Regulatory T Cells Overexpress a Subset of Th2 Gene Transcripts[1]

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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 $\alpha_E\beta_7$. 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 (T*reg*)[6] 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 $CD4^+$ thymocytes and spleen 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/T*reg* 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 downmodulate 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 naive $CD4^+$ 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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[6] Abbreviations used in this paper: T*reg*, regulatory T; GITR, glucocorticoid-induced TNFR superfamily member 18; GM2a, GM2 ganglioside activator protein; HPRT, hypoxanthine-guanine phosphoribosyl transferase; ppENK, prepro-enkephalin; MHC-II, MHC class II; SAGE, serial analysis of gene expression; LEF, lymphoid enhancer binding factor.

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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 T*reg*) 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 T*reg* 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 T*reg* 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 177.9.6 and YTS 105.18.10 per injection) over a period of 1 wk starting on the day of transplantation (1). After >100 days mice received a second B10.BR skin transplant that was harvested 7 days postgrafting. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986.

*Generation of Th1, Th2, T*reg*, and T*skin *$CD4^+$ T cell clones*

Spleen cells were taken from a primed A1(M) × RAG-$1^{-/-}$ TCR female transgenic mouse that had been grafted three times previously with male tail skin, and 5×10^5 cells were cultured with mitomycin C-treated male CBA/Ca stimulators in 2 ml of RPMI 1640 and 10% FCS plus either 50 U/ml mouse rIL-2 (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 cloned by limiting dilution in the presence of Ag. The Tr1D1 clone was generated from naive A1(M) × RAG-$1^{-/-}$ spleen cells according to the method of Groux et al. (8). Briefly, 5×10^5 cells were cultured with 5×10^6 mitomycin C-treated male CBA/Ca spleen cells in 2 ml of RPMI 1640 containing 10% FCS and 50 ng/ml IL-10 (Genzyme, Cambridge, MA) for 7 days, at which time spent medium was removed and fresh stimulator cells and medium containing IL-10 were added. After three cycles of polarization in IL-10, viable cells were harvested and cloned at limiting dilution on anti-CD3-coated plates (50 μg/ml 145.2C11) in the presence of mitomycin C-treated female CBA/Ca cells and 20 U/ml IL-2. Cells were expanded with IL-2 (20 U/ml), IL-4 (20 U/ml), and mitomycin C-treated male spleen cells every 2 wk. The clone A1MP was similarly generated from A1(M).CBA naive spleen cells by stimulation with mitomycin C-treated male CBA/Ca spleen stimulators in the presence of 10 μg/ml anti-CLTA4 mAb (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-E^k peptide (REEALHQFRSGRKPI) (20), IL-2 (20 U/ml), and IL-4 (20 U/ml) every 2 wk. The T*skin* lines were generated by removing secondary challenge male CBA/Ca skin grafts from female A1(M).CBA mice that had previously accepted male skin for >60 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-E^k 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 day the spleen cells were harvested and erythrocytes were lysed by isotonic shock. The spleen cell suspension was labeled with a mix of anti-mouse Igκ (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 Fc block (BD PharMingen), incubated with streptavidin 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), T*reg* (clone Tr1D1), or T*skin* cells, 7 days after Ag stimulation, by density gradient centrifugation on Histopaque-1083, and in the case of the T*reg* and T*skin* 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 DTT, 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 SMARTII oligonucleotide (5′-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3′) and the anchoring primer (5′-GACTCGAGTTGACATCGAGG$(T)_{20}$V-3′; Clontech Laboratories, Palo Alto, CA). The cDNAs were preamplified with the forward (5′-AGTGGTAACAACGCAGAGTAC-3′) and reverse (5′-GACTCGA GTTGACATCGAG-3′) primers using the Advantage-GC cDNA PCR enzymes (Clontech Laboratories) with 1 M GC-Melt, following the manufacturer's protocol. cDNAs were subjected to 16 cycles of preamplification at 94°C for 30 s and at 68°C for 7 min. The preamplification steps 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$(dT)_{25}$ Dynabeads (Dynal Biotech). Double-stranded cDNAs were generated using the cDNA synthesis kit from Roche (Lewes, U.K.). SAGE was performed on all these cDNAs using *Nla*III as the anchoring enzyme, *Bsm*F1 as the tagging enzyme, and *Sph*I 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 $f(x) = x^c(1 - x)^c$, where $c = 3$, 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-CD3 plus anti-CD28 (clone 37.51 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-αE (CD103-biotin, M290; BD PharMingen) were used to label live T cells in PBS containing 0.1% NaN_3, 1% BSA, 10 μg/ml Fc block (BD PharMingen), and 5% heat-inactivated normal rabbit serum at 4°C. The cells were then washed, fixed in 2% paraformaldehyde, permeabilized in PBS containing 0.5% saponin, and stained with Alexa-488 (Molecular Probes, Eugene, OR)-conjugated rabbit anti-Ly116 C-terminal

