaid.nih.gov/david/]; Ref. 34) was used to identify significant enrichment of cellular functional gene classes identified to be over- or underepressed using SAM. EASE provides statistical significance of gene families identified using standardized Kyoto Encyclopedia of Genes and Genomes or Genome Ontology databases terms. A normalized gene enrichment score and Fisher r test are reported for each functional category. Representative clones from these functional areas were identified using an independent set of 718 cDNA clones selected using high-stringency unsupervised gene filtering (less than four observed values with a minimum 2-fold expression difference on arrays derived from among five paired samples with greatest cytotoxicity (three CN4 and two TN12)). Average linkage clustering was applied to the expression data using Genelinker Platinum version 4.5 (Predictive Patterns).

QRT-PCR

QRT-PCR analysis was performed with the ABI Prism 7900HT thermocycler (Applied Biosystems) using SYBR Green detection. Briefly, two of the RNA sample sets used for microarray analysis were used initially to validate QRT-PCR. Later, when these samples were depleted, RNA from independent cultures of CN4, TN12, CN4.8, and TN12.8 were obtained as described above reverse transcribed using random hexamers. Each reaction was performed in a 10 μL reaction containing 3 mM MgCl₂, 50 nM dNTP, 20 μM primers, 40 ng of cDNA, 1× SYBR green reagent, and 0.125 U/μL Jumpstart Taq polymerase (Sigma-Aldrich). The following protocol was used: denaturation program (95°C for 3 min), followed by the amplification and quantification program (95°C for 10 s, 60°C for 15 s, 72°C for 20 s) repeated for 40 cycles, with one cycle of a finishing program (72°C for 1 min). Amplification was followed by melting curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) to ensure the presence of a single PCR product. The expression of β-actin was used to normalize starting cDNA concentrations. The primers used for all QRT-PCR are listed in Table I. A standard curve consisting of five 3-fold dilutions of cDNA from a pool of all four samples (1:3:9:27:81) was used for linear regression analysis of all samples.

Cell surface marker staining

Regulatory and mutant clone cells were collected at day 3 poststimulation and incubated with fluorescence-conjugated mAbs CD3ε-FITC, CD44-FITC, TLR4-FITC, and CD80-PE from eBioscience, biotinylated CXCR5 from Becton-Dickinson, as well as being detected by mAb 1B2. They are αβ TCR CD3ε NK1.1 CD4 CD8, as well as being negative for CD44 and CD28 (Table II) by flow cytometric analysis. Long-term cultivation of the DN Treg clones CN4 and TN12 resulted in the generation of two phenotypic variants (CN4.8 from CN4 and TN12.8 from TN12), which significantly underexpressed their TCR and simultaneously acquired CD8 expression (Table II). During the subsequent cultures, the phenotype of these cells remained stable and never reverted to that of parental regulatory clones; therefore, we designated these variants as mutant clones.

To further examine whether DN Treg and mutant clones differ in their function in vivo, we compared the ability of DN Treg clones to prolong allograft survival with that of the mutant clones. (2C×dm2)F₁ mice were infused with either syngeneic DN Treg clone TN12 or its natural mutant TN12.8 clone cells followed by transplantation of cardiac allografts from CB6F₁ mice. A group of (2C×dm2)F₁ mice received cardiac transplantation without pretransplant infusion of clone cells as a control. Fig. 1B shows the graft survival in the control, TN12.8-treated, and TN12-treated (2C×dm2)F₁ mice. The allogeneic heart grafts survived over 100 days in all the animals that were preinfused with TN12 regulatory clones. In contrast, infusion of mutant clones had no beneficial effect on allograft survival (19.5 ± 11.1 days) and no significant difference in mean graft survival time compared with untreated controls (22.8 ± 10.5 days survival). These data clearly demonstrate that DN Treg clones have a potent regulatory function, whereas their natural mutants have lost the regulatory function both in vitro and in vivo (p < 0.001). The availability of DN Treg clones and their natural mutants provides us with unique tools to further identify the molecules that are expressed by DN Treg cells and are important for their immunoregulatory function.

