Fingerprints of anergic T cells

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Peripheral T cell tolerance may result from activation-induced cell death [1], anergy [1], and/or immune response modulation by regulatory T cells [2] . In mice that express a transgenic receptor specific for peptide 111–119 of influenza hemagglutinin presented by E^d class II MHC molecules as well as hemagglutinin under control of the immunoglobulin-κ promoter, we have found that anergic T cells [3] can also have immunoregulatory function and secrete IL-10 [4]. In order to obtain information on molecular mechanisms involved in anergy and immunoregulation, we have compared expression levels of 1176 genes in anergic, naive, and recently activated CD4$^+$ T cells of the same specificity by gene array analysis. The results provide a plausible explanation for the anergic phenotype in terms of proliferation, provide new information on the surface phenotype of in vivo-generated anergic CD4$^+$ T cells, and yield clues with regard to new candidate genes that may be responsible for the restricted cytokine production of in vivo-anergized CD4$^+$ T cells. The molecular fingerprints of such T cells should enable the tracking of this small population in the normal organism and the study of their role in immunoregulation.

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Received: **20 November 2000**
Revised: **31 January 2001**
Accepted: **5 March 2001**

Published: **17 April 2001**

Current Biology 2001, 11:587–595

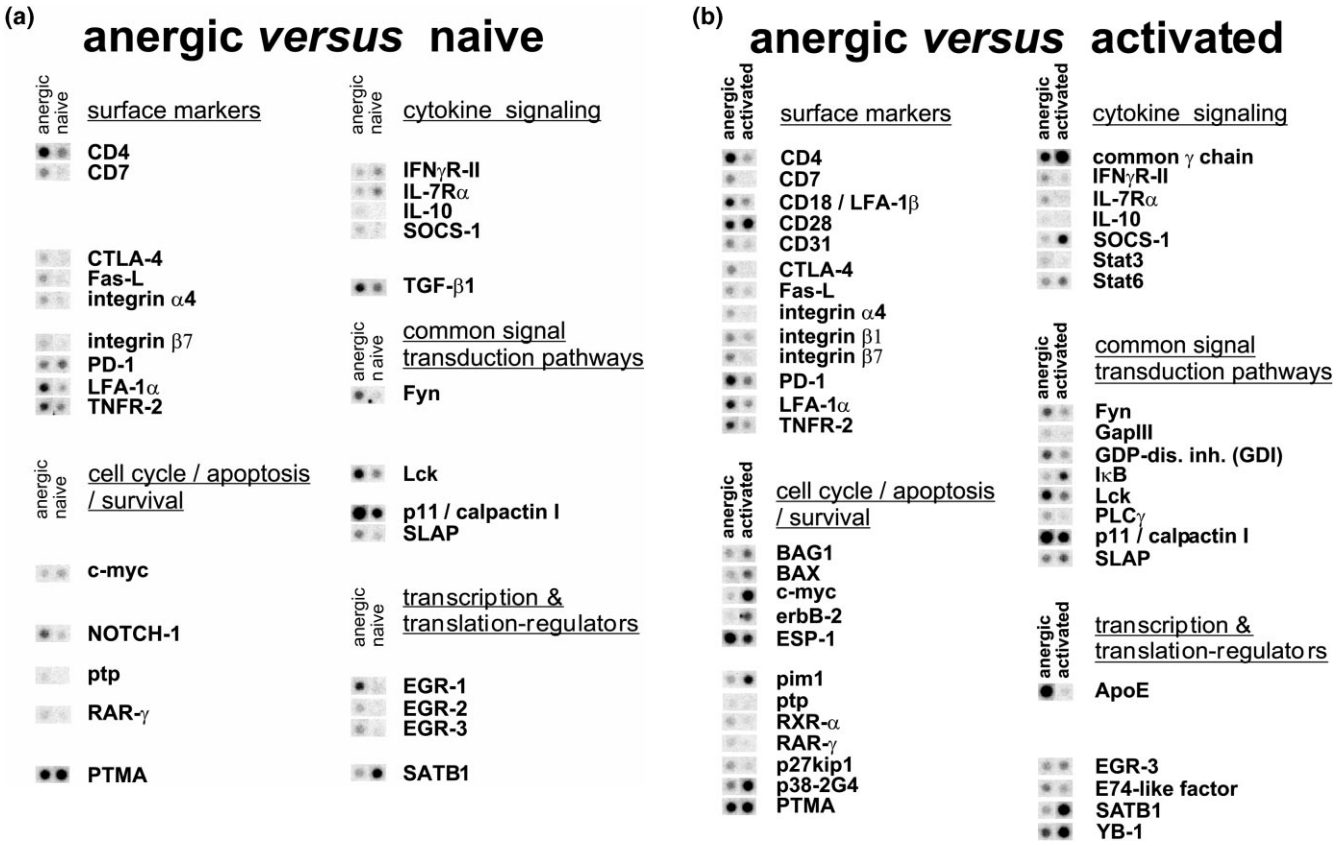
0960-9822/01/$ – see front matter

Results and discussion

Surface molecules

In view of the likely physiological significance of the in vivo-generated anergic and regulatory T cells described in the *TCR-HA* × *IG-HA* mice, we have further analyzed their phenotype by comparing differentially expressed genes by gene array analysis between anergic, naive, and freshly activated T cells with the same specificity. CD4$^+$ T cells with the transgenic TCR-HA receptor, which can be identified by the clonotypic 6.5 antibody, were sorted from either single transgenic *TCR-HA* mice or double transgenic *TCR-HA* × *IG-HA* mice as a source of naive and anergic T cells, respectively. To obtain freshly activated cells, naive 6.5$^+$ cells obtained from *RAG-2*$^{-/-}$ *TCR-HA* mice were stimulated in vitro for 16 hr by a mitogenic dose of anti-CD3 antibodies. RNA was prepared from all populations, ^{32}P-labeled cDNA was obtained by reverse transcription, and cDNA probes were hybridized to identical replicas of gene array membranes (Atlas Mouse 1.2 Array, Clontech). The differential expression of 1176 genes was analyzed. The blot analysis of differentially expressed genes is shown in Figure 1. Numerical values of signal strength after background correction (see Materials and methods) are given in Table 1. Whenever feasible, duplicate analysis was performed, and the values are indicated in Table 1. The differences in gene expression between different cells were, as a rule, well reproducible. With regard to surface marker expression, i.e., CD4, CTLA-4, PD-1, CD7, TNFR-2, and Fas-L, it is clear that with the exception of CD7, anergic cells resemble more closely recently activated CD4$^+$ T cells than naive CD4$^+$ T cells, even though some of these genes are expressed at levels even higher than those seen in recently activated T cells. The higher levels of PD-1 