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16. Animals were treated with vehicle or LY354740 before they were injected with a moderate dose of PCP (1 and 5 mg/kg ip injection). Measurements were made of (i) in vivo extracellular glutamate concentrations in the prefrontal cortex and nucleus accumbens, (ii) in vivo extracellular dopamine concentrations in the prefrontal cortex and nucleus accumbens, (iii) locomotor activity, (iv) stereotypy, and (v) performance in a T-maze discrete-trial delayed alternation task, a rodent working memory paradigm. Animal use procedures were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, 1996) and were approved by the Yale University Animal Care and Use Committee. Microdialysis and behavioral measures were performed in awake, freely moving rats (male Sprague-Dawley, 275 to 325 g) as described in (*12*). Microdialysis and locomotor data were analyzed by repeated measures of analysis of variance (ANOVA). The delayed alternation data were compared by multifactorial ANOVA followed by Tukey post hoc analysis. Significance was set at $P < 0.05$.
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Independent and Epigenetic Regulation of the Interleukin-4 Alleles in CD4$^+$ T Cells

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How an individual effector T cell acquires a particular cytokine expression pattern from many possible patterns remains unclear. CD4$^+$ T cells from F_1 mice, which allowed assignment of the parental origin of interleukin-4 (IL-4) transcripts, were divided into clones that expressed IL-4 biallelically or monoallelically from either allele. The allelic pattern was transmitted as a stable epigenetic trait. Regulation of cytokine expression by a mechanism that treats each allele independently suggests a probabilistic process by which a diverse repertoire of combinatorially assorted cytokine gene expression patterns could be generated among the clonally related daughters of a single precursor cell.

Descriptions of T helper (T_H) cell types that express cytokine patterns distinct from the classic T_H1 and T_H2 subsets are not readily explained by current models of T cell differentiation from naïve to cytokine-expressing effector cells (*1*). The unusual cytokine patterns appear as if generated by combinatorial assortment of probabilistically expressed genes (*2*, *3*). We hypothesized that a probabilistically regulated gene would have two chances to be expressed in diploid cells and that, if the two alleles were regulated independently, a mixture of cells that used either one or both alleles should exist within a population expressing the gene.

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Although the IL-4 gene was nonpolymorphic among a number of traditional *Mus musculus* inbred strains, a polymorphism in exon 1 allowed discrimination of the IL-4 cDNA of inbred strains from the CAST/Ei strain, by differential sensitivity to the restriction enzyme Bsg I (Fig. 1A) (*4*). CD4$^+$ T cells from (129 × CAST/Ei)F_1 hybrid mice were stimulated in vitro under conditions that favored the generation of IL-4–expressing effector cells (*5*). Even under such conditions, the frequency of IL-4–expressing cells is less than 5% (*3*). We used a limiting-dilution approach to screen for monoallelic IL-4 gene expression (*6*), a strategy used to demonstrate monoallelic expression among olfactory receptor genes (*7*).

Under these conditions of limiting template, the semi-nested polymerase chain reaction (PCR) approach was, on average, capable of detecting IL-4 transcripts 30% of the time from a repeatedly screened cDNA aliquot. Consequently, the PCR assay was done multiple times on each of the nine samples that revealed the presence of a single allele (*8*). As demonstrated by differential sensitivity to Bsg I digestion, three samples screened repeatedly revealed only the CAST/Ei allele, whereas six revealed only the BALB/c allele [Fig. 1B and (*9*)]. These data were consistent with monoallelic expression and prompted further studies with cloned cells.