Table I. *$CD4^+$ T cell lines with identical TCR against DBY-E^k peptide of male Ag*

$CD4^+$ T Cell Clone	Source	Polarized In	Clone Type	ELISA			FACS		Ref.
				IFN-γ	IL-4	IL-10	CD25	CD152	
R2.2	A1(M) × RAG-$1^{-/-}$	IL-2	Th1 clone	+	−	−	−	−	7
R2.4	A1(M) × RAG-$1^{-/-}$	IL-4	Th2 clone	−	+	+	−/+	−/+	7
Tr1D1	A1(M) × RAG-$1^{-/-}$	IL-10	T*reg* clone	−	−/+[a]	+	+	+	13
A1MP	A1(M).CBA	α-CTLA4+DBY-E^k	T*reg* clone	−	−/+[a]	+	+	+	13
SkA	Male skin→female A1(M).CBA	CD4 sorted+DBY-E^k	T*skin* line	−	−	+	+	+	This paper

[a] IL-4 secretion is clearly detectable only after stimulation with male bone marrow-derived dendritic cells or by stimulation with 100 nM DBY-E^k peptide.

peptide (NVPGNVYKNHPGEIV; AbCam, Cambridge, U.K.), anti-CTLA-4-PE (4F10; BD PharMingen), and streptavidin-allophycocyanin (BD PharMingen) in the dark at 4°C. Four-color analysis was performed using a FACSort (BD Biosciences, Oxford, U.K.) with dual laser (488 and 633 nm) excitation in combination with data acquisition and cross-beam color compensation using CellQuest 3.1 software (BD Biosciences). The analysis gate was set on the forward and side scatters to eliminate cell debris and dead cells.

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, 2.5 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 T*reg* $CD4^+$ T cell clones with identical specificity for a male (H-Y)-derived peptide (REEALHQFRSG RKPI) in association with H2-E^k (DBY-E^k) (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). Tr1-like T*reg* 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 nonresponding 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 T*reg* clones without apparently modifying the phenotype (13). This line was cloned on anti-CD3 (clone A1MP), then maintained on 100 nM DBY-E^k peptide. Finally, we generated T cell lines against DBY-E^k peptide from male skin grafts that had been permanently accepted by A1(M).CBA females (here called T*skin* lines). All these $CD4^+$ T*reg* clones and the T*skin* 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 T*reg* 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 T*reg* 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

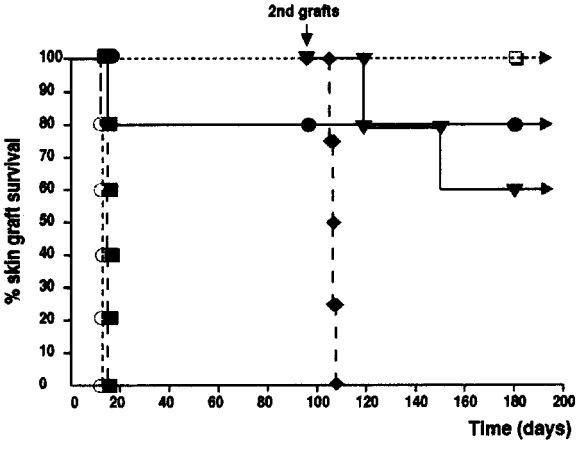

FIGURE 1. Th1 and Th2 clones reject male skin grafts, while a T*reg* clone suppresses. Female RAG-$1^{-/-}$ CBA/Ca recipients were all grafted with male and female CBA/Ca skin grafts on day 0. T cell clones were injected i.v. the day before grafting; 5×10^6 Th1 (R2.2; ○; $n = 5$) or 5×10^6 Th2 (R2.4; ■: $n = 5$) cells both caused rapid rejection of the male, but not the female (□), skin grafts. Four of five mice given 1×10^7 T*reg* (Tr1D1; ●) cells, and those given only 0.5×10^6 Th1 (R2.2; ♦) did not reject their grafts up to day 95. These latter two groups were then regrafted with male and female skin and given sufficient Th1 (5×10^6 R2.2) cells to normally cause rapid rejection. The group that had initially received insufficient Th1 cells to elicit rejection lost both their first and second grafts rapidly (♦) at the same tempo as control RAG-$1^{-/-}$ mice given the same high number for the first time (a repeat of the first group; data not shown). None of the remaining four grafts in the mice originally receiving Tr1D1 was rejected (●), and only two of five of the fresh male grafts on the same mice were rejected slowly (▼; compared with ♦, $p < 0.02$).

