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Coordinate Regulation of the IL-4, IL-13, and IL-5 Cytokine Cluster in Th2 Clones Revealed by Allelic Expression Patterns

Ben L. Kelly and Richard M. Locksley

The cytokines IL-4, IL-13, and IL-5 are markers for the Th2 subset of effector T cells and are often expressed together. These cytokine genes are organized within 140 kb of orthologous DNA in both mouse and human. Using IL-4-expressing CD4+ T cell clones derived from F1 mice, we identified allelic polymorphisms for each of these cytokines and assessed the parental identity of the cytokine mRNAs. Both monoallelic and biallelic expression occurred for each gene and for an additional gene, IL-3, that lies with GM-CSF over 450 kb telomeric on the same chromosome. When coexpressed in T cell clones, IL-4 was expressed from the same allele as IL-13 or IL-5 in 81% of instances. In contrast, there was only 52% concordance of these three cytokines at the allelic level among clones that expressed IL-3. Independent expression of the cytokine alleles occurs commonly in T cells, but the clustered locus encompassing IL-4, IL-13, and IL-5 is subject to coordinate regulation.

Methods


Mechanisms that mediate patterning of the cytokine repertoire are important in understanding how the T cell immune response becomes matched with a given antigenic challenge. Naive T cells must synthesize signals from the peptide-specific TCR within the context of cytokine receptors that provide information regarding the physiologic microenvironment to undergo proliferation and commitment to various effector phenotypes (1, 2). Such signals result in the reorganization of chromatin structures surrounding cytokine gene loci with the establishment of stable effector cytokine patterns that become epigenetically fixed (3–8).

Analysis of the IL-4 gene revealed both monoallelic and biallelic expression among stable, IL-4-expressing, Th2 cells (3, 9). Activation of one or both alleles was suggested to reflect the degree and/or duration of stimulation through the TCR (9), but raised the possibility that monoallelic expression might constitute a common mechanism of expression at cytokine loci serving to enhance effector cell diversity. The IL-4 gene lies in an area of 140 kb with the IL-13 and IL-5 genes, cytokines expressed, like IL-4, in Th2 lymphocytes (10). We analyzed the expression patterns of these linked genes in a panel of IL-4-expressing clones that contained allelic differences at these various loci and compared expression with an additional cytokine, IL-3, which resides almost 500 kb telomeric on the same chromosome in close association with GM-CSF.

Materials and Methods

T cell clones

Alloreactive (anti-H-2b) CD4+ IL-4-expressing clones were generated from (BALB/c × CAST/Ei) F1 mice in three separate experiments as previously described (3). Clones were established by single-cell flow cytometry or by limiting dilution. Individual clones were activated for 17 h by culture on plates coated with anti-TCR (H57-597, 10 µg/ml) and anti-CD28 (37N51.1, 10 µg/ml) mAbs before harvesting RNA using RNAzol B (Biotecx, Houston, TX) and preparation of cDNA using Advantage RT-for PCR (Clontech, Palo Alto, CA).

Cytokine expression analysis

Primers for PCR amplification were selected to span introns and to contain an internal polymorphism between the parental BALB/c and CAST/Ei alleles for each cytokine (Table I). Analysis of a constitutively expressed gene, hypoxanthine phosphoribosyltransferase, was included to monitor the general abundance of cellular mRNAs in the samples. Amplification conditions (GeneAmp PCR System 9700 Cycler, ABI/PE Biosystems, Foster City, CA) were as follows: for IL-4: 94°C for 5 min; 45 cycles of 94°C for 40 s, 62°C for 40 s, 72°C for 1 min; for IL-5, 94°C for 5 min; 45 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 40 s; and for IL-13, 94°C for 5 min. PCR-amplified cDNAs were visualized on 1–2% agarose gels and purified using a Qiaex II gel extraction kit (Qiagen, Hilden, Germany) as directed. Dye terminator sequencing was performed in bulk on the purified cDNAs using a PE Biosystems ABI Prism 377 DNA Sequencer (ABI/PE Biosystems) using the sequencing primers in Table I.