Microarray analysis reveals differences in gene expression between DN Treg and mutant clones

To characterize the genetic changes associated with the change in function from DN Treg cells to nonregulatory mutants, we used cDNA microarray technology for global gene expression profiling. Unsupervised clustering of ~10,000 genes differentially expressed between the DN Treg and mutant clones revealed parallel expression analysis across both regulatory cell lines (Fig. 2A), despite the fact that 12 independent RNA preparations were used on each array. Consistency in the informative expression profile was confirmed using one-class response in SAM (32) to identify a subset of 1099 cDNAs significantly over- or underepressed in the DN Treg clones (Fig. 2B; q = 0.021% and 90th percentile false positive rate < 4.5% or 49 clones). To better understand the functional pathways predominantly impacted by these differences, gene ontology annotations of the known unique genes (187 underepressed and 356 overexpressed genes) in this list were analyzed using the EASE-online ([http://apps1.niaid.nih.gov/david/]; Ref. 34), and results are summarized in Table III. Redundant pathways are represented clearly on this intrinsic gene list, and consequently, genes that are differentially expressed between DN Treg and mutant clones can be categorized into eight functional groups, including TCR associated, Ag presentation, chemotaxis, IFN-inducible
genes, survival and proliferation markers, cytotoxicity related, apoptosis, and immunomodulators. The average expression change in DN Treg cells relative to mutant cell lines is summarized in Table IV column A, and representative genes from each of these functional classifications were selected for confirmatory studies. Far more cDNAs (1099 vs 22) were found to have similarities in expression changes between the two DN Treg cell lines than to differentiate them. Only 10 known genes (mapping to the 22 differentiating cDNAs) were found to segregate the CN4 and TN12 DN Treg cell lines (Fig. 2D). Qualitative differences in the expression of an apoptosis marker ( annexin 4A), proliferation/growth-related genes, myeloid-associated differentiation marker and insulin-like growth factor (IGF)-2R, and two H2 class II genes Aβ1 and DMα are among this differentiating gene set.

QRT-PCR validation of cDNA microarray data
To validate the differences in gene expression observed by cDNA microarray screen, 57 representative genes (33 overexpressed and 24 underexpressed in both DN Treg cell lines) were selected for confirmation using QRT-PCR assays. Table IV column B shows confirmation using QRT-PCR assays. Table IV column B shows

### Table I. QRT-PCR primer sequences for genes selected for cDNA microarray validation

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<th>Gene Name</th>
<th>5’-3’ Primer Sequence</th>
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<td></td>
<td></td>
<td>β-Actin</td>
<td>CGGAGGATGTTGCTGCTTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>GCCAGGAGGCTGCTGCTGCTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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fold increase and decrease of genes in regulatory clones compared with their mutant progenies. Similar expression differences in the two DN Treg strains were observed using QRT-PCR as determined by cDNA microarrays (Fig. 3).

As shown in Table IV, genes that showed overexpression in DN Treg cells with both cDNA microarray and QRT-PCR methods could be classified into groups consistent with the EASE gene ontology enrichment: 1) TCR-associated genes, including the FcerIγ subunit and TCRβ variable region 13 (TCRβ-v13); 2) chemokinesis such as CXCR5, IGF-binding protein (IGFBP)5, Decorin (Dcn), gelsolin (Gsn), prostaglandin E2 receptor (Ptger2), CCR2 and CCR3, aplysia ras-related homologue 9 (RhoC), and integrin β1 (Itgb1); 3) the IFN-γ-associated genes: T cell-specific GTPase (TGTP), IFN regulatory factor (IRF)1, IFN-γ-inducible GTPase (IGTP), and IFN-γ-inducible (IFI) proteins 30 and 47 (IFI30 and IFI47); 4) survival and proliferation genes decay accelerating factor (CD55), granzyme (Gzm)M and TNF ligand superfamily, member 6 (Fas ligand (Fasl)); 5) apoptosis-associated genes growth arrest and DNA damage-inducible 45γ (Gadd45g), retinoic acid receptor (RAR)γ, caspase 3 (Casp3), and Bcl2 interacting domain death agonist (Bid); 7) immunomodulatory genes osteopontin (Opn), CSF2Rβ1, GM-CSF, suppressor of cytokine signaling 3 (Socs3), IL-1α, leukemia inhibitory factor (LIF), and TLR4.