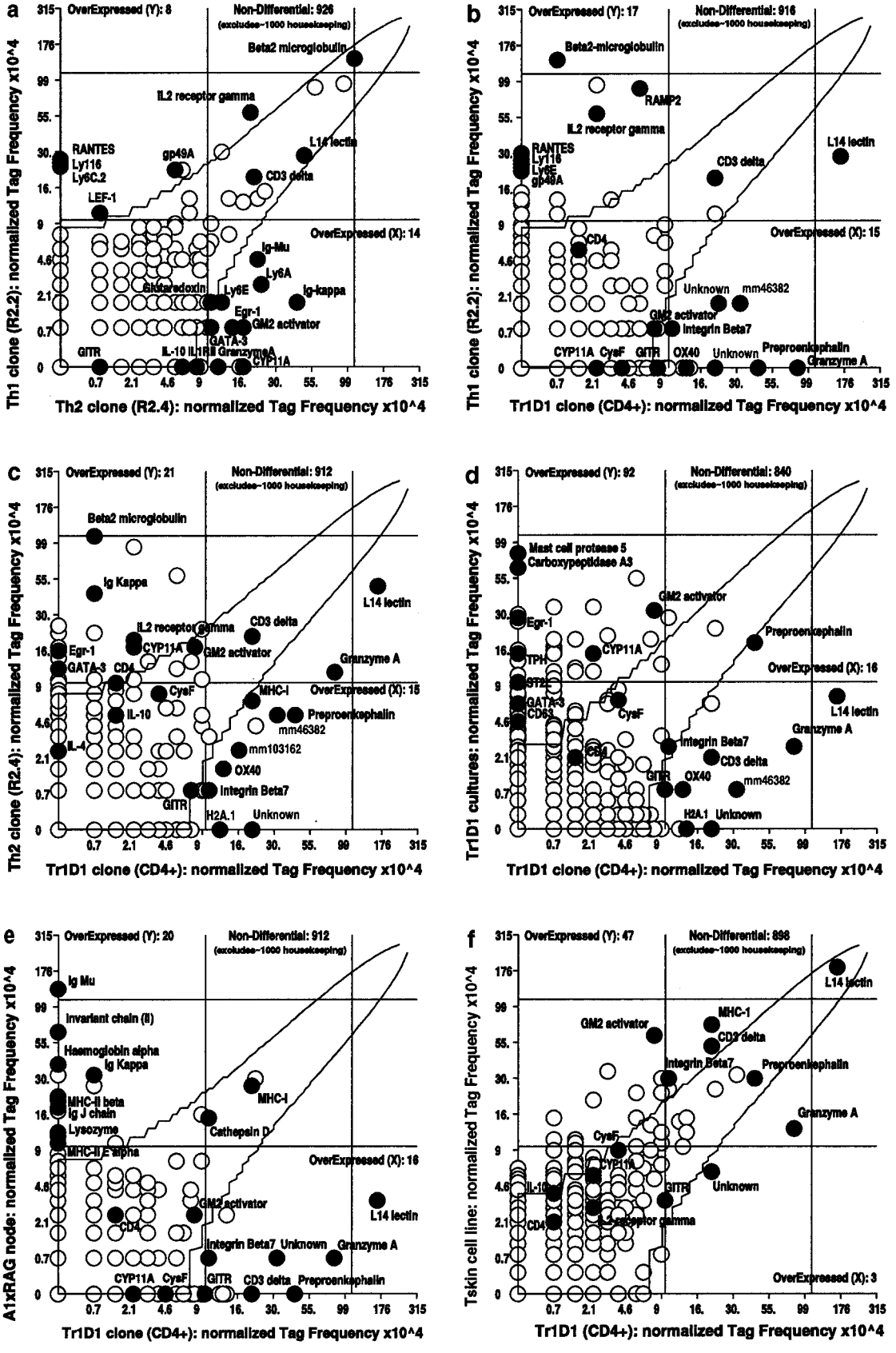

FIGURE 2. Analysis of SAGE libraries by scatter plots. The SAGE libraries were compared in pairs using scatter plots where each SAGE gene tag is represented by a point plotted at the coordinates corresponding to the tag frequency per 10,000 tags (note the logarithmic scale). Tags with a statistically 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 $\leq$ mean) were removed from all the plots ($\sim$1000 tags mostly mapping to housekeeping and ribosomal protein genes). The following gene transcripts were identified by their SAGE tags

capable of causing acute graft rejection. More significantly, the T*reg* 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 T*reg* (Tr1D1) $CD4^+$ T cell clones and the T*skin* 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 T*reg* cultures (13) had found an association with mast cells that was a consistent finding with all the independent T*reg* clones and lines described in this work, so the analysis presented here is from new libraries derived from highly purified (>99%) $CD4^+$ T*reg* and T*skin* 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-T*reg* 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, T*reg*, $CD4^+$ T*reg*, and T*skin* cell lines and lymph nodes), providing a total of 97,690 tags corresponding to 5,257 different unique gene tags detected three times or more in the combined libraries. Our intention was to obtain sufficient tags to identify a reasonable number of candidate genes with a statistically significant differential expression between libraries. The differential analyses are depicted graphically in Fig. 2 as pairwise scatter plots.

