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Stochastic Monoallelic Expression of IL-10 in T Cells

Dinis Pedro Calado, Tiago Paixão, Dan Holmberg, and Matthias Haury

IL-10 is a potent anti-inflammatory and immunomodulatory cytokine, exerting major effects in the degree and quality of the immune response. Using a newly generated IL-10 reporter mouse model, which easily allows the study of IL-10 expression from each allele in a single cell, we report here for the first time that IL-10 is predominantly monoallelic expressed in CD4+ T cells. Furthermore, we have compelling evidence that this expression pattern is not due to parental imprinting, allelic exclusion, or strong allelic bias. Instead, our results support a stochastic regulation mechanism, in which the probability to initiate allelic transcription depends on the strength of TCR signaling and subsequent capacity to overcome restrictions imposed by chromatin hypoacetylation. In vivo Ag-experienced T cells show a higher basal probability to transcribe IL-10 when compared with naive cells, yet still show mostly monoallelic IL-10 expression. Finally, statistical analysis on allelic expression data shows transcriptional independence between both alleles. We conclude that CD4+ T cells have a low probability for IL-10 allelic activation resulting in a predominantly monoallelic expression pattern, and that IL-10 expression appears to be stochastically regulated by controlling the frequency of expressing cells, rather than absolute protein levels per cell. The Journal of Immunology, 2006, 177: 5358–5364.

Interleukin-10 was first described in 1989 (1) and has since been shown to be a potent anti-inflammatory and immunomodulatory cytokine, exerting major effects in the degree and quality of the immune response (2).

Although several cell types express IL-10 (2), specific inactivation of this gene in T cells, similarly to ubiquitous IL-10 deletion, generates a phenotype of spontaneous enterocolitis that resembles Crohn’s disease in humans (3, 4). This observation underlines the general importance of IL-10 in the immunoregulation mediated by CD4+ T cells (5–7).

IL-10 has been subject of intense investigation; however, most studies aimed to understand its biological function, and only recently the regulation of IL-10 gene expression by transcription factors (8–10) and chromatin accessibility (9, 11–13) has gathered more attention.

PCR-based approaches using polymorphisms to distinguish transcripts from each allele at the single cell level or by generating allelic reporter mice revealed that some cytokines are predominantly monoallelic expressed in CD4+ T cells (14–21). Monoallelic expression of cytokine genes was unexpected, because a biological function for such an expression pattern is not directly obvious, in contrast to the monoallelic expression of genes like the Ig or TCR (22, 23), which is clearly important for the biological specificity of the adaptive immune system.

Experiments using two different IL-4 reporter mice suggested that the production of this cytokine is regulated via a probabilistic transcriptional regulation mechanism (15, 16), thus via the control of the actual frequency of expressing cells rather than the absolute level of cytokine expression per cell. These findings supported a hypothesis that monoallelic expression is the reflection of the low probability of such a mechanism, an assertion still to be verified with similar studies for other cytokines.

Due to the immunological importance of IL-10 and since its allelic expression patterns have not been addressed so far, we decided to generate an IL-10 reporter mouse in which expression from individual alleles at the single cell level could easily be distinguished. Using this newly generated IL-10-YFP knock-in mouse, we report here for the first time that IL-10 is predominantly monoallelic expressed in CD4+ T cells, and provide compelling evidence that this expression pattern is not due to parental imprinting, allelic exclusion or strong allelic bias. Instead, our results suggest a stochastic transcriptional allelic activation mechanism, in which the probability for allelic transcriptional initiation at the IL-10 locus is clearly dependent on the strength of the signal delivered by the TCR, and its ability to overcome chromatin hypoacetylation states. Furthermore, we confirm that in vivo Ag-experienced CD4+ T cells have a higher probability to initiate transcription when compared with naive cells, correlating memory for IL-10 expression with a higher basal probability to transcribe IL-10 (24, 25). Nevertheless, these cells still mainly express IL-10 from one allele.

Although we do not exclude the existence of additional regulatory mechanisms, our findings fully support the hypothesis that stochastic regulation of cytokine gene expression could be a more general phenomenon, rather than a single peculiarity of the IL-4 gene (15, 16).