Analysis of cloned cells allows the direct investigation of gene expression in individual cells and thus avoids the statistical imprecisions of the limiting-dilution approach. We examined a panel of 30 CD4$^+$ T cell clones generated from (BALB/c × CAST/Ei)F_1 hybrid mice by stimulation with allogeneic H-2^b cells in the presence of recombinant IL-4 and IL-12 monoclonal antibody (mAb), conditions that favor the establishment of IL-4–producing clones (*10*). Of these alloreactive clones, 25 expressed IL-4, and 12 could be expanded and maintained long-term. Seventeen hours after activation of resting clones with immobilized mAbs to the T cell receptor (TCR) and CD28 (*11*), RNA was isolated and screened for the parental origin of the IL-4 transcripts. Twelve (48%) of the 25 clones revealed monoallelic expression (eight BALB/c, four CAST/Ei) and 13 revealed biallelic expression [Fig. 2A and (*9*)]. The twofold bias in favor of monoallelic expression of the BALB/c rather than the CAST/Ei allele was not statistically significant. These data confirmed the suggestion from the limiting-template analysis that monoallelic IL-4 expression does occur. Because some clones expressing IL-4 from either one or both alleles were obtained from a single animal [experiment A (*10*)], the data suggested a process distinct

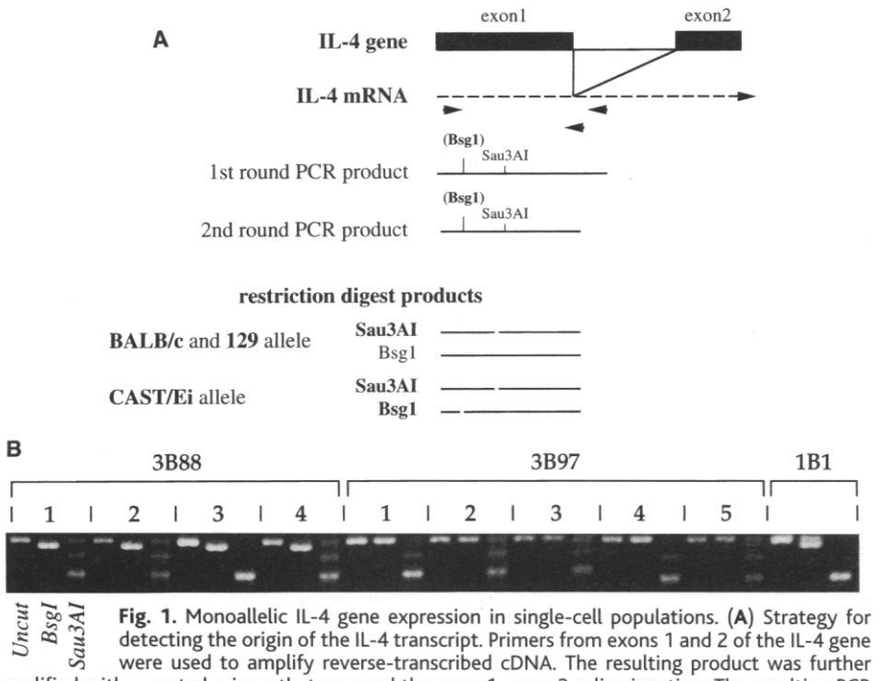

Fig. 1. Monoallelic IL-4 gene expression in single-cell populations. (**A**) Strategy for detecting the origin of the IL-4 transcript. Primers from exons 1 and 2 of the IL-4 gene were used to amplify reverse-transcribed cDNA. The resulting product was further amplified with a nested primer that spanned the exon 1–exon 2 splice junction. The resulting PCR products were discriminated by their sensitivities to the endonucleases Bsg I (CAST/Ei gene product sensitive) and Sau 3AI (all gene products sensitive) as resolved after agarose gel electrophoresis. (**B**) Limiting-dilution analysis of stimulated CD4$^+$ T cells purified from (129 × CAST/Ei)F_1 mice. Pools 3B88 and 3B97 were amplified and the resultant product resolved without (Uncut) or with (Bsg I or Sau 3AI) digestion with the indicated restriction endonucleases in the three consecutive lanes as indicated. Amplicons were detected in 6/16 (3B88) or 7/14 (3B97) separate experiments, of which four and five digestions are shown. 1B1 represents cDNA from undiluted material from the F_1 cells demonstrating the capacity to discriminate both alleles in mixed populations. The rightmost three samples were run on a separate gel, accounting for the increases in mobility of the restriction fragments.

from parental imprinting wherein the same parental allele is used by almost all expressing cells of the individual. Control experiments, in which reconstitutions of discrete mRNA or cDNA pools were used, confirmed that the reverse transcriptase (RT)–PCR assay could discriminate at least a 16- to 32-fold difference in transcript abundance (Fig. 2B). Simple sequence length polymorphisms on either side of the IL-4 gene were used to confirm that both parental chromosomes were maintained in the clones that expressed single alleles *(12)*.