at the RNA and protein level is of special interest because this molecule plays an essential, but unknown, role in the prevention of autoimmune disease. The ligand of PD-1 (PD-L1) has recently been shown to be a member of the *B7* gene family. Engagement of PD-1 by PD-L1 leads to the inhibition of TCR-mediated lymphocyte proliferation and cytokine secretion. In addition, PD-1 signaling has been shown to inhibit at least suboptimal levels of CD28-mediated costimulation [5]. Increased protein levels of both CTLA-4, a negative regulator of immune activation, and PD-1 among anergic cells were evident by FACS staining and are shown in Figure 2. There was, however, no complete concordance with upregulated molecules on activated cells, as the CD28 costimulatory molecule corresponded to the levels found on naive T cells. This confirms that the method is reliably detecting differences in RNA content, which can be reflected in different protein expression patterns. In the view of recently published data [2], it should be noted that the anergic cells are heterogeneous with regard to CD25 expression, which is not included in the analysis; about 30% of the cells are CD25$^+$. With regard to the

Figure 1



(a) Genes differentially regulated in anergic versus naive $CD4^+$ T cells. Total RNA of in vivo-anergized T cells from *TCR* × *Ig-HA* and naive T cells from *TCR-HA* mice, respectively, were used as templates to generate specific ^{32}P-labeled cDNA probes for expression array analysis (Clontech). RT probes were hybridized to individual cDNA blots overnight and washed, and gene expression was determined by exposing the arrays to Phosphor Imaging screens (Fuji) and analyzed using a Fuji Bas 2500 16-bit image analysis system. Expression levels were determined by measuring the spot density for each gene using Array-Vision software (Version 5.1). To compare density values among the two cell populations, the level of each gene was normalized to the total of all genes measured (Table 1). One out of two independent array analyses results are shown. **(b)** Genes differentially regulated in anergic versus activated $CD4^+$ T cells. Total RNA of in vivo-anergized T cells from *TCR* × *Ig-HA* mice and naive T cells from *Rag-2*$^{-/-}$ *TCR-HA* mice, respectively, were used as templates to generate ^{32}P-labeled cDNA probes for expression array analysis (Clontech). For further details see Figure 1.

analyzed cell surface markers, a combination of PD-1, CD7, CD28, and CD4 antibodies should be well suited for the identification of the particular anergic cell type and should be able to distinguish it from naive and recently activated T cells.

Signal transduction

Regarding TCR signaling, proximal signaling molecules, such as CD4 and the src-kinase, were overexpressed when compared to both naive and recently activated HA-specific T cells. While these findings could be interpreted to indicate that anergic cells somehow represent hyperactivated cells, a closer inspection of the expression levels of genes encoding intracellular proteins yields a different picture; here, several inhibitors of signal transduction, i.e., GDP-dissociation inhibitor (GDI), GapIII, or CD31, exhibited levels comparable to naive $CD4^+$ T cells but were upregulated with regard to recently activated cells. This was the first indication that anergic cells also exhibited features typical of resting naive $CD4^+$ T cells.

