

Regulation of IL-4 Expression by Activation of Individual Alleles

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Summary

To study the *in vivo* role of IL-4-expressing cells, we developed a strategy to tag these cells, by generating mice in which one *IL-4* allele was replaced with a cDNA encoding the human CD2 (huCD2) cell-surface molecule. Expression of the huCD2 reporter was, like IL-4, restricted to the appropriately polarized T helper 2 cells. However, most of the cells expressed only the *IL-4* or the targeted allele. Analysis of the frequency of monoallelic versus biallelic expression suggests that the activation of each individual allele is regulated by a stochastic process whose probability can be augmented by increasing the strength of signal delivered through the TCR. Allele-specific activation may be a general feature of cytokine regulation that contributes to the functional diversity within T helper cell subpopulations.

Introduction

Among the factors that control the differentiation of T lymphocytes, interleukin-4 (IL-4) and interferon- γ (IFN γ) play pivotal roles in promoting the differentiation of CD4⁺ naive T lymphocytes toward T helper 2 (Th2) and T helper 1 (Th1) effector cells, respectively. Both Th subsets are subjected to cross-regulation, such that Th2 development is inhibited by IFN γ , whereas Th1 development is inhibited by IL-4. IL-4 is itself the hallmark cytokine of the Th2 effectors, insofar as cells that become competent to express IL-4 concomitantly acquire the ability to produce other Th2-specific cytokines such as IL-5, IL-9, IL-10, or IL-13 (reviewed by O'Garra, 1998; Paul and Seder, 1994). For Th1 effectors, the hallmark cytokine is IFN γ , although these cells also express lymphotoxin and IL-2. Th0 populations and clones secreting combinations of Th1- and Th2-type cytokines have also been described (Bucy et al., 1994; Openshaw et al., 1995; Murphy et al., 1996; Miner and Croft, 1998). Numerous studies that have examined the differentiation of Th1 and Th2 effectors suggest that these two segregated phenotypes represent endpoints of chronic stimulation (reviewed by O'Garra, 1998). *In vitro* studies show that Th1 and Th2 populations can still revert to the reciprocal subset after 1 week of stimulation but

appear irreversibly polarized after repeated stimulations (Murphy et al., 1996; Huang and Paul, 1998).

Many studies suggest that naive T cells are not pre-programmed to become Th1 or Th2 cells. In the absence of selective pressure enjoined by the cytokine micro-environment, the expression of a given cytokine in a "naive" T cell may arise stochastically upon TCR stimulation. Originally, it was demonstrated that Th1- and Th2-like cells could be derived from a bipotential IL-2-producing precursor CD4⁺ T cell (Sad and Mosmann, 1994). Using transgenic mice that express the herpes simplex virus 1 thymidine kinase under the control of the IL-4 promoter, it was also shown that Th1- and Th2-type cells emerge from a common precursor capable of expressing both IL-4 and IFN γ (Kamogawa et al., 1993). In addition, antigenic stimulation with KLH also revealed that the coexpression of many pairs of cytokines occurs at the same frequency as if the expression of each cytokine is independently regulated by a stochastic process (Kelso et al., 1995). Upon infection with *Leishmania major*, RNA transcripts encoding IL-2, IL-4, IL-10, IL-13, and IFN γ are also detectable in both resistant and susceptible strains of mice (Reiner et al., 1994). In conjunction with cytokines, other factors such as the nature of the antigen, the antigen dose (Bretscher et al., 1992; Constant et al., 1995; Hosken et al., 1995), the route of antigen administration, the type of antigen-presenting cells (APCs) (Macatonia et al., 1995; Macaulay et al., 1997), and the genetic background (Hsieh et al., 1995) influence the outcome of the immune response (reviewed by Constant and Bottomly, 1997; Fitch et al., 1993; O'Garra, 1998). Ultimately, the ability of these factors to promote the differentiation of naive T cells toward the Th2 or Th1 pathways relies in large part on their propensity to provide a bias for induction of either IL-4 or IFN γ in T cells and of IL-12 in APCs. However, upon immunization of IL-4^{-/-} mice with soluble antigens or parasites, there is evidence that IL-5, IL-13 (Chensue et al., 1997; Hogarth et al., 1998; Urban et al., 1998), and IL-9 (Monteyne et al., 1997) expression nevertheless occurs, indicating that IL-4 is not the sole determinant for Th2 cytokine gene expression.

The genes encoding IL-4, IL-5, IL-13, GM-CSF, and IL-3 are clustered within a 1000 kb array on mouse chromosome 11 and may have arisen by gene duplication (Boulay and Paul, 1992; McKenzie et al., 1993). Because Th2 cells can express various combinations of these cytokines, it is likely that, even if there is some shared regulation of the linked genes, independent regulation of cytokine transcription can also result as a consequence of specific signals or of appropriate selective environments. Selection of T helper cells that express appropriate cytokines would imply that the cytokine program of a particular cell is the result of a stochastic program to turn on any particular gene. There is currently no molecular evidence to distinguish between stochastic versus instructed mechanisms of cytokine gene expression.

Several features of IL-4 transcriptional control have been recently elucidated. Naive T cells do not express

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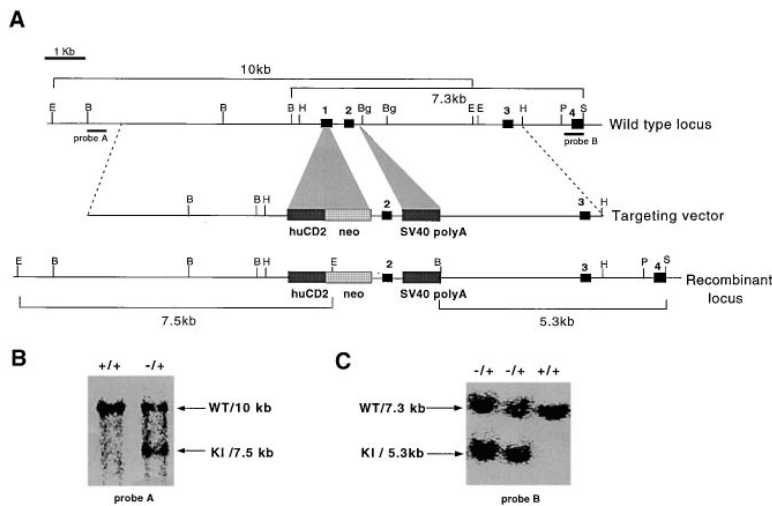


Figure 1. Strategy to Knock-In the huCD2 Reporter Gene at the *IL-4* Locus

(A) A map of the targeting vector is shown along with the wild-type (WT) and the predicted recombinant *IL-4* loci. The neo box stands for the *neomycin resistance* gene and huCD2 box for the tailless huCD2 cDNA. Homologous recombination between the WT locus and the targeted construct results in the replacement of the first exon with the huCD2 cDNA and insertion of the SV40 polyA cassette downstream of the second exon. Restriction enzyme sites: E, EcoRI; B, BamHI; H, HindIII; Bg, BglII; P, PstI; S, Scal.

(B) Southern blot analysis of recombination on the 5' side of the targeting vector in a targeted ES cell clone. Genomic DNA was extracted from ES cells, digested with EcoRI, and hybridized with probe A. The WT locus generates a 10 kb fragment and the recombinant locus a 7.5 kb fragment.

