Regulatory T Cells Overexpress a Subset of Th2 Gene Transcripts

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Regulatory T Cells Overexpress a Subset of Th2 Gene Transcripts

Diana Zelenika,2,3 Elizabeth Adams,2 Sue Humm, Luis Graca, Sara Thompson, Stephen P. Cobbold,4 and Herman Waldmann4,5

There is now compelling evidence for subpopulations of CD4+ T cells whose role is to prevent immune pathology in both autoimmunity and transplantation. We have cloned CD4+ T cells against a male transplantation Ag that, unlike Th1 or Th2 clones, suppresses the rejection of male skin grafts and are therefore considered examples of regulatory T cells. We have identified, using serial analysis of gene expression, transcripts that are overexpressed in regulatory T cells compared with Th1 and Th2 clones. Some of these transcripts are increased in tolerated rather than rejecting skin grafts and in addition are expressed by the natural regulatory CD4+CD25+ subpopulation of naive mice. These genes include prepro-enkephalin, GM2 ganglioside activator protein, glucocorticoid-induced TNFR superfamily member 18, and integrin α6β1. They seem to represent a subset of transcripts shared with Th2 cells, suggesting that transplantation tolerance and normal immunoregulation may represent a unique form of Th2-like differentiation. The Journal of Immunology, 2002, 168: 1069–1079.

It is now possible to generate Ag-specific transplantation tolerance in adult rodents using nondepleting mAbs to CD4 and CD8 (1) or by costimulation blockade (2). This form of tolerance is maintained by powerful regulatory CD4+ T cells whose ability to suppress graft rejection and to recruit other T cells via linked suppression and infectious tolerance can readily be demonstrated in vivo (3, 4). There is as yet no clear in vitro correlate for this tolerant state, as T cells from tolerant mice often proliferate and secrete Th1 and Th2 cytokines upon donor Ag stimulation in a manner similar to graft-primed recipients (5). It is for this reason that we sought to identify genes selectively expressed by regulatory T cells in vitro and in vivo so that these might provide both markers to monitor the tolerant state and some indications of molecular mechanisms.

The immune system is primarily orchestrated by a range of T cells with diverse functions. CD8+ T cells are largely involved with cytotoxicity, while CD4+ T cells primarily direct the differentiation and effector functions of other cells through both cell surface interactions and secreted mediators. CD4+ T cells have been subdivided into at least three functional Th subsets on the basis of their cytokine production. Th1 cells are associated with cell-mediated immunity and characteristically produce IL-2, IFN-γ, and TNF-β. Th2 cells regulate humoral immunity, can moderate Th1 responses (6), and produce cytokines IL-4, IL-5, and IL-10. Both Th1 and Th2 cells can act as effectors for acute skin graft rejection (7). A third, as yet poorly defined, subpopulation, variously termed Tr1 (8), Th3 (9), or regulatory T (Treg) cells (10), has been shown capable of regulating T cell proliferation in vitro and autoimmune pathology in vivo, in the latter case seeming to require cytokines such as IL-10 and TGF-β (8, 10). In addition, there is accumulating evidence of a subpopulation of CD4+ T cells that is thought to be required to regulate anti-self responses in normal animals and that is able to suppress autoimmune diseases after adoptive transfer in vivo (11) and to inhibit T cell proliferation in vitro (12). It is not clear how, or even if, these natural regulatory T cells are related to Tr1/Th3/Treg cells, although they share some features (13), including the expression of CD4, CD25 (12), CTLA4 (14), and low CD45RB (15).

We currently have little understanding of the molecular mechanisms by which regulatory T cells act. The suppression of T cell proliferation that is observed with some regulatory T cell populations in vitro may in some cases be via anti-inflammatory cytokines such as IL-10 (8) and TGF-β (15), possibly acting to down-modulate APC functions (16), while in other cases it has been shown to be dependent on direct contact, possibly through cell surface molecules such as CTLA4 (14) or surface TGF-β (17). We have shown that naïve CD4+ T cell proliferation and IFN-γ production can be suppressed not only by Tr1-like but also by Th2 and Th1 clones in vitro (13). In the case of Th1 cells this can be completely reversed by inhibitors of NO synthase, but these had no effect on suppression by Th2 or Tr1-like clones (13). Therefore, there is no clear consensus about what defines immune regulation in vitro or how this relates to a regulatory T cell population in vivo, but we hypothesized that there might be common patterns of gene expression that distinguish effector T cells from regulatory populations that are independent of their source or test system.

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In this work we describe how we used serial analysis of gene expression (SAGE) (18) to search for gene transcripts that are selectively expressed in various regulatory T cell clones (hereafter collectively identified as Treg) compared with the other T cell subpopulations. We then identified, using quantitative real-time RT-PCR, whether any of the markers associated with these cultured Treg clones were also present on natural CD4^+ CD25^+ regulatory T cells in the spleens of naive mice. We additionally showed that some, but not all, of these candidate Treg markers were indeed increased in tolerant grafts compared with rejecting grafts, and that there was generally a close correlation between genes expressed in tolerant and syngeneic grafts, suggesting a link between allogeneic and self-immune regulatory processes.