Regulation of transcription and cytokine secretion

Of special interest was the specific downregulation of SATB1 in anergic cells with regard to both of the other cell types. SATB1 can act as a transcriptional repressor, but recent evidence suggests that it may regulate the expression of multiple gene clusters, either positively or negatively, by binding to matrix attachment regions that are implicated in the loop domain organization of chromatin [6]. SATB1 may, thus, be involved in the control of lineage-specific expression of multiple genes. These may include cytokine genes or cytokine receptor genes that are differentially expressed in anergic versus naive and recently activated T cells.

Table 1

Genes regulated in CD4$^+$ T cells that might be critical for the induction and maintenance of anergy in vivo.

mRNA	Naive	Anergic	Activated	Putative function
		Surface antigens		
CD4	85	171	30	Expressed on most thymocytes and a subset of mature T cells. Interacts with MHC class II molecules and participates in T cell activation.
CD7	4 7	45 27	−2	Expressed early in T cell development and induced upon T cell activation. Ligand unknown. Plays a role in the regulation of integrin expression.
CD18	100 62	110 55	27	Common β subunit of LFA-1.
CD28	80 10	115 20	248	Costimulatory receptor engagement of CD28 either by B-7 or by Ab can strongly enhance TCR signaling responses. Blocking CD28 engagement inhibits T cell activation and may result in T cell anergy.
CD31	71 13	46 5	12	ITIM-containing inhibitor of PTK-dependent signal transducers; dampens signals transduced by ITAM-containing receptors, which delays Ca^{2+} mobilization after TCR-ligation.
CTLA-4	2 15	23 29	11	Coligand on T cells. Binds to B7 on APCs; delivers a negative signal leading to cell cycle arrest.
Fas-L	6 4	29 13	16	Binds to Fas (CD95) and participates in T cell development, regulation of immune responses, and T cell-mediated cytotoxicity. Fas-L inhibits CD4 T cell proliferation, cell cycle progression, and IL-2 secretion.
Integrin α4	16 12	28 13	1	Together with integrin β1, forms the VLA4 receptor for fibronectin and VCAM-1. Role in leukocyte adhesion and migration expressed on the cell surface as a heterodimer with one of the integrin α chains.
Integrin β1	30 13	43 15	17	Together with integrin β1, forms the VLA4 receptor for fibronectin and VCAM-1; important in leukocyte adhesion and migration.
Integrin β7	103 17	51 8	6	Expressed on the cell surface as a heterodimer with one of the integrin α chains.
PD-1	22 4	145 65	68	Induced upon lymphocyte activation. Contains an immunoreceptor tyrosine-based inhibitory motif. Involved in the maintenance of peripheral self-tolerance by serving as a negative regulator of immune responses.
LFA-1α	31 8	101 18	30	Member of the integrin β2 family. Expressed on all leukocytes; mediates interactions with ICAM-1,2,3; involved in leukocyte adhesion and transendothelial migration.
TNFR-2	44 17	84 75	32	Together with TNFR-1, it can induce a context-dependent apoptosis or cell activation; lacks a death domain (unlike TNFR-1 and CD95).
Common signal transduction pathways				
Fyn	20 9	77 20	21	Member of the Src-kinase family; involved in TCR-stimulated signaling and proliferation of T cells.
GapIII	25 9	19 5	1	Negative regulator of Ras; Ras is activated by TCR ligation and coordinates important signaling pathways for T cell activation.
GDP-diss. Inh (GDI)	93	82	25	Blocks dissociation of GDP from G proteins, which keeps the G-protein in an inactive state, preventing the activation of adenylate cyclase. Substrate for caspase-3.
NFκB-Ia/IκB	23 9	20 6	86	IκB-nuclear factor of κ-light chain protein enhancer in B cells inhibitor alpha; blocks NF-κB transition to the nucleus; thus, it inhibits NF-κB transcription factor.

(*continued*)

