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$CD4^+CD25^+$ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor

Rebecca S. McHugh,[1] Matthew J. Whitters,[2] Ciriaco A. Piccirillo,[1] Deborah A. Young,[2] Ethan M. Shevach,[1,3] Mary Collins,[2,3] and Michael C. Byrne[2]

[1]Cellular Immunology Section
Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892

[2]Genetics Institute/Wyeth Research
Cambridge, Massachusetts 02140

Summary

$CD4^+CD25^+$ immunoregulatory T cells represent a unique lineage of thymic-derived cells that potently suppress both in vitro and in vivo effector T cell function. We analyzed $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells by DNA microarray, identifying 29 genes differentially expressed in the resting subpopulations, and 77 that were differentially expressed following activation. Most of these genes were elevated in the $CD4^+CD25^+$ population, suggesting a previously activated phenotype. Among these were a number of genes that antagonize signaling, including members of the SOCS family, which may contribute to their anergic phenotype. Multiple cell surface receptors also had increased expression in $CD4^+CD25^+$ cells, including GITR, a member of the TNF receptor superfamily. Importantly, antibodies to GITR abrogated suppression, demonstrating a functional role for this receptor in regulating the $CD4^+CD25^+$ T cell subset.

Introduction

Thymic-derived regulatory T cells have been shown to be critical in the regulation of self-reactive T cells in the periphery (Shevach, 2000). These cells represent a small population (~10%) of $CD4^+$ T cells constitutively expressing the α chain (CD25) of the IL-2R complex (Sakaguchi et al., 1995). Transfer of $CD4^+CD25^-$ T cells readily induced the development of autoimmune disease in immunodeficient recipients and cotransfer of $CD4^+CD25^+$ cells suppressed the development of disease. $CD4^+CD25^+$ cells were also shown to prevent autoimmune disease induced by thymectomy of certain strains of mice on day 3 of life, as well as to inhibit the capacity of both autoreactive Th1 and Th2 clones to induce autoimmune gastritis (Suri-Payer et al., 1998, 1999). $CD4^+CD25^+$ T cells appear to inhibit the development of autoimmune disease by both cytokine-dependent (Asseman et al., 1999; Seddon and Mason, 1999) and cytokine-independent pathways (Suri-Payer and Cantor, 2001; McHugh et al., 2001).

Development of in vitro model systems has facilitated analysis of the functional properties of the $CD4^+CD25^+$ subset (Thornton and Shevach, 1998; Takahashi et al., 1998). $CD4^+CD25^+$ T cells are anergic when stimulated via their TCR but proliferate when costimulated with IL-2. Importantly, $CD4^+CD25^+$ T cells inhibit the proliferative responses of $CD4^+CD25^-$ T cells by suppressing the capacity of the responders to transcribe IL-2. Suppression requires stimulation of the $CD4^+CD25^+$ T cells via their TCR and, in contrast to the in vivo studies, is mediated exclusively by a cell contact-dependent, cytokine-independent mechanism. Once activated, $CD4^+CD25^+$ T cells acquire nonspecific suppressor activity and this suppressive function is maintained in the absence of further stimulation via the TCR (Thornton and Shevach, 2000). As suppression is only observed upon activation of the $CD4^+CD25^+$ cells, this suggests that TCR activation induces the expression of a cell surface protein(s) that mediates suppressor function by binding to its receptor(s) constitutively expressed or induced on the responder cells by antigen stimulation.

Very little is known about the requirements for the development and physiologic regulation of $CD4^+CD25^+$ T cell function in vivo. The survival and/or expansion of the $CD4^+CD25^+$ population in the periphery appears to require the availability of IL-2, as mice deficient in IL-2 (Papiernik et al., 1998), components of the IL-2R (CD25, CD122), as well as cell surface antigens that play critical roles in the costimulation of IL-2 production (CD80/CD86, CD28, and CD40) (Salomon et al., 2000; Kumanogoh et al., 2001) manifest major deficiencies in the $CD4^+CD25^+$ T cells. Some of these mice also develop lymphadenopathy, hyperproliferation, and autoimmunity that may be a direct result of the deficiency in $CD4^+CD25^+$ T cells.

Taken together, both the in vivo and in vitro studies strongly support the view that the $CD4^+CD25^+$ population represents a unique lineage of regulatory T cells. However, CD25, the most suitable marker for identification of this population, is also expressed by all recently activated T cells. Although the mere acquisition of CD25 expression does not induce the suppressor phenotype (Suri-Payer et al., 1998; Thornton and Shevach, 2000), it is difficult to rule out the possibility that this population of T cells, constitutively expressing CD25, also contains a certain percentage of normal activated or memory T cells. Furthermore, it is also unknown whether the expression of CD25 is stable in vivo, if induction of CD25 under certain conditions may generate the suppressor phenotype, or how $CD4^+CD25^+$ T cells are related to other immunoregulatory T cells that function primarily by producing the suppressor cytokine, IL-10 (Groux et al., 1997; Cottrez et al., 2000; Jordan et al., 2000).

We have employed DNA microarray technology to compare patterns of gene expression in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. This technique allows a systematic analysis of gene expression differences between cell groups with a single hybridization (Staudt and Brown, 2000). It has proven to be very valuable in the analysis of differences that occur upon malignant transformation of cells (Alizadeh and Staudt, 2000), following

[3]Correspondence: eshevach@niaid.nih.gov (E.M.S.), marycollins@genetics.com (M.C.)

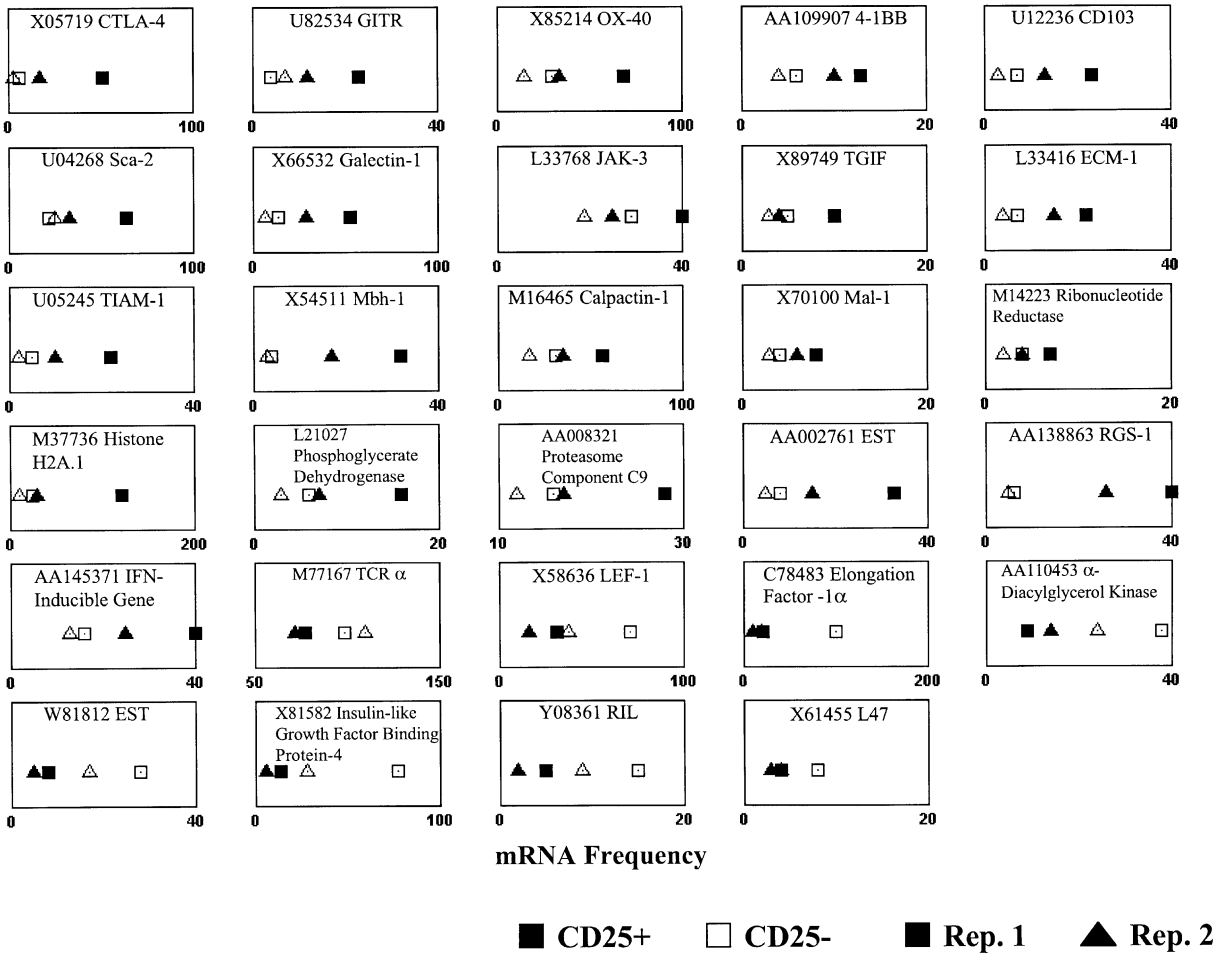

Figure 1. Genes Differentially Expressed between Resting $CD4^+CD25^+$ and $CD4^+CD25^-$ T Cells