Th1 and Th2 T cell differentially expressed gene transcripts

The comparison of the Th1 and Th2 libraries (Fig. 2*a*) highlighted eight tags overexpressed in Th1 cells and 14 in Th2 cells. In the case of the Th1 clone these tags mapped to transcripts known to be associated with Th1 cells, including RANTES, Ly116 (26), the transcription factor lymphoid enhancer binding factor (LEF)-1, and IL-2Rγ. The preferential Th1 expression of the first three was confirmed by TaqMan quantitative real-time RT-PCR (data not shown). Additionally, we identified transcripts previously associated with NK cells: Ly6-C.2 and gp49A. The Th2 clone also generated appropriate up-regulated tags, including IL-10, GATA-3, and IL-1RII, that were also confirmed by TaqMan RT-PCR (data not shown). Transcripts for both IgM H and Igκ L chain constant regions (presumably germline in the RAG-$1^{-/-}$ cells and unlikely to be from contaminating B cells, as these could not be detected by immunofluorescence and no MHC-II-associated tags were present) may be in response to cytokines such as IL-4 and IL-5 that we know are made by the Th2 cells (data not shown). Other Th2 transcripts identified by SAGE included glutaredoxin and Ly6E.

*Differentially expressed gene transcripts in Th2 and T*reg *cells*

To identify T*reg*-specific gene transcripts we compared the highly purified $CD4^+$ Treg clone Tr1D1 with both Th1 (Fig. 2*b*) and Th2 (Fig. 2*c*) SAGE libraries. The first point to note is that while T*reg* cells express a number of different genes compared with Th1 cells, for example, granzyme A, prepro-enkephalin (ppENK), OX40, Unigene cluster mm46382, and integrin β_7, 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 T*reg* cells are closely related. However, a considerable number of Th2-expressed genes were down-regulated on the T*reg* cells, including the Ig germline transcripts, β_2-microglobulin (but not MHC class I), Egr-1, and GATA-3, with a further down-regulation of IL-2Rγ from Th1 to Th2 to T*reg*. 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-committed lineage (27). The only two tags unique to this CD4-purified T*reg* library were a tag (CATGCGCCGCGGCT) that we could not assign to any known transcript and a histone-associated gene (H2A.1; Fig. 2*c*).

The comparison shown in Fig. 2*d* of the unmanipulated T*reg* culture and that from highly purified $CD4^+$ T*reg* 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 within the whole Tr1D1 cultures but were clearly associated only with the mast cell-containing SAGE library. We have discussed this association between T*reg* and mast cells in more detail previously (13). Similarly, it can be seen that the mix of cell types from draining lymph nodes of grafted A1(M)×RAG-$1^{-/-}$ mice, despite being numerically dominated by $CD4^+$ T cells, is strongly biased to tags from macrophages (and possibly germline B-lineage cells), with a high expression of MHC-II α-, β-, 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.

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), cystatin F, integrin β_7, 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 were similarly expressed when the SAGE libraries of the Tr1D1 clone and the T*skin* line from the tolerated grafts were compared

as follows (each tag is prefixed with the *Nla*III site CATG): β_2-microglobulin, TTTTCAAAAA; carboxypeptidase A3, AAGTCCTGCA; cathepsin D, CCTCAGCCTG; CD3δ, AGACCGGAAG; CD4, CTGGGGTCCT; CD63, GAGTGGATTC; Cyp11A, GGGCATTTGA; cystatin F (CysF), AG CAGATTCT; Egr-1, GGATATGTGG; GATA-3, AAGGACGCCA; GITR (TNFRsf18), CTCTGCACCC; glutaredoxin, GATCTGTAGA; GM2 activator, ACAACTTCCT; gp49a, TGTTATCAGA; granzyme A, ATTTGTGCAG; hemoglobin α, CCCTTCTTCT; histone gene 2A.1, TCCGGGCGAG; Ig J chain, TCTGCTCAAG; Igκ L, CTAATATTTG; Igμ H, TCAGAGTGAG; IL-1RII, GACGATGCAG; IL-2Rγ, GTCCTTCTCT; integrin β_7, CAGCCAGCGG; IL-10, GGTCTTGGGA; invariant chain (Ii), GTTCAAGTGA; L14 lectin, GCGGCGGATG; LEF-1, GTGGTAAGAG; Ly116/Chandra, GCAGTGGTTC; Ly6A, TATGCCTGTC; Ly6C.2, TGTGCCTGTC; Ly6E, TATCCTGAAT; lysozyme, TGTCAGTCTG; mast cell protease 5, CGATCTGGCC; MHC class I (K; D; L), GATTGAGAAT; MHC-II β, GAAGAAGTGG; MHC-II Eα, GCACTATTGT; OX40, CTAGCAGCTG; ppENK, CTGCTTTGTG; RAMP2, AAGGCTTATT; RANTES, AAGATCTCTG; ST2L, TGTGTTTGAA; tryptophan hydroxylase (TPH), AATGAGTTGC; unigene mm46382, GGCTTCACTG; unigene mm103162, TGGCTCACAA.