Materials and Methods

Mice, surgery, and tolerance induction

A1(M) × RAG-1^−/− and A1(M): CBA TCR-transgenic mice (7), CBA/Ca, CBK (CBA transgenic for K^b) (19), and B10.BR mice were bred and maintained in specific pathogen-free conditions at Sir William Dunn School of Pathology (Oxford, U.K.). Skin grafting was conducted as described previously (1). Tolerance was induced in CBA/Ca recipients by giving three i.p. injections of anti-CD4 and anti-CD8 nondepleting Abs (1 mg each of YTS 156.7 and YTS 169.1.2 Abs (21), and antibodies to the spleen cells from A1(M) TCR-transgenic mice (22) to induce an immediate but transient rejection that caused the loss of the graft due to severe ischemic shock. The spleen cell suspension was labeled with a mix of anti-mouse IgG (187.1) and MHC-II (M5/114) mAbs, followed by sheep anti-rat coupled Dynabeads (Dynal Biotech, Oslo, Norway) and magnetic depletion according to the manufacturer’s instructions. The unbound cells were then labeled with biotin-conjugated anti-mouse CD25 (BD Pharmingen) in the presence of Fe block (BD Pharmingen), incubated with streptavidin and MACS microbeads (Miltenyi Biotec), and separated according to the manufacturer’s recommendations on an AutoMACS using the two-column positive selection program. The positive fraction was used as the CD4^+ CD25^+ fraction and was generally >90% pure by FACS analysis. The negative fraction was further purified by adding excess streptavidin beads, followed by further negative selection on the AutoMACS, and finally a positive selection of the CD4^+ cells using CD4-conjugated MACS microbeads. This fraction was usually >98% CD4^+ CD25^+ cells. To obtain activated CD4^+ CD25^+ and CD4^+ CD25^− cells these purified fractions were incubated overnight with 1 μg/ml plate-bound anti-CD3 (KT3) at 37°C.

SAGE libraries

Viable cells were harvested from cultures of Th1 (clone R2.2), Th2 (clone R2.4), Treg (clone TR1D1), or Tskin cells, 7 days after Ag stimulation, by density gradient centrifugation on Histopaque-1083, and in the case of the Th1 and Tskin lines CD4^+ cells were purified by two-step MACS separation and the cell pellets were snap-frozen. Total RNA was isolated using thiocyanate buffer (4 M guanidinium thiocyanate, 20 mM NaOAc, 0.1 mM EDTA, and 0.5% sodium lauroyl sarcosine), and RNA was pelleted through a 5.7-M CsCl cushion. First-strand cDNAs were prepared from 1 μg of total RNA from each of the cell lines using Superscript II (Life Technologies, Gaithersburg, MD) annealed with SMART oligonucleotide (5′-GTGTTGAACAGCGAGATGACGG-3′) and the anchoring primer (5′-GACTCGAGTTGAGATCAGG(T)20) and the Advantage-GC cDNA PCR enzymes (Clontech Laboratories) with 1 μM Gelt, following the manufacturer’s protocol. cDNAs were subjected to 16 cycles of preamplification for 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C. The PCR products were monitored by RT-PCR using various housekeeping and cytokine cDNAs as tests. SAGE was applied to these samples. The SAGE lymph node libraries were generated from 40 μg of total RNA, prepared the same way as described above. The poly(A)^+ fraction was purified using oligo(dt)20 Dynabeads (Dynal Biotech). Double-stranded cDNAs were generated using the DNA synthesis kit from Roche (Lewes, U.K.). SAGE was performed on all these cDNAs using NbIII as the anchoring enzyme, BsmF1 as the tagging enzyme, and SpIII as the cloning enzyme, as previously described (18). DNA sequencing was performed using the 377 ABI automated sequencer (PE Applied Biosystems, Foster City, CA). Sequence analysis software SAGE 3.04 β was provided by K. W. Kinzler (Johns Hopkins Oncology Center, Baltimore, MD). A conservative estimate of the differential up-regulation of each gene within the given library compared with a pool of the other three libraries was calculated using a Bayesian statistics model developed by S. Altschul (22, 23) using the β function )x (−x + y, where x = 3, i.e., β (4,4). The differential ratio for each tag that could then be assigned with 95% confidence was obtained by iteration.