Genes that showed underexpression in DN Treg cells could also be classified into significant functional groups: 1) Ag presentation genes histocompatibility 2, class II Ags Eβ1 (H2-Eβ1) and Dβ1 (H2-Dm1); 2) chemokinesis genes vimentin (Vim) and homing-associated cellular adhesion molecule (CD44); 3) IFN-associated genes IRF4, IIFI transmembrane protein 3-like (IFITM3l), IIFI proteins 202a (IFI202a) and 204 (IFI204), and IFN consensus sequence-binding protein 1 (Icsbp1); 4) survival and proliferation genes IGF-1 and cell division cycle-like kinase 4 (Cdk4); 5) cytokine genes lymphotixin α, TNF-α, GzmA, GzmB, and GzmC; 6) the apoptosis-associated gene RARα; and 7) immunomodulatory genes Campath-1 (CD52), CD8α subunit (CD8α), Notch gene homologue 1 (Notch1), CD80, CD48, and IL-4.

Flow cytometry correlates surface protein expression to cDNA microarray data

As both cDNA microarray and QRT-PCR detect only RNA transcript levels, it is also desirable to determine whether these gene expression patterns also reflect changes in protein expression. To test this, flow cytometric analysis of FceRIγ, TLR4, CXCR5, CD8, CD44, and CD80 was performed to validate expression at the protein levels (Fig. 4). FceRIγ, TLR4, and CXCR5 protein were over-expressed in both regulatory clone strains relative to their mutant progeny, whereas CD8, CD44, and CD80 were noticeably under-expressed in the regulatory clones. These data suggest that the gene expression patterns observed using cDNA microarray and QRT-PCR are consistent with their corresponding protein expression for this subset of the genes.

**Discussion**

In this study, we have developed clonal populations of DN Treg cells and natural mutants and used these clones in both functional and expression-based assays. Our results demonstrate that DN Treg clones are able to suppress immune responses in vitro through killing of activated syngeneic CD8+ T cells as seen in human DN Treg cells (15, 17, 18). Furthermore, a single-dose infusion of DN Treg clones was found to induce permanent survival of MHC class I-mismatched cardiac allografts. The nonregulatory mutant clones, generated through serial cultivation of the DN Treg clones, have lost regulatory capacity both in vitro and in vivo. Global gene expression profiling using cDNA microarray revealed 1099 differentially expressed genes. Statistical analysis of gene function enrichment in this gene list and QRT-PCR analyses validated the differential expression of genes that may contribute to DN Treg cell function.

We and others (15, 17, 18, 27) have shown that both human and mouse DN Treg cells and clones down-regulate immune responses, at least in part, through killing of activated syngeneic CD8+ and CD4+ T cells. T cells mediate cytotoxicity through two independent mechanisms, including expression of perforin/Gzm.
FIGURE 2. Genes differentially expressed between DN Treg clones and their mutants identified by cDNA microarray analysis. Six DN Treg:mutant clones are compared by hierarchical clustering of 10,199 differentially expressed transcripts (A). Array experiments consist of three DN Treg CN4 clones (blue) and three TN12 clones (turquoise) each labeled in Cy5 (red) that have been cohybridized with mutant clones CN4.8 and TN12.8, respectively, labeled in Cy3 (green). The relative expression scale ranged ± 2log₂, or 256-fold, and missing data are displayed in gray. Thus, genes displayed in red are expressed in higher levels in the parental DN-Treg lines, whereas those displayed in green are expressed in higher levels in the noncytotoxic mutant lines. B, Supervised analysis across the six arrays using SAM across the genes in A reveals that ~10% (or 1099 transcripts) show highly significant differential expression between the DN Treg clones relative to the noncytotoxic mutant clones. The false positive rate beyond the 95% confidence interval in the SAM plot was observed to be <0.025% or under one gene. Unsupervised clustering was used to identify candidate genes among the five arrays with highest level of cytotoxicity (the array for clone TN12# was excluded) and 718 differentially expressed transcripts identified (representative data and 2-fold expression difference across four of five samples). Samples were clustered on the basis of the array expression results (C). These genes were all either over- or underexpressed in parallel between the two parental lines, as well as in TN12#. Overall similarity in the global expression profiles in the CN4 and TN12 clones was confirmed using Predictive Analysis of Microarray class prediction. A set of only 22 cDNA clones representing 10 known genes can differentiate the two parental DN Treg cell lines (D).