Table 1

Continued

mRNA	Naive	Anergic	Activated	Putative function
			Surface antigens	
Lck	75 23	139 28	48	Membrane-associated Src-family kinase; primarily expressed in T cells; involved in early phosphorylation events of T cell activation.
PLCγ	25 1.4	32 1.8	8	Involved in tyrosine kinase-dependent signaling for mitogenic responses; phosphorylated during T cell activation; anergic Th1 lymphocytes differ in TCR-mediated tyrosine kinase activity; associated with alterations in PLCγ, IP, and intracellular free calcium.
Calpactin I/pII	242	471	233	Involved in downregulation of phospholipase-A2 (PLA2); forms CD44/calpactin-I lipid raft complexes that interact with the underlying cytoskeleton; blocking of PLA2 leads to an inhibition of IL-2 stimulated PKC stimulation.
SLAP	24 13	45 13	82	Src-like adaptor protein (SLAP) expressed in T lineage cells; inhibits TCR signaling when expressed in the Jurkat T cell line.
Regulators of transcription and translation				
ApoE	568	408	9	Antagonizes posttranslational events in mitogen-activated T cells that are required for the secretion of bioactive IL-2; thus, inhibits T cell proliferation.
EGR-1	20 16	89 34	98	Early growth response protein 1 regulates IL-2 transcription by synergistic interaction with the nuclear factor of activated T cells.
EGR-2	0	25	37	EGR-2 and EGR-3 regulate Fas-L expression in activated T cells; EGR-2 is likely to play a role in Fas-L upregulation in lpr/lpr and gld/gld CD4- and CD8-T cells.
EGR-3	4	24	49	EGR-3 (unlike EGR-1) can directly mediate TCR-induced transcription of Fas-L; transcription upregulated after TCR activation.
E74-like factor 1	41 11	44 6	18	ETS family transcription factor, binds to conserved DNA sequence domains including the GM-CSF promoter, the IL-2, and the CD4-enhancer.
SATB1	251 77	32 5	430	Represses apoptosis and proliferation-associated genes (e.g. PD-1); peripheral $CD4^+$ T cells from $SATB1^{-/-}$ mice fail to proliferate in response to activating stimuli and upregulate CD44; SATB1 orchestrates temporal/spatial expression of genes during T cell development.
YB1	89 19	86 17	301	Class II Y-box binding nuclear factor induced by IFNγ; regulates various genes involved in cell proliferation.
Cytokine signaling				
Common γ chain	25	25	655	Shared by multiple cytokine receptors (IL-2, 4, 7, 9, and 15); unresponsiveness in anergic $CD4^+$ T cells is due to defective signaling through the IL-2R γ chain.
IFNγ-RII	45 2.4	13 1.1	7	Receptor subunit for the proinflammatory cytokine IFNγ; downregulated upon TCR-stimulation of primary T cells.
IL-7Rα	70 22	20 9	7	Ligand binding part of the IL-7R; defective IL-7 signal transduction leads to severe immunodeficiency.
IL-10	0	6	0	Regulatory cytokine; inhibits macrophage-mediated cytokine synthesis and proinflammatory T cell-mediated immunity; inhibits IL-2-mediated proliferation by blocking the degradation of the cell cycle inhibitor p27kip1.
SOCS-1	1	14	186	Short lived, negative feedback-regulator of cytokine receptor signaling (e.g. IL-2, IL-4) via the JAK/STAT pathway; deletion causes perinatal lethality; peripheral T cells in these mice express activation antigens and proliferate to IL-2 in the absence of anti-CD3.

(*continued*)