(C) Analysis of offspring tail DNA for recombination on the 3' side of the targeting construct. Genomic DNA was prepared from tails, digested with BamHI and Scal, and hybridized with probe B. The WT locus generates a 7.3 kb fragment and the recombinant locus a 5.3 kb fragment. In (B) and (C), the genotypes are indicated as WT (+/+) and heterozygous (-/+).

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any detectable IL-4, but upon appropriate antigenic stimulation IL-4 expression is established within 48 hr (Le Gros et al., 1990; Lederer et al., 1996). Initiation of this cellular differentiation program involves the activation of numerous transcription factors and possibly chromatin remodeling (Ho et al., 1996; Hodge et al., 1996; Lederer et al., 1996; Zheng and Flavell, 1997; Yoshida et al., 1998). IL-4 expression, like that of IL-2, is thought to be regulated by an all-or-none mechanism that leads to a relatively fixed level of cytokine expression per cell (Bucy et al., 1994; Itoh and Germain, 1997). Although this mechanism has not been confirmed at the molecular level as extensively as for IL-2 (Garrity et al., 1994), its existence would suggest that a threshold of TCR signaling is essential to promote the activation and the simultaneous assembly of all necessary transcription factors on the IL-4 promoter. Limiting availability of any one factor may determine if the all-or-none transcriptional phenomenon is achieved. As previously reported for the *IL-2* gene, it may also restrict the transcriptional activation to a single allele (Hollander et al., 1998).

To facilitate experimental manipulation of T helper cell subsets and to study mechanisms that control IL-4 gene expression, we developed a strategy to tag IL-4-expressing cells at the single cell level. Homologous recombination was used to replace one of the *IL-4* alleles with a cDNA encoding an inert form of the cell-surface human CD2 (huCD2) molecule. Mice with a huCD2-targeted *IL-4* allele expressed the cell surface marker only on appropriately polarized Th2 cells. However, analysis of huCD2 and IL-4 expression in Th2-enriched cell lines revealed that most cells expressed only one of the two *IL-4* alleles. The frequency of biallelic expression suggests that the activation of each individual allele is independently regulated by a stochastic process. We show that the ratio of monoallelic- versus biallelic-expressing cells is independent of the stage of T cell development and of TCR specificity but does correlate with the strength of TCR-mediated signaling. Thus, our results uncover an additional level of complexity in the regulation of IL-4 expression in T lymphocytes involving allele-specific activation.

Results

Targeting of the huCD2 cDNA into the Murine *IL-4* Locus

To mark cells that express IL-4, we designed a vector for homologous recombination of a cDNA encoding an inert form of huCD2 into the murine *IL-4* locus (Figure 1A). Expression of the huCD2 molecule can be monitored by flow cytometry at the single cell level and does not interfere with normal murine development (Sawada et al., 1994). We further engineered the huCD2 cDNA to delete its cytoplasmic domain and to eliminate its ligand binding activity (see Experimental Procedures). The mutant huCD2 cDNA was introduced at the translation start point of the *IL-4* gene, such that the initiating codon of the huCD2 cDNA replaced that of the target gene. This strategy aims to optimally preserve the regulation of huCD2 expression under the *cis*-regulatory transcriptional control elements of the *IL-4* gene. The deletion of exon 1 also fully disrupts the expression of IL-4 from the targeted allele. A polyadenylation signal was introduced in intron 2, upstream of the previously identified mast cell enhancer (Henkel and Brown, 1994), to prevent premature destabilization of the huCD2 mRNA. This targeting vector was used to electroporate E14 embryonic stem (ES) cells, and G418-resistant clones that had undergone homologous recombination were identified by Southern blot analysis with flanking probes (Figures 1A–1C). Targeted ES cell clones were injected into C57BL/6 blastocysts, and, following transmission of the mutation to the germ line, a line of mice heterozygous for the “knock-in” (KI) of huCD2 at one of the two *IL-4* alleles was established (huCD2-KI mice).

The Majority of Th2-Polarized Cells Express Either the *IL-4* or the huCD2 Allele

To characterize the pattern of reporter gene expression in the huCD2-KI mice, Th1 and Th2 polarized cell lines were established from the lymph nodes of huCD2-KI and WT littermates. Cell lines derived from both WT and huCD2-KI animals were highly enriched for intracellular

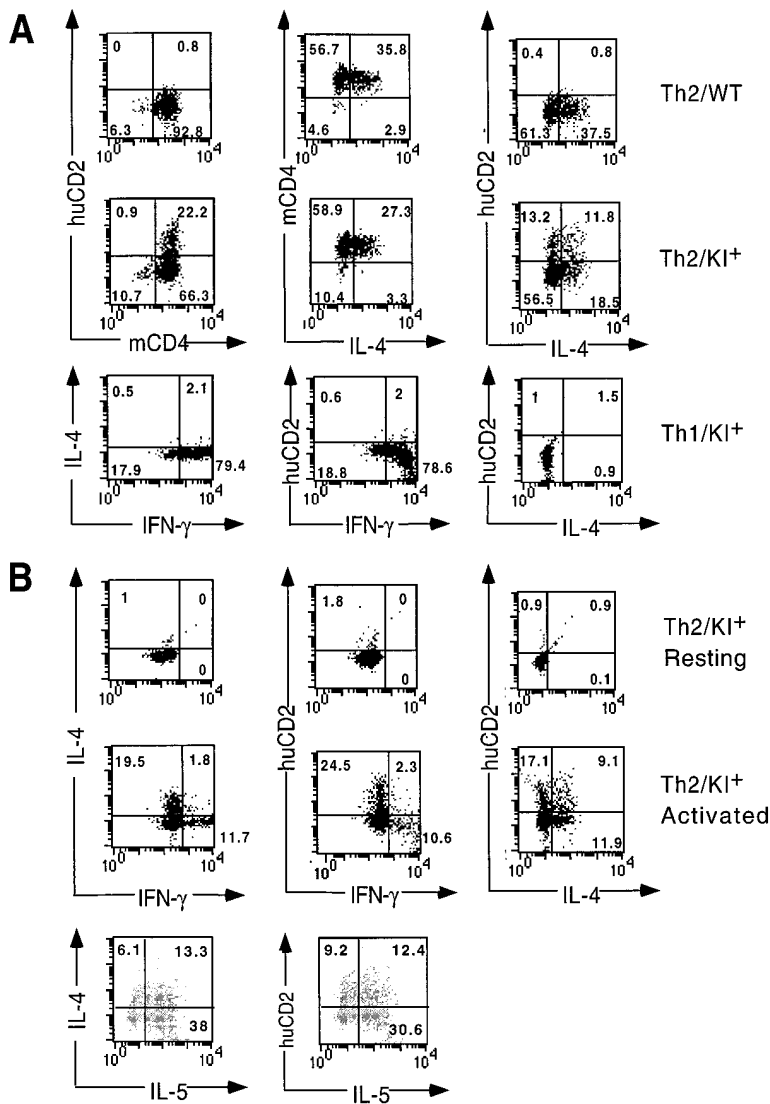


Figure 2. Characterization of huCD2 and IL-4 Expression in Th2 Enriched Cell Lines

(A) CD4⁺ enriched T cell lines were generated from lymph nodes of F1(129xC57BL/6) WT and huCD2-KI littermates by depletion of CD8⁺ T cells and B cells (see Experimental Procedures).