Two independent isolations of lymph node T cells were sorted for expression of CD4 and either the presence or absence of CD25. RNA was isolated and analyzed on DNA microarrays. mRNA frequency values (mRNA molecules per million) are displayed for genes differentially expressed in both replicates.

infection of a population with a pathogen (Ichikawa et al., 2000), or after disease onset (Wilson et al., 2000). Our goals in the application of this technology were 3-fold: (1) the identification of genes differentially expressed by resting $CD4^+CD25^+$ T cells whose products might be used to develop more specific reagents to facilitate functional studies; (2) analysis of differential gene expression at two time points following TCR activation to search for molecules (cell surface or secreted) that may be involved in the effector phase of suppression; and (3) determination of genes expressed in the resting or activated state of $CD4^+CD25^+$ cells that may maintain their anergic phenotype. We find that only a small number of genes (29) are differentially expressed between the resting $CD25^-$ and $CD25^+$ T cells and that a larger number (77) are differentially expressed following activation. Nine of these genes are shared between the resting and activated state, making the total number of genes differentially expressed 97. One gene encodes a cell surface receptor that appears to be involved in the generation of suppressor effector function and several genes encode factors that may be related to the anergic state of $CD4^+CD25^+$ cells.

Results

Comparison of Gene Expression in Resting $CD4^+CD25^+$ and $CD4^+CD25^-$ T Cells by DNA Microarray Analysis

$CD4^+CD25^+$ T cells have been distinguished from $CD4^+CD25^-$ T cells on the basis of a small number of differentially expressed cell surface markers (Shevach, 2000). To further characterize $CD4^+CD25^+$ T cells, we compared the results of hybridization of two independent isolations of RNA from $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells to DNA microarrays that monitor the expression of 11,000 murine genes and ESTs. Genes differentially expressed between the two resting populations in both replicates are shown in Figure 1. Of the 29 genes identified, 21 genes were expressed at higher levels and 8 were expressed at lower levels in resting $CD4^+CD25^+$ cells relative to resting $CD4^+CD25^-$ T cells.

A wide variety of functional gene classes are represented, including cell surface receptors, secreted molecules, transcription factors, signaling molecules, small G proteins, and kinases, in addition to a number of uncharacterized ESTs. Eight cell surface receptors were

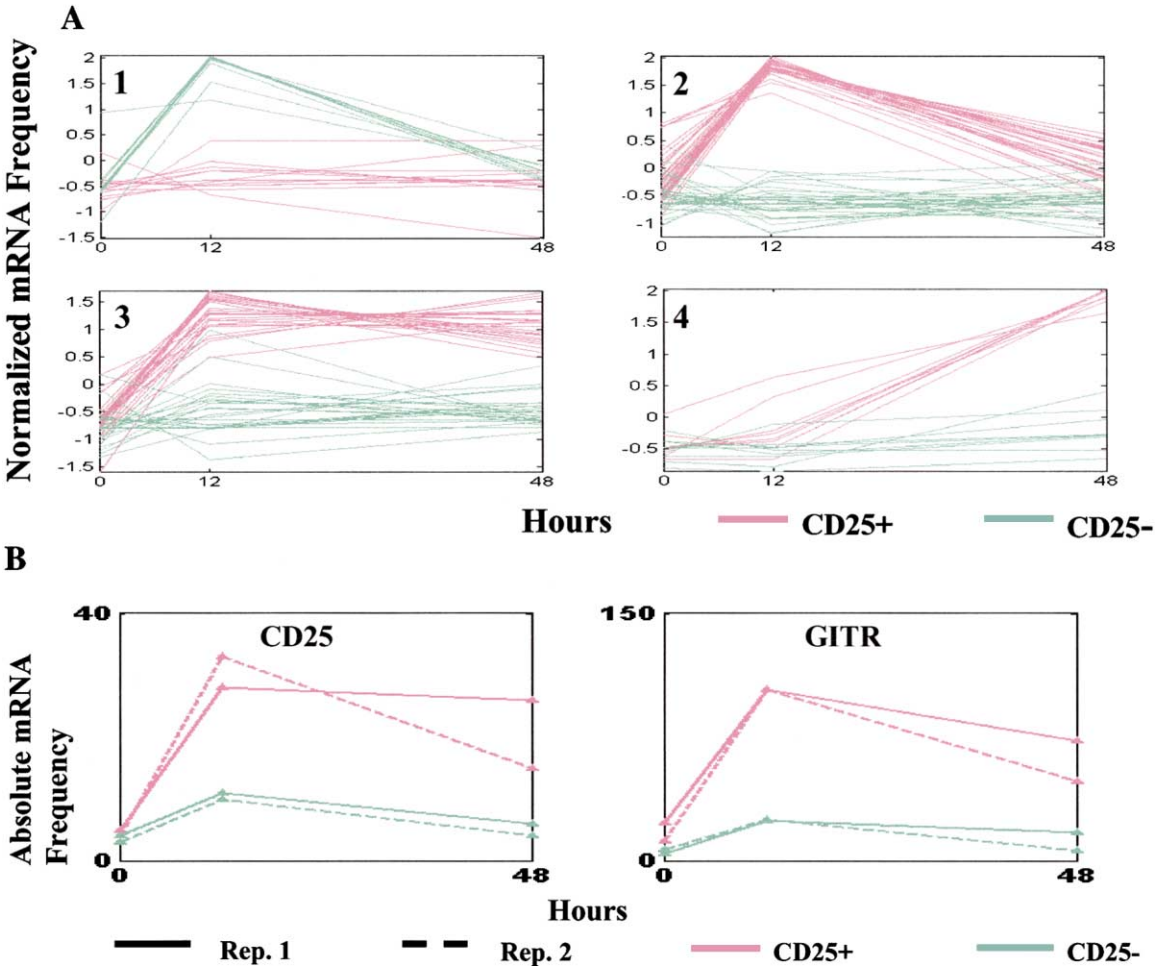

Figure 2. Kinetic Expression Patterns of Genes Differentially Expressed between CD4+CD25+ and CD4+CD25− T Cells after Stimulation

Two independent purifications of CD4+CD25+ and CD4+CD25− T cells were stimulated with plate-bound anti-CD3 and IL-2 for 12 or 48 hr. RNA was isolated and analyzed on DNA microarrays. (A) Genes differentially expressed in both replicates at either one or both of the two stimulation time points were selected for clustering analysis using the self-organizing map (SOM) algorithm. Hours of anti-CD3 stimulation are indicated on the *x* axis and normalized mRNA frequency (a log transformation of absolute frequency values, which allows clustering independent of expression magnitude) is displayed on the *y* axis. Genes populating each of the four clusters are listed in Table 1. (B) Kinetic mRNA expression of CD25 and GITR was analyzed for CD4+CD25− and CD4+CD25+ T cells at the 0, 12, and 48 hr time points for the two independent purifications and stimulations.

differentially expressed, including three members of the TNF receptor superfamily (TNFRSF). CTLA-4 (CD152), Galectin-1, CD103, Ly6, Glucocorticoid-induced TNF receptor (GITR/TNFRSF18), OX-40 (CD134/TNFRSF4), and 4-1BB (CDw137/TNFRSF9) had increased mRNA levels in the CD4+CD25+ T cells, whereas TCR α chain had decreased mRNA levels in the CD4+CD25+ T cells. In addition, LEF-1, a transcription factor that binds to the TCRα enhancer to regulate expression (Okamura et al., 1998), was also expressed at lower levels in CD4+CD25+ T cells. Although CD25 surface expression is readily detected by flow cytometry, CD25 mRNA levels were not identified as increased in comparison to the CD25− subset, suggesting that levels of transcription for CD25 in resting CD4+CD25+ cells are below the detection limits of the arrays. In agreement with this interpretation, semiquantitative PCR revealed an 11-fold increase in RNA transcription of CD25 in the CD25+ subset compared to the CD25− subset in the resting state (data not shown), consistent with the differential protein expression.