Table 1

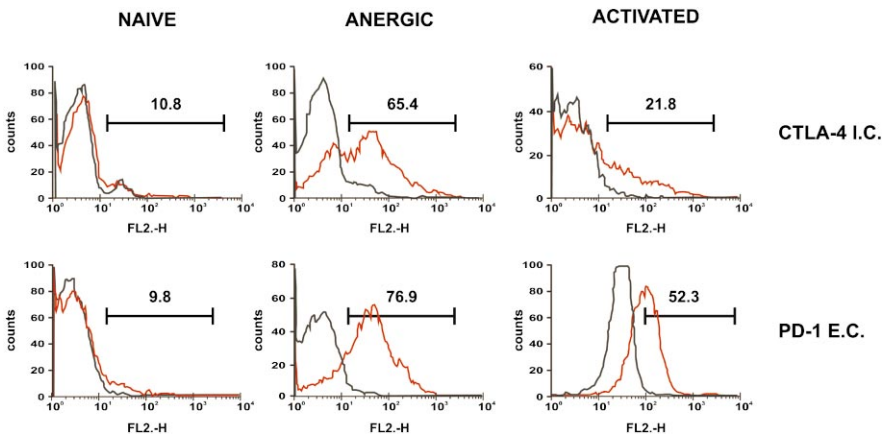
Continued

mRNA	Naive	Anergic	Activated	Putative function
			Surface antigens	
STAT3	2 30	9 34	4	Crucial role in cell growth, suppression of apoptosis, and cell motility; important role in cytokine receptor signal transduction (IL-2R, IL-10R).
STAT6	25 26	37 26	81	Activated in response to IL-4 stimulation; critical for the activation or enhanced expression of many IL-4 responsive genes.
TGFβ-1	65 52	102 38	116	Effector cytokine of Th3; increased TGF-β1 production correlates with protection and/or recovery from autoimmune diseases. TGF-β1 and CTLA-4 act together to terminate immune responses.
Cell cycle/apoptosis/survival				
Bag1	40 14	35 13	93	Induced by IL-2R binding; in combination with Bcl-2, BAG-1-induction is a key event for the antiapoptotic function of IL-2.
Bax	32 9	31 11	102	Integral membrane protein that promotes apoptosis; its function is countered by Bcl-2 and Bcl-XL.
C-myc	34 27	14 8	395	Protooncogene, expression in T cells induced by IL-2R binding through STAT5; c-myc, together with cyclin E/CDK2, inhibits p27kip1 and enables progression through the cell cycle.
ErbB-2	9 3	4 3	116	ERBB-2/c-neu/Her-2, oncogene, receptor tyrosine kinase involved in DNA repair mechanisms and G1/S-phase progression. ErbB2 signals reduce p27kip1 stability, thereby enabling cell cycle progression.
ESP1 protein	221	280	106	GRG protein, interacts with transcription factors, represses transcription; suppressor of pim1; required for cyclin destruction; controls anaphase and mitotic exit.
NOTCH-1	29	74	67	Important role in controlling levels of cell death in T cells.
Pim1	36 13	33 10	169	Protooncogene, protease expressed in primarily in hematopoietic cells, implicated in intracellular signaling processes accompanying lymphocyte activation; induced by IL-2 as well as CD3 cross linking.
Ptp	0 1	6 9	1	Protein tyrosine phosphatase-1 associated with Fas (FAP-1); inhibits Fas-mediated cell death.
RXR-α	s18	25	4	Receptor for all-*trans* and 9-*cis* retinoic acids (RAs) that functions as a transcription factor. RAs inhibit TCR/CD3-mediated (activation induced) but enhance glucocorticoid-induced apoptosis in thymocytes. Costimulation of RXR-α leads to inhibition of RAR-γ-mediated apoptosis.
RAR-γ	0	10	0	Receptor for RAs; functions as a transcription factor (see also RXR-α).
P27(kip1)	41 26	34 14	7	Tumor suppressor, inhibits activity of cyclin D-, Ep, A-, and B-dependent kinases and of cyclin-activating kinases; in anergic T cells, it inhibits AP-1 transactivation and IL-2 transcription; IL-10 inhibits proliferation by blocking the downregulation of p27kip1.
P38-2G4	63	56	303	Nuclear protein that is active in cell cycle control, highly expressed between G1 and mid S-phase.
PTMA	228 58	159 43	331	Prothymosin alpha; nuclear protein required for proliferation; induces the unfolding of chromatin fibers.

Differential expression of 1176 mRNA was investigated by cDNA array hybridization in naive, in vivo-anergized, and recently activated $CD4^+$ T cells. Spot density for each gene was measured as described in Materials and methods. Anergic versus naive populations were compared in two independent array experiments. Local backgrounds were computed for each spot. Net density (presented in this table after normalization to the total of all mRNA measured for a particular sample) was determined by subtraction of this background from average density for each spot. All nonflagged array elements (see Materials and methods) for which the density value was greater than 1.4 × the local background were considered well measured and were included in this table if they were up or downregulated in the studied T cell populations by a factor ≥1.75 and a density value ≥5 compared to naive and/or recently activated $CD4^+$ T cells.

Figure 2

FACS analysis of intracellular CTLA-4 and surface PD-1 on in vivo naive, anergic, and in vitro anti-CD3-activated *TCR-HA* T cells. Histograms were obtained by gating on $CD4^+6.5^+$ T cells. Broken lines represent background staining (I.C. indicates intracellular, and E.C. indicates extracellular).



Considering cytokine expression, it was previously noted that these in vivo-generated anergic cells produced little IL-2, IL-4, and IFNγ ex vivo [4]. Generally, downregulation of IL-2 secretion seems to be one major pathway to achieve anergy in several in vitro and in vivo models. At least four molecules that are directly implicated in the regulation of IL-2 synthesis and secretion were differentially regulated in anergic cells when compared to freshly activated cells. YB-1, a transcription factor reported to be necessary for stabilizing the mRNA of IL-2, was downregulated, while ApoE was upregulated as in naive cells. Fas-L as well as CTLA-4 were specifically upregulated in anergic cells when compared to both naive and recently activated T cells. The latter three molecules were reported to inhibit the secretion of bioactive IL-2 at different levels [7–10].

Previous data from our group as well as the present array analysis revealed that both naive and recently activated $CD4^+$ T cells did not produce any IL-10, whereas ex vivo anergic cells expressed significant levels of IL-10 mRNA. It is known that IL-10 blocks the IL-2-mediated downregulation of p27kip1 and upregulation of D cyclins in T cell activation and entry into the cell cycle [10]. This may represent another pathway contributing to the anergic phenotype (see below). Also, one additional regulatory cytokine, TGF-β1, was detected in all three populations, but there was no difference between the three populations, confirming previous quantitative PCR data (unpublished data). Cytokine signaling pathways seem to represent another important target in the regulation of anergy. Signal transduction pathways of the IL-2R, IL-4R, and IL-7R are affected by the common γ-chain, which was downregulated in anergic T cells when compared to activated T cells. The correlation between the regulation of the common γ chain and induction of anergy has previously been described by Grundstrom et al. [11]. The anergic cells analyzed in the above mentioned study were characterized by a weaker activation and tyrosine phosphorylation of Janus-associated kinase (Jak)-3 and signal transducers and activators of transcription (Stat)5 and reduced DNA binding ability to Stat5-specific elements.