Statistical analysis

Probability of variance was calculated using Fisher’s exact test.

Results

Cytokine expression among IL-4-expressing clones

A total of 23 clones with stable IL-4 expression were analyzed for the presence of mRNAs for IL-13, IL-5, and IL-3 (Fig. 1). Within the limits of the PCR-based assay, the most common cytokine patterns were IL-4 alone (nine clones) or all four cytokines (seven clones). IL-4 and IL-13, sequential genes separated by ~13 kb, were coexpressed in 10 clones. IL-5, separated from the IL-4/IL-13 genes by the RAD50 gene over a distance of ~120 kb, was not expressed independently with IL-4 in any clone, but was coexpressed with IL-4 and the intervening IL-13 gene in seven clones. Thus, substantial heterogeneity occurs among Th2 clones, even when established from the same culture.

Allele-specific expression of chromosome 11 cytokine genes

Because the clones were derived from F1 (BALB/c × CAST/Ei) mice, individual cytokine cDNAs were sequenced from the two...
parental strains to identify polymorphisms that would allow assignment of the parental alleles as previously described (11). Artificial templating with varying ratios of the parental cDNAs established that this technique could reliably detect either allele at a minimum of 4:1 excess (B. Kelly, unpublished observations; data not shown).

Among the 23 clones, 14 (61%) expressed IL-4 from both alleles; the remainder were monoallelic from either the BALB/c (six clones) or CAST/Ei alleles (three clones). Monoallelic and biallelic expressions were observed for each of the cytokines examined, suggesting a general mechanism of gene expression applicable to each of these loci (Fig. 2). No clones that expressed solely the CAST/Ei IL-5 allele were detected among the 8 IL-5-expressing clones, although this might reflect the small number of cells analyzed. Each of the potential expression patterns, BALB/c allele, CAST/Ei allele, and both alleles, was otherwise represented for the other cytokines. The predilection for expressing the BALB/c allele among monoallelic clones seen for each of the proximal cytokine cluster genes did not extend to IL-3, where the CAST/Ei allele was more frequently recovered.

Allelic concordance among expressed chromosome 11 cytokine genes

Fourteen of the 23 clones expressed cytokines in addition to IL-4 and could be analyzed for allelic concordance among IL-4 genes (Fig. 3). Twelve of the 14 could be sampled repeatedly to assess the stability of allelic expression patterns using this method. For the four genes/eight alleles sampled, only 2 of 128 (1.6%) gave discordant results (i.e., BALB/c vs CAST/Ei) during repetitive sampling (B. Kelly, unpublished observations). All recovered PCR products were scored positive for the subsequent analysis.

Cytokines were compared pairwise to assess whether expression of these genes might be linked to activation of IL-4, the marker used for selecting the clones. Thus, each cytokine was examined for the likelihood that the upstream cytokine(s) had been activated from the same allele. When assessed in this manner, cytokines in the proximal cytokine cluster, IL-4, IL-13, and IL-5, were expressed from the same alleles more often than with the more distally positioned gene, IL-3 (Fig. 4). Thus, pairwise expression of either IL-13 or IL-5 with upstream expression of IL-4 from a given allele or of IL-5 with upstream expression of IL-13 was concordant from the same allele in 80% (28 of 35) of cases. Conversely, expression of the IL-3 gene from a given allele was concordant with the upstream expression of IL-4, IL-13, or IL-5 from the same allele in 52% (22 of 42) of cases, consistent with independent regulation of the more distant cytokine locus (p < 0.01). Similar relationships were apparent in the expression of IL-5, the most distally positioned cytokine in the proximal cluster. Expression of IL-5 was concordant with the upstream IL-13 allele in seven of nine cases (78%), but with the downstream IL-3 allele in only 5 of 14 cases (36%; Fig. 4).