(B) A CD4⁺ enriched T cell line was generated from lymph nodes of N3 C57BL/6 huCD2-KI mouse as described in (A).

In (A) and (B), Th2-enriched cell lines were generated in the presence of IL-4 and Th1 cell lines in the presence of IFN γ and anti-IL4 MAbs. After the fourth overnight stimulation on plate-bound anti-CD3 MAbs, the cells were treated for 6 hr with PMA (50 ng/ml) and ionomycin (500 ng/ml) and stained for huCD2, mCD4 and IL-4, IL-5, or IFN γ .

expression of IFN γ if they had been polarized toward the Th1 lineage, but there was no expression of huCD2 (Figure 2A; data not shown). In contrast, a large proportion of Th2 cells expressed IL-4, and more than 20% of cells from the KI animals but not from the littermate controls expressed the huCD2 reporter molecule. Because both huCD2 and IL-4 were expressed only in CD4⁺ cells polarized to the Th2 lineage, we expected that these molecules would be coexpressed in these cells. However, most of the cells cultured under Th2-promoting conditions expressed either IL-4 or huCD2, and only a minority of cells expressed both (Figure 2A, right). This pattern of expression was confirmed in all Th2-enriched cell lines derived from all animals examined ($n > 10$) (Figures 2A and 2B; data not shown).

To ensure that the phenotype of the huCD2-expressing cells was similar to that of IL-4 expressing cells, we examined the patterns of expression of IFN γ and IL-5 in the Th2-enriched cell lines. Such polarized populations were intentionally generated in the absence of anti-IFN γ MAbs so that IFN γ -expressing cells could develop and provide an internal control upon staining for huCD2 and

IL-4. As expected, the vast majority of lymphocytes that produced IFN γ did not coexpress any IL-4 or huCD2 (Figure 2B, left and center). Furthermore, huCD2 and IL-4 were down-modulated and induced in parallel when polarized populations of Th2 cells were rested or stimulated, respectively (Figure 2B, Th2 resting or activated). The proportion of cells that coexpressed either IL-4 and IL-5 (13.3%) or huCD2 and IL-5 (12.4%) were also comparable (Figure 2B, lower). IL-5 was also expressed in "Th2" cell populations derived from mice homozygous for the *huCD2-KI* allele (data not shown). In conclusion, in the huCD2-KI mice the reporter gene is specifically induced by activation of T cells in the presence of added exogenous IL-4 and not in the presence of IL-12. The expression of huCD2 is thus induced in parallel with that of IL-4.

To further verify that expression of huCD2 does not intrinsically modify the frequency of Th2-like cells that develop in polarized cultures, we compared the frequency of these cells in CD4⁺ T cell lines derived from huCD2-KI animals versus WT littermates. To be able to control the strength of stimulation, we used T cell lines

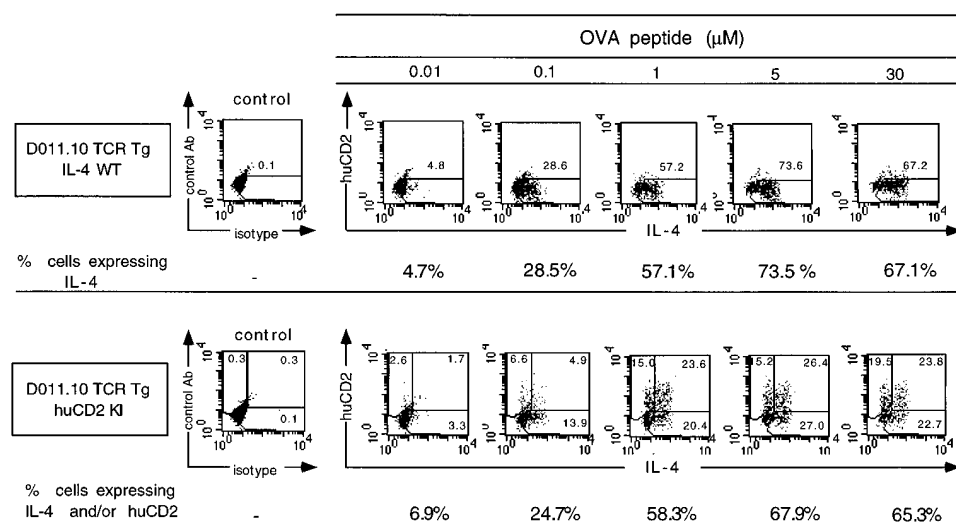


Figure 3. T Helper 2 Lymphocytes Are Present at the Same Frequency in T Cell Lines Derived from Either WT or huCD2-KI Animals
Flow cytometry-sorted CD4⁺Vβ8⁺ T lymphocytes (5×10^5 /well) derived from D011.10 TCRαβ WT and huCD2-KI transgenic mice were cultured with irradiated splenocytes (2×10^6 /well) and increasing doses of antigen (0.01 to 30 μM of OVA 323–339 peptide) in 24-well plates. Cells were restimulated every 5 to 7 days. Within 7 hr following the fifth stimulation, the cells were stained for huCD2, IL-4, and Vβ8. Anti-huCD2 was used as a control antibody for huCD2 expression, and rat IgG2b was the isotype control for the anti-IL-4 MAb. The gate was set on Vβ8⁺ T cells.

derived from animals crossed to the D011.10 TCRαβ transgenic mice. WT and huCD2-KI-derived CD4⁺Vβ8⁺ T cell lines were cultured under identical conditions and restimulated in parallel with APCs and increasing amounts of ovalbumin peptide (OVA_{323–339}), in the absence of exogenous IL-4. Th2-like cells were enumerated after the fifth stimulation. In T cells derived from the WT animals, we found that increasing numbers of T cells were recruited to express IL-4 with increasing antigen doses, reaching a peak between 1–5 μM (Figure 3, top). In each T cell line derived from the huCD2-KI littermate, the sum of cells expressing IL-4 and/or huCD2 was similar to the fraction of IL-4-expressing cells in the WT T cell line stimulated with the same amount of antigen (Figure 3, bottom). Thus, CD4⁺ T lymphocytes polarized toward the Th2 pathway are present at the same frequency in T cell lines derived from either WT or huCD2-KI animals. This result further confirms that the three discrete phenotypes (IL-4⁺huCD2⁻, IL-4⁺huCD2⁺, and IL-4⁻huCD2⁺) that we observed in T cell lines polarized in the presence of exogenous IL-4 are bona fide Th2 cells. Taken together, these results show that expression of the huCD2-KI allele is highly Th2-specific, but not paired with that of its WT IL-4 counterpart, suggesting that IL-4 expression is regulated by a mechanism that involves a limited probability of activating each allele.

Analysis of animals that had inherited the huCD2-tagged allele either paternally or maternally showed that expression of the individual alleles is independent of parental imprinting (data not shown). Our results also show that monoallelic versus biallelic expression of the IL-4 loci is not dictated by the specificity of the TCR. Indeed, we find monoallelic- and biallelic-expressing cells in comparable ratios in both polyclonal T cell populations (derived from non-TCR transgenic mice; Figure

2) as well as in T cell populations bearing a unique TCR (derived from TCRαβ transgenic mice; Figure 3).