Identification of Genes Differentially Expressed in CD4+CD25+ T Cells upon Activation

Immunoregulatory activity of CD4+CD25+ T cells is dependent upon stimulation through the TCR. Preactivation of CD4+CD25+ T cells, for a minimum of 2 days with anti-CD3 and IL-2, results in the generation of a suppressive bioactivity that is TCR nonspecific and stable for several weeks. To identify genes whose products may contribute to this functional activity, we compared RNA isolated from CD4+CD25+ and CD4+CD25− T cells after stimulation for 12 or 48 hr from two independent purifications. Genes identified as increased or decreased in expression after activation in both replicates were included for further analysis. Of 11,000 genes and ESTs analyzed, approximately 1500 responded similarly to stimulation in both subsets of cells (data not shown).

Table 1. Genes/ESTs Differentially Expressed between $CD4^+CD25^+$ and $CD4^+CD25^-$ T Cells after Stimulation with Anti-CD3 and IL-2

Functional Category	Accession Number	Common Name	Cluster[a]
Secreted			
	M16762	IL-2	1
	U28493	Lymphotactin	1
	M37897	IL-10	2
	U43088	IL-17	2
	L33416	ECM-1	2
	M13227	Enkephalin	2
	M17015	Lymphotoxin	3
	X12531	MIP-1α	4
	M35590	MIP-1β	4
	X51834	ETA-1	4
Surface Receptor			
	L38971	ITM-2	1
	M28052	IL2Rβ	2
	M80481	GIR	2
	X16834	Mac-2	2
	U18797	H-2M3	2
	X06143	CD2	2
	K02891	IL2Rα	3
	L29441	TMS-1	3
	U82534	GITR	3
	X85214	OX40	3
	U04268	Ly-6	3
	X03151	Thy-1	3
	X66532	Galectin-1	4
Signaling			
	L16956	JAK-2	1
	M25811	PKC-α	2
	AF001863	SLAP-130	2
	U88327	SOCS-2	2
	D31943	CIS	3
	AB000677	JAB	3
Nucleotide Metabolism			
	AA407018	Thy. DNA glycos.	1
	X17459	Jκ RS BP	3
	M14223	Ribo. reductase	4
General Metabolism			
	L25885	GM2/GD2 Synth.	1
	U59488	p40-phox	2
	AA186047	Glutaredoxin	3
	AF030343	Ech-1	3
	X61600	β-enolase	3
	AA174394	Phos.inos.glyc.	3
	M26270	SCD-2	4
Transcription Factor			
	U43788	OBF-1	1
	M12848	Myb	2
	M60285	CREM	2
	U06924	STAT-1	2
	AA048098	B-Myc	2
	AF027963	XBP-1	3
Protein Metabolism			
	AA008321	Prot. Comp. C9	1
	X71642	GEG-154	1
	AA118121	Isoleu. tRNA Synth.	1
	AA396357	Ubiq. Conj. Enz.	2
	M65270	Cathepsin B	2
	X95818	Synaptophysin	2
	D85561	Prot. Sub. MECL-1	3
	L11145	Prot. Sub. LMP-7	3
	L11613	Prot. Sub. LMP-2	3
	M64085	Spi-2	3
	M12302	Granzyme B	4
Small G Protein/Cytoskeleton			
	U72519	Ena-VASP	1
	AAAA415898	IIGP	2
	U05245	TIAM-1	2
	U44731	GBP-3	2

(*continued*)

Table 1. Continued

Functional Category	Accession Number	Common Name	Cluster[a]
Calcium			
	M37761	Calcyclin	2
	M16465	Calpactin I	2
	M96823	Nucleobindin	3
Cell Cycle			
	AA138777	GADD45-γ	3
	X58708	Cyclin B	4
Unknown			
	AA620163	EST	1
	AA163876	EST	2
	AA289168	EST	2
	AA726223	EST	2
	U13371	EST	2
	U38196	Palmityol.p55	2
	W08322	EST	2
	W98255	EST	3
	C78378	EST	3
	AA016708	EST	3
	W10606	EST	3
	AA117227	EST	4

[a] Genes/ESTs populating the four expression clusters of Figure 2 are listed by functional class.

In contrast, only 77 genes were significantly and reproducibly differentially expressed after activation between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, and these genes showed similar expression patterns in both experiments at all three time points. These genes were clustered using the self-organizing map (SOM) algorithm, a statistical method for grouping genes based on expression patterns independent of expression magnitude (Tamayo et al., 1999) (Figure 2A). Four basic patterns of expression in the $CD25^+$ subset relative to $CD25^-$ subset were revealed. Cluster 1 contains genes that are induced almost exclusively in the $CD25^-$ subset at 12 hr, but for which induction is transient, with expression levels dropping to baseline by 48 hr. This group includes molecules characteristic of the productive immune response, including IL-2, lymphotactin, and JAK-2. The lack of induction of these mRNAs in the $CD4^+CD25^+$ T cells is consistent with an anergic phenotype. The other three clusters contain genes preferentially expressed at a higher level in $CD25^+$ cells relative to $CD25^-$ cells. Cluster 2 identifies genes that are exclusively induced in the $CD25^+$ subpopulation at 12 hr, with expression returning to baseline by 48 hr. Cluster 3 contains genes that are elevated in the $CD25^+$ subpopulation at 12 hr and remain elevated at the 48 hr time point. The genes in cluster 4 are elevated in $CD25^+$ cells, but only at the later time point. Of the genes found to be differentially expressed between $CD25^+$ and $CD25^-$ cells, 84% (65/77) were elevated in the $CD25^+$ subset. Table 1 lists the genes and functional classes populating the four clusters.

$CD4^+CD25^+$ T cells have certain markers characteristic of memory/activated T cells, particularly $CD45RB^{low}$ (Thornton and Shevach, 2000) and CTLA-4 (Read et al., 2000; Takahashi et al., 2000). The propensity of the $CD25^+$ subpopulation to upregulate so many genes upon activation of the T cell receptor is consistent with a previously activated/memory phenotype. This is supported by the heightened expression of a group of cell surface receptors, several of which have been implicated as activation markers. The activation markers IL-2Rα (CD25), IL-2Rβ, CD2, and OX-40, although induced upon activation in the $CD25^-$ subset, are more highly induced in the $CD25^+$ subpopulation. In addition, we reproducibly observed upregulation of Glucocorticoid induced receptor (GIR), a G protein coupled receptor (Harrigan et al., 1991) whose ligand is unknown, and GITR, a TNFRSF member, that when engaged by its ligand (GITR-L), causes activation of the NF-κB pathway and protection from apoptosis (Gurney et al., 1999; Nocentini et al., 1997). The levels of induction of both CD25 and GITR in $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells are displayed in Figure 2B.