Materials and Methods
Targeting of eYFP cDNA into murine IL-10 locus

The targeting construct was comprised of a 1.2-kb short homology arm (SHA) and a 4.5-kb long homology arm (LHA). As SHA we used a 3’ 1.2-kb fragment of IL-10’s promoter and 5’ untranslated region (UTR) amplified by genomic PCR using a 5’ oligo with a engineered BamHI site.

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4 Abbreviations used in this paper: SHA, short homology arm; LHA, long homology arm; NaBU, sodium butyrate; neoR, neomycin resistance; rm, recombinant murine; TSA, trichostatin A; UTR, untranslated region.

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(eg gga tcc AGA TTC TTT TCC GTG CGA AGA GTG) and a 3′ oligo with a Nhel site (ccc tta gct acg TCT TCT TCT CAA GGC TGC TCT GTT GC). This fragment plus the eYFP cDNA obtained by restriction digest of peYFP-C1 vector (BD Clontech), with the enzymes Nhel and AccI, were cloned in the restriction sites BamHI and Ciol in the multiple cloning site 1 of the targeting vector by a triple ligation. The 4.5-kb LHA arm was obtained by two genomic PCRs and includes the last 42 nt of IL-10′s first exon and the first 334 nt of the 3′ UTR region of the murine IL-10 loci. In the first PCR, we used a 5′ oligo with an engineered Nhel site (cta gct acg TAG AGC TGC AGA CTA AGC AAG A) and a 3′ oligo with a SacII site (tcc cgg gga CAC TAG GCG CCC CCT TGA CTA). This fragment was cloned in the SpeI/SacII restriction sites in the multiple cloning site 2 of the targeting vector. The second PCR was amplified with a 5′ oligo (GCA GTA CAG TGC TGA GCC AGG CAT) and the other to eYFP cDNA (oligo3 = GTA GCC GAA GGT GGT CAC GAG GGT) and the other to eYFP cDNA (oligo3 = GTA GCC GAA GGT GGT CAC GAG GGT) (see Fig. 1). One of the double resistant clones obtained was used to generate chimeric mice by injection into C57Bl/6 blastocysts, and the neoR resistance gene was removed by crossing to a general Cre-expressing “deleter” mouse strain (26). Mice were backcrossed to C57BL/6 to remove the Cre recombinase from the genome as confirmed by Southern blot analysis (our unpublished data).

**Mice**

Specific pathogen free IL-10<sup>-/-</sup> knock-in mice, aged between 6 and 12 wk old, backcrossed for four to five generations to C57BL/J6 (4 wk old in the experiments of Fig. 1E).

**Abs and reagents**

The following Abs and reagents were obtained: CD3<sub>145.2</sub> (house production); CD28, 37.51, CD62L-CyChrome MEL-14 (eBioscience); IL-10-PE JES5-16E3 (BD Pharmingen); GFP-Alexa 647 (Molecular Biosciences) with CellQuest software (BD Biosciences), and analyzed using FlowJo software (Tree Star). Fluorescence intensities in one- and two-dimensional histograms are presented in log<sub>10</sub> scale.

**Cell purification and staining**

Splenic CD<sub>4</sub><sup>+</sup> T cells either from wild-type mice (IL-10<sup>-/-</sup>), heterozygous (IL-10<sup>+/</sup>), or homozygous IL-10<sup>-/-</sup> knock-in mice were MACS (Miltenyi Biotec) purified with a consistent purity > 95%. Within MACS-purified CD<sub>4</sub><sup>+</sup> T cells, CD4<sub>62L<sup>low</sup></sub> and CD4<sub>CD62L<sup>high</sup></sub> T cells were detected by staining with CD62L Ab; CD4<sub>eYFP<sup>+</sup></sub> or CD4<sub>eYFP<sup>-</sup></sub> detected by eYFP fluorescence; eYFP<sub>IL-10<sup>-</sup></sub>, eYFP<sub>IL-10<sup>+</sup></sub>, and eYFP<sub>IL-10</sub><sup>+/</sup> populations identified by MACS IL-10 Detection kit (Miltenyi Biotec), and eYFP fluorescence were all sorted using a MoFlo High Speed Cell Sorter (DakoCytometry). Cell culture medium and stimulation protocols