The Probability of *IL-4* Allelic Activation Is Influenced by the Strength of TCR-Mediated Signaling

To assess the parameters regulating monoallelic versus biallelic activation at the *IL-4* locus, we sought to determine if differential allelic activation depends on TCR signaling and/or the stage of mature T cell development. Hence, we examined the effect of varying strength of T cell activation in precursor T helper cells (Th0 cells) and in Th2 cells and also determined if memory versus naive T helper cells differ in their activation of individual *IL-4* alleles.

T cell lines derived from huCD2-KI or WT animals crossed to the D011.10 TCRαβ transgenic mice were prepared as described above. The CD4⁺Vβ8⁺ T cells were stimulated in the absence of exogenous IL-4 with increasing doses of peptide antigen to deliver quantitatively different signals. In this experiment, the T cells were analyzed after the third stimulation, as soon as huCD2 and IL-4 molecules became readily detectable by FACS analysis. Because we expected a bias toward the Th2 differentiation pathway (see Figure 3 and below), we reasoned that performing the analysis as early as possible would result in the optimal detection of the Th0 population whose stability seems to be dependent upon the relative amounts of IL-4, IFNγ, and IL-12 present in the cultures (Openshaw et al., 1995; Murphy et al., 1996; Miner and Croft, 1998). By performing three-color staining with anti-IFNγ MAbs, we were able to identify not only the Th1 cells but to further distinguish Th2 from the IFNγ-expressing Th0 cells (Figure 4A). The relative frequencies of Th1, Th0, and Th2 cells were assessed for each peptide concentration (Figure 4A, bottom).

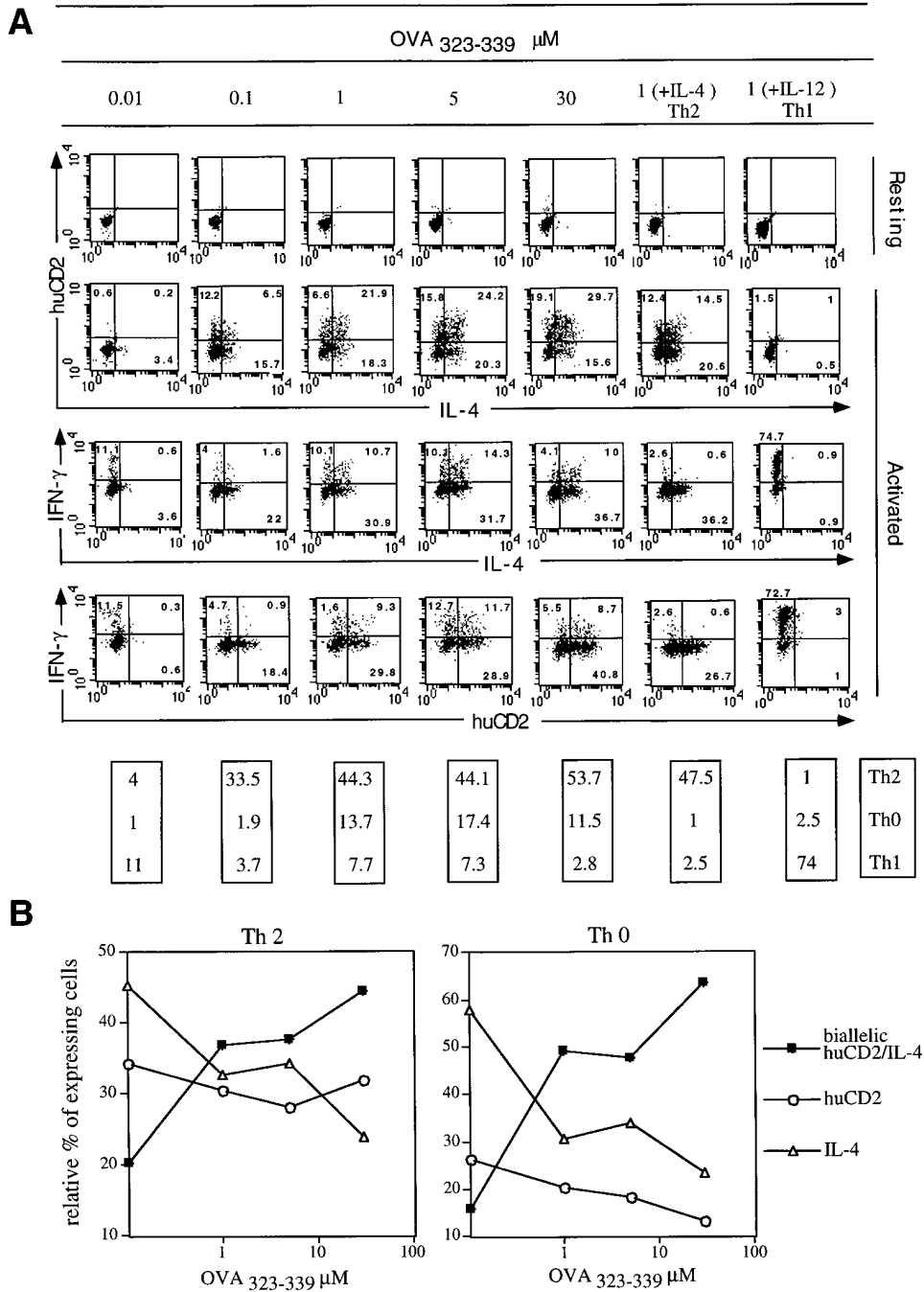


Figure 4. The Probability of *IL-4* Allelic Activation Is Influenced by the Strength of TCR-Mediated Signaling

(A) Flow cytometry-sorted $\text{CD4}^+\text{V}\beta 8^+$ T lymphocytes (5×10^6 /well) derived from D011.10 $\text{TCR}\alpha\beta$ huCD2-KI transgenic mice were cultured as in Figure 3. The Th2 control cell line was initiated in the presence of $1 \mu\text{M}$ OVA₃₂₃₋₃₃₉ peptide, IL-4, and anti-IFN γ MAbs, and the Th1 cell line was initiated in the presence of IL-12 and anti-IL-4 MAbs. All cell lines were restimulated 6 and 12 days after initiating the cultures with OVA₃₂₃₋₃₃₉ peptide. Within 10 hr following the third stimulation, the cells were stained for huCD2, IL-4, and IFN γ (activated). Cells not restimulated were stained in parallel (resting).

After performing three-color staining for IFN γ , huCD2, and IL-4, the percentages of Th0, Th1, and Th2 cells (bottom numbers) were deduced from the FACS data for each peptide concentration. Cells that coexpressed IFN γ with either huCD2 or IL-4 or with both IL-4 and huCD2 belong to the Th0 subset. Cells that expressed huCD2, IL-4, or both in the absence of IFN γ are classified as Th2 cells. Cells that expressed only IFN γ are Th1 cells.

(B) Correlation of the relative frequency of biallelic expression with the strength of TCR-mediated signaling. The relative percentages of Th2 and Th0 cells expressing either a single or both alleles were deduced from data in Figure 4A.