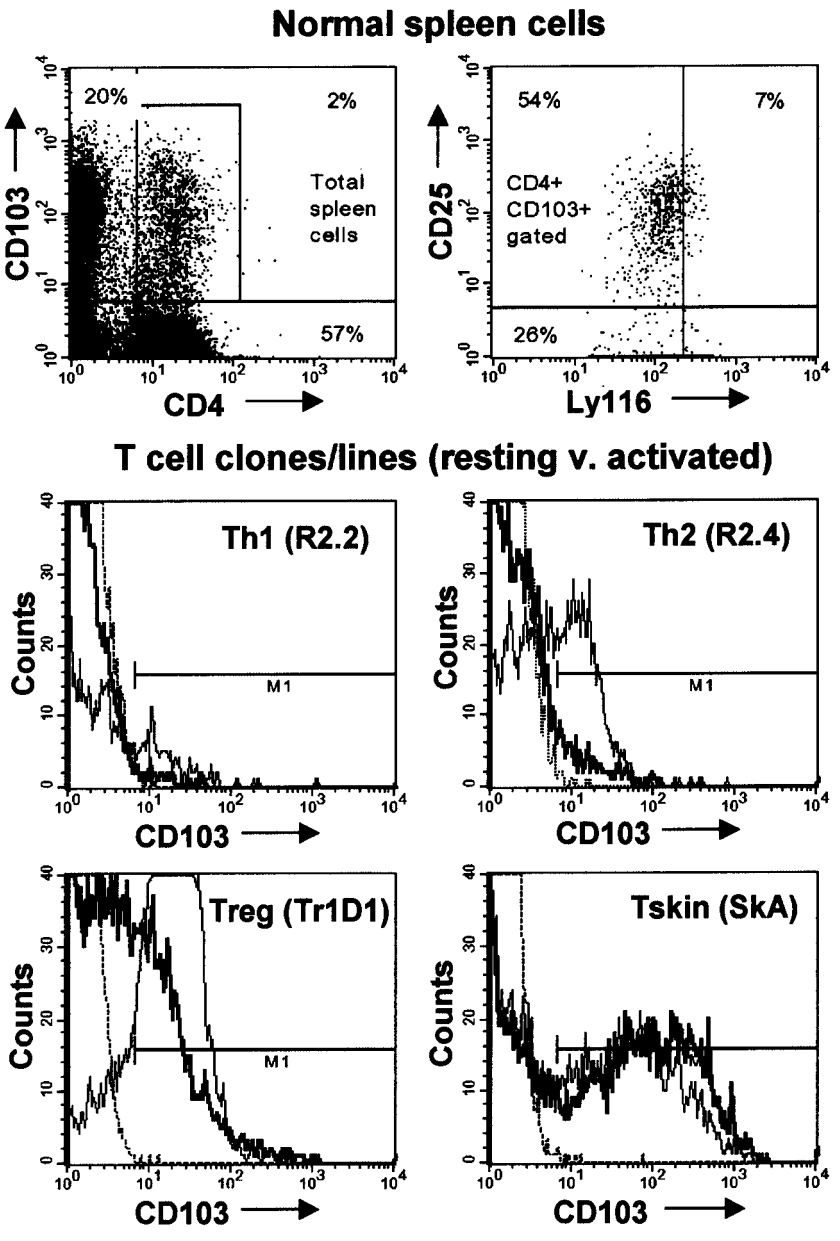

FIGURE 3. Immunofluorescence of $\alpha_E\beta_7$ expression on $CD4^+$ T cells. *A*, Spleen cells from normal CBA/Ca mice were depleted of red cells by water lysis and surface-stained with anti-CD4-PerCP and anti-CD103-biotin-streptavidin allophycocyanin, followed by fixation and permeabilization with anti-Ly116-Alexa488 and anti-CTLA4-PE. Lymphocytes were gated on forward and side scatters, and the *left panel* shows the dot plot of CD103 (vertical axis) vs CD4 (horizontal axis). The $CD4^+CD103^+$ gated lymphocytes (2% of total) are then plotted in the *right panel* for CD25 (vertical axis) vs Ly116/TM4 (horizontal axis). *B*, Th1, Th2, TR1D1 (T*reg*), and T*skin* cells were harvested either as resting cells (14 days plus 1 day since Ag stimulation) or as activated cells (PMA plus ionomycin overnight) and stained as described above. Histograms are plotted for live (forward and side scatter), $CD4^+$-gated cells, with background (no conjugate) staining as dashed lines, resting cells as thick lines, and activated cells as thin lines.