Four clones demonstrated allelic discordance between the interposed IL-13 gene and the expression of either the upstream IL-4 or downstream IL-5 genes. A single gene, the DNA repair enzyme RAD50, is positioned over a region of 110 kb between the IL-13 and IL-5 genes (Fig. 5). There was sufficient material from two of the clones to repeatedly assess the expression of the RAD50 gene. Clone BR1, although monoallelic BALB/c at both the IL-4 and IL-5 genes, was consistently biallelic at the interpositioned IL-13 gene. Clone BR2 was similarly monoallelic BALB/c at the IL-4 and IL-5 genes, but failed to score a product for IL-13. Analysis of the RAD50 gene demonstrated biallelic expression in both clones, suggesting that expression of the RAD50 gene might impart structural consequences on flanking genes.

![FIGURE 1. Cytokine expression patterns among 23 CD4+ Th cell clones selected for expression of IL-4.](http://www.jimmunol.org/)
Discussion

We used a panel of IL-4-expressing CD4\(^+\) T cell clones to examine the expression of cytokine genes linked on chromosome 11 in the mouse and on the orthologous region of chromosome 5 in the human (10). The use of F\(_1\) mice bearing allelic polymorphisms at each of the cytokine loci allowed extension of the analysis to each of the two alleles. Several findings were noteworthy. First, even among clones picked from common culture conditions, substantial heterogeneity in expression patterns was evident. Second, the evidence for allele-independent expression of the IL-2 and IL-4 genes (3, 9, 12, 13) was extended to each of the additional cytokines analyzed, suggesting a general principle that might apply to all cytokine loci. Third, although heterogeneity was apparent among clones, expression patterns suggested allelic concordance among the closely linked proximal Th2 locus, which includes the IL-4, IL-13, and IL-5 genes, that did not extend to the more distal locus, which includes the IL-3 and GM-CSF genes.

Prior studies using labeled mRNA probes, intracellular cytokine detection, and PCR-based strategies comparable to those used here have demonstrated heterogeneity in cytokine expression among CD4\(^+\) and CD8\(^+\) T cell clones, even when generated from the same cultures (14–21). Analysis of the IL-4/IL-13/IL-5 cluster in Th2 clones has not been systematically assessed, however. The coexpression of IL-4, IL-13, and IL-5 in conditions associated with the appearance of Th2 cells suggests coregulation of these cytokines, consistent with their clustered position over 140 kb of orthologous DNA in both human and mouse (10). Of 23 IL-4-expressing clones analyzed here, 10 coexpressed IL-13 and eight coexpressed IL-5. Coexpression of these cytokines correlates with the appearance of DNase hypersensitivity sites that appear in this region under the appropriate Th2-inducing conditions (4, 6). Indeed, recent studies have identified a highly conserved region at a DNase hypersensitivity site between the IL-4 and IL-13 genes that effects the regulation of each of the three cytokine genes in this cluster (22).

Studies of IL-2- and IL-4-expressing CD4\(^+\) T cells have documented stable expression from either or both the maternal or paternal cytokine alleles (3, 8, 12, 13). Indeed, as assessed here using clones from F\(_1\) mice bearing allele-specific markers for each of the cytokines, expression of three additional cytokines, IL-13, IL-5,
and IL-3, occurred from either allele. Thus, independent expression of the cytokine alleles is common in T cell clones. If chromatin remodeling is linked to expression of these genes, expression from the type 2 cytokine cluster would presumably occur in cis from the same allele. We compared allelic expression concordance for the IL-4, IL-13, and IL-5 genes, which sit in the proximal cytokine cluster, with expression of the IL-3 gene, which is clustered in close association with the GM-CSF gene over 460 kb telomeric to IL-5. Indeed, if IL-13 or IL-5 was expressed, the likelihood that IL-4 was expressed upstream from the same allele was 81%. Conversely, expression of IL-3 was associated with expression of either IL-5 or IL-4 from the same upstream allele in 50% of cases, a finding no different from randomness. The data are consistent with coordinate regulation of the proximal cytokine cluster on chromosome 11 (5q in human) that does not extend to the more distal IL-3/GM-CSF locus despite the sharing of a common signaling receptor chain by IL-5, GM-CSF, and IL-3 (23).