Table 1. Correlation between Observed Versus Calculated Biallelic Expression Frequencies

Cell Lines	Cells Expressing IL-4 and/or huCD2 (%)	Experimental Frequencies for Each or Both Alleles			Calculated Biallelic Frequency $a \times b$
		huCD2 ^a	IL-4 ^b	huCD2/IL-4	
Th #21	28.9	0.32	0.49	0.19	0.16
Th #299	35.9	0.47	0.28	0.25	0.13
Th #243	16	0.51	0.31	0.18	0.16
Th #236	24.8	0.52	0.32	0.16	0.17
Th KI ⁺ (Figure 2A)	43.5	0.3	0.42	0.27	0.13
Th 0.1 μ M (Figure 3)	24.7	0.26	0.56	0.2	0.15
Th 0.1 μ M (Figure 4)	33.5	0.34	0.45	0.2	0.15
CD62L ⁻ sorted (1)	14.8	0.31	0.52	0.17	0.16
CD62L ⁻ sorted (2)	24.2	0.41	0.36	0.23	0.15

huCD2^a, observed frequency of huCD2-expressing cells; IL-4^b, observed frequency of IL-4-expressing cells; huCD2/IL-4, observed frequency of cells expressing both huCD2 and IL-4; $a \times b$, predicted frequency of cells expressing both huCD2 and IL-4.

Exposure of cells to antigen within the range of 0.01 to 30 μ M resulted in a dose-dependent increase in development of Th2 cells (from 4% to 53.7%). Some Th1 and Th0 cells also developed, although at lower frequencies. We further observed that the frequency of T cells that expressed both *IL-4* locus alleles within the Th2 population was augmented in parallel with the peptide concentration (from 20.3% with 0.01 μ M to 44.4% with 30 μ M OVA) (Figure 4B, left). As was observed with Th2 cells, the frequency of Th0 cells in which both alleles were transcriptionally active also rose upon stimulation with increasing amounts of antigen (from 15.8% with 0.01 μ M to 63.5% with 30 μ M) (Figure 4B, right).

In the control Th2 cell line generated in the presence of exogenous IL-4 during primary stimulation, there was no significant difference in the relative frequency of biallelic expression (30.5%) from cells prepared by antigen stimulation in the absence of IL-4 (36.8%) (Figure 4A). This observation is in accordance with the finding that the production of IL-4 by differentiated Th2 cells as a result of TCR-mediated stimulation is independent of IL-4 itself (Bucy et al., 1995; Huang et al., 1997).

Our results suggest that two mechanisms contribute to the augmentation of IL-4 production with increased strength of signal delivered through the TCR. There is an increase in both the relative frequency of Th2-like cells in which IL-4 is expressed and in the frequency of IL-4-expressing cells in which both alleles at the *IL-4* locus are transcriptionally active. The latter observation also applies to Th0 cells, suggesting that the ratio of biallelic:monoallelic expression is independent of the endogenous production of IFN γ in these cells (Figures 4A and 4B).

In memory cells sorted on the basis of the level of CD62L expression (CD4⁺CD62L^{lo}), we found that the frequency of biallelic expression (23%) was not significantly different from that observed in unfractionated cell populations (16 to 27%) (data not shown). Memory cells were able to produce IL-4 or huCD2 more rapidly than naive cells, but this response was not the result of an increase in biallelic activation (data not shown). The frequency of cells in which both alleles are transcriptionally active thus appears to be dependent on the strength of signal delivered through the TCR rather than on either the presence in the microenvironment of cytokines such as IL-4 or IFN γ or the stage of mature T cell differentiation.

Stochastic Versus Regulated Allele-Specific Activation at the IL-4 Locus

The finding that activation of individual *IL-4* alleles is independent of maternal or paternal inheritance, of TCR specificity, and of stage of mature T cell differentiation suggests that expression is governed by a stochastic mechanism. If each allele is independently regulated by a stochastic process, the fraction of cells that express both alleles should be roughly equal to the product of the frequencies of cells expressing either allele, at least in T cell lines maintained by stimulation with low concentrations of peptide (<1 μ M OVA) or moderate concentrations of plate-bound anti-CD3 MAbs (1 μ g/ml). A summary of the relative frequencies of monoallelic versus biallelic expression in the various cell lines stimulated under these conditions is shown in Table 1. The predicted frequencies of biallelic expression deduced from the product of the frequencies of monoallelic expression generally match the observed frequencies. Hence, our results are consistent with a model in which allele-specific activation at the *IL-4* locus is initially regulated by a stochastic mechanism (Table 1) and is subsequently regulated by the strength of signal delivered through the TCR (Figure 4).

Stability of Allele-Specific Expression

Monoallelic activation of *IL-4* could be stable, implicating epigenetically regulated expression of a specific allele, or unstable, with random reestablishment of allele-specific transcription following each T cell activation or cell division event. To distinguish between these two mechanisms, we sorted the huCD2-expressing (ThCD2⁺) and nonexpressing (ThCD2⁻) cells from a CD4⁺V β 8⁺Th2 cell population derived from a D011.10 TCR $\alpha\beta$ transgenic animal (Figure 5). These cells were stimulated in the presence of APCs and 5 μ M OVA peptide and sorted within 10 hr after restimulation. The pattern of huCD2 and IL-4 expression was determined prior to sorting the cells (Figure 5, left). The purity of the sorted ThCD2⁺ and ThCD2⁻ was greater than 98% (Figure 5, center), and less than 0.7% contaminating cells expressing only IL-4 were detected in ThCD2⁺ cells after the sort (data not shown).

As expected, within 48 hr after the cells were sorted, expression of huCD2 was down-modulated in the ThCD2⁺ cell populations (Figure 5, peak 2 in center top). ThCD2⁺