Immunofluorescence analysis and Abs

Cells were Ag-stimulated for 14 days in the presence of the appropriate cytokines (as described above), and viable cells were collected from Histopaque-1083 centrifugation. The cells were then stimulated with anti-CD3 (100 ng/ml 145.2C11 absorbed to 24-well plates), anti-CD28 (cluster 57.51 absorbed to wells at 1 μg/ml), or PMA (50 ng/ml) plus ionomycin (500 ng/ml) or were left unstimulated in RPMI 1640/10% FCS at 37°C overnight. For FACS staining, CD4-CyChrome (CD4-PerCP; BD Pharmingen) and anti-oE (CD103-biotin, M290; BD Pharmingen) were used to label live T cells in PBS containing 0.1% NaN_3, 1% BSA, 10 μg/ml mouse rIL-2 plus 100 ng/ml mouse rIL-4 (to generate the R2.2 Th1 line) or 200 U/ml mouse rIL-4 (to generate the R2.4 Th2 line) as previously described (13). Cells were expanded using female mitomycin C-treated spleen cells together with 100 ng/ml 145.2C11 absorbed to 24-well plates, anti-CD3 plus anti-CD28 (ab (clone 4F10), BD Pharmingen, San Diego, CA) plus 20 U/ml each of IL-2 plus IL-4. After cloning on anti-CD3 as described above, the cells were maintained and expanded using female mitomycin C-treated spleen cells together with 100 nM DBY-Eβ peptide (REELHLQFRSSQPKL) (20), IL-2 (20 U/ml), and IL-4 (20 U/ml) 1 every 2 wk. The Tskin lines were generated by removing secondary challenge male A1(M): CBA skin grafts from female A1(M): CBA mice that had previously accepted male skin for >30 days, cutting them into small pieces, digesting with trypsin at 37°C for 1 h, and removing dead cells on nylon wool. Viable lymphocytes were enriched by Histopaque-1083 centrifugation, followed by AutoMACS (Miltenyi Biotec, Auburn, CA) positive selection of CD4^+ T cells according to the manufacturer’s instructions. These T cells were then maintained and expanded using female mitomycin C-treated spleen cells together with 100 nM DBY-Eβ peptide, IL-2 (20 U/ml), and IL-4 (20 U/ml) every 2 wk.

MACS enrichment of T cell clones

T cell cultures were stimulated for 7 or 14 days with mitomycin-treated male spleen cells from CBK (K^b^+ ) transgenic mice. Viable cells were collected by Histopaque-1083 centrifugation, washed, labeled with biotin-anti-mouse K^b^ conjugate (clone 28-8-6, BD Pharmingen), washed, incubated with MACS streptavidin microbeads (Miltenyi Biotec), and run through the AutoMACS using the slow depletion program. The negative fraction was then further purified using automated positive selection with MACS anti-mouse CD4 microbeads (Miltenyi Biotec). The purity of all fractions was monitored by four-color FACS immunostaining, and the T cell fraction was in all experiments >97% CD4^+ K^b^- cells.

**MACS enrichment of spleen CD4^+ CD25^+ cells**

Normal CBA/Ca mice were first depleted of CD8^+ T cells in vivo by administration of 1 mg each of YTS 156.7 and YTS 169.1.2 Abs (21), and after 1 week the spleen cells were harvested and used for the MACS protocol with the same results as in the Treg cultures. The purity of the CD4^+ CD25^+ regulatory T cells in the spleens of naive mice. We additionally showed that some, but not all, of these candidate Treg markers were indeed increased in tolerant grafts compared with rejecting grafts, and that there was generally a close correlation between genes expressed in tolerant and syngeneic grafts, suggesting a link between allogeneic and self-immune regulatory processes.
Table 1. CD4+ T cell lines with identical TCR against DBY-Eκ peptide of male Ag

<table>
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<tr>
<th>CD4+ T Cell Clone</th>
<th>Source</th>
<th>Polarized In</th>
<th>Clone Type</th>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>IFN-γ</td>
<td>IL-4</td>
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<td>R2.2</td>
<td>A1(M) × RAG-1-/-</td>
<td>IL-2</td>
<td>Th1 clone</td>
<td>+</td>
<td>–</td>
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<tr>
<td>R2.4</td>
<td>A1(M) × RAG-1-/-</td>
<td>IL-4</td>
<td>Th2 clone</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tr1D1</td>
<td>A1(M) × RAG-1-/-</td>
<td>IL-10</td>
<td>Tr1 clone</td>
<td>–/–</td>
<td>+</td>
</tr>
<tr>
<td>A1MP</td>
<td>A1(M), CBA</td>
<td>α-CTL4A4+DBY-Eκ</td>
<td>Tr2 clone</td>
<td>–/–</td>
<td>+</td>
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<tr>
<td>Ska</td>
<td>Male skin—female</td>
<td>CD4 sorted + DBY-Eκ</td>
<td>Tskin line</td>
<td>–</td>
<td>–</td>
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<tr>
<td>A1(M), CBA</td>
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* IL-4 secretion is clearly detectable only after stimulation with male bone marrow-derived dendritic cells or by stimulation with 100 nM DBY-Eκ peptide.

Real-time quantitative RT-PCR

Total RNA from grafted tissues or from purified populations from the T cell clone cultures was prepared using the SV Total RNA isolation system (Promega, Madison, WI), followed by DNase I treatment. Rever transcription was performed using the proStar kit with random hexamers (Stratagene, Cedar Creek, TX). From a total volume of 50 μl/cDNA, 25 μl were used in the PCR reactions. Real-time quantification was performed using gene-specific, fluorogenic probes and the Universal MasterMix Kit (PE Applied Biosystems) in a final volume of 25 μl. The reaction mixture contains all primers at 300 nM and the probe at 200 nM. The enzyme was heat-activated for 10 min at 95°C. A two-step PCR procedure of 15 s at 95°C and 60 s at 60°C was applied for 40 cycles. PCR and TaqMan analysis were performed using the ABI/PRISM 7700 sequence detector system (PE Applied Biosystems). The multiplex PCR reactions were performed using VIC-labeled CD3γ or hypoxanthine-guanine phosphoribosyltransferase (HPRT) probes and FAM-labeled test probes, as shown in Table II. Standard curves of cDNAs from the R2.2, R2.4, or Tr1D1 clones were used to calibrate the threshold cycle to amounts of test and normalizing cDNAs on each 96-well plate run. Normalized values for mRNA expression were calculated as (1000 × test mean)/(normalizer mean), except where stated otherwise. All samples were run in triplicate. Significance between values for skin graft groups was calculated by a Mann-Whitney U test.