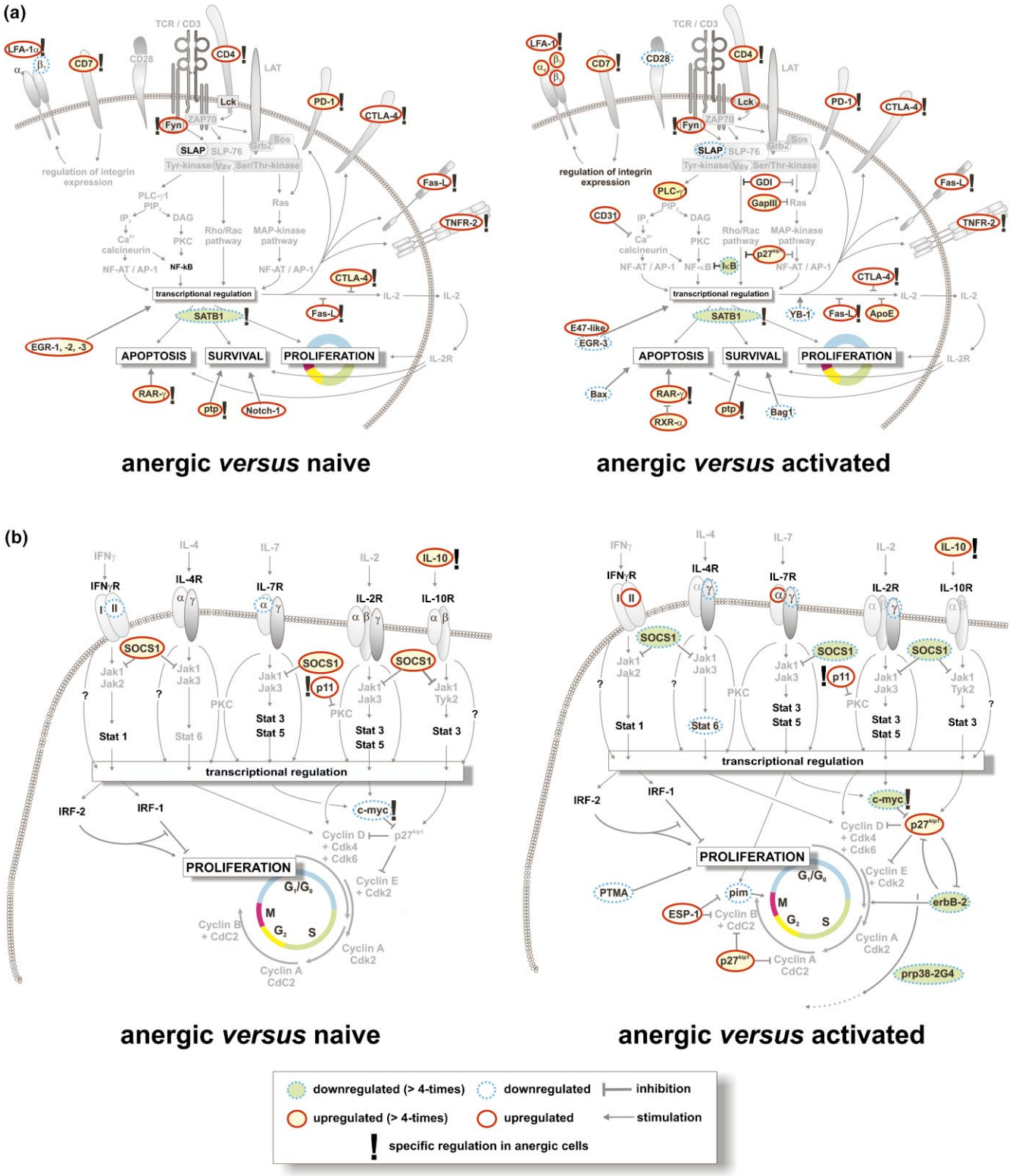
Cell proliferation

IL-2, IL-4, and IL-7 have important roles in cell activation and proliferation, and it is of interest that in anergic cells, the decreased signaling through these receptors results in low level expression of c-myc and pim-1, two key molecules of cell activation and cycle regulation [12]. In addition to the regulation of signaling molecules in common with the TCR (e.g., Lck, MAPK-pathway), cytokine receptor-specific signaling appears also to be modulated at the level of Jak's and Stat's when compared to activated cells.

Considering proliferation and cell cycle progression, it is obvious that anergic cells more closely resemble the naive than the activated $CD4^+$ T cells in that they express high levels of the cell cycle inhibitors p27kip1 and ESP-1 [13] and low levels of cell cycle promoters pim-1 [12] and p38–2G4. C-myc, a direct promoter of proliferation [14], is specifically downregulated in anergic cells. In the context of these data, it is of interest that recently Boussiotis et al. showed direct inhibition of cyclins D and E by p27kip1, which is also responsible for the defective transactivation of AP-1 and IL-2 transcription in anergized T cells [15]. In these in vitro-anergized T cells, p27kip1 levels were specifically upregulated. While these cells could be rescued upon coculture with a specific antigen and IL-2, our in vivo-anergized T cells differ since they remain anergic under these conditions.

An additional feature regarding cell cycle regulation that may explain functional differences between in vivo- and in vitro-anergized cells is the observation that CD28, c-myc, and ErbB2 levels are downregulated. CD28-medi-

Figure 3



(a) Interrelationships between differentially regulated genes at the level of TCR signal transduction, regulation of transcription, and cell fate in anergic CD4$^+$ T cells relative to naive or resting cells.