A number of mRNAs for secreted molecules were induced to a higher level in the $CD25^+$ subpopulation, including the chemokines MIP-1α and MIP-1β, which are involved in cellular recruitment to sites of immune activation (Moser and Loetscher, 2001) and have been reported to be expressed in anergic cells (Lerner et al., 2000). In addition, the inflammatory protein IL-17 and the immunosuppressive cytokine IL-10 exhibited an increase in expression in the $CD25^+$ subset following activation. mRNA for enkephalin, a neuropeptide shown to be expressed in Th2 cells (Yahata et al., 2000), was elevated after 12 hr of stimulation in $CD4^+CD25^+$ T cells. We also observed elevated induction of early T cell activation-1 (ETA-1), a cytokine reported to regulate the expression of IL-12 and IL-10 in macrophages (Ashkar et al., 2000). mRNA for extracellular matrix protein-1 (ECM-1), an 85 kDa secreted protein that possesses angiogenic activity (Han et al., 2001) and has not previously been reported in immune cells, was consistently induced in the $CD25^+$ subset.

Activated $CD4^+CD25^+$ cells also expressed high levels of mRNA for factors that play important roles in downregulation of cytokine production and cytokine mediated activation. Several members of the suppressors of cytokine signaling family (Chen et al., 2000) CIS, SOCS-1/JAB, and SOCS-2, were expressed at higher

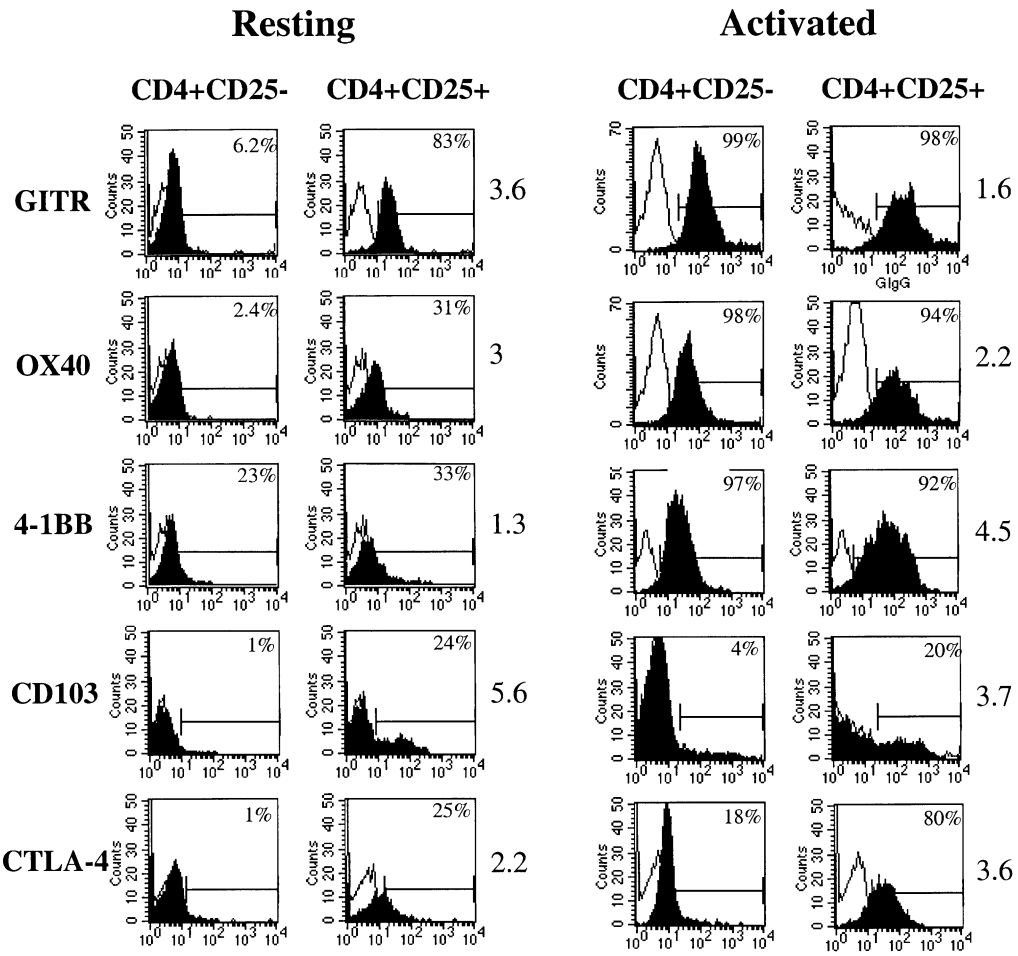

Figure 3. Resting and Activated CD4$^+$CD25$^+$ T Cells Have Increased Protein Expression of Cell Surface Receptors

Freshly isolated peripheral lymph node cells, stained for CD4 and CD25, were analyzed for cell surface expression of GITR, OX40, 4-1BB, CD103, and CTLA-4. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were purified and stimulated for 48 hr with plate-bound anti-CD3 and IL-2. These cells were stained for CD4 and CD25 and analyzed for expression of GITR, OX40, 4-1BB, CD103, and CTLA-4. The various cell subsets were compared to their isotype control Ig and analyzed by CellQuest software for percent positive expression. The CD25$^+$ to CD25$^-$ mean fluorescence intensity ratio is indicated to the right of each group of histograms.

levels in CD4$^+$CD25$^+$ cells. Another signaling molecule elevated in the CD25$^+$ subset was SLAP130, shown to have a negative regulatory effect on SLP-76/NFAT enhanced IL-2 production (Musci et al., 1997). The expression of these proteins may account, in part, for the failure of the CD25$^+$ cells to produce IL-2.

Differential Expression of Cell Surface Receptors

Differential mRNA expression for cell surface molecules was extended to the protein level using flow cytometry. Comparison of CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells showed that the molecules GITR, OX40, and CTLA-4 were expressed at a higher level on resting CD4$^+$CD25$^+$ cells (Figure 3 left panels, CD25$^+$/CD25$^-$ mean fluorescence intensity (MFI) ratio 3.6, 3, and 2.2, respectively). In addition to the previously reported constitutive expression of CTLA-4 by the CD4$^+$CD25$^+$ subpopulation of T cells, only CD4$^+$CD25$^+$ cells expressed the TNFRSF members GITR and OX40 without in vitro activation. CD103, an integrin expressed by intraepithelial lymphocytes (IEL) (Agace et al., 2000), was expressed on 20%–30% of CD4$^+$CD25$^+$ cells, and was not found on the CD25$^-$ subset. Although the percentage of cells expressing 4-1BB on the cell surface was not dramatically different between to the two subsets, the MFI was higher in the CD4$^+$CD25$^+$ cells in the resting state. Differential expression of Ly6 genes was detected in the CD25$^+$ subpopulation; however, we found that only Ly6A/E (Sca-1) was preferentially expression on the cell surface of resting CD4$^+$CD25$^+$ T cells (data not shown).

As expected, molecules expressed on activated T cells (GITR, 4-1BB, OX40, and CTLA-4) were upregulated on both cell populations after 48 hr of stimulation with plate-bound anti-CD3 and IL-2 (Figure 3 right panels). The levels of GITR, OX40, 4-1BB, and CTLA-4, however, were elevated on CD25$^+$ cells, even after activation (CD25$^+$/CD25$^-$ MFI ratio, 1.6, 2.2, 4.5, and 3.6, respectively). Ly6 was also induced on both subsets, with Ly6A/E still being expressed at a higher level on the CD25$^+$ cells

(data not shown). Interestingly, the expression of CD103 was not significantly upregulated after activation for 48 hr, and the percentage of $CD4^+CD25^+$ cells that expressed CD103 in the resting and activated state was comparable (Figure 3).