Results

Functional Th1, Th2, and Treg CD4+ T cell clones with identical specificity

We derived Th1, Th2, and Treg CD4+ T cell clones with identical specificity for a male (H-Y)-derived peptide (REELHQFRSG RKPI) in association with H2-Eκ (DBY-Eκ) (20) from the A1(M) TCR-transgenic mouse on the RAG-1-/- background as previously described (Table I). Both the Th1 (R2.2) and Th2 (R2.4) clones were stable over many months and produced the appropriate cytokines whether stimulated with male spleen cells, peptide-pulsed bmDCs, CD3 cross-linking, or PMA plus ionomycin (data not shown). We have shown previously that both Th1 and Th2 clones elicit rapid rejection of male skin grafts after adoptive transfer to T cell-depleted recipients (7). Th1-like Treg cells were derived from the spleen cells of naive A1(M) × RAG-1-/- mice following the protocol of Groux et al. (8). Alternatively, spleen cells from nonresponsive A1(M),CBA female mice were repeatedly stimulated by male Ag in the presence of anti-CTLA4 mAb (13). We had previously found that this enhanced the proliferation of Treg clones without apparently modifying the phenotype (13). This line was cloned on anti-CD3 (clone A1MP), then maintained on 100 nM DBY-Eκ peptide. Finally, we generated T cell lines against DBY-Eκ peptide from male skin grafts that had been permanently accepted by A1(M),CBA females (here called Tskin lines). All these CD4+ Treg clones and the Tskin cell lines from tolerated skin grafts shared a similar phenotype (CD25+ and CTLA-4+), secreted IL-10 and variable IL-4 but no IFN-γ (13), and could suppress the proliferation and IFN-γ production of naive or Th1 cells in vitro (13) (data not shown).

Treg clones derived in vitro can suppress skin graft rejection in vivo

It was important to demonstrate that cultured Treg clones could suppress skin graft rejection in vivo, as there is still no proven surrogate assay for regulatory T cell activity in vitro. We adoptively transferred each of the Th1, Th2, or Treg clones into RAG-1-/- female recipients of a male skin graft. As expected, both the Th1 and Th2 clones caused rapid rejection of the male, but not control female, skin grafts (Fig. 1). This result confirmed what we had previously demonstrated in T cell-depleted mice (7) and also work by others (24, 25), i.e., that both Th1 and Th2 responses are...
FIGURE 2. Analysis of SAGE libraries by scatter plots. The SAGE libraries were compared in pairs using scatter plots where each SAGE gene tag is represented by a point plotted at the coordinates corresponding to the tag frequency per 10,000 tags (note the logarithmic scale). Tags with a statistically differential expression (95% confidence of >1.2-fold up-regulation) are those plotted outside the diagonal area shown. To improve the clarity of presentation, all tags that were nondifferentially expressed across all the libraries (defined by SDev ≤ mean) were removed from all the plots (~1000 tags mostly mapping to housekeeping and ribosomal protein genes). The following gene transcripts were identified by their SAGE tags:

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...
capable of causing acute graft rejection. More significantly, the Treg clone (Tr1D1) failed to reject four of five male grafts but was subsequently able to suppress the rejection of fresh male grafts given to these same mice together with sufficient new Th1 cells to cause rapid rejection in controls (Fig. 1). Previously, we have also demonstrated that the A1MP clone was able to suppress the rejection of male B10.BR grafts by adoptively transferred CBA/Ca spleen cells (13). Therefore, we have evidence that two independently derived CD4+ T cell clones with similar phenotype and growth characteristics in vitro are able to act as regulatory T cells to suppress skin graft rejection in vivo.

Generation of SAGE libraries

SAGE libraries were first produced from the Th1, Th2, and Treg (Tr1D1) CD4+ T cell clones and the Tskin line 7 days after their last stimulation. The intention was to minimize the APC contribution and to bias the search to stable lineage markers rather than transitory activation Ags. Our first SAGE analysis of genes in Treg cultures (13) had found an association with mast cells that was a consistent finding with all the independent Treg clones and lines described in this work, so the analysis presented here is from new libraries derived from highly purified (>99%) CD4+ Treg and Tskin cells. We also made SAGE libraries from the draining lymph nodes of equivalent A1(M) × RAG-1−/− TCR-transgenic mice 7 days after a second-set challenge with male skin, as this would act as a control to subtract many housekeeping and non-Treg cell gene transcripts from our T cell clone-derived libraries. We sequenced a minimum of 10,000 tags in each of the six libraries (Th1, Th2, Treg, CD4+ Treg, and Tskin cell lines and lymph nodes), providing a total of 97,690 tags corresponding to 5,257 different unique transcripts previously associated with NK cells: Ly6-C.2 and gp49A. The Th2 clone also generated transcripts positively associated with the Tr1D1 clone were ppENK, granzyme A, and OX40, the glucocorticoid-induced TNFR superfamily member 18 (GITR; also known as TNFRSf18) (28), and the cytochrome P450 enzyme Cyp11a, which is the rate-limiting step in glucocorticoid synthesis (29). Significantly, all these candidates for known genes that may be positively associated with the Tr1D1 clone were ppENK, granzyme A, GM2 ganglioside activator protein (GM2a), cytokin F, integrin β7, and invariant chains. The fact that these tags were not observed in the T cell clone libraries makes it unlikely that the present analysis is contaminated with any residual APCs.