(35) and Fas-FasL interaction (as reviewed by Hahn and Erb (36)). We have previously shown that DN Treg cells do not mediate killing of syngeneic CD8⁺ T cells through the perforin/Gzm pathway (15). Rather, these cells kill activated CD4⁺ and CD8⁺ T cells at least partially through Fas-FasL-mediated interaction (15, 27). Blocking Fas-FasL interaction significantly decreases DN Treg cell cytotoxicity (15, 17). Our examination of cytotoxicity-associated genes revealed significant increases in the expression of Egr-1, FasL, and GzmM in DN Treg clones. In contrast, lymphotoxin α, TNF-α, GzmA, GzmB, and GzmC were underexpressed. Egr-1 has previously been shown to up-regulate expression of FasL in both lymphoid and nonlymphoid cells (37, 38). Although FasL is widely accepted as an inducer of cell death upon ligation with the Fas receptor (36), this interaction may also be a mechanism by which immune privilege is achieved in certain sites (39). These results validate previous observations concerning Fas-FasL-mediated cytotoxicity being the primary mechanism by which DN Treg cells act upon activated syngeneic CD8⁺ T cells (15, 17, 27).

Both mouse and human DN Treg cells produce high levels of IFN-γ (15, 18), and constitutive IFN-γ expression has been demonstrated in CD4⁺CD25⁺ T cells (40). Additionally, we demonstrated that neutralization of secreted IFN-γ abrogates the cytotoxic capacity of DN Treg cells (J. Pun and L. Zhang, unpublished data). The mechanism by which IFN-γ regulates DN Treg-mediated cytotoxicity is not clear. Several IFN-regulated genes (TGTP, IGTP, IRFI, IFI47, and IFI30) were found to be overexpressed in this study, whereas others (IRF4, IFI204, IFI202a, IFITM3l, and Icsbp1) were underexpressed. Whether these IFN-regulated genes are involved in DN Treg cell-mediated suppression requires further study.

It has been shown that both human and murine DN Treg cells are able to acquire allo-MHC-peptides from APCs, and this process is critical for DN Treg cell-mediated Ag-specific suppression (15, 18). Furthermore, we have shown previously that the TCR on DN Treg cells is required for the acquisition of allo-MHC-peptide and that blocking the interaction between the TCR and allo-MHC-peptide abrogates DN Treg cell-mediated cytotoxicity to CD8⁺ T cells (15). However, the molecules that are involved in the acquisition remain unclear. Two genes that are associated with TCR (FceRIγ and TCRβ-v13) were identified to be differentially expressed between DN Treg and mutant cell lines. FceRIγ was identified originally as a subunit of the high-affinity IgE receptor (FceRI) and is expressed on a variety of cells, such as mast cells, basophils, neutrophils, macrophages, NK cells, and some T cells (41). More recently, FceRIγ has been demonstrated to be an alternate component of the TCR complex in place of CD3ζ (42). FceRIγ is functionally and structurally very similar to CD3ζ which is associated as a homodimer within the TCR of the majority of peripheral T cells. FceRIγ was found to be the most significantly overexpressed molecule in both functional DN Treg clones relative to the nonfunctional mutant clones (~100-fold higher in DN Treg clones relative to their mutants by cDNA array analysis and over...
DN Treg cells relative to mutants as indicated in Table II and may correlate with the observed increased expression of TCR in signaling in DN Treg cells (43). Increased expression of TCR \( \mu \alpha \beta \) (47–49). Several reports indicated no expression of CXCL13 (47–49). Thus, CXCL13 may indicate an alternate method of TCR signaling in DN Treg cells (43). Increased expression of TCR\( \mu \alpha \beta \) in place of CD3\( \varepsilon \) as part of the TCR complex to acquire allo-MHC-peptides. Alternatively, expression of Fc\( \varepsilon \)RI may indicate an alternate method of TCR signaling in DN Treg cells (43). Increased expression of TCR\( \mu \alpha \beta \) may correlate with the observed increased expression of TCR in DN Treg cells relative to mutants as indicated in Table II and may represent a general increase in genes associated with Ag detection.