(Fig. 2*f*). 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 β_7, which can be associated with either α_4 or α_E 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 T*reg* and T*skin* cells stained highly for β_7 (data not shown) and, more interestingly, a variable proportion of the cells were also positive for α_E/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 T*skin* 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 $\alpha_E{}^+CD4^+$ cells from normal CBA spleens were contained within and made up ~20% of the spleen $CD4^+CD25^+$ population (Fig. 3). These $\alpha_E{}^+CD4^+CD25^+$ 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%

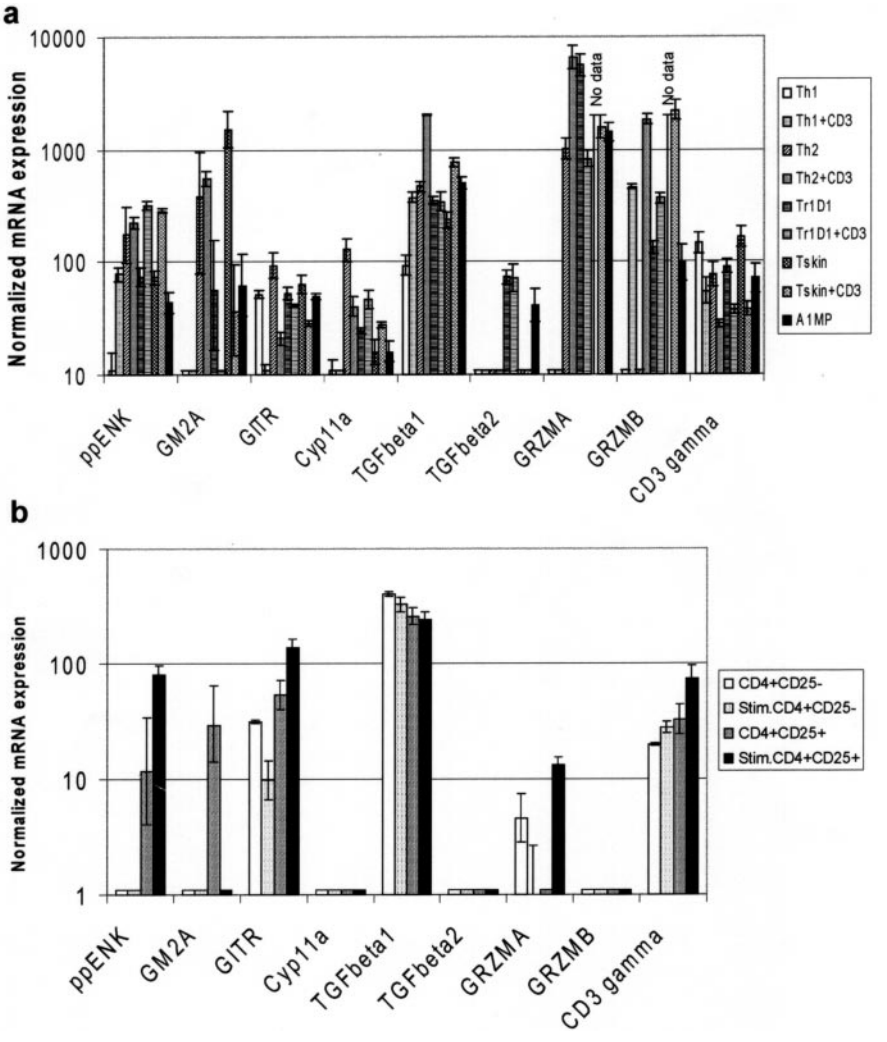

FIGURE 4. Quantitative RT-PCR validation of T*reg* gene candidates on $CD4^+$ T cell subpopulations. *a*, Real-time quantitative PCR analysis of mRNA samples was performed on T cell clones and lines 14 days after Ag stimulation or after overnight activation with anti-CD3 (100 ng/ml 145.2C11 on plastic), using primers and FAM-labeled probes as listed in Table II. All data are presented as arbitrary units ($\times 100$) normalized to HPRT using a multiplex reaction with a VIC-labeled TaqMan probe. *b*, Real-time quantitative PCR analysis similar to that in *A* was performed using AutoMACS-fractionated $CD4^+CD25^+$ or $CD4^+CD25^-$ subpopulations of normal CBA/Ca spleen cells either before or after stimulation with anti-CD3 (145.2C11 at 1 μg/ml on plastic) overnight. Arbitrary units are the same as in *A*.

of $\alpha_E{}^-CD4^+CD25^+$ cells (data not shown). This combination of α_E and Ly116 staining further demonstrates the heterogeneity of the $CD4^+CD25^+$ subset that is thought to contain the predominant natural T*reg* population, but that may also contain other memory subpopulations.