The comparison shown in Fig. 2d of the unmanipulated Treg culture and that from highly purified CD4+ Treg cells demonstrate that even as few as 5% mast cells can dominate the mRNA pool, as shown by high frequencies of mast cell genes (mast cell protease 5, carboxypeptidase A3, tryptophan hydroxylase, and CD63). Interestingly, the tags for GATA-3, ST2L, and Egr-1 were found generally at a lower frequency, with the Th2 clone, suggesting that even as few as 5% mast cells can dominate the mRNA pool, as shown by high frequencies of mast cell genes (mast cell protease 5, carboxypeptidase A3, tryptophan hydroxylase, and CD63). Interestingly, the tags for GATA-3, ST2L, and Egr-1 were found generally at a lower frequency, with the Th2 clone, suggesting that these tags were not observed in the T cell clone libraries makes it unlikely that the present analysis is contaminated with any residual APCs.

Therefore, the strongest candidates for known genes that may be positively associated with the Tr1D1 clone were ppENK, granzyme A, GM2 ganglioside activator protein (GM2a), cytokin F, integrin β7, and invariant chains. The fact that these tags were not observed in the T cell clone libraries makes it unlikely that the present analysis is contaminated with any residual APCs.

as follows (each tag is prefixed with the NiloIII site CATG): β2-microglobulin, TTTTCAAAA; carboxypeptidase A3, AAGTGCTGCA; cathepsin D, CTCAGCCCTG; CD18, AGAGCCGAAG; CD4, CTGGGCTCTT; CD63, GAGTGATTG; Cyp11A, GGGCATTTGA; cystatin F, CYPF; AG CAGATTCT; Egr-1, CGTAAATGTTG; GATA-3, AAGGACCCCA; GITR (TNFRsf18), CTCTGACC; glutaredoxin, GATCTGTA; GM2, activator, ACAACTCTCT; gp49A, TGTGTACGAG; granzyme A, ATTTTGTCGAG; hemoglobin α, CCCCCTTCTCT; histone gene 2A1, TCCGGGCGGAG; Ig1 chain, TCTGCTCAAG; Igκ L, CTAAATGTTG; Igκ H, TCAGATGTGAG; IL-1RI, GACGATGCG; IL-1β, GTCCTCTCT; integrin β3, CAGCCAGCCCG; IL-10, GCTCTGGGA; invariant chain (Ii), GTTCAAGTTG; IL-12, CTGGCATAG; IL-19, CTGCGAG; L-selectin, CTAGCTGATT; lysozyme, TCGTACGATC; mast cell protease 5, CGATCAGCGGC; MHC class I (K; D; L), GATTTGGAAT; MHC-II β, GAAGAAGTGG; MHC-II αE, GCACATTGT; OX40, CTACAGCTG; ppENK, CTGGCTTTTG; RAMP2, AAGGCTATT; RANTES, AAGCTCTTCT; ST2L, TGGTTTGGAA; tryptophan hydroxylase (TPH), AATAGTGTCG; unigene mm46382; GCCTCAGT; unigene mm103162, TGGCTCACA.

Differentially expressed gene transcripts in Th2 and Treg cells

To identify Treg-specific gene transcripts we compared the highly purified CD4+ Treg clone Tr1D1 with both Th1 (Fig. 2b) and Th2 (Fig. 2c) SAGE libraries. The first point to note is that while Treg cells express a number of different genes compared with Th1 cells, for example, granzyme A, prepro-enkephalin (ppENK), OX40, Unigene cluster mm46382, and integrin β7, these were shared, but generally at a lower frequency, with the Th2 clone, suggesting that despite their different abilities to promote or suppress graft rejection, the Th2 and Treg cells are closely related. However, a considerable number of Th2-expressed genes were down-regulated on the Treg cells, including the Ig germ line transcripts, β2-microglobulin (but not MHC class I), Egr-1, and GATA-3, with a further down-regulation of IL-2Ry from Th1 to Th2 to Treg. It may be that this specific gene down-regulation, particularly of the two transcription factors GATA-3 and Egr-1, is associated with the loss of graft rejection ability, as they may be required to initiate appropriate patterns of effector gene expression in the Th2-committeled lineage (27). The only two tags unique to this CD4-purified Treg library were a tag (CATGCGCCCGGCT) that could not assign to any known transcript and a histone-associated gene (H2A.1; Fig. 2c).