Cells were cultured in GlutaMax II DMEM, plus 10% FCS, 2-ME, nontoxic amino acids (all from Invitrogen Life Technologies), and sodium pyruvate (Sigma-Aldrich). In general, total CD<sub>4</sub><sup>+</sup> T cells were stimulated with 10 μg · ml<sup>-1</sup> plate-bound anti-CD3 and 1 μg · ml<sup>-1</sup> anti-CD28 plus 10E<sub>5</sub> M dexamethasone and 4E<sub>5</sub> M vitamin D<sub>3</sub> and then restimulated in the same way for 3–4 more days, before flow cytometric analysis or IL-10 ELISA quantitative assay. In some experiments, the concentration of plate bound anti-CD3 was varied as indicated. We have performed IL-10 ELISA in cell culture supernatants at day 6 of stimulation, i.e., the supernatant from the last 3 days of culture. IL-10 protein values measured by ELISA were then normalized according to the number of cells recovered in each well. These normalized values were then divided by the absolute number of IL-10-expressing cells (including double-expressing cells in the IL-10<sup>+/</sup>) to obtain an average level of IL-10 expression per cell. For analysis of CD4<sub>CD62L<sup>low</sup></sub> and CD4<sub>CD62L<sup>high</sup></sub> T cells or chromatin hyperacetylation studies, cells were stimulated as above, using 15 μg · ml<sup>-1</sup> plate-bound anti-CD3 and in the last case adding 540 μM NaBu or 20 nM TSA to the culture 12 h before analysis. Sorted CD4<sub>eYFP<sup>-</sup></sub> and CD4<sub>eYFP<sup>+</sup></sub> or eYFP<sub>IL-10<sup>-</sup></sub>, eYFP<sub>IL-10</sub><sup>+</sup>, and eYFP<sub>IL-10</sub> cell populations were restimulated as before plus the addition of 20 ng·ml<sup>-1</sup> recombinant murine (rm)IL-2, with or without 10 ng · ml<sup>-1</sup> rmIL-10, for a period of 7 days or 3 wk. In some cultures, we did 4 h of stimulation with 50 ng·ml<sup>-1</sup> PMA and 500 ng·ml<sup>-1</sup> ionomycin in cells previously treated or not 1 h with 1–5 μg · ml<sup>-1</sup> actinomycin D.

**Cell staining and flow cytometry**

Cytokine secretion was blocked during 4 h with 10 μg · ml<sup>-1</sup> brefeldin A, and cells were fixed in 2% formaldehyde and permeabilized with 0.1% of saponin. Data acquisition was performed on a FACS Calibur cytometer (BD Biosciences) with CellQuest software (BD Biosciences), and analyzed using FlowJo software (Tree Star). Fluorescence intensities in one- and two-dimensional histograms are presented in log<sub>10</sub> scale.

**Statistical analysis**

To test for independence of the allelic expression we used Pearson’s χ<sup>2</sup> independence test with two-tail analysis (χ<sup>2</sup> = 2(O−E)<sup>2</sup> E<sup>−1</sup>), where O is the observed frequency and E the expected (theoretical) frequency, asserted by the null hypothesis. To remove any artificial correlation between alleles, we first defined quadrant gates on data produced from IL-10<sup>wt</sup> and IL-10<sup>+/</sup> mice. These gates were then applied to data from IL-10<sup>−/−</sup> mice. A value of ρ ≤ 0.05 was considered significant.

φ correlation coefficient between IL-10 alleles was calculated from dot plots by using the test for correlation coefficients according to the equation φ = (ad-bc)/(a+b+c+d) as described in Ref. 21, where a, b, c, and d are the frequencies of cells in the lower left, lower right, upper left, and upper right quadrant, respectively. The φ range is −1.00 to +1.00, where −1.00 shows complete negative and +1.00 a complete positive correlation between the two alleles. A φ value between −0.100 and +0.100 is indicative of full independence between the two alleles. φ values approaching −1.00 or +1.00 show increasingly negative or positive correlation between the two alleles.

**Results**

**IL-10<sup>-/-</sup> knock-in mouse reveals predominant monallelic IL-10 expression in CD4<sup>+</sup> T cells**

We have generated an IL-10 reporter mouse model, replacing just the first 121 nucleotides of the first exon (including the secretory signal sequence) with eYFP cDNA by homologous recombination (Fig. 1). Our homozygous IL-10<sup>-/-</sup> knock-in mice (IL-10<sup>−/−/−</sup>) are thus IL-10 deficient and do develop inflammatory bowel disease at 8–10 wk, similarly to conventional IL-10 knockout mice. Heterozygous mice (IL-10<sup>−/−/+</sup>), on the contrary are indistinguishable from their wild-type littermates (IL-10<sup>−/−/−</sup>) (4) (our unpublished data).

