Supporting information for Diehn *et al*. (2002) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.092284399.

Supporting Materials and Methods

Isolation and Stimulation of Primary T Cells. Primary T cells were isolated from whole blood of normal donors by using Ficoll–Paque (Pharmacia Biotech) followed by magnetic depletion of non-T cells (MACS Pan-T Cell isolation kit, Miltenyi Biotech). Purity was assessed by FACS analysis for each isolation, and all preparations were at least 98% pure. Cells were allowed to rest overnight in RPMI 1640 media with 10% FCS and 1% penicillin/streptomycin at 5×10^6 cells/ml. T cells were stimulated in parallel at 1.1×10^6 cells/ml in media. Time courses for the first set of experiments consisted of the following stimuli: mock, tosyl-activated polystyrene beads (Dynal, Great Neck, NY) coated with a 1:1 mixture of anti-CD3 antibody (OKT3) and anti-MHCI antibody (W6/32) (anti-CD3 beads), beads coated with a 1:1 mixture of anti-CD28 antibody (9.3) and anti-MHCI antibody (W6/32) (anti-CD28 beads), beads coated with a 1:1 mixture of anti-CD3 antibody and anti-CD28 antibody (costimulatory beads), 1 μM Ionomycin/25 ng/ml of phorbol ester phorbol 12-myristate 13-acetate (PMA), 5 μg/ml of phytohemagglutinin, and 24 nM FK506 (pretreated 1 h) in combination with costimulatory beads. For the cytokine time courses shown in Fig. 7, T cells were treated with 100 ng/ml of TNF-α or 10 ng/ml of IFN-γ. Time points were harvested at 0, 1, 2, 6, 12, 24, and 48 h, except for the cytokine time courses, which were harvested at 2, 6, and 24 h. All beads were a generous gift of James L. Riley (University of Pennsylvania), were used at a density of three beads per cell, and were prepared as previously described (1). For harvesting, cells were diluted 2:5 in ice-cold PBS and centrifuged at $1{,}100 \times g$ at 4°C for 6 min. The supernatant was retained for subsequent IL-2 ELISA analysis. Cell pellets were flash frozen on liquid nitrogen and stored at –80°C prior to RNA isolation. The second set of array experiments consisted of the following stimuli: mock, beads coated with 100% anti-CD28 antibody and beads coated with 100% B7.2 protein (CD86). Experimental details were as above, except that cells were harvested at 0, 1, 2, 6, and 12 h.

RNA Isolation and aRNA Amplification. Total RNA was isolated by using QIAshredder and RNeasy columns (Qiagen, Chatsworth, CA). RNA quality and quantity were assessed via electrophoresis and UV spectrophotometry. The resulting RNA was amplified by using a previously published *in vitro* transcription-based amplification method (2) with minor modifications (http://cmgm.stanford.edu/pbrown/protocols/ampprotocol_3.html).

cDNA Microarray Manufacture and Hybridization. The cDNA microarrays were manufactured and hybridized as previously described (3, 4) (also see http://cmgm.stanford.edu/pbrown/). Arrays contained 37,632 elements, representing ≈18,000 different genes, and were scanned by using a 4000B GenePix scanner at 10-μm resolution (Axon Instruments, Foster City, CA). Equal amounts of the experimental and reference probes were pooled and hybridized to microarrays. The reference pool of mRNA was prepared from human primary T cells and the human Jurkat leukemia cell stimulated for varying times with ionomycin and PMA. Comparison of all experimental samples to the same reference allowed the relative expression level of each gene to be compared across all of the experiments. The resulting images were processed by using GENEPIX PRO 3.0. The data were then normalized and indexed in the Stanford Microarray Database (http://genome-www4.stanford.edu/MicroArray/SMD/).

IL2-ELISA. Luminescence-based IL-2 ELISA (R & D Systems) was performed on the cell culture supernatants from each of the time courses per manufacturer's instructions.

Data Selection and Analysis. Microarray elements (i.e., spots representing uniquely arrayed cDNA clones) were selected for analysis in two stages. In the first stage, we applied a high-stringency data selection, allowing only elements for which at least 80% of the measurements within a set of experiments had fluorescence intensity in either channel at least 3-fold over background intensity. In the second stage, additional array elements with low signal intensity (at least 1.5-fold over background intensity in one of the two channels for at least 70% of the arrays) were added if their expression profile had a correlation of at least 0.9 to any gene selected in the first stage. All data points in this

combined dataset were extracted, and each array was normalized by using an array-specific normalization coefficient. This coefficient was calculated by subtracting in log base 2 space the average Cy5/Cy3 ratio of all elements with an intensity/background ratio greater than 3-fold in both channels for at least 80% of the arrays. Only cDNAs that had high-quality measurements for 80% or more of the measured time points across all of the experiments were used for further analysis. Measurements from multiple samples (six) taken at the $t = 0$ time point were averaged, with the exclusion of those measurements that fell beyond 1 standard deviation from the mean. This average $t = 0$ measurement was then subtracted from each subsequent time point measurement to depict the temporal response patterns of expression relative to $t = 0$ as the baseline. We then filtered the data on the basis of the variation of each element from the baseline across all experiments, as described in the figure legend (Fig. 1). Hierarchical clusters and self-organizing maps were generated by the CLUSTER program and analyzed with the TREEVIEW program (M. Eisen, http://www.microarrays.org). Additional calculations and transformations were performed as described in the figure legends (Figs. 1 and 2).

Aggregate enhancement of gene expression responses to αCD3 stimulation by costimulation with αCD28 was analyzed by using a customized distance metric. In Fig. 2*B*, the abscissa represents the difference in the response between monostimulation with αCD3 and costimulation with αCD3/αCD28-coated beads by using a customized distance metric. Specifically, for each gene, the distance between the two time series was calculated as

$$\left[\frac{\sum_{i=1}^{6} X_i}{\left|\sum_{i=1}^{6} X_i\right|}\right] \bullet \left[\sum_{i=1}^{6} \left\{ \begin{matrix} 1-\frac{1}{2^{X_i}} \\ 2^{X_i}-1 \end{matrix} \right\}_{X_i \geq 0}^{X_i<0} - \left\{ \begin{matrix} 1-\frac{1}{2^{Y_i}} \\ 2^{Y_i}-1 \end{matrix} \right\}_{Y_i \geq 0}^{Y_i<0}\right],$$

where X_i and Y_i represent $\log_2$ of the ratio of the expression level of a given gene at time point i in the compared pair of time series X (costimulated) and Y (CD3 stimulated).

Only genes exhibiting a 2-fold change at two time intervals for either treatment were included.

Protein Studies. All protein studies, including stimulations, PAGE, Western blots, isolation of nuclear extracts, and gel shifts, were performed by using standard methods and as described in the respective figure legends and *Materials and Methods* in the text.

References

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