Regions that coordinately regulate the distal cytokine locus have been identified using DNase hypersensitivity and footprinting analyses, suggesting independent regulation of the IL-3/GM-CSF cluster in T cells (24, 25). As with the IL-4, IL-13, and IL-5 genes, T cell clones can express IL-3 and GM/CSF either together or independently (18), reflecting aspects unique to their regulation despite the localization of these two genes within 10 kb (24–27).

Although concordance among the more proximal cytokine loci was frequent, it was not absolute. In four clones that could be sampled at least three times, expression of IL-13 was discordant from either upstream IL-4 or downstream IL-5 genes. IL-4 and IL-13 are adjacent, but IL-13 and IL-5 are separated by a single large gene, the DNA repair enzyme, RAD50. Prior analysis using Northern blots suggested that RAD50 was expressed at lesser amounts in Th1 clones, which do not express any of the Th2 cytokines, than in Th2 clones, consistent with an effect of expression of the cytokines on the adjacent RAD50 gene (6). Two of our discordant clones had sufficient material to allow analysis of the RAD50 gene three or more times. In both clones, RAD50 was expressed biallelically, suggesting that transcription at the RAD50 locus might influence expression of the adjacent IL-13 or IL-5 genes. Deletion of the RAD50 gene is lethal in mice (28), but further study will be required to determine whether its expression is required for lymphocyte viability.

Although these data are consistent with allele-specific regulation of the Th2 cytokine cluster, several caveats are noteworthy. First, PCR-based strategies rely on sufficient material to amplify the target. The cytokine genes typically encode messages of low abundance in T cells, perhaps reflecting the rapid degradation established by sequences within the 3’-untranslated regions of the RNAs (29, 30). We did not consistently achieve a signal in all replicate assays, raising questions about whether negative results absolutely reflected failure to express a gene. Among the 14 clones analyzed most extensively, however, concordance was high in repeated experiments, although we recognize the relatively small numbers of cells used for study. Additionally, we analyzed a single

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**Figure 4.** Allelic concordance of chromosome 11 cytokine genes. Orientation of designated chromosome 11 cytokine genes is shown at the top. Percentages along the lines connecting the shaded boxes indicate the likelihood that the expression of the given downstream gene was allelically concordant with the expression of the upstream gene. Overall allelic concordance among the proximal cytokine genes or compared with the distal IL-3 gene is shown at the left.

**Figure 5.** Biallelic expression of RAD50 in clones BR1 and BR2. Expressed genes are depicted by shading. ☐, nonexpressed genes. B, BALB/c allele; C, CAST/Ei allele.
time point after T cell activation, leaving open the possibility that expression patterns might vary at different times after stimulation. Second, artificial dilution experiments confirmed that this approach could reliably establish a 4:1 ratio of expression between either of the two alleles; more discordant ratios of expression could not always be discerned. Third, these studies do not discard the possibility that a given allele was expressed but subsequently silenced during propagation. Finally, the clones were selected based on expression of IL-4. It remains unknown whether analysis of clones selected for the expression of IL-13 or IL-5 would give comparable results, nor is it clear whether this analysis of alloreactive clones can be extended to clones established through alternative methods of stimulation. It should be emphasized, however, that these studies were conducted examining the endogenous wild-type alleles. Similar results obtained from cells containing gene-targeted alleles support these findings (9, 12, 13) and suggest that allele-specific regulation is a common expression strategy for cytokine genes. The finding that the cytokine modules are regulated in independent, but coordinate, fashion at the IL-4, IL-13, IL-5, IL-3, and GM-CSF clusters suggests that identification of molecules that establish such coordinate expression may be important targets for modifying patterns of effector function.

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