Furthermore, we wanted to examine whether eYFP expression faithfully paralleled endogenous IL-10 production. Since eYFP expression was nearly undetectable in freshly isolated lymphocytes (our unpublished data), we decided to stimulate purified CD4<sup>+</sup> T cells in vitro (5) with anti-CD3, anti-CD28, vitamin D<sub>3</sub>, and dexamethasone. Comparing such T cells from IL-10<sup>−/−/−</sup> and IL-10<sup>−/−/+</sup> mice, we observe similar frequencies of eYFP and IL-10-expressing cells, and as expected, IL-10<sup>−/−/−</sup> and IL-10<sup>−/−/+</sup> only show IL-10 or eYFP-positive cells, respectively.

Surprisingly, most of the IL-10<sup>−/−/−</sup> CD4<sup>+</sup> T cells do express the IL-10 gene monoleially, either from the wt allele (IL-10<sup>−/−/−</sup>) or the ki allele (eYFP<sup>−/−/−</sup>, either with only cells expressing from both alleles (~2%) (Fig. 1E). Nevertheless, the combined frequency of expressing cells is comparable to IL-10<sup>−/−/−</sup> mice. Therefore, the IL-10 gene is predominantly monoleially expressed in T cells, similar to what has been reported for other cytokines, suggesting that such an allelic expression pattern might be a feature common to several cytokines (14–20).

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IL-10 allelic expression pattern is not due to parental imprinting, allelic exclusion, or strong allelic bias

We selectively analyzed CD4\(^+\) T cells from animals inheriting the eYFP-tagged allele either paternally or maternally to verify if the inserted eYFP sequence would interfere with a possible imprinting mechanism. We did not find any differences in allelic expression frequencies in either case, thus eliminating genetic imprinting as a possible reason for the observed monoallelic expression (our unpublished data).

We next sought to determine whether IL-10 allelic expression would be conserved during cell division. We sorted CD4\(^+\)eYFP\(^+\) T cells from 6 day stimulated CD4\(^+\) T cells of IL-10\(^{wt}\) mice (Fig. 2, A and B), and restimulated them in the presence of rmIL-2. Already after 48 h a clear reduction in eYFP\(^+\)IL-10\(^+\) cells and a concomitant increase of eYFP\(^-\)IL-10\(^+\) cells was visible, converging to similar percentage of cells expressing monoallelically from either allele after 7 days of restimulation (Fig. 2C). Similar results were obtained using purified CD4\(^+\)eYFP\(^+\) cells (our unpublished data).

To further eliminate the possibility of allelic exclusion or strong allelic bias, we sorted 100 cells from each population (eYFP\(^+\)IL-10\(^+\), eYFP\(^-\)IL-10\(^+\), and eYFP\(^-\)IL-10\(^+\)) directly into 96-well plates and restimulated them as before in the presence of rmIL-2. After 3 wk, we analyzed those wells with sufficient cell numbers (~7% of the wells) and obtained in all of them the original expression pattern. Most cells were found to be double negative, and the remainder transcribing predominantly from one of the alleles (Fig. 3).

In both experiments, the addition of exogenous IL-10 did not have any visible effect (our unpublished data). Together, these results show that allelic exclusion or strong biasing mechanisms do not cause IL-10 allele specific expression, analogously to what was observed in the case of IL-4 (15, 16).
TCR signal strength modulates the probability of IL-10 transcriptional activation

The observed low frequencies for IL-10 allelic activation, and the randomization of allele specific expression, led us to suspect that the monoallelic expression of IL-10 might be due to a probabilistic regulation mechanism, as has been suggested for IL-4 (15, 16) and partially for IL-2 (18, 27). The probability of each cell to express a gene in such a system seems to be set by both, intrinsic (basal probability, e.g., loci accessibility for different differentiation states) and extrinsic factors (dynamic probability e.g., strength of extracellular signals) (28).