Separation of $CD4^+CD25^+$ Cells by CD103 Expression

Since the discovery that $CD4^+$ cells expressing CD25 were capable of exerting a suppressive effect in vivo and in vitro, many groups have worked to find another marker to identify these cells. We previously attempted to functionally subdivide $CD4^+CD25^+$ cells based on the differential expression of CD45RB, CD62L, CD69, and CD38 (Thornton and Shevach, 2000); however, all subpopulations were equally suppressive when tested in vitro. As $CD4^+CD25^+$ cells also displayed a bimodal pattern of expression of CD103, it was of interest to test whether $CD103^+CD25^+$ and $CD103^-CD25^+$ cells would exhibit activity in an in vitro suppression assay. $CD4^+CD25^+$ cells were sorted based on the highest and lowest 20% of CD103 expression (Figure 4A) and tested functionally in an in vitro suppression assay with $CD4^+CD25^-$ responding T cells (Figure 4B). Both $CD103^+CD25^+$ and $CD103^-CD25^+$ cells were able to suppress anti-CD3 induced proliferation of $CD4^+CD25^-$ T cells. Consistently, however, the $CD103^+CD25^+$ cells were more efficient, on a per cell basis, at suppressing the proliferation of the responders. In addition, $CD4^+CD103^+$ cells, without selection for $CD25^+$, were able to suppress in vitro proliferation (data not shown). Analysis of $CD103^+CD25^+$ cells revealed that they have a phenotype of recently activated cells, showing higher levels of CD69 and lower levels of CD45RB and CD62L by flow cytometry than $CD25^+CD103^-$ T cells (data not shown). Acquisition of suppressive activity was independent of CD103 expression, as $CD25^+CD103^-$ cells, although suppressive, did not express CD103 upon activation (data not shown).

Reversal of Suppression with Anti-GITR Antibody

Analysis of differential expression using DNA microarrays identified many candidate genes that may be involved in the suppressive function of the $CD4^+CD25^+$ cells or involved in the acquisition/regulation of the suppressive phenotype. As monoclonal or polyclonal antibodies are available for many of the products of the differentially expressed genes, we tested the capacity of these antibodies to reverse suppression in cocultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Antibodies to CD103, CTLA-4, 4-1BB, OX40, CD2, IL-17, and IL-17R (10 μg/ml) had no effect on the ability of the $CD4^+CD25^+$ cells to exert suppression (data not shown). In contrast, a polyclonal Goat antiserum to the mouse GITR extracellular domain was able to reverse suppression induced by freshly isolated $CD4^+CD25^+$ cells from normal BALB/c animals in response to anti-CD3 (Figure 5A). The specificity of this polyclonal antibody for mouse GITR was confirmed by staining of Cos cells transfected with the mouse GITR cDNA (data not shown). In addition, anti-GITR reversed the capacity of $CD4^+CD25^+$ T cells isolated from HA Tg mice to inhibit the responses of HA Tg $CD4^+CD25^-$ T cells to their specific peptide (Figure 5B). As $CD25^+$ T cells can also suppress the responses of $CD8^+$ T cells, we also tested whether anti-GITR could

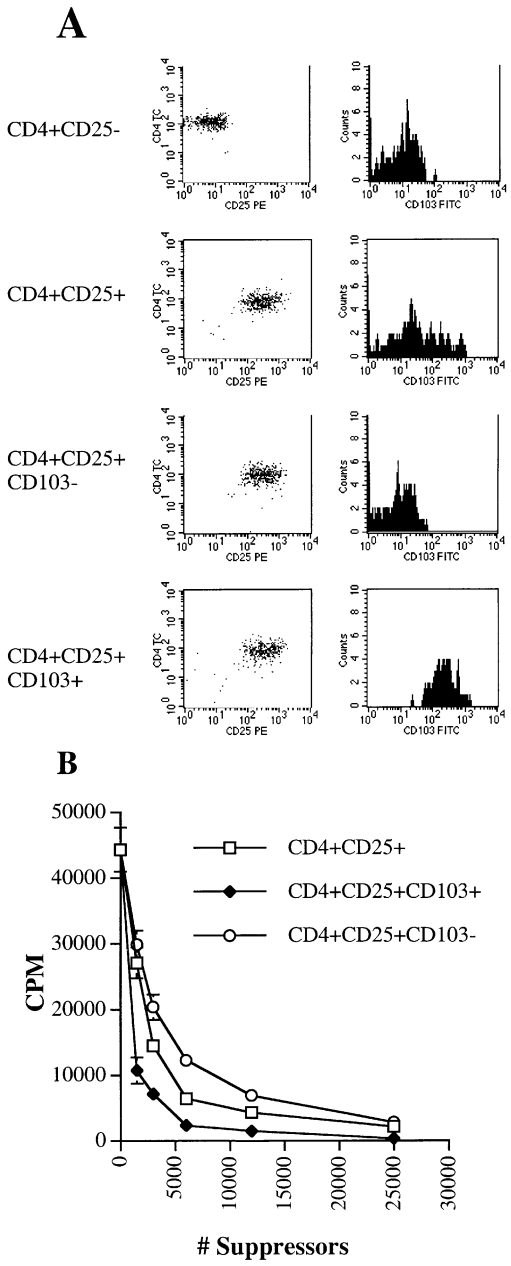

Figure 4. Suppression by $CD4^+CD25^+$ Cells Does Not Segregate with CD103 Expression

(A) $CD4^+CD25^+$, $CD4^+CD25^+CD103^+$, and $CD4^+CD25^+CD103^-$ T cells were cell sorted as described in Experimental Procedures and (B) cocultured with $CD4^+CD25^-$ T cells in the presence of 0.5 μg/ml anti-CD3 and irradiated T cell depleted splenocytes. This figure is representative of four independent experiments.

reverse $CD25^+$ mediated suppression of the activation of $CD8^+$ cells. These results are identical to those seen with $CD4^+$ responders (Figure 5C).

We next evaluated the capacity of anti-GITR to reverse suppression by $CD25^+$ T cells that had been preactivated to confer the suppressive phenotype (Figure 5D). Reversal of suppression in the response to anti-CD3 was consistently observed in these studies, but only at lower numbers of $CD4^+CD25^+$ T cells. Suppression was

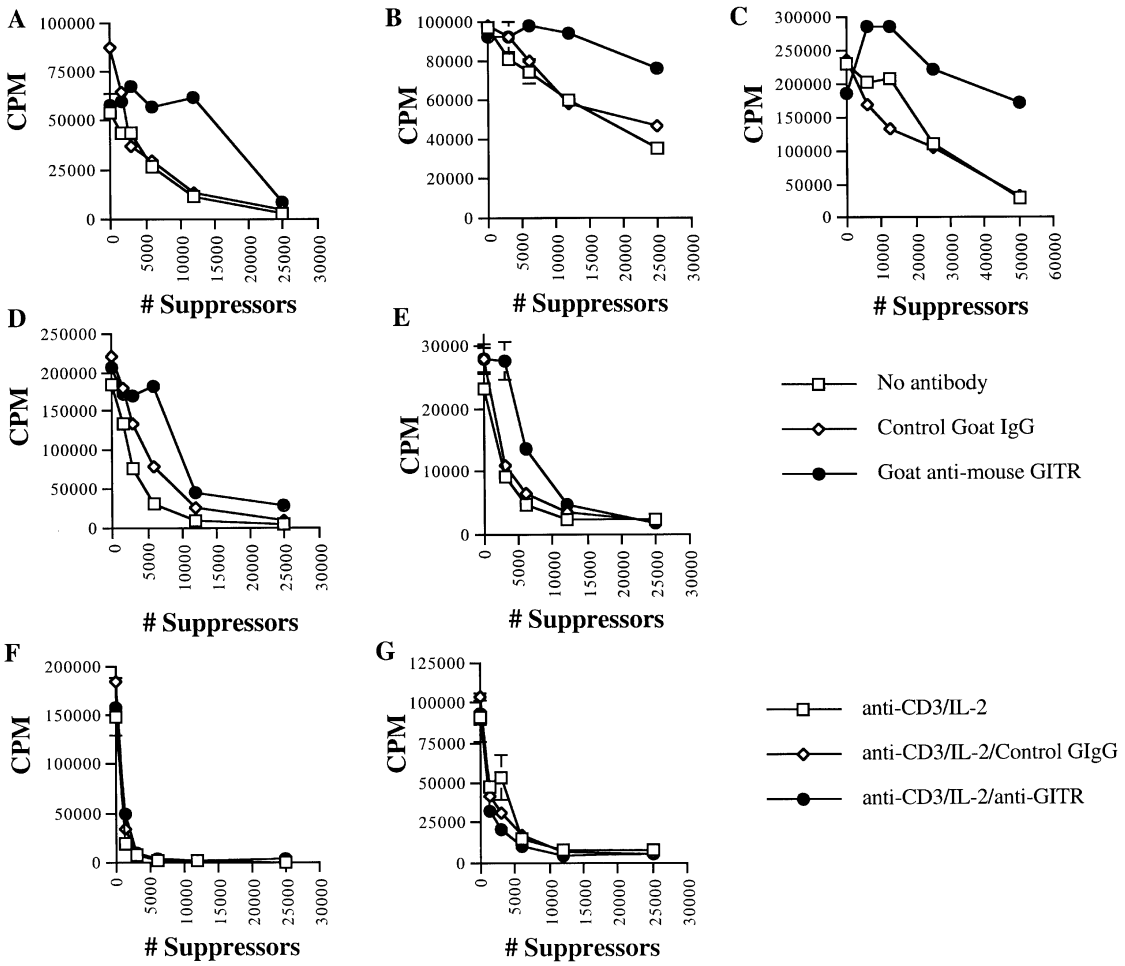

Figure 5. Polyclonal Antibodies to GITR Reverse Suppression Mediated by Fresh $CD4^+CD25^+$ T Cells, but Do Not Prevent Induction of Suppressive Activity