(b) Interrelationships between differentially regulated genes at the level of cytokine signaling and proliferation in anergic CD4$^+$ T cells relative to naive or resting cells.

ated signaling as well as c-myc and ErbB2 activities were reported to block p27kip1 activity and, thus, to permit entry into the cell cycle [12, 16, 17]. Thus, with the down-regulation of these three genes in in vivo-anergized CD4$^+$ cells, p27kip1 has lost its antagonists. Moreover, it was shown that upregulation of CTLA-4, as observed in anergic cells, inhibits progression through the cell cycle by inhibiting the production of cyclin D3, cyclin-dependent kinase (cdk)4, and cdk6. This indicates that CTLA-4 signaling inhibits events early in T cell activation both at the IL-2 transcription level as well as at the level of the IL-2-independent events of the cell cycle [8].

Overall, the data indicate that anergic/regulatory T cells exhibit a quite unique gene expression program (see Figure 3, Table 1) that should enable one to trace these cells in normal mice and help promote understanding of the anergic phenotype in terms of proliferation. A closer investigation of differentially expressed genes involved in regulating transcription and perhaps cytokine secretion is required in order to address the regulatory function of these cells that they exhibit both in vivo as well as in vitro (unpublished data).

Material and methods

Mice

The *TCR-HA* transgenic mice expressing a TCR-α/β specific for peptide 111–119 from influenza HA presented by I-E^d are on the Balb/c background and have been previously described, as have those on the recombination-activating gene *(RAG)-2*$^{-/-}$ background [18]. Mice expressing HA under the *Igκ* promoter and enhancer elements are also on the Balb/c background. *TCR-HA* × *IG-HA* mice were bred in our animal facility at Necker.

Antibodies and flow cytometry

The following mAbs were used for staining: biotinylated F23.1 (specific for the TCR-β chain of the HA-reactive TCR), FLUOS-labeled 6.5 clonotypic mAb streptavidin-PE (Southern Biotechnology), Cy-Chrome-coupled anti-CD4 (Pharmingen), biotinylated anti-PD-1 (a kind gift of Dr. T. Honjo, Kyoto University, Japan), and PE-labeled anti-CTLA-4 (CD152) (Pharmingen).

All stainings were done in round bottom 96-well plates (1 × 10^6 cells/well) in 20 μl of mAb in PBS/2% FCS for 20 min on ice. For cell sorting experiments, splenocytes from *TCR-HA* or *TCR-HA* × *IG-HA* mice (pooled from several mice) were depleted of surface Ig$^+$ cells with Dynabeads (Dynal) and stained with F23.1 and 6.5 mAbs. F23.1$^+$6.5^{hi} cells were sorted on a FACS Vantage (Beckton Dickinson). Sorted populations were >98% pure, as determined by FACS analysis. For intracellular staining of CTLA-4, cells were stained with anti-CD4-Cy-Chrome and 6.5-FLUOS, washed, fixed in 0.1% paraformaldehyde for 15 min at room temperature, and then permeabilized in 0.1% saponin (Sigma) for 10 min at room temperature. Cells were then incubated on ice with anti-CTLA-4-PE diluted in saponin buffer, or with anti-CD4-PE or anti-CD19-PE as positive and negative controls for intracellular staining.

In vitro T cell activation

Lymph node cells from a *RAG-2*$^{-/-}$ *TCR-HA* mouse were isolated and incubated with biotinylated anti-CD4 (GK1.5) mAb on ice. Subsequently, cells were washed, incubated with streptavidin-coupled magnetic microbeads, and passed through a MS2+ column (Miltenyi Biotec) according to manufacturer's instructions. CD4$^+$6.5$^+$ cells (>95% purity) were incubated in 24-well plates coated with 5 μg/ml anti-CD3 (2C11) for 16 hr and then recovered for RNA preparation.

cDNA array procedures

Differential expression of 1176 genes was investigated by cDNA array hybridization in naive, recently activated, and in vivo-anergized CD4+ T cells. Total RNA was extracted using Tri-Reagent LS (Molecular Research Center) according to the manufacturer's instructions. After a DNase I digestion for 30 min at 37°C, a second extraction with Tri-Reagent LS was carried out in order to obtain high quality total RNA. Total RNA (3.5 μg) from each population was used as a template for the specific synthesis of ^{32}P-radiolabeled cDNA probes according to the manufacturer's recommendations (Clontech), except that the RT enzyme was replaced by Superscript II (GIBCO). cDNA probes were subsequently hybridized side-by-side to identical gene array membranes containing various mouse cDNAs, nine housekeeping control cDNAs, and negative controls immobilized on a nylon membrane (a complete list of cDNAs and controls immobilized on the Atlas Mouse 1.2 Array from Clontech can be found at www.clontech.com). After hybridization and extensive washes, membranes were exposed to Phosphor Imaging screens (Fuji). Radioactivity was detected using a Fuji Bas 2500 16-bit image analysis system (Fuji).