Thus, we decided to investigate the influence of the strength of TCR stimulation on the allelic transcription of IL-10. Purified CD4⁺ T cells from IL-10⁺⁺ mice were stimulated as above, but with increasing concentrations of plate bound anti-CD3. As illustrated in Figs. 4 and 5, the frequency of monoallelically, as well as biallelically expressing cells, increases with higher anti-CD3 concentrations, and the observed frequencies are compatible with a probabilistic model of transcriptional regulation. As expected the overall amount of IL-10 produced in IL-10⁺⁺ CD4⁺ T cell cultures is twice the amount produced in IL-10⁺⁺ cultures. The increase in the frequency of IL-10-expressing cells is mainly due to de novo transcription, since it can be completely blocked by pretreatment with actinomycin D (our unpublished data).

Noticeably, varying levels of cytokine expression are observed in monoallelically expressing cells for either low (1 μg · ml⁻¹) or high (45 μg · ml⁻¹) anti-CD3 concentrations (Figs. 4 and 5), indicating that IL-10 is regulated at the level of transcriptional initiation, controlling frequencies of expressing cells rather than levels of protein expression in individual cells. Confirming this hypothesis, we observed a clear increase in the level of IL-10 production concurrent with increased anti-CD3 stimulus (Fig. 5C); however, the average amount of IL-10 produced by each IL-10-expressing cell does not change significantly with increased stimulation (Fig. 5D).

Thus, the initiation of transcription at each IL-10 allele appears independent, and the level of TCR triggering affects the probability for this initiation to take place.
that in vivo Ag experienced CD4+ T cells with respect to IL-10 expression, and it has previously been shown a stimulation-dependent increase in the frequency of IL-10 and IL-10 from either allele.

Both NaBU and TSA considerably increase the frequency of IL-10-expressing cells, compared with the controls (nearly three times in the case of NaBU at day 4), suggesting that chromatin accessibility is a strong modulator of the actual probability for transcriptional initiation induced via TCR signaling (Fig. 6).

In vivo Ag-experienced CD4+ T cells stochastically express IL-10 from either allele

In the experiments displayed in Figs. 4 and 5, we have observed a stimulation-dependent increase in the frequency of IL-10 and eYFP single expressing, as well as double expressing, CD4+ T cells. However, CD4+ T cells are a heterogeneous populations with respect to IL-10 expression, and it has previously been shown that in vivo Ag experienced CD4+ T cells, characterized by low expression of CD62L, show “memory” for IL-10 expression (24, 25). Naive cells, on other hand, require long-term TCR stimulation in the presence of IL-4 (25) or immunosuppressive drugs (5) to establish such “memory” for IL-10.

To clarify weather the in vivo Ag-experienced CD4+ T cells are indeed the main IL-10 producers in our cultures, we purified CD4+ T cells based on their level of CD62L expression (CD62Llow—memory T cells and CD62Lhigh—naive T cells) (Fig. 7A) and stimulated them for 5 days in vitro. IL-10 is almost exclusively produced by CD62Llow cells, early on, as well as after 5 days in culture (Fig. 7B), confirming that CD62Llow cells are the main producers of IL-10, and have an intrinsically higher probability to express IL-10 compared with naive cells. Nevertheless, activation of IL-10 allelic transcription in these cells also appears to be probabilistic and with low efficiency, since it occurs without any visible allelic bias and most cells still express the gene from one allele.

Statistical analysis supports probabilistic model for IL-10 gene regulation

To verify if our data does indeed support a probabilistic model of transcriptional regulation (21), we applied two statistical tests on allelic expression data of IL-10/wtki mice. Using the Pearson’s χ2 independence test (32) we always obtained a p value higher then 0.05 (p = 0.34 in Fig. 1E data sets), favoring the null hypothesis. We also did not observe any significant ϕ correlation coefficient between IL-10 alleles (21) for experiments in Fig. 1E (ϕ = 0.076 for IL-10/wtki), Figs. 4–6 (Tables I and II), and Fig. 7 (ϕ = 0.086 for CD62Lhigh and ϕ = 0.073 for CD62Llow), where a statistical relevant number of cells express IL-10 and at least four independent experiments were performed. Together, these results strongly support a probabilistic model of IL-10 gene expression, with both alleles being independent with respect to the probability of transcriptional activation.

Discussion

Strong inflammation sometimes accompanies an immune response against pathogens. Nevertheless counter inflammatory responses are initiated to prevent substantial collateral damage originated by the potent antimicrobial effectors mechanisms. Achieving a balance between protection and pathology appears to be the major function of IL-10 (2), thus suggesting the requirement for a tight regulation of its expression.