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from (A) BALB/c or (B) HA Tg mice and cocultured in the presence of (A) anti-CD3 or (B) $HA_{(110\text{-}120)}$ and irradiated T cell depleted splenocytes. $CD8^+$ T cells were also cocultured with $CD4^+CD25^+$ T cells (C) in the presence of anti-CD3 and irradiated T cell-depleted splenocytes. BALB/c $CD4^+CD25^+$ T cells were also purified and prestimulated for 3 days in the presence of soluble anti-CD3, IL-2, and irradiated T cell depleted splenocytes. These cells were washed and cocultured with (D) BALB/c $CD4^+CD25^-$ T cells in response to anti-CD3 or (E) HA Tg $CD4^+CD25^-$ T cells in response to $HA_{(110\text{-}120)}$. Suppression assays were cultured in media alone or in the presence of 2 μg/ml Control Goat Ig (open diamonds) or Goat anti-GITR (closed circles). For (F) and (G), $CD4^+CD25^+$ T cells were preactivated for 3 days with soluble anti-CD3, irradiated T cell depleted splenocytes, and IL-2. During the preactivation step the cells were cultured without antibody (open squares), in the presence of 2 μg/ml control Goat IgG (open diamonds), or Goat anti-GITR (closed circles). These cells were then cocultured with BALB/c CD4 cells responding to anti-CD3 (F) or HA Tg CD4 cells responding to peptide (G). Results are representative of three to eight independent experiments.

not abrogated at higher suppressor to responder cell ratios. One difficulty in interpreting this experiment is that the possibility remained that the preactivated $CD25^+$ T cells were restimulated by anti-CD3 in the suppression assay. In order to determine whether anti-GITR would reverse suppression under conditions that precluded restimulation of the activated $CD25^+$ cells in the suppression assay, we tested the ability of anti-GITR to reverse suppression of HA-specific $CD4^+$ T cells, in response to their specific peptide, by activated $CD25^+$ T cells from normal BALB/c mice (Figure 5E). Again, at low suppressor to responder ratios, suppression was consistently reversed. Thus, anti-GITR treatment could reverse suppression mediated by preactivated $CD25^+$ T cells, although this inhibition could be overcome with the addition of more activated suppressor cells.

A possible interpretation of these results is that the antibody engages GITR on $CD4^+CD25^+$ T cells and inhibits the induction of suppressor activity, but has minimal effect once suppressor function has been induced. To test this possibility, we preactivated $CD4^+CD25^+$ T cells from wild-type BALB/c mice with soluble anti-CD3, irradiated T-depleted splenocytes, and IL-2 in the presence of anti-GITR or control GIgG. These activated cells were then tested for suppressor activity. Preactivation of $CD4^+CD25^+$ T cells under these conditions, however, did not affect their subsequent ability to suppress responding T cells where either suppressor activity was

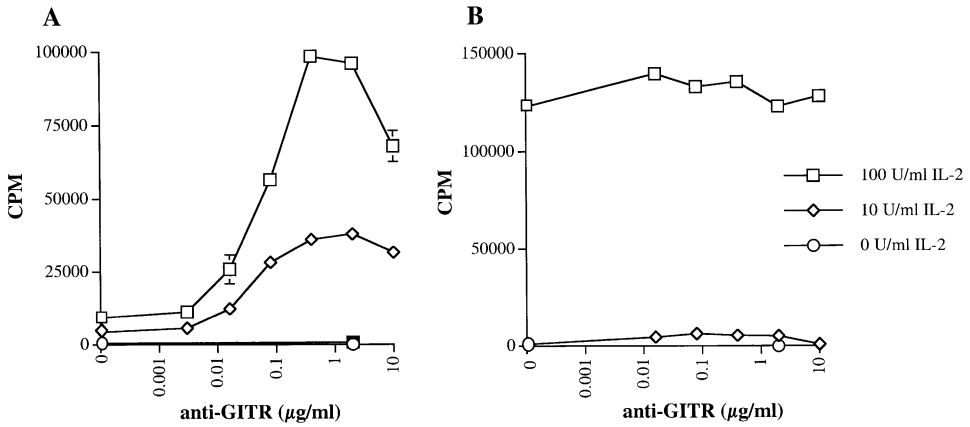

Figure 6. Anti-GITR and IL-2 Induce Proliferation of $CD4^+CD25^-$ T Cells

(A) Purified $CD4^+CD25^-$ (closed symbols) or $CD4^+CD25^+$ (open symbols) cells (5×10^4) were cultured with Goat anti-GITR and irradiated T cell-depleted splenocytes in the presence of different doses of IL-2. (B) Purified $CD4^+CD25^-$ T cells were preactivated with soluble anti-CD3, irradiated T cell depleted splenocytes and IL-2. These cells were then cultured with Goat anti-GITR in the presence of different doses of IL-2. Results are representative of three independent experiments.

restimulated (anti-CD3, Figure 5F) or restimulation was precluded (HA peptide, Figure 5G).

Anti-GITR Acts on $CD4^+CD25^+$ T Cells, Not $CD4^+CD25^-$ T Cells, to Enhance IL-2 Responsiveness

The results presented above are consistent with the possibility that interaction of the antibody with GITR on $CD4^+CD25^+$ T cells modulates suppressor function. However, the flow cytometry studies (Figure 3) demonstrate that GITR is also induced on $CD25^-$ T cells after activation. It was important to rule out the possibility that reversal of suppression mediated by anti-GITR was not due to engagement of GITR on the responder T cells providing a costimulatory signal similar to anti-CD28. While anti-CD28 increased the proliferation of the $CD4^+CD25^-$ cells to anti-CD3, anti-GITR had no effect (data not shown). Treatment of $CD4^+CD25^-$ cells with anti-GITR does not appear to allow protection from apoptosis, as a similar percentage of apoptotic cells are found when culturing with anti-CD3 alone or in the presence of control Ig or anti-GITR (20, 22, and 19% respectively, data not shown).

Although $CD4^+CD25^+$ T cells express the α chain of the IL-2R, they are unable to proliferate even when cultured with high concentrations of IL-2 (Thornton and Shevach, 1998). The nonresponsiveness to IL-2 may be secondary to a failure of expression of the β and/or γ chains of the IL-2 R, although mRNA for both chains is easily detectable by real-time PCR (H. Mizuhara and E.M.S., unpublished data). Alternatively, the nonresponsiveness to IL-2 may be related to their anergic state and suppressive activity. We cultured $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in the presence of anti-GITR and IL-2 in the absence of TCR stimulation. In the presence of anti-GITR, the $CD25^+$ subpopulation (open symbols) proliferated to both a high and low concentration of IL-2, while the $CD25^-$ subset (closed symbols) failed to proliferate (Figure 6A). The responses of activated $CD4^+CD25^-$ T cells that express both CD25 and GITR to both low and high concentrations of IL-2 were also not augmented by the presence of anti-GITR (Figure 6B). Taken together, these studies strongly favor the view that the reversal of suppression by the presence of anti-GITR is mediated by an effect of the antibody on the $CD4^+CD25^+$ population.