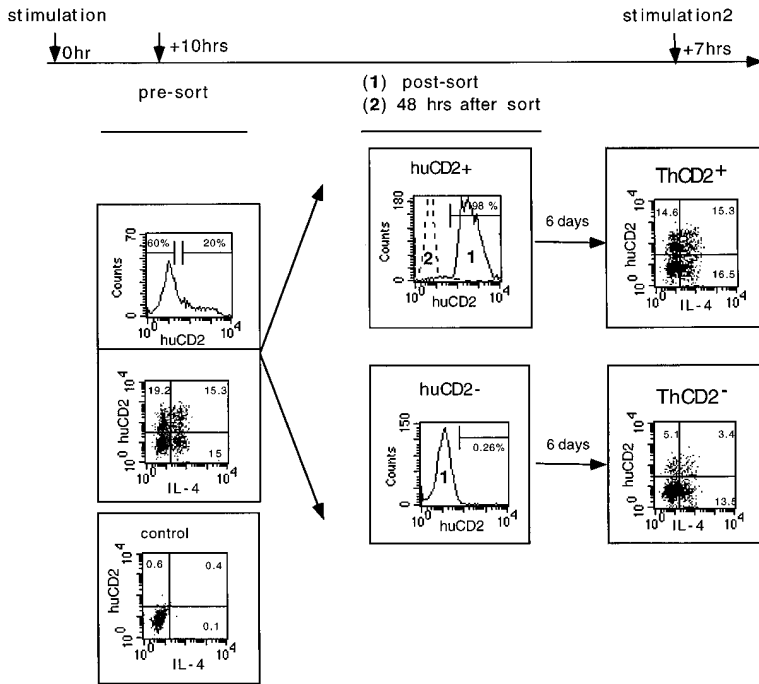


Figure 5. Stability of the Flow Cytometry-Sorted huCD2⁺ T Cells

A CD4⁺Vβ8⁺T cell line derived from a D011.10 TCR-αβ transgenic huCD2-K1 animal was generated by sorting CD4⁺Vβ8⁺ T cells prepared from lymph nodes. Cells were stimulated every 5 to 7 days with irradiated splenocytes and 5 μM of OVA₃₂₃₋₃₃₉ peptide. After the fourth stimulation, huCD2⁺ (ThCD2⁺) and huCD2⁻ (ThCD2⁻) T cells were sorted by flow cytometry at 98% purity (center panels, peaks 1) and put back in culture. ThCD2⁺ cells were stained for huCD2 48 hr after the sort (center top panel, peak 2). Both ThCD2⁺ and ThCD2⁻ T cells were restimulated with irradiated splenocytes and 5 μM of OVA peptide 6 days after the sort. Within 7 hr after the restimulation, the cells were stained with antibodies for huCD2, IL-4 and Vβ8⁺. The cells are gated on Vβ8⁺ T cells.

and ThCD2⁻ cells were restimulated 6 days after the sort, and the pattern of expression of huCD2 and IL-4 was determined by FACS analysis. Upon restimulation, a significant proportion of the previously sorted ThCD2⁺ cells (16.5%) had lost the ability to express huCD2 and only produced IL-4, while about 30% of the cells expressed huCD2 (Figure 5, right top). The overall pattern of huCD2 and IL-4 expression was similar to that observed in the presorted population, which suggests that the allelic expression pattern of the sorted cells was unstable. Restimulation of the huCD2⁻ cells 6 days after the sort also resulted in the appearance of some cells that expressed only huCD2 (~5%) (Figure 5, right bottom). However, this result is difficult to interpret, due to the fact that the ThCD2⁻ sorted population consisted of cells expressing IL-4 as well as cells that did not

express either IL-4 or huCD2 at the time of sorting. The huCD2⁺ cells could thus have arisen from the IL-4⁻huCD2⁻ population and not from cells already committed to expressing IL-4, a distinction that could only be made in clonal analyses.

To further explore the stability of IL-4 allelic expression, we analyzed CD4⁺ T cell clones generated either by limiting dilution or FACS cell sorting. The analysis of representative clones is shown in Table 2. Most of the clones fell into one of four categories: either each cell within a clone mainly expressed a single allele, IL-4 (clones #6, #7, #8, and #9), or huCD2 (clones #1 and #2); or in some clones, a majority of the cells expressed a single allele while the rest of the cells expressed both alleles [huCD2⁺ (±) IL-4, clones #3, #4 and #5; IL-4⁺ (±) huCD2, clones #10 and #11].

Table 2. Phenotype and Stability of the CD4⁺ T Cell Clones

Clones	Expressing Cells (%)						
	huCD2	1st Stimulation		2nd Stimulation		IL-4	stability
	huCD2	huCD2/IL-4	IL-4	huCD2	huCD2/IL-4		
1*	36	0.8	0.1	70.4	2.8	0.4	+
2*	21.6	2.5	1.8	28.5	2.7	2	+
3*	48.9	21.3	3.6	56.9	19.6	0.9	+
4*	44.8	14.9	2.5	11.6	25.6	24.8	-
5*	47.3	18.6	3.6	0.1	2.1	31.5	-
6#	0.6	0.9	17	0.3	1.1	23	+
7#	1.1	1.9	12.5	1.3	2.6	23.1	+
8#	1.6	1.5	16.6	0.4	1.9	23.4	+
9#	1.4	4.3	26.9	2.9	3.1	6.1	-
10#	7.1	12.8	15.6	1.6	14.1	44.9	+
11#	2.7	10.1	19.5	nd	nd	nd	nd

CD4⁺ Th2 clones derived from (129/huCD2-K1 × C57BL/6)F1 animals were generated as described in Experimental Procedures. Clones were analyzed for huCD2 and IL-4 expression after overnight stimulation on anti-CD3 MAb (1 μg/ml)-coated plates and treatment with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hr. Analyses were performed either 7 days (*) or 18 days (#) apart. In the latter case, the clones were restimulated at day 9 without being analyzed.

To assess the stability of allele-specific expression, we determined the pattern of expression of huCD2 and IL-4 after restimulation of clones at least 7 days later. We observed that clones that expressed IL-4 were rather stable (with the exception of clone #9). Among the clones that expressed huCD2 (\pm IL-4), some clones appeared stable (#1, #2, and #3), but others displayed partial (#4) or complete (#5) switching to predominant expression of IL-4. More than five other clones behaved similarly to clones #4 and #5 (data not shown). The phenotype of these latter clones (#4 and #5) can account for the appearance of cells expressing only IL-4 upon restimulation of sorted huCD2⁺ cells, as demonstrated in the study of sorted populations (Figure 5). While we have observed switching within clones from expression of the *huCD2 KI* allele to expression of the intact *IL-4* allele, we have not yet isolated clones that revert from producing IL-4 to expressing mainly or only huCD2.

Our results thus suggest that, at the clonal level, Th2 cells can express at least one and in some instances two alleles in a stable, fixed manner upon restimulation. Although some of the huCD2 clones are stable, some switch back to producing IL-4. This instability, also illustrated upon restimulation of the cells sorted on the basis of huCD2 expression (Figure 5), may be due to an intrinsic instability of the modified allele as compared to the wild-type *IL-4* allele. It is also possible that IL-4 expression is more stable and that IL-4 itself may exert a yet uncharacterized feed-back mechanism preventing the second allele from becoming active.

Discussion

The above results show that introduction of a modified huCD2 cDNA into the *IL-4* locus resulted in faithful expression of the huCD2 reporter gene in Th2 and Th0 cells and not in Th1 cells, that only express IFN γ . As observed with IL-4, huCD2 expression was inducible upon T cell activation and down-modulated within 48 hr after stimulation. Another Th2-specific cytokine, IL-5, was produced at the same frequency in cells expressing either huCD2 or IL-4.

Because this model renders each allele distinguishable from the other, we were led to the surprising finding that IL-4 expression is controlled by allele-specific activation. Initially, each allele appears to be independently activated by a stochastic mechanism, at least upon low or moderate strength of stimulation as reflected by the frequencies of monoallelic and biallelic expression (Table 1). We tested whether biallelic expression was an acquired feature associated with T cell differentiation, enabling repeatedly stimulated lymphocytes or memory lymphocytes to promptly secrete large amounts of IL-4. Our findings clearly establish that it is the TCR strength of signaling that exerts the most profound effect on biallelic expression and not the developmental stage of the responding T cells (Figure 4). It was demonstrated previously that increasing the strength of TCR stimulation can increase the number of IL-4 expressing cells according to an all-or-none phenomenon (Bucy et al., 1994). Our results provide an additional mechanism by which greater amounts of IL-4 per cell could be produced. The antigen dose thus appears to differentially

influence the utilization of *IL-4* alleles, such that the frequency of biallelic expression increases with the strength of signal delivered through the TCR.