T*reg* *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 T*reg*, T*skin*, 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, T*reg*, and T*skin* 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 T*reg* and T*skin* 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 T*reg* 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, CD3γ, 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 T*reg* and $CD4^+CD25^+$ subpopulations, ppENK, GITR, and granzyme A were most highly expressed on activated T*reg* and $CD4^+CD25^+$ cells, with generally lower levels on Th2 and undetectable expression on Th1 cells, even after activation. In addition, GM2 activator was present in resting Th2, T*reg*, 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

Table II. *Sequences of primers and probes used for quantitative real time RT-PCR (TaqMan)*

Target Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	TaqMan probe (5′–3′)
CD3γ	TTACAGAATGTGTGAAAACTGCATTG	CACCAAGAGCAAGGAAGAAGATG	FAM (or VIC)-ACATAGGCACCATATCCGGCTTTTCTTCG-TAMRA
Cyp11a	CCAAGTTCAGCCTCATCCTGAT	ACAGAGGATACCACCCTCAAATGC	FAM-CCCAGCCGTGACCAGAAAAGACAACAC-TAMRA
CysF	CGCAGAGAGTCCTTTGATGACA	GACCAGACGGCTGCAGAATC	FAM-CACGTGCAGAGCAAAGCAGATACAGTCAAC-TAMRA
ppEnk	AAATCTGGGAGACCTGCAAGG	GATCTCTCCTCCGTTCGCTTC	FAM-TCCAGGCCCGAGTTCCCTTGG-TAMRA
GATA-3	TCGAGGTGGTGTCTGCATTC	TTACAGCTATCCAGGTACAATAAAGTCTTC	FAM-ATCCGGATCCCCATTTGTGAATAAGCCA-TAMRA
GITR (TNFR18)	GTCTTCCTCTGTGCCCCAAG	ACCGCTCTCATACACCCACTTC	FAM-AGACTTGCCCAGCTATACCCTTGGTGAGAG-TAMRA
GM2a	TGGCTGCTTTTCCTCACATCT	GGTGGGATCTACTCCAGTGTGAAC	FAM-CCAACTTCCGAGCCATCACCACCA-TAMRA
Granzyme A	TGTAATTGGACTAAACACATGATTTGTG	ATAGCAGAGGGCTGCCAGAAT	FAM-TTGCAGGAGTCCTTTCCACCACGG-TAMRA
Granzyme B	CATGCTGTGACAACCCAACTG	GCAACTTGGGTGCAACTGTATG	FAM-ATGCTCTGCTTCCCCTCAGTGCCC-TAMRA
HPRT	GACCGGTCCCGTCATGC	ACCCGCAGTCCCAGCGTCGTG	VIC-TCATAACCTGGTTCATCATCGC-TAMRA
IL-1RII	GGAGACCCCACACGCCTATT	GCCATTGTAGGTAAATGTCATAACACA	FAM-CACGTCCATGGACGATGCAGGCTATT-TAMRA
LEF-1	TCAGGTACAGGTCCCAGAATGAC	GTCGCTGCCTTGGCTTTG	FAM-CCTACATCTGAAACATGGTGGTAAGAAGCTCCT-TAMRA
Ly116	CTGCTCTGGAAACCATGCAA	TTCTCTTTCTTCTCATTCCACATTTG	FAM-TCTTTGAAGGCACAGCAACTCCAGGAAC-TAMRA
RANTES	CATATGGCTCGGACACCACTC	CGACTGCAAGATTGGAGCAC	FAM (or VIC)-CTGCTGCTTTGCCTACCTCTCCCTCG-TAMRA
TGF-β1	AAGAGGTCACCCGCGTGCTA	GCACTGCTTCCCGAATGTCTG	FAM-TGGTGGACCGCAACAACGCCATCTA-TAMRA
TGF-β2	CGAGCAGCGGATTGAACTG	AGGAGAGCCATTCACCCTCC	FAM-AATCCAAAGACTTAACATCTCCCACCCAGC-TAMRA

T*reg* 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 in such mice tolerance is dependent on and mediated by $CD4^+$ T cells (3). We performed a quantitative 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 cystatin F. However, some of the other Th2- and T*reg*-associated markers did show differential expression between tolerant and rejecting skin tissue (Fig. 5). TGF-β2, ppENK, GM2a, GITR, and IL-1R2 showed clear up-regulation in tolerated compared with rejecting challenge grafts of 14.8-, 624-, 32.6-, 2.8-, and 3.4-fold, respectively, while Cyp11a was down-regulated by 7-fold. Of particular interest was that most genes that were up-regulated in 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 ($r^2 = 0.9935$; Fig. 5*b*), while there was much less concordance between tolerant and rejecting grafts ($r^2 = 0.1414$; Fig. 5*c*). 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 self-tissues. 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 T*reg* cells, with some Th2-expressed genes being up-regulated in T*reg*, 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 T*reg* cells to cause rejection. The remaining genes that are shared but up-regulated from Th2 to T*reg* 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 T*reg* clones and T cells from tolerated skin grafts and that are also expressed in normal splenic