Discussion

A number of different technologies have been used to study gene expression in different subpopulations of T lymphocytes, including subtraction techniques and differential display (Marrack et al., 2000). Microarray technology has recently been used to compare patterns of gene expression in vivo in resting T cells and following activation with superantigen (Mitchell et al., 2001), as well as T cells that have been rendered anergic by exposure to self-antigen in vivo (Lechner et al., 2001). We have applied the microarray technology to compare gene expression in the resting and activated state of two highly purified subpopulations of the $CD4^+$ lineage of lymphocytes that have different functional properties in vivo and in vitro. While $CD4^+CD25^-$ lymphocytes represent cells that can produce IL-2, proliferate in vitro, and differentiate into effector Th1/Th2 cells, $CD4^+CD25^+$ T cells fail to respond to stimulation via the TCR by producing IL-2 and have the unique capacity to suppress the production of IL-2 and other effector cytokines by both $CD4^+$ and $CD8^+$ $CD25^-$ T cells. Our studies support the view that these two populations of $CD4^+$ T cells differ in only a small number of the 11,000+ genes and ESTs tested, as well, over 1000 genes/ESTs were modulated similarly in the two populations in response to anti-CD3 (data not shown), but only 97 were found to be consistently differentially expressed. In addition, the patterns of genes expressed in the $CD25^+$ cells are not consistent with the hypothesis that $CD25^+$ cells simply represent a population of previously activated T cells, as many of the observed differences are closely correlated with the distinct functional properties of these subpopulations.

One of the goals of this study was the identification

of genes that encode cell surface antigens that distinguish the $CD25^+$ subpopulation. Several genes were identified that fit this description, including CTLA-4, Ly6, OX40, 4-1BB, CD103, and GITR. The availability of antibodies to all of these antigens has facilitated a comparison of the mRNA data with protein expression at the cell surface. Four antigens, OX-40, GITR, CD103, and CTLA-4, were readily detectable and exclusively expressed on the cell surface of resting $CD4^+CD25^+$, but not $CD4^+CD25^-$ T cells. Several groups have previously shown that $CD4^+CD25^+$ T cells were the only T cells in the normal lymphocyte pool that expressed CTLA-4 (Read et al., 2000; Takahashi et al., 2000). Following activation, CTLA-4 is induced on the $CD25^-$ subpopulation. Indeed, OX-40 and GITR are also induced on the $CD25^-$ subset and thus resemble CD25 and CTLA-4.

Expression of CD103 was, interestingly, only expressed on a minor ($\sim$30%) subpopulation of $CD25^+$ T cells, and the level of expression was not modulated by T cell activation. Both $CD25^+CD103^+$ and $CD25^+CD103^-$ T cells were capable of inhibiting the activation of $CD25^-$ cells in vitro. This result is similar to our observation with other antigens (CD45, CD62L, CD69, CD38) that subdivided the $CD25^+$ population. If the cells constitutively expressed CD25, they always inhibited T cell activation in vitro. As CD103 plays a role in the maintenance of lymphocytes in the intestine (Schon et al., 1999), it would be of interest to compare the capacity of $CD103^+$ and $CD103^-$ T cells to suppress inflammatory bowel disease with their capacity to suppress autoimmune disease of another organ system.

The $CD25^+$ subpopulation is unique in that activation via the TCR fails to result in IL-2 production and proliferation, even in the presence of potent costimulatory signals such as agonistic anti-CD28. It was therefore of interest that three members of the suppressors of cytokine signaling (SOCS) family, CIS, SOCS-1/JAB, and SOCS-2, appeared to be more highly induced after activation of the $CD25^+$ T cells. As IL-2 is required for the survival/maintenance of the $CD25^+$ population, the SOCS proteins may be induced in response to this cytokine or other cytokines needed for the homeostatic control of these cells. Indeed, IL-2 induces the expression of SOCS-1 (Sporri et al., 2001), perhaps resulting in negative feedback in response to IL-2 through inhibition of STAT5 (Ram and Waxman, 1999). CIS, similar to SOCS-1, is induced by IL-2, IL-3, and erythropoietin and inhibits STAT5 activation in response to these cytokines (Chen et al., 2000). Elevated levels of SOCS expression may be required to carefully control the size of the $CD25^+$ population in vivo to achieve a fine balance between the necessity to suppress autoreactivity and the ability to allow appropriate responses to foreign antigens. SOCS-1 has also been shown to bind to all four JAK kinases, therefore having additional inhibitory effects on multiple cytokines (Chen et al., 2000). In addition to STAT5, SOCS-1 is also a negative regulator of STAT3 and STAT6 activation and may play a role in regulating responses to IL-4 and IL-6 (Starr et al., 1997; Losman et al., 1999). IFNγ is also a potent inducer of SOCS-1 and it has been suggested that SOCS-1 can serve as a negative regulator of IFNγ/STAT1 (Yasukawa et al., 2000). Indeed, SOCS-$1^{-/-}$ mice were found to have constitutive activation of STAT1 resulting in hyperresponsiveness to IFNγ in certain cell types. As large amounts of IFNγ may be produced during an immune response to an infectious agent, the capacity of the $CD25^+$ T cells to preferentially upregulate this inhibitor may diminish their suppressive function during protective immune responses.

We, along with others, have postulated that activation of the $CD4^+CD25^+$ T cells results in induction of a cell surface molecule(s) that contributes to $CD25^+$ T cell function. We investigated the role of a number of identified genes by using neutralizing antibodies to both cytokines and cell surface antigens that were either selectively expressed on resting $CD25^+$ T cells or induced during activation. A polyclonal Goat antiserum to GITR reversed $CD25^+$ T cell-mediated suppression, suggesting that GITR plays a critical role in the suppression process.

A comparison of the structure of GITR with other members of the TNFRSF raises the possibility that GITR may play a costimulatory role in the activation of $CD25^+$ T cell-mediated suppressor function. The murine GITR is a 228 amino acid type I transmembrane protein with three cysteine pseudorepeats in the extracellular domain and resembles TNFRSF members CD27 and 4-1BB in the intracellular domain (Nocentini et al., 1997). Importantly, 4-1BB and CD27 molecules provide strong costimulatory signals for T cell proliferation when ligated with their respective ligands or agonistic antibodies (DeBenedette et al., 1997; Kobata et al., 1994). Four different splicing products of murine GITR have been identified and one of the variants, GITR-B, bears a unique cytoplasmic domain due to a reading frame shift (Nocentini et al., 2000). A region of the GITR-B cytoplasmic domain has significant homology with the cytoplasmic region of CD4 and CD8 that interacts with $p56^{lck}$. Thus, interaction of GITR with the GITR-L, perhaps expressed on an APC, may deliver a signal to regulate the suppressive function of $CD25^+$ T cells.

As GITR was upregulated on the $CD25^-$ subset following activation, it was necessary to determine whether addition of anti-GITR was mediating reversal of suppression by acting on the $CD25^+$ T cell or the responding $CD25^-$ T cell. Importantly, culture of the $CD25^+$, not the $CD25^-$ T cells, with anti-GITR and IL-2 resulted in a vigorous proliferative response. Thus, anti-GITR is capable of directly inducing a signal in the $CD25^+$ population that enables it to respond to IL-2. Additionally, this suggests that anti-GITR functions as an agonist for GITR in this assay, providing a signal to the $CD25^+$ cells, rather than blocking the interaction of GITR with its ligand.

Anti-GITR was not as potent in reversal of suppression once the suppressor cells were fully activated, suggesting that it acts by modulating the induction of suppressor cell function. Surprisingly, addition of anti-GITR to $CD25^+$ T cells during the preactivation phase did not inhibit suppressor function in a subsequent responder assay. Preactivation of $CD25^+$ T cells requires engagement of the TCR in the context of IL-2. Thus, high levels of IL-2 during preactivation may override the effect of GITR engagement. These studies suggest that engagement of GITR on $CD25^+$ suppressor cells in the presence of limiting amounts of IL-2 results in the abrogation of suppressor cell function. In the presence of IL-2, how-

ever, GITR engagement does not permanently prevent the induction of their suppressive activity.