Recent studies indicate that CD4\( ^{+} \) Treg cells accumulate in autoimmune diseased organs (2), accepted allografts (44), and at tumor sites (6). We have previously shown that DN Treg cells preferentially accumulate in accepted skin allografts (28). Furthermore, DN Treg clones, but not mutant clones, are able to migrate to cardiac allografts and induce tolerance. However, the mechanisms by which these cells migrate to the graft are as yet unclear. In the present study, we report that CXCR5 gene expression is increased ~50-fold in both of the DN Treg clones compared with their mutant progeny. CXCR5 was described initially in Burkitt’s lymphoma as a G protein-coupled receptor (45) and subsequently found in B cells and a subset of CD4\( ^{+} \) T cells (46). More recently, CXCR5 expression has also been shown in DN T cells from MRL-Lpr mice (47). CXCR5 responds to its exclusive ligand CXCL13 by initiating chemotaxis toward an increasing gradient in vitro and to B cell zones in lymph nodes that express CXCL13 (47–49). Several reports indicated no expression of CXCL13 in rejecting allografts (50). However, it is not known whether tolerant allografts express this chemokine or whether this expression occurs at early stages of posttransplantation. It is possible that grafts expressing elevated CXCL13 may preferentially attract DN Treg cells expressing high levels of CXCR5, thereby homing these cells into donor tissue. Our recent studies have indicated that graft-infiltrating DN Treg cells are able to suppress anti-graft CD8\( ^{+} \) T cells that could otherwise destroy the graft (28). We are currently testing this hypothesis in a model of transplant tolerance. In addition to CXCR5, regulatory clones also showed overexpression of other genes associated with chemotaxis, including the chemokine receptors CCR2 and CCR3. The ligands of CCR2 and CCR3, such as MCP-1, MCP-2, and MCP-3, Eotaxin, and RANTES, have been detected in transplanted allografts in both humans and animal models (51–54). This suggests that DN Treg cells could home in to the transplanted graft through CCR2 and/or CCR3, possibly in conjunction with CXCR5. Moreover, we found increased expression of genes that are involved in cell mobility, such as Gsn, Ptgser2, RhoC, and Itg\( \beta 1 \). These molecules may facilitate the mobility of DN Treg cells. Although they may aid in chemotaxis, it is necessary to determine under what conditions they function. Furthermore, whether they respond exclusively to the stimulation of a specific receptor or whether they represent a general increase in migratory capacity remains to be tested.

As previously described, DN Treg cells do not appear to undergo activation-induced cell death in response to TCR cross-linking (55, 56). Consequently, we focused on genes involved in cell survival and proliferation. We found that DN Treg cells overexpressed CD55 and VEGF, whereas mutant clones did not show significant increases in expression of any survival genes. It has been demonstrated that CD55 prevents cell death through complement activation (57, 58), whereas VEGF has been implicated in cell proliferation (59, 60). This supports the observation that DN Treg cells are highly resistant to activation-induced cell death (55, 56). Although this data suggests that CD55 and VEGF may contribute to this DN Treg cell resistance to activation-induced cell death, additional studies are still required.

In nature, DN Treg cells are anergic, much like CD4\( ^{+} \) Treg cells (7, 61). An important mechanism for anergy in DN Treg cells may

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### Table III. Gene enrichment analysis using EASE

<table>
<thead>
<tr>
<th>Category</th>
<th>Genes</th>
<th>Genes on Array in This Category</th>
<th>EASE Score</th>
<th>Fisher’s Exact Test</th>
</tr>
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<tr>
<td>Cytokine metabolism/synthesis</td>
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<td>12</td>
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<td>0.000705</td>
<td>0.0000184</td>
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<td>189</td>
<td>0.001666</td>
<td>0.0000615</td>
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<tr>
<td>Cell communication/chemotaxis</td>
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<td>235</td>
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<td>0.000931</td>
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<td>Signal transduction</td>
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<td>763</td>
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<td>Transcriptional activator activity</td>
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<td>0.0908</td>
<td>0.014</td>
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\(^5\) Lee, B. P.-L., W. Chen, R. Forster, and L. Zhang. CXCR5 is important for migration of double-negative regulatory T cells.
result from a finding of potentially altered Csk-Lck interaction. There is increased expression of Csk, the COOH-terminal Src kinase (data not shown), which is a strong repressor of TCR signaling and is known to directly inhibit TCR-induced tyrosine protein phosphorylation and lymphokine production (62). Csk is important for dephosphorylating and inactivating the protein tyrosine kinases responsible for T cell activation, such as Lck (63). Lck is responsible for proximal TCR signal transduction (63) and is down-regulated 16-fold on the array in DN Treg cells compared with their mutants (data not shown). TCR-based signaling is required at
We report here that a large percentage of genes (~10% of the transcriptome) are differentially expressed between regulatory DN T cell clones and nonregulatory mutants. Furthermore, many of these genes can be associated with various functional properties of DN Treg cells. The gene expression differences identified in this study may underlie the molecular mechanisms involved in DN Treg cell-mediated immune regulation and potentially also for Treg cells in general.

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Disclosures
The authors have no financial conflict of interest.

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