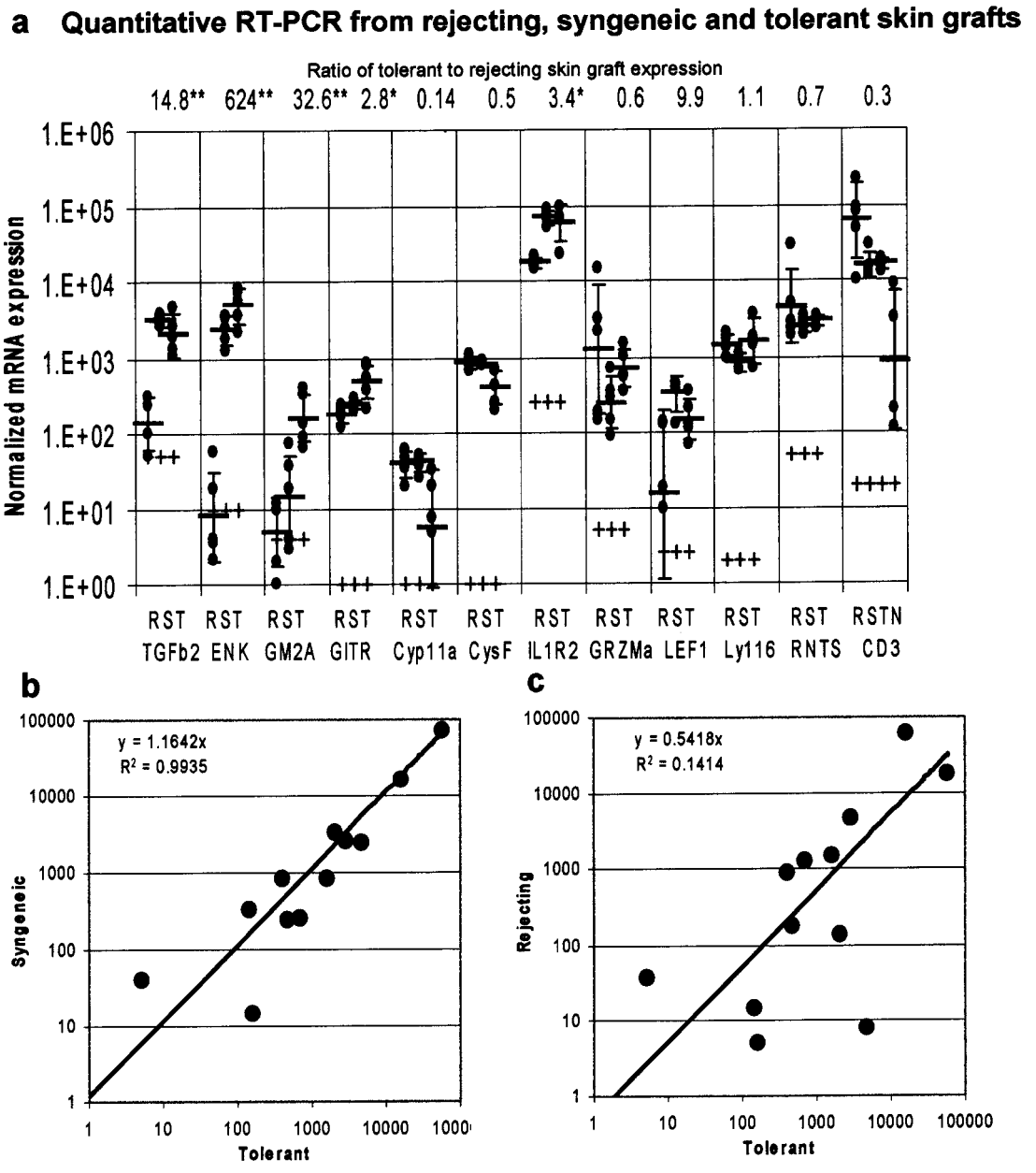

FIGURE 5. Gene expression in skin grafts measured by quantitative real-time RT-PCR. B10.BR skin grafts were harvested 7 days after challenge grafting onto CBA/Ca mice. These had previously either been allowed to reject their first B10.BR graft (group R, $n = 5$), or were still carrying a tolerated B10.BR 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.

subpopulations of $CD4^+CD25^+$ cells. The first of these, the α_E/β_7 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 α_E-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 α_E/β_7 (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 T*reg* cells and activated $CD4^+CD25^+$ populations. Pre-pro-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

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 T*reg* cells have mRNA for the critical rate-limiting enzyme, Cyp11a, for the synthesis of glucocorticoids from cholesterol. It has recently been reported that thymocytes 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 T*reg* 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 T*reg*-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 syngenic 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 T*reg* 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 T*reg* 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 unfractionated spleen cells but was again lost on stimulation. This suggests that ppENK may be a good marker to detect Th2 and T*reg* activity within local sites of tolerance, while GM2 activator may be a better marker for the T*reg* resting state, e.g., in spleen, which is a good source of T*reg* 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 T*reg* 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, and -β4; data not shown) was up-regulated in tolerant and syngeneic 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 vitro and in vivo, and these suggest that there are multiple molecular mechanisms involved in the maintenance of tolerance in vivo.

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