The detection of a single IL-4 allele from the RNA pooled from more than 10^4 F_1 CD4$^+$ T cells suggested that most (and perhaps all) of the cells in a given clone expressed the same allelic pattern. To examine this issue, we reanalyzed 12 clones, representing all three possible allelic expression patterns, by sequential sampling over an extended period of continuous growth (Table 1) *(11)*. The allelic expression pattern for each clone remained constant. Thus, as early as 23 days after primary stimulation, the allelic expression pattern had become fixed as a heritable, epigenetic trait.

The experiments here suggest that IL-4 belongs to a category of genes whose allelic expression pattern is random and established developmentally late *(13)*. By analogy with some other members of this category, which belong to gene families *(7, 14)*, we speculate that other cytokine genes also might be probabilistically expressed. The IL-2 gene was shown to be expressed monoallelically in CD4$^+$ T cells *(15)*. A relatively small number of individual cells were analyzed at an early time after activation, leaving open the possibility that biallelic expression for IL-2 may also occur. Indeed, expression of LY49 NK cell receptors was first reported to be allelically excluded and only later shown to be biallelic in some cells, consistent with a probabilistic expression mechanism *(16)*. We have preliminary evidence that granulocyte-macrophage–colony-stimulating factor can also be expressed from one or both alleles in CD4$^+$ T cells *(9)*. Thus, at least some cytokine genes are regulated by a mechanism that treats the alleles independently.

Why might cytokines be expressed in a probabilistic manner? Such a mechanism would allow combinatorial assortment of distinct cytokine genes among the clonal progeny of individual precursor T_H cells sharing the same antigen receptor specificity. Once the probabilistic gene-activation mechanism ceas-