Chromatin hypoacetylation restricts IL-10 expression induced by TCR

Chromatin structure plays a crucial role in the regulation of eukaryotic gene transcription. Transcriptional activators and the transcription machinery must gain access to DNA and posttranslational acetylation of histones is thought to facilitate this process (29). We therefore decided to study the consequence of a short-term chromatin hyperacetylation in the transcriptional activation of individual IL-10 alleles induced by TCR. CD4+ T cells were stimulated with a fixed anti-CD3 concentration, and exposed (12 h) to NaBU (30) or TSA (31), compounds well known for their ability to specifically inhibit histone deacetylase (HDAC) activity.

NaBU (30) or TSA (31), compounds well known for their ability to restrict IL-10 expression induced by TCR. Splenic CD4+ T cells from IL-10wtki mice were MACS sorted for CD62Lhigh and CD62Llow, where a statistical correlation coefficient 0.076 for CD62Llow and ϕ = 0.073 for CD62Llow), where a statistical relevant number of cells express IL-10 and at least four independent experiments were performed. Together, these results strongly support a probabilistic model of IL-10 gene expression, with both alleles being independent with respect to the probability of transcriptional activation.

To clarify weather the in vivo Ag-experienced CD4+ T cells are indeed the main IL-10 producers in our cultures, we purified CD4+ T cells based on their level of CD62L expression (CD62Llow—memory T cells and CD62Lhigh—naive T cells) (Fig. 7A) and stimulated them for 5 days in vitro. IL-10 is almost exclusively produced by CD62Llow cells, early on, as well as after 5 days in culture (Fig. 7B), confirming that CD62Llow cells are the main producers of IL-10, and have an intrinsically higher probability to express IL-10 compared with naive cells. Nevertheless, activation of IL-10 allelic transcription in these cells also appears to be probabilistic and with low efficiency, since it occurs without any visible allelic bias and most cells still express the gene from one allele.

### FIGURE 6. Chromatin hypoacetylation restricts IL-10 expression induced by TCR.

Splenic CD4+ T cells from IL-10/wtki mice were MACS purified with high purity (>95%). Cells where stimulated as before but with a fixed concentration of anti-CD3, with or without the presence of histone deacetylase inhibitors (NaBU and TSA) added just 12h before each analysis. Numbers represent relative percentages. Top, Representative results obtained at day 7 for this experimental set up. Bottom, Daily compiled results of four independent experiments (each composed at least of duplicates). Bars indicate SDs between experiments.

### FIGURE 7. In vivo Ag-experienced cells stochastically express IL-10 from either allele.

A. Splenic CD4+CD62Llow—memory T cells and CD4+CD62Lhigh—naive T cells from IL-10/wtki were sort purified with high purity. B. Sorted cells were stimulated in vitro for 5 days. Numbers represent relative percentages. Data representative of two independent experiments.
Using a newly generated IL-10\textsuperscript{eYFP} knock-in mouse model, that easily allows the study of IL-10 gene expression at the allele level, we observed for the first time that IL-10 is expressed in a predominantly monoallelic pattern in CD4\textsuperscript{+} T cells. We provide evidence that the observed expression pattern is not due to parental imprinting, allelic exclusion or strong allelic bias, but the result of stochastic transcriptional allelic activation mechanism. Statistical analysis clearly supports this hypothesis since analysis of allelic expression data shows that transcription activation from each IL-10 allele is an independent process.

The probability for allelic transcriptional initiation at the IL-10 locus is clearly dependent on the strength of signal delivered by the TCR and its ability to overcome inhibitory chromatin hypoacetylation states.

Analogous approaches yielded similar results in the case of the IL-4 gene. The fact that both IL-4 and IL-10 production seems to be regulated via a probabilistic allelic activation mechanism supports the idea that the observed predominant monoallelic expression is the reflection of the low probability of such mechanism. These results, along with other reports (14–21), indicate that such an expression pattern might be a common phenomena to several cytokine genes.