Although two groups have identified a ligand for human GITR, the murine GITR-L has not been cloned. The human GITR-L was not expressed by unstimulated or stimulated T or B cells, but was expressed in umbilical vein endothelial cells (Gurney et al., 1999). It has therefore been postulated that GITR/GITR-L interactions are important in the interaction of lymphocytes with the vascular endothelium. However, it is possible that the murine GITR-L is expressed on other cell types including subpopulations of antigen-presenting cells or responder T cells. Induction of GITR-L expression during a rigorous inflammatory response to a pathogen may be necessary to regulate the suppressive activity of CD4$^+$CD25$^+$ T cells in a situation requiring a vigorous effector immune response.

As CD4$^+$CD25$^+$ T cells have recently been identified in human thymus and peripheral blood and appear to closely resemble their murine counterparts in all their functional properties in vitro (Shevach, 2001), one major issue that remains is the development of technologies to therapeutically manipulate regulatory T cell function. The gene expression data presented here strongly support the view that furthering our understanding of the normal cellular physiology of regulatory T cells may yield important insights into how to control both their numbers and functional activity in vivo. For example, inhibition of the function of one of more of the SOCS family members expressed by the CD25$^+$ population may be needed to successfully expand the numbers of CD25$^+$ cells. The model we have proposed for the role of GITR in regulating CD25 function suggests that enhancement of CD25$^+$ T cell-mediated suppression in autoimmunity, as well as downmodulation of CD25$^+$ T cell-mediated suppression in conjunction with development of vaccines to tumors or infectious agents, may be achieved by manipulating GITR/GITR-L interactions or modulating signaling through GITR or GITR-L.

Experimental Procedures

Mice, Antibodies, and Reagents

BALB/c mice (6- to 8-week-old females) were purchased from NCI Frederick animal facility. C57BL/6 (B6) mice (6- to 8-week-old females) were purchased from Jackson (Bar Harbor, ME). B10.D2 expressing a transgenic TCR specific for HA$_{(100\text{-}120)}$ (HA Tg) (Kirberg et al., 1994) were purchased from the NIAID/Taconic Contract. All mice were housed in SPF conditions. PE labeled Rat anti-CD25 (clone PC61), FITC labeled Rat anti-CD25 (clone 7D4), FITC labeled Rat anti-CD8a (clone 53-6.7), purified Hamster anti-CD28 (clone 37.51), FITC or PE labeled Rat anti-Ly6 family members (Ly6A/E, Sca-1 clone D7; Ly6C clone AL-21; Ly6D, ThB clone 49-H4; Ly6G, Gr-1 clone RB6-8C5; Sca-2, Ly-6E, clone MTS35), purified and biotinylated Rat anti-CD103 (integrin αIEL, clone M290), purified Hamster anti-CD3e (clone 145-2C11), purified and PE labeled Hamster anti-CTLA-4 (CD152, clone UC10-4F10-11), purified Rat anti-OX40 (CD134, clone OX-86), purified Hamster anti-4-1BB (CDw137, clone 1AH2), purified Rat anti-CD2 (LFA-2, clone RM2-5) and Streptavidin (SA)-FITC and -PE and all the directly labeled or biotinylated-isotype controls were purchased from PharMingen (San Diego, CA). Purified and FITC labeled Rat anti-OX40 (clone OX-86) were purchased from Serotec (Oxford, United Kingdom). Tri-Color labeled Rat anti-CD4 (clone CT-CD4) was purchased from Caltag (Burlingame, CA). Normal goat IgG (control Ig), purified polyclonal Goat anti-GITR, purified Rat anti-IL-17 (clone 50104.11), and purified polyclonal Goat anti-IL-17R were purchased from R&D Systems (Minneapolis, MN). Biotinylated Hamster anti-4-1BB (clone 17B5) was purchased from eBioscience (San Diego, CA). FITC labeled Donkey anti-Goat was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-CD8 and anti-PE magnetic beads were purchased from Miltenyi (Auburn, CA).

Cell Purification, Stimulation, and RNA Isolation

Peripheral lymph nodes (axillary, inguinal, salivary, and mesenteric) were harvested from 6- to 8-week-old female BALB/c or HA Tg mice. In brief, T cells were purified using T cell enrichment columns (R&D Systems), and then depleted of CD8$^+$ cells by incubation with anti-CD8 microbeads followed by sensitive depletion on AutoMACS (Miltenyi). CD8$^-$ T cells were incubated with PE labeled anti-CD25 (PC61) for 20 min, washed, and incubated with anti-PE microbeads for 15 min and purified by double column positive selection on AutoMACS. Purity was confirmed by flow cytometry. CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cells were greater than 98% and 96%, respectively, with no CD8$^+$ cell contamination. All CD4$^+$CD25$^+$ cells were tested in a suppression assay immediately or after preactivation to assess their suppressive function. FACS was also used for purification of cells. Lymph node cells were incubated with appropriate amounts of TC labeled anti-CD4, PE labeled anti-CD25, and for some applications biotinylated anti-CD103 followed by SA-FITC. CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells were selected, and in addition CD4$^+$CD25$^+$ were further separated using BD FACSVantage Turbo sorter based on the highest and lowest 20% of CD103 expression. Sorter profiles for all populations are shown in Figure 4A.

For cell stimulations, purified cells (10^6/ml) were cultured in complete RPMI/10% FBS supplemented with 100U/ml IL-2 at 37°C for 0, 12 or 48 hr in 24 well plates precoated with 5 μg/ml anti-CD3. RNA was purified using RNeasy columns (Qiagen, Valencia, CA) according to manufacturer's instructions. Flow cytometry was also performed using TC labeled anti-CD4, PE or FITC labeled anti-CD25 in combination with various antibodies indicated in Figure 4. Intracellular staining was performed to analyze CTLA-4 expression, by first incubating with TC labeled anti-CD4 and FITC labeled anti-CD25. They were washed, fixed in 4% paraformaldehyde, and washed again in staining buffer containing 0.5% saponin. The cells were then counterstained with PE labeled-anti-CTLA-4 in this same buffer. Positive staining of each antibody was compared to staining of its respective control antibody and analyzed using CellQuest software (BDIS, San Jose, CA).

DNA Microarray Hybridization and Analysis

Ten micrograms of total RNA was quantitatively amplified and biotin-labeled (Byrne et al., 2000). In brief, RNA was converted to double-stranded cDNA using an oligo dT primer that has a T7 RNA polymerase site on the 5′ end (5′-GGCCAGTGAATTGTAATACGACTCACTA TAGGGAGGCGG-(T_{24})-3′). The cDNA was then used directly in an in vitro transcription reaction in the presence of biotinylated nucleotides Bio-11-UTP and Bio-11-CTP (Enzo, Farmingdale, NY). To improve hybridization kinetics, the labeled antisense RNA was fragmented by incubating at 94°C for 35 min in 30 mM MgOAc, 100 mM KOAc. Hybridization to Genechips (Affymetrix, San Jose, CA) displaying probes for 11,000 mouse genes/ESTs was performed at 40°C overnight in a mix that included 10 μg fragmented RNA, 6X SSPE, 0.005% Triton X-100, and 100 μg/ml herring sperm DNA in a total volume of 200 μl. Chips were washed, stained with SA-PE, and read using an Affymetrix Genechip scanner and accompanying gene expression software. Labeled bacterial RNAs of known concentration were spiked into each chip hybridization mix to generate an internal standard curve, allowing normalization between chips and conversion of raw hybridization intensity values to mRNA frequency (mRNA molecules per million). Chip sensitivity for RNA detection in each run was determined to be 5 RNA molecules/10^6.

In Vitro Proliferation Assays

Suppression assays were performed as previously described (Thornton and Shevach, 1998; Piccirillo and Shevach, 2001). Also, BALB/c CD4$^+$CD25$^+$ T cells (10^6/ml) were prestimulated for a minimum of 3 days in 24-well plates with 0.5 μg/ml soluble anti-CD3 in the presence of 100 U/ml rIL-2, irradiated T cell depleted splenocytes with or without anti-GITR or control Goat IgG. These activated

$CD4^+CD25^+$ cells were used in suppression assays as described (Thornton and Shevach, 2000). All cultures were pulsed with 1 μCi ^{3}H-thymdine for the last 6–12 hr of 65–72 hr cultures.

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