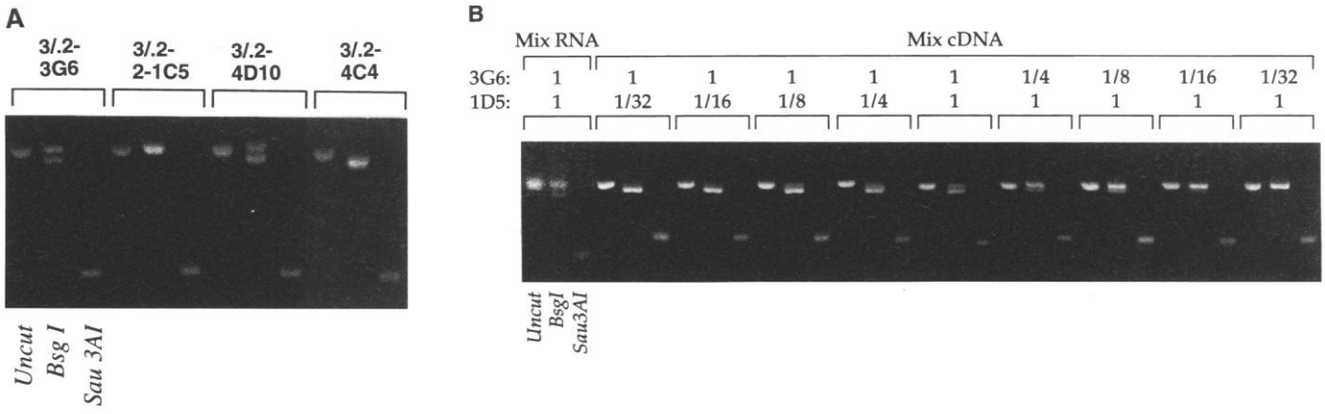

Fig. 2. Monoallelic IL-4 expression in CD4$^+$ T cell clones. (**A**) Four CD4$^+$ T cell clones derived from (BALB/c × CAST/Ei)F_1 mice were analyzed as described in the legend to Fig. 1. Groupings of three consecutive lanes represent IL-4 amplification products resolved in the absence or presence of the indicated restriction endonucleases. (**B**) Reconstruction experiments to assess fidelity of the RT-PCR amplification procedure. Either mRNA (Mix RNA) or reverse-transcribed cDNA (Mix cDNA) derived from the stable monoallelic IL-4–expressing clones 3G6 (CAST/Ei allele) and 1D5 (BALB/c) was mixed at the indicated ratios and used to template the RT or PCR assays. The resulting IL-4 amplification products were analyzed with or without the designated restriction enzymes before resolution on agarose gels.

Table 1. Stability of IL-4 allelic expression patterns over time in individual clones.

Clone	Exp.*	IL-4 allele† (alleles) detected (number of times tested)									
		Days since cloning‡									
		16	21	23	37	55	73	80	140	150	221
1D5	A					**B**(2)	**B**(1)	**B**(2)			
2F2	A					**B**(2)	**B**(1)				
2G7	A					**B/C**(2)	**B/C**(1)				
3/2-4B1	A					**C**(2)	**C**(1)	**C**(2)			
2-1A6	B	**B/C**	(1)		**B/C**(3)	**B/C**(1)					
3/.2-4G12	B			**B/C**(3)	**B/C**(1)					**B/C**(2)	
3/.2-3G6	C		**B/C**(1)						**B/C**(2)		
3/.2-21C5	C		**B**(1)						**B**(2)		
3/2-4E1	C		**B**(1)						**B**(2)		
3/.2-4D10	C		**B/C**(1)								**B/C**(2)
3/.2-4F7	C		**B/C**(1)						**B/C**(2)		
3/.2-4C4	C		**C**(1)						**C**(2)		

*Experiments A, B, and C are as described (*10*). †**B**, B BALB/c allele; and **C**, CAST/Ei allele. ‡Day of cloning is defined as the day cells were first plated at limiting dilution.

es, the cytokine expression pattern among the clonally related but phenotypically diverse daughter cells would become fixed as a heritable epigenetic trait. Depending on microenvironmental signals like IL-12 or IL-4, selective growth or death could act to influence the prevalence of cells that express distinct cytokine patterns, presumably in a manner promoting successful resolution of different immunological challenges. Specific cytokines would thus act to support the survival and proliferation of committed cells rather than to mediate effector commitment, consistent with the role of cytokine growth factors in lineage commitment of hematopoietic precursors (*17*). This process may underlie a fundamental strategy by which the immune system ensures that a diverse repertoire of cytokine-producing effector cells can be generated from limited numbers of antigen-specific precursors, thus allowing selection of the appropriate immune response to any given pathogen.