The comparison shown in Fig. 2d of the unmanipulated Treg culture and that from highly purified CD4+ Treg cells demonstrate that even as few as 5% mast cells can dominate the mRNA pool, as shown by high frequencies of mast cell genes (mast cell protease 5, carboxypeptidase A3, tryptophan hydroxylase, and CD63). Interestingly, the tags for GATA-3, ST2L, and Egr-1 were found generally at a lower frequency, with the Th2 clone, suggesting that these tags were not observed in the T cell clone libraries makes it unlikely that the present analysis is contaminated with any residual APCs.

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Indeed, there was an overall very high correlation between the SAGE data from these two independent sources of mRNA, confirming the repeatability of the SAGE methodology and also suggesting the two cell types are functionally related.

Validation of SAGE data by quantitative RT-PCR and immunofluorescence

The SAGE data from T cells was obtained 7 days after Ag stimulation to deliberately try and bias the search away from transient activation-related transcripts and toward potential stable differentiation markers, so we wished to test whether the above candidate genes would remain preferentially associated with T_reg cells under both resting and recently activated conditions. We first examined the expression of OX40 by immunofluorescence but found it to be expressed on all T cell populations after activation (data not shown), behaving similarly to CD25 and CTLA4 as being common activation markers that are constitutively expressed by T_reg cells.

We next looked at the integrin αE/β7, which can be associated with either αα or αE chains, with the latter (also known as CD103) generally being thought to be a marker for intraepithelial T cells (30). We did not find recognized SAGE tags for either of these integrin α-chains within our SAGE libraries, but this could be due to expression below the level of detection with the number of tags sequenced or to the fact that the 3’untranslated regions of these genes have not been fully characterized, so the correct SAGE tag may not be known. However, immunofluorescence demonstrated that the Treg and Tskin cells stained highly for αE/β7 (Fig. 3), and, more interestingly, a variable proportion of the cells were also positive for αE/CD103 (Fig. 3). We then activated the clones with anti-CD3, anti-CD3 plus anti-CD28, or PMA plus ionomycin (Fig. 3) and found that although there was some weak expression in the activated Th2 clone, the T_reg and Tskin cells maintained, if not increased, their CD103 expression, while Th1 cells remained negative under all conditions. Also of considerable interest was the observation that the majority (61%) of αE/CD4+ cells from normal CBA spleens were contained within and made up 20% of the spleen CD4+CD25+ population (Fig. 3). These αE/CD4+ cells were also found to be negative when permeabilized and stained with an Ab to the C-terminal peptide of Ly116 (Chandra) that is considered a Th1 marker (26), which is expressed on a different subset of ~30%
of αE−CD4+CD25+ cells (data not shown). This combination of αE and Ly116 staining further demonstrates the heterogeneity of the CD4+CD25+ subset that is thought to contain the predominant natural Treg population, but that may also contain other memory subpopulations.

**Treg gene expression in T cell clones and normal CD4+ spleen cell subsets**

We then proceeded to compare the expression of the remaining gene candidates, by TaqMan quantitative RT-PCR, on resting and activated T cell clones and fractionated subpopulations of normal spleen CD4+ T cells (Fig. 4). Of particular interest was the finding that ppENK was not expressed in resting Th1 or CD4+CD25+ cells, and even after activation it remained low or undetectable, respectively, while it was highly expressed and further up-regulated after activation in Treg, Tskin, and normal spleen CD4+CD25+ T cells. GM2a was not expressed on Th1 or CD4+CD25+ cells, but it was detected at high levels in the resting Th2, Treg, and Tskin lines. However, these GM2a transcripts were lost on all populations after CD3 stimulation. The GITR transcript was detected on all resting T cell clones and CD4+ subpopulations, although it was lost after activation of the Th1 clone, but increased after CD3 stimulation of the CD4+CD25+ cells. Immunofluorescence staining with a polyclonal Ab to mouse GITR (BAF 524; R&D Systems, Minneapolis, MN) was positive on Th2 and Tr1D1 clones regardless of activation, but negative on Th1 (data not shown), confirming the SAGE analysis. The steroid synthetic enzyme Cyp11a was not detected on Th1 cells or normal spleen CD4+ subpopulations, but the SAGE data were confirmed by a strong signal on resting Th2 cells and weaker signals from the Treg and Tskin lines. While granzyme B was up-regulated after stimulation of all T cell lines and clones, granzyme A remained undetectable in Th1 cells. The two granzyme transcripts also differed in their expression on normal spleen CD4+ subpopulations, with granzyme B remaining undetected, while granzyme A was highest in the stimulated CD4+CD25+ cells. TGF-β1 mRNA, which was not identified as differential from the SAGE analysis but has previously been associated with Treg cells (15), was detected in all T cell populations, while TGF-β2 was at the lower limit of detection in Tr1D1 and A1MP cells. Finally, CD3, which was used as an additional control, was similarly present in all samples, as expected.

In summary, while we were unable to identify any genes that were unique markers for Treg and CD4+CD25+ subpopulations, ppENK, GITR, and granzyme A were most highly expressed on activated Treg and CD4+CD25+ cells, with generally lower levels on Th2 and undetectable expression on Th1 cells, even after activation. In addition, GM2a activator was present in resting Th2, Treg, and CD4+CD25+ cells but was lost after activation.