It is worth noting that in the case of IL-10 we did not observe transmission of the allelic expression pattern as a stable epigenetic trait, contrary to what others observed for IL-4, in clones derived from prolonged cultures under Th2 differentiation stimuli (16). This discrepancy might derive from different protocols used to activate cells or different periods of stimulation. However, it could also reflect different characteristics of these two cytokines, as for example the involvement of DNA methylation in the regulation of IL-10 gene expression is still very controversial (11, 33), in contrast to what has been shown for IL-4 (34).

The mechanistic elements governing TCR-dependent IL-10 transcription are still rather little understood. Sp1 and Sp3 transcription factors positively induce IL-10 production (8), but their ubiquitous expression led to the conclusion that IL-10 expression would be predominantly posttranscriptionally controlled (35). Although not excluding the existence of such regulatory mechanisms, we and others (9, 10) clearly demonstrate that fundamental control of IL-10 expression takes place at the transcriptional level. c-Jun and JunB proteins have been shown to bind to an AP1 binding site 3' to the IL-10 locus, whose accessibility is TCR signaling dependent (9, 13), and correlated with a higher IL-10 production. Ets-1, on other hand, seems to act as inhibitor of IL-10 expression, but it is unknown how its action is affected by external stimuli (10). Further studies will be necessary to elucidate the individual regulation of these transcription factors and their respective contribution toward the activation of IL-10 expression.

Experiments showing that Th2 and Tr1 "like" cells have a transcriptionally more permissive chromatin configuration at the IL-10 locus, compared with Th1 or naive CD4\textsuperscript{+} T cells (9, 11–13), clearly correlate long-term changes in locus accessibility to cytokine gene expression, as evidenced by in vivo Ag experienced CD4\textsuperscript{+} T cells in our study. The precise chromatin changes affecting IL-10 expression in our system remain to be elucidated, but the fact that even CD62L\textsuperscript{low} cells mostly express from one allele, suggests that the probability for IL-10 allelic activation is low and determined by both, TCR signaling pathways and chromatin accessibility state. Further investigation will be necessary to clearly dissect how extrinsic and intrinsic factors modulate the probabilities for IL-10 expression in particular cell types and differentiation stages. It would be of extreme interest to understand if possible chromatin changes induced by the TCR signaling are dynamically modulated, possibly relating to the ability of monoallelic producers to quickly revert to nonproducers, change the expressing allele, or to express from both alleles (Figs. 2 and 3).

The biological significance of such a regulation mechanism in vivo is, however, not directly obvious. Little is known about the in vivo secretion of IL-10 by CD4\textsuperscript{+} T cells with immunoregulatory functions. It is not clear whether IL-10 activity is controlled at the overall expression levels in the tissue, or rather regulated via the frequency of expressing cells. Although we do not exclude the existence of other additional regulatory mechanisms, our data are very indicative of the latter, as we see a large variation in the expression levels of IL-10 or eYFP in exclusively monoallelically expressing cells and stronger TCR stimulation directly increases the frequency of expressing cells, rather than significantly affecting the average expression levels per cell (Figs. 4 and 5). Controlling IL-10 production via the frequency of expressing cells may be an attractive solution for immune regulation. Local fine-tuning of the immune response, requiring close cell-to-cell interactions between regulatory T cells and their targets for IL-10 properties to be effective, may avoid deleterious endocrine side effects observed in certain mouse model systems (36).

To what extend these mechanisms do operate in vivo remains to be investigated, and future experiments should aim to elucidate the

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<th>TCR Stimulus\textsuperscript{a} Anti-CD3 (μg \textsuperscript{-} ml\textsuperscript{-1}) (1) (2) (4) (5) (6) (15) (16) (45)</th>
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<td>Expt. D</td>
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<td>0.097</td>
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<tr>
<td>Expt. E</td>
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<td>0.043</td>
<td>0.056</td>
<td>0.049</td>
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</table>

\(\phi\) Using the data obtained in Fig. 4 (Expt. A to D) and Fig. 5 (Expt. E) at day 6 of culture, we have calculated the \(\phi\) correlation coefficient between alleles according to the formula \(\phi = \frac{(ad - bc)}{(a + b)(c + d)}\) as indicated in Materials and Methods. For various concentrations of plate-bound anti-CD3. Values can vary between -1 to 1, and values in the range of -0.1 and +0.1 are generally considered to indicate independence of the factors; nd, nondetermined.
biological consequences of probabilistic cytokine expression for the immune system response and regulation.

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Disclosures

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References