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4. Cellular RNA was isolated through use of RNAzol-B (Biotecx, Houston, TX). cDNA was prepared with a kit from Clontech Laboratories (Palo Alto, CA). PCR primers spanned the first intron of the IL-4 gene to avoid contributions by DNA contamination. PCR was done with a primer from exon 1 (ACTTAATTGTCTCTCGTCACT) together with a primer from exon 2 (ACGTTTGGCACATCCATCTCC) or a nested primer that spanned the exon 1–exon 2 splice junction (CATGGCGTCCCTTCTCCTGT). The first-round PCR reaction (one cycle: 94°C for 2 min, 56°C for 4 min, 72°C for 2 min; 38 cycles: 94°C for 1 min, 56°C for 1 min, 72°C for 2 min) with exon 1 and exon 2 primers yielded a 203–base pair (bp) product. The second-round reaction (one cycle: 94°C for 2 min; 30 cycles: 94°C for 1 min, 56°C for 1 min, 72°C for 2 min) with exon 1 and the nested primer yielded a product 26 bp smaller. Sequence analysis of the IL-4 gene revealed a polymorphism in exon 1 that allowed discrimination of 129 and BALB/c from CAST/Ei strain cDNA by differential sensitivity to Bsg I endonuclease. CAST/Ei PCR products were sensitive to Bsg I, liberating two restriction fragments of 9 and 194 bp (and for nested reactions, 9 and 168 bp). PCR products from all strains were sensitive to Sau 3AI, liberating two restriction fragments of 134 and 69 bp (for nested reactions, 134 and 43 bp). Restriction fragments were resolved through 3.5% Metaphor agarose (FMC BioProducts, Rockland, ME) in 1× tris-borate–EDTA buffer.
5. $CD4^+$ T cells enriched from the lymph nodes and spleen of (129 × CAST/Ei)F_1 hybrid mice were stimulated with soluble mAbs to TCRβ (H57, 20 μg/ml) and CD28 (37.51, 5 μg/ml) together with irradiated T cell–depleted BALB/c spleen cells, recombinant murine IL-4 (10 ng/ml), recombinant human IL-2 (50 U/ml), and IL-12 mAb (C17.15, 100 μg/ml). Three days later F_1 T cells were recovered by purification over Ficoll.
6. Stimulated F_1 cells were distributed in five serial, 10-fold dilutions in which the total cell number of each dilution was normalized by the addition of thymocytes from IL-4 gene-knockout mice (*18*). IL-4 null thymocytes did not generate a signal in these assays and thus served to normalize the conditions. From each dilution, 100 samples, each containing 1000 cells, were frozen for later analysis. Ten aliquots from each of the five dilutions were then sampled for the presence of IL-4 mRNA. RNA isolated from each aliquot was used to make cDNA, from which a sensitive semi-nested IL-4 PCR reaction was performed (Fig. 1A). A dilution that yielded a frequency of one positive IL-4 PCR signal out of 10 sampled aliquots was chosen for further analysis, because positive aliquots from such a dilution were unlikely to result from the RNA of two or more distinct, IL-4–producing cells. Calculation of the estimated numbers of IL-4–expressing cells in the starting population yielded a frequency of 3.6%, in agreement with previous studies (*3*). The remaining 90 aliquots from each positive dilution were then processed into cDNA and analyzed for IL-4 expression by semi-nested PCR, followed by Bsg I digestion to discern the parental source of the transcripts, and, as a control, with Sau 3AI, which recognizes the products of both alleles.
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10. A panel of 30 alloreactive (anti-$H2^b$) $CD4^+$ T_H2 clones were generated from (BALB/c × CAST/Ei)F_1 mice in three separate experiments, designated A, B, and C, yielding 4, 3, and 23 clones, respectively. Series A clones were derived from a single F_1 mouse immunized intraperitoneally 1 month earlier with 50 × 10^6 female C57BL/6 β_2-microglobulin (β_2M)–deficient ($\beta_2M^{-/-}$) spleen cells, whereas B and C clones were derived from the same pool of two unimmunized F_1 mice. The $\beta_2M^{-/-}$ mice, which do not express major histocompatibility complex class I molecules (*19*), were used to avoid the outgrowth of $CD8^+$ alloreactive T cell clones. Clones were generated in cultures with irradiated C57BL/6 $\beta_2M^{-/-}$ spleen cells, IL-4, IL-2, and anti–IL-12 (all at the above concentrations). Two days after the second in vitro culture, $CD4^+$, L-selectin^{lo}, $H2\text{-}A^{d+}$ cells were distributed by flow cytometry (Becton Dickinson Vantage, Mountain View, CA) as single cells (series A or B) or by limiting dilution (series C) into 96-well plates containing irradiated T cell–depleted C57BL/6 spleen cells, IL-4, and IL-2. Clones were picked 16 to 37 days after plating. Cloning efficiency ranged from 0.4 to 3%.
11. Clones containing 10^4 to 10^5 cells were activated for 17 hours by culture on plates coated with mAbs to TCRβ (H57-597, 10 μg/ml) and CD28 (37N51.1, 10 μg/ml) before harvesting for RNA.
12. DNA was prepared from clones by standard methods and used to template primers that distinguish sequence length polymorphisms between BALB/c and CAST/Ei at positions D11Mit20, D11Mit271, and D11Mit242 flanking the IL-4 gene on chromosome 11.
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