At this time, only a few genes are known to be regulated at the level of allele-specific transcription. These include the genes encoding T and B cell receptors (Malissen et al., 1992; Melchers et al., 1995), olfactory receptors (Chess et al., 1994), natural killer (NK) cell receptors (Held et al., 1995), X-linked genes (Riggs and Pfeifer, 1992), and imprinted genes (Pfeifer and Tilghman, 1994). These genes exhibit either random activation or silencing of one of the two copies of a gene or parental imprinting. By analogy to IL-4, the expression of the NK receptor Ly49A gene is predominantly but not exclusively monoallelic (Held and Raulet, 1997). The activation of only one of the Ly49A alleles also seems to be the consequence of a stochastic mechanism. More recently, the expression of the IL-2 gene was suggested to be regulated by random allele-specific activation (Hollander et al., 1998). Curiously, all of the clones examined had silenced one of the two alleles, although the result may have been biased by measuring IL-2 expression at only one time point after activation and in a limited number of clones. Like IL-4, this cytokine gene is tightly regulated during development and its expression is restricted to a T helper subset (Th1 in this case). IL-2 expression has been shown to be regulated by an all-or-none mechanism. This implies that a threshold in TCR signaling is essential to promote the activation and the simultaneous assembly of all of the necessary transcription factors at the IL-2 promoter. This phenomenon leads to a relatively fixed level of IL-2 expression per cell (Garrity et al., 1994; Itoh and Germain, 1997). A similar process was suggested for IL-4 expression in D011.10 TCR $\alpha\beta$ transgenic Th2 clones. Upon increasing concentrations of peptide antigen, greater numbers of cells were shown to express IL-4, yet each cell expressed the same amount of IL-4 mRNA (Bucy et al., 1994). Thus, spatial and time constraints similar to those proposed for IL-2 could apply for coordinated recruitment of the multiple transcription factors required to achieve IL-4 expression. The limiting availability of these factors might not only set the specific threshold responsible for the all-or-none transcription phenomenon but might also limit the activation of a single allele at a time. Our results are compatible with this hypothesis and with a model that at higher antigen doses ($\geq 1 \mu\text{M}$ in our experiments) greater amounts of factors can be generated and assembled, leading to a higher fraction of the cells in which both alleles are transcriptionally activated (Figure 4). It will be of interest to determine if additional cytokines beside IL-4, such as IL-2, are regulated by the same TCR signaling-dependent mechanism.

The finding that the frequency of biallelic expression is independent of IL-4 levels but depends on TCR signaling suggests that the strength of signal might determine at the clonal level the frequency of cells in which both alleles will be transcriptionally active (Figure 4). The latter frequency could be determined by the strength of stimulation received at the time of the establishment of IL-4 expression and remain unmodified upon further stimulations. Subsequently, the successful establishment of IL-4 expression on one allele might prevent

the second allele from becoming active by an unknown feed-back mechanism. The ability to generate stable clones in which cells express either IL-4 or huCD2 with a certain frequency of biallelic expression is compatible with this hypothesis (Table 2). However, the instability of some of the clones, particularly those that express only huCD2, suggests that in some cells there can be resetting of the frequency of biallelic expression upon restimulation or that selective pressure may be favoring cells that express IL-4. Alternatively, it is possible that the absence of IL-4 in huCD2-expressing cells prevents the establishment of a feed-back mechanism necessary to stabilize the expression of the activated allele. Consequently, these cells are prone to revert to expressing IL-4. Further studies at the clonal level are necessary to clarify these issues.

The physiological relevance of limiting the majority of Th2 cells to activating a single *IL-4* allele is not immediately obvious. However, it is tempting to speculate that the levels of IL-4 need to be finely tuned, as slight decreases or increases may dramatically modify the outcome of an immune response. Indeed, it has been shown that BALB/c animals infected with *L. major* are exquisitely sensitive to IL-4 gene dosage. Heterozygous IL-4^{+/-} mice are more resistant to *L. major* infection than the susceptible WT animals, as reflected by the smaller lesions and the reduced ulceration and necrosis of the foot pads (Kopf et al., 1996). This implies that small variations in the amounts of available IL-4 influence the outcome of the disease. IL-4 is also a growth factor for thymocytes during ontogeny (Carding et al., 1989). However, high levels of transgene-encoded IL-4 are deleterious to T cell development and induce allergic inflammatory disease (Tepper et al., 1990).

The TCR-regulated activation of one versus both *IL-4* alleles may be important not so much for regulating the amount of IL-4 produced by a single cell as for signaling that the cell has achieved terminal differentiation. Doubling the amount of IL-4 produced by a single cell is rather insignificant when compared to the 100-fold induction of IL-4 expression observed upon commitment of a naive T cell toward the Th2 phenotype (Le Gros et al., 1990). In vitro, high doses of IL-4 have been shown to superinduce molecules that mediate TCR-induced apoptosis (Lerner et al., 1996; Zheng et al., 1998). This observation, along with our results suggesting that the frequency of biallelic expression increases with the strength of the TCR signal, raises the interesting possibility that the effector cells in which both alleles are transcriptionally active are programmed to undergo apoptosis. The transcriptional activation of both alleles would signal the IL-4-expressing cells that they have reached a point of terminal differentiation. Although we can identify the cells in which both alleles are activated, we cannot yet test this hypothesis, as the cells express huCD2 instead of doubling their production of IL-4. However, we would predict that cells in which both alleles are active would be present at higher frequencies in our model (huCD2⁺IL-4⁺) than in WT mice. IL-2 also induces molecules that mediate TCR-induced apoptosis. This could explain the absence of cells in which both alleles are transcriptionally active among the IL-2-expressing cells (Hollander et al., 1998).

An intriguing feature of the *IL-4* gene is related to its chromosomal localization adjacent to two other genes, *IL-5* and *IL-13*, which are also expressed specifically in the Th2 effector subset. These three genes colocalize within a region of 200 kb. Although there is no evidence at this time for a common transcriptional regulatory *cis* element such as an enhancer or a locus control region, the existence of such an element is quite conceivable. It would be of interest to determine if the *IL-5* and *IL-13* genes are also regulated by a mechanism involving single allele activation. If these three genes compete for an upstream or downstream *cis* regulatory element, it could be important that IL-4 be expressed from a single allele to ensure the expression of IL-5 or IL-13 from the other allele. In that instance, the expression of IL-4 could be fixed on one chromosome. Alternatively, the genes could be sequentially transcribed in a stochastic manner if the *cis* regulatory element can switch back and forth between the promoters of several genes in a flip-flop mechanism, as previously suggested for the β - and γ -globin genes (Wijgerde et al., 1995). There may also be a hierarchy of sensitivity for stochastic activation of each locus in response to variable strength or length of TCR signaling. This may account for variations in cytokine production among different Th2 cells.

In conclusion, our findings demonstrate that IL-4 expression is regulated by allele-specific activation. This may be a mechanism for controlling the level of cytokine expression in each cell or could, instead, be an important regulatory feature of the commitment of a precursor T helper cell to a differentiated lineage. Further experiments are required to elucidate the mechanism and potential physiological relevance of limited allelic activation.