**Transcripts associated with Treg cells are selectively overexpressed in tolerated grafts**

We then turned to a well-established in vivo model of transplantation to test whether there was any evidence for our candidate
Treg genes being associated with donor-specific tolerance. CBA/Ca mice can be made tolerant of a B10.BR multiple minor mismatched skin graft by using a short course of nondepleting CD4 and CD8 T cell Abs. We have previously shown that such mice tolerance is dependent on and mediated by CD4+ T cells (3). We performed a qualitative TaqMan RT-PCR analysis of the mRNA expression in the transplanted tissue of genes representative of the three T cell subsets. First we assessed the degree of T cell infiltration by measuring CD3γ in a multiplex reaction standardized to HPRT. Very little CD3γ was detected in normal skin, but by comparison it was readily detectable (but variable and low compared with HPRT) in all skin grafts regardless of whether they were allogeneic or syngeneic (Fig. 5). This allowed us to normalize all subsequent mRNA measurements in syngeneic, tolerant, or primed skin grafts to the level of the CD3γ T cell message (Fig. 5), although we found comparable results when normalizing to other nondifferential transcripts of similar abundance to those tested (e.g., RANTES; data not shown).

The three Th1-associated markers, Ly116, RANTES, and LEF-1, were found in all challenge grafts, with no significant difference among tolerant, rejecting, or syngeneic skins. Similarly, there were no differences between any groups for granzyme A or B, but by comparison it was readily detectable (but variable and low compared with HPRT) in all skin grafts regardless of whether they were allogeneic or syngeneic (Fig. 5). This suggests that the mechanisms of transplantation tolerance induced by CD4 and CD8 mAb treatment are similar to those involved in the acceptance of tolerated allogeneic skin grafts were expressed similarly in syngeneic grafted skin. Indeed, there was a high correlation between the genes expressed in tolerant and syngeneic skin (r² = 0.9935; Fig. 5c), while there was much less concordance between tolerant and rejecting grafts (r² = 0.1414; Fig. 5c). This suggests that the mechanisms of transplantation tolerance induced by CD4 and CD8 mAb treatment are similar to those involved in the acceptance of tolerated allogeneic grafts. This may not be surprising with the accumulating evidence for the role of CD4+ regulatory T cells in the prevention of autoimmunity and inflammatory bowel disease but may also reflect a role for cells of this type in so-called protective autoimmunity (31).

**Discussion**

We have previously demonstrated that both Th1 and Th2 clones can elicit acute skin graft rejection in T cell-depleted recipients, and we confirm this here in RAG-1−/− mice that have no other possible source of T or B cell effectors. We have also shown that two different CD4+ clones with the same specificity can suppress, rather than elicit, rejection in the same system (Tr1D1 in this study) and A1MP previously (13). SAGE libraries of these clones reveal considerable similarity between the Th2 and purified Treg cells, with some Th2-expressed genes being up-regulated in Treg, while others were lost. It may be that this loss is associated with certain critical effector functions normally elicited upon activation of Th2 cells, perhaps those programmed by the transcription factors GATA-3 and Egr-1 (27, 32), and that this is then responsible for the inability of the Treg cells to cause rejection. The remaining genes that are shared but up-regulated from Th2 to Treg may be involved in the regulatory/suppressive activity that can be associated with both populations, especially under conditions where the Th2 cells are unable to fulfill their normal effector functions.

We have identified a number of genes that are expressed by Th2 cells that are further up-regulated in Treg clones and T cells from tolerated skin grafts and that are also expressed in normal splenic
subpopulations of CD4⁺CD25⁺ cells. The first of these, the αε/β7 integrin, has generally been associated with effector cell activity of CD8⁺ intraepithelial cells in the skin and gut (30). However, it is interesting to note that αε-deficient mice develop autoimmune-like skin lesions when crossed to susceptible backgrounds (33), suggesting a role in immune regulation within these tissues. The finding that αε/β7 (CD103) is expressed on ~20% of the CD4⁺CD25⁺ population, which is thought to contain the natural regulators of autoreactivity, is intriguing in light of studies that suggested the first wave of thymic emigrants in young mice have a different capacity to migrate through tissues such as the skin compared with adult naive T cells (34), where they may become tissue-specific regulatory T cells.

Also unexpected was the high level expression of transcripts for ppENK in Treg cells and activated CD4⁺CD25⁺ populations. Prepro-ENK belongs to the opioid family and can be cleaved into different active enkephalin peptides by proteases. Remarkably, although brain and adrenal gland are the classical sites of expression of this family of molecules, ppENK cDNA was originally cloned from stimulated T cells (35). Although Abs are available to the two main products of ppENK (Met and Leu enkephalins), and these have been reported to stain Th2 cells by immunohistology (36), we