Data analysis

cDNA array analysis of gene expression was essentially done as described [19]. Each spot was defined by positioning of a grid of circles over the array image. Spot density for each gene was measured using Array-Vision software (Version 5.1, Imaging Research), along with numerous quality control parameters (see Array-Vision manual). Local backgrounds were computed for each spot. The net density was determined by subtraction of this background from the average density for each spot. Data files generated by Array-Vision were entered into a custom database. Single spots or areas of the array with obvious blemishes were flagged and excluded from subsequent analyses. All nonflagged array elements for which the density value was greater than 1.4× the local background were considered well measured. Most obtained results were confirmed in two independent array analyses (see Table 1). To compare density values among the different cell populations, the level of each gene was normalized to the total of all genes measured for that sample.

Acknowledgements

We would like to thank Sebastien Lavie for animal care and Corinne Garcia, Patricia Gatzlaff, and Carole Zober for excellent technical assistance. O. L. is a recipient of a Marie Curie Training Grant of the European Commission, A. F. is supported by the Förderverein für Tumorzentren in Niedersachsen, A. S. is supported by a fellowship from the Juvenile Diabetes Foundation, H. v. B. is supported by the Institut Universitaire de France and the Körber Foundation, and J. B. is supported by a grant from the Deutsche Forschungsgemeinschaft and by the Deutsche Krebshilfe. In addition, this work was funded by the Juvenile Diabetes Foundation, the Institut National de la Santé et Recherche Médicale, and the Faculté Necker Enfants Malades in Paris.

References

1. Rocha B, von Boehmer H: **Peripheral selection of the T cell repertoire.** *Science* 1991, **251:**1225-1228.
2. Suri-Payer E, Amar AZ, Thornton AM, Shevach EM: **CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells.** *J Immunol* 1998, **160:**1212-1218.
3. Lanoue A, Bona C, von Boehmer H, Sarukhan A: **Conditions that induce tolerance in mature CD4+ T cells.** *J Exp Med* 1997, **185:**405-414.
4. Buer J, Lanoue A, Franzke A, Garcia C, von Boehmer H, Sarukhan A: **Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized in vivo.** *J Exp Med* 1998, **187:**177-183.
5. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, *et al.*: **Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.** *J Exp Med* 2000, **192:**1027-1034.
6. Alvarez JD, Yasui DH, Niida H, Joh T, Loh DY, Kohwi-Shigematsu T: **The MAR-binding protein SATB1 orchestrates temporal and**

spatial expression of multiple genes during T-cell development. *Genes Dev* 2000, **14:**521-535.
7. Kelly ME, Clay MA, Mistry MJ, Hsieh-Li HM, Harmony JA: **Apolipoprotein E inhibition of proliferation of mitogen-activated T lymphocytes: production of interleukin 2 with reduced biological activity.** *Cell Immunol* 1994, **159:**124-139.
8. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP: **CTLA-4-mediated inhibition of early events of T cell proliferation.** *J Immunol* 1999, **162:**5813-5820.
9. Desbarats J, Duke RC, Newell MK: **Newly discovered role for Fas ligand in the cell-cycle arrest of CD4+ T cells.** *Nat Med* 1998, **4:**1377-1382.
10. Perrin GQ, Johnson HM, Subramaniam PS: **Mechanism of interleukin-10 inhibition of T-helper cell activation by superantigen at the level of the cell cycle.** *Blood* 1999, **93:**208-216.
11. Grundstrom S, Dohlsten M, Sundstedt A: **IL-2 unresponsiveness in anergic CD4+ T cells is due to defective signaling through the common gamma-chain of the IL-2 receptor.** *J Immunol* 2000, **164:**1175-1184.
12. Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T: **Synergistic roles for pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis.** *Immunity* 1999, **11:**709-719.
13. Tinker-Kulberg RL, Morgan DO: **Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage.** *Genes Dev* 1999, **13:**1936-1949.
14. Beerli RR, Dreier B, Barbas CF III: **Positive and negative regulation of endogenous genes by designed transcription factors.** *Proc Natl Acad Sci USA* 2000, **97:**1495-1500.
15. Boussiotis VA, Freeman GJ, Taylor PA, Berezovskaya A, Grass I, Blazar BR, *et al.*: **p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes.** *Nat Med* 2000, **6:**290-297.
16. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE: **ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency.** *Mol Cell Biol* 2000, **20:**3210-3223.
17. Yang HY, Zhou BP, Hung MC, Lee MH: **Oncogenic signals of HER-2/neu in regulating the stability of the cyclin-dependent kinase inhibitor p27.** *J Biol Chem* 2000, **275:**24735-24739.
18. Kirberg J, Baron A, Jakob S, Rolink A, Karjalainen K, von Boehmer H: **Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor.** *J Exp Med* 1994, **180:**25-34.
19. Eisen MB, Brown PO: **DNA arrays for analysis of gene expression.** *Methods Enzymol* 1999, **303:**179-205.