Experimental Procedures

Mice

Mice were housed in the pathogen-free facility at the Skirball Institute of Biomolecular Medicine. To generate chimeric mice, targeted E14 ES cells were injected into C57BL/6 blastocysts. The resulting chimeric mice were bred to C57BL/6 WT, and transmission of the mutation to the germ line was tested in mice of 3 weeks of age by Southern blotting of tail DNA. Mice carrying the mutation were further crossed to either C57BL/6 WT animals or D011.10 TCR- $\alpha\beta$ BALB/c transgenic mice specific for the OVA323–339 peptide and restricted by I-A^d (Murphy et al., 1990).

Generation of the Targeting Vector

The IL-4 genomic DNA isolated from a 129 genomic library (Stratagene) was kindly provided by C. Davis and was subcloned into pBluescript (pBS, Stratagene). The arms of the construct consist of a 3.9 kb XbaI/HindIII fragment of the IL-4 genomic DNA on the 3' side and a 5.1 kb fragment on the 5' side of the first exon (see Figure 1A). The inert huCD2 reporter cDNA was derived from the pNeoCD2 vector containing the Δ 227–307 cytoplasmic domain mutation (Hahn and Bierer, 1993) and from the π H3M-CD2 vector containing the extracellular mutation Y91D (Peterson and Seed, 1987). A NcoI site was created at the ATG initiation codon of the mutant huCD2 cDNA by PCR mutagenesis. The 1 kb fragment PstI/BglIII encompassing exon1 and exon2 of IL-4 genomic DNA was cloned into pBS. Within this vector, a NcoI site was created at the ATG initiation codon in exon 1 of the IL-4 genomic DNA and a Sall cloning site was created by PCR mutagenesis 22bp upstream of the 3' end of Exon 1. huCD2 cDNA was linked to the 1.3 kb BamHI/Sall fragment containing a *neomycin resistance* gene (driven by the herpes simplex virus thymidine kinase promoter) flanked by two lox P sites

derived from the pL2neo vector, kindly provided by N. Killeen (Gu et al., 1993). The resulting 2.2 kb NcoI/SalI fragment containing the mutant huCD2 cDNA and the "neo cassette" was cloned in place of the PCR mutated NcoI/SalI fragment of exon 1 (pZaz1 vector). The 0.8kb SpeI/XbaI fragment containing three SV40 polyadenylation sites (Maxwell et al., 1989) was inserted in the XbaI site downstream of exon 2 in the pZaz1 construct. Both arms of the targeting construct were sequentially introduced into the vector. All cloning steps were performed according to standard procedures (Sambrook et al., 1989).

E14.1. ES cells (Kuhn et al., 1991) were transfected with the linearized targeting vector and selected in presence of 350 $\mu\text{g}/\text{ml}$ of G418 (Geneticin, Gibco). Targeted ES cell clones and tail DNA from mice were screened for homologous recombination by Southern blotting (Figures 1A–1C).

Generation of T Cell Populations

All the T helper cell populations were cultured in 10 mM HEPES-buffered RPMI 1640 media (GIBCO-BRL) supplemented with 10% FCS (Hyclone), L-glutamate (2 mM), β -2 mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), and antibiotics. Recombinant mouse IL-2 (Boehringer Mannheim) was added to all cultures at 20 U/ml to increase the viability of the cells. Single cell suspensions were generated from lymph nodes or spleen by passing the tissues through 70 μm nylon cell strainers. To generate T cell populations derived from nontransgenic mice, cells were incubated with rat anti-mouse CD8 MAbs (supernatant from the hybridoma 53–6.72) and subsequently depleted of CD8⁺ and B cells by adherence depletion on plates coated with 10 $\mu\text{g}/\text{ml}$ of goat anti-rat IgG (Jackson ImmunoResearch). Cells were harvested, washed, and stimulated on plates coated with 1 $\mu\text{g}/\text{ml}$ of anti-mouse CD3 MAbs (145-2C11) (Pharmingen), in presence of cytokines added in the primary stimulation (see below). To generate T cell populations from transgenic animals, CD4⁺V β 8⁺ T cells from D011.10 TCR $\alpha\beta$ transgenic mice were sorted by flow cytometry. CD4⁺ T cells were restimulated with OVA_{323–339} peptide (0.01–30 μM) and irradiated splenocytes (2000 rads). OVA_{323–339} peptide was synthesized at the Skirball Institute of Biomolecular Medicine (Protein Chemistry Laboratory).

Th1 enriched populations were generated in presence of 100 U/ml of IL-12 kindly provided by J. Lafaille (gift of M. Gately, Roche). Th2 enriched cell lines were established in presence of 1000 U/ml of recombinant IL-4 (Sigma). Some cultures (as indicated in the text and in figure legends) received purified 11B11 rat anti-mouse IL-4 (5 $\mu\text{g}/\text{ml}$) (Pharmingen) or XMG1.2 rat anti-mouse IFN γ (5 $\mu\text{g}/\text{ml}$) (Pharmingen).

Th2 CD4⁺ T cell clones from nontransgenic (129huCD2-KI \times C57BL/6)F1 mice were generated by limiting dilution or by single cell sorting in 96-well plates. Clones were restimulated every 7 to 11 days and expanded with splenic allogeneic BALB/c APCs (irradiated at 2000 rads) in the presence of anti-CD3 MAbs (0.1 $\mu\text{g}/\text{ml}$). 200 U/ml of IL-4 (Sigma) was added in the primary stimulation. Clones were analyzed for huCD2 and IL-4 expression after overnight stimulation on anti-CD3 MAb (1 $\mu\text{g}/\text{ml}$)-coated plates and treatment with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hr. Clonality of the cells was demonstrated by analysis of V β cell surface expression.

Flow Cytometry Analysis, Intracellular Staining, and Antibodies

Intracellular staining was performed according to the procedures recommended by Pharmingen. In brief, monensin (Sigma) was added at 3 μM to the cultures 2 hr before harvesting the cells. Extracellular stainings were performed following standard procedures in staining buffer (PBS 1% FCS and 0.1% Na Azide) at 4°C. Cells were fixed in 2% paraformaldehyde (Sigma) for 20 min at 4°C and washed and incubated in permeabilizing buffer (PBS 1% FCS, 0.1% saponin, and 0.1% Na Azide). Intracellular stainings were performed in permeabilizing buffer for 30 min at 4°C. The following antibodies were used for the stainings: PE-anti-huCD2 (UMCD2, Ancell), TC-anti-mCD4 (Caltag), PE- or FITC-anti-IL-4 (BVD4-1D11, Pharmingen), PE- or FITC rat IgG2b isotype control (R35-38, Pharmingen), FITC-anti-IL-5 (TRFK5, Pharmingen), biotin anti-IFN γ (XMG1.2, Pharmingen), PE-anti-huCD4 (Caltag), and TC-streptavidin (Caltag). Cells were

analyzed using a Becton Dickinson FACScan flow cytometer and Cell Quest software.

Acknowledgments

The authors thank Juan Lafaille, David Raulet, Michel Sadelain, Antonio Lanzavecchia, and Derya Unutmaz for helpful discussions and critical reading of the manuscript. We also thank Shana Marmon for technical assistance and John Hirst for cell sorting. I. R. is supported by a postdoctoral fellowship from the Cancer Research Institute. D. R. L. is an investigator of the Howard Hughes Medical Institute.

Received June 30, 1998; revised July 6, 1998.

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