FIGURE 5. Gene expression in skin grafts measured by quantitative real-time RT-PCR. B10.BR skin grafts were harvested 7 days after challenge grafting onto CBA/Ca mice. These had previously either been allowed to reject their first B10.BR graft (group R, n = 5), or were still carrying a tolerated B10.BR skin graft given with initial treatment with anti-CD4 and anti-CD8 nondepleting mAbs (group T, n = 5). Group S (n = 5) mice received only syngeneic CBA/Ca grafts. After total RNA extraction the samples were subjected in triplicate to quantitative real-time RT-PCR, with each sample being simultaneously tested for the expression of the gene of interest and a normalizing gene using different fluorescent-labeled probes. CD3γ was normalized to HPRT, and all other tests were normalized to CD3γ. Normal tail skin was also tested (group N, n = 4), but is only shown for CD3γ. The limit of reliable detection estimated as (test cDNA at the threshold cycle of 40)/(mean CD3γ of group). a, The mean normalized value for each sample is plotted as a single point, and the geometric mean and SD of the group are shown as horizontal and error bars, respectively. *, p < 0.05; **, p < 0.01 (by Mann-Whitney U test). The geometric means for each transcript are also plotted to compare tolerant groups with syngeneic (b) or rejecting (c) groups.
have been unable to demonstrate convincing staining of any T cells or grafted tissues with such Abs. This may be because proENK can be secreted intact and processed by a number of different proteases from other cells (e.g., mast cell carboxypeptidase) to generate a range of peptides in addition to the Met and Leu enkephalins, including the non-opioid synenkephalin moiety (37). Mechanistically, there is one report showing that Met-enkephalin peptides can inhibit induced chemotaxis of Th1 cells through receptor desensitization (38), and this may be a means for regulatory cells to act locally by inhibiting a further influx of inflammatory cells. This might relate to the observation that tolerated skin grafts or regrafts, while often having similar levels of T cell infiltration, do not have the massive influx of polymorphonuclear cells associated with rejecting skin grafts (our unpublished observation).

The adrenal glands are not only a source of enkephalins but also have a major influence on the immune system through the production of glucocorticoids. This is most clearly demonstrated in acutely induced autoimmune models such as experimental allergic encephalomyelitis, where the steroids produced by the adrenal glands are required for the resolution of the disease and subsequent T cell-mediated resistance to further induction (39). Both the natural steroid-dependent remission and the use of artificial steroids in vivo and in vitro seem to be associated with promoting a form of immune deviation from Th1 to Th2 (40). Therefore, it is surprising to find that Th2 and Treg cells have mRNA for the critical rate-limiting enzyme, Cyp11a, for the synthesis of glucocorticoids from cholesterol. It has recently been reported that thyromyces may also express this gene (40) and that this may play a role in thymocyte selection, although the area remains controversial. Regulatory T cells have also been suggested to modulate dendritic cell maturation and Ag presentation in favor of tolerance, and one of the most potent ways to achieve this experimentally is using the artificial steroid dexamethasone (29, 40). In addition to the potential to produce glucocorticoids, the Treg cells express the mRNA for TNFRsf18, also known as the GITR protein (41), suggesting that there may be an autocrine loop of steroid production and responsiveness. It is interesting to speculate that this might provide a mechanism for maintaining regulatory T cell activity after an initial external pulse of glucocorticoids from the adrenal glands. However, unlike most of the other Th2- and Treg-associated markers that were associated with tolerance in vivo, RT-PCR of the Cyp11a transcripts at the single time point of 7 days after regrafting suggested that synergenic and rejecting grafts had higher levels of expression than the tolerated skin. While this seems counterintuitive, it may be that the timing of Cyp11a expression is an important factor, and this will need to be further investigated.

Two of the genes, ppENK and GM2 activator, while expressed on Treg cells in vitro and in vivo, were seen to respond differently to CD3-mediated T cell activation. While ppENK was markedly increased in all Th2 and Treg populations and was found to be positive on T cell lines and fresh T cells extracted from tolerated skin grafts (data not shown), GM2 activator was lost by activation. Although it was apparently up-regulated in whole tolerated skin compared with rejecting grafts, it was not detected on freshly isolated graft infiltrating cells (data not shown). GM2 activator was also detected in normal, unstimulated CD4+CD25+ and unfractinated spleen cells but was again lost on stimulation. This suggests that ppENK may be a good marker to detect Th2 and Treg activity within local sites of tolerance, while GM2 activator may be a better marker for the Treg resting state, e.g., in spleen, which is a good source of Treg cells that suppress rejection after adoptive transfer. However, both ppENK and GM2a were increased in the tolerated skin grafts 7 days after regrafting, so there may be further complexity in the timing of regulatory gene activity in vivo. It is not clear whether GM2 activator could have a functional role in immunoregulation, but there is one report that it is able to act as an inhibitor of platelet-activating factor, which is a potent proinflammatory mediator (41).

Although the gene transcripts we used to test for by RT-PCR of whole skin grafts were selected on the basis of a restriction to Th2 and Treg cells, we could not be sure that there were not other additional, non-T cell sources of these transcripts as a result of nonspecific inflammation within the grafted tissues. Indeed, although we only found evidence for TGF-β1 being highly expressed by T cells, we found that TGF-β2 (as well as TGF-β1, -β3; data not shown) was up-regulated in tolerant and synergetic grafts. This raises the possibility that there may be considerable interplay between regulatory T cell-mediated tolerance and tissue protection and repair mechanisms. Indeed, there may be a precedent for this where encephalitogenic, autoimmune T cells have been implicated in the processes of nerve repair (31).

In summary, we have identified gene transcripts that may be indicative of regulatory T cell populations in vivo and in vitro, and these suggest that there are multiple molecular mechanisms involved in the maintenance of tolerance.

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