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Biallelic expression of the IL-2 locus under optimal stimulation conditions

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Recent experiments have suggested that the IL-2 locus is monoallelically expressed. We tested this hypothesis using TCR-transgenic mice carrying one inactivated IL-2 allele. The frequency in single-cell assays of IL-2-producing cells following optimal stimulation by antigen and antigen-presenting cells was equivalent to that from wild-type mice, but the amount of IL-2 produced per cell was twofold less. Similar observations were made by intracellular staining for IL-2, although stimulation in bulk culture was less optimal, showing only a 1.7-fold difference. Importantly, the frequency of responding cells from the heterozygotes was less than from the wild-type mice if the IL-2 assay was performed after only 24–30 h of activation, suggesting that the targeted allele could compete with the normal allele early after stimulation and give the misimpression that the heterozygotes had fewer IL-2-producing cells. These data strongly argue that the IL-2 locus can be expressed biallelically under optimum stimulation conditions.

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1 Introduction

IL-2 was first discovered as a T cell growth factor [1]. It is produced by naive T cells and a subset of previously activated T cells. It is important in both autocrine and paracrine proliferative responses and plays a role in the growth, death and differentiation of helper and cytotoxic T cells [2] as well as B cells [3]. The production of IL-2 is under tight regulation. There is no basal level of transcription and the necessary transcription factors must be assembled in a coordinate fashion by binding to multiple specific sites on the IL-2 gene in an all-or-none process before transcription can be initiated [4, 5]. There is also evidence for control at the post-transcriptional level by CD28 signaling, which promotes stability of the IL-2 mRNA [6].

During an immune response it is crucial to regulate both the amount and duration of IL-2 production. Recently, Holländer et al. [7, 8] have suggested that one way the organism does this is by imposing monoallelic expression at the IL-2 locus. They demonstrated this with a variety of techniques using one allele from an IL-2 knockout (KO) mouse and by looking at individual alleles in an F_1 cross between *Mus musculus* and *Mus spretus* which have a detectable genetic difference at the IL-2 locus. They found that each cell only expressed one IL-2 allele following stimulation with Con A or PMA and ionomycin. Monoallelic expression has also previously been shown for the B cell receptor [9], the TCR [10], olfactory receptors [11] and imprinted genes [12], as well as during X-inactivation [13].

Subsequent work by Naramura et al. [14], however, has not supported the idea of monoallelic expression at the IL-2 locus. These experiments used a green fluorescent protein (GFP) "knock-in" (GFP^{ki}) gene as one allele at the IL-2 locus and compared its expression to the wild-type allele in heterozygotes. They found that at least one half of the responding T cells expressed both alleles following stimulation with anti-CD3 and anti-CD28 antibodies. In agreement with these results, our older studies with single T cells in limiting dilution assays (LDA) had suggested that $CD4^+$ Th1 mouse clones make either a 1X or 2X amount of IL-2 [6]. Thus, the idea of monoallelic expression at the IL-2 locus remains controversial.

Recent experiments from our laboratory [15] and those of Chen et al. [16] have shown that IL-2 response ele-

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Abbreviation: KO: Knockout **GFP^{ki}:** Green fluorescent protein knock-in **Tg:** Transgenic **WT:** Wild type **HET:** Heterozygote **LDA:** Limiting dilution assay **DC:** Dendritic cells **MFI:** Mean fluorescence intensity **PCC:** Pigeon cytochrome *c* **RAG:** Recombinase-activating gene

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ments affecting mRNA stability are located in the coding region of the IL-2 gene. Thus, it is formally possible that control elements affecting monoallelic expression also reside in the coding regions. Since some of these elements were deleted in the construction of the GFP^{ki}, this possibility could account for the failure to see monoallelic expression in the experiments by Naramura et al. [14]. Therefore, we decided to re-examine the issue of monoallelic expression at the IL-2 locus using a variation of one of the models of Holländer et al. [7]. We generated TCR-transgenic (Tg) mice, which were either wild type (WT) or heterozygotes (HET) for the gene-targeted null allele at the IL-2 locus [17]. Cells from these mice were stimulated at limiting dilution with an optimum amount of Ag and APC instead of the Con A used in the previous studies. Under these conditions, we demonstrate that the precursor frequencies of IL-2 producers are comparable for cells from both the IL-2 HET and WT mice, and that the WT T cells make twice as much IL-2 per cell. These data clearly show that bialleleic expression at the IL-2 locus can occur under optimal stimulating conditions.

2 Results

2.1 LDA demonstrates equivalent precursor frequencies for IL-2 production by LN T cells from WT and HET mice

We generated recombinase-activating gene (RAG)-$2^{-/-}$ mice that carried a Tg TCR and that were either WT or HET at the IL-2 locus for a deletion of the third exon of the gene. As a sensitive method to measure IL-2 production by individual T cells, we used an LDA in which a single T cell was surrounded by 10^3 activated dendritic cells (DC) in the presence of high concentrations of peptide [18]. Seven experiments were done on LN cells from a total of nine WT and ten HET mice. In every instance, the precursor frequency of responding T cells from the HET was similar to that of the WT mice (Table 1). In one experiment (no. 6), they were equal even at lower Ag concentrations, although the mean amount of IL-2 produced per cell was reduced in both populations. Overall, the frequency of responding cells ranged from 1 in 1.15 to 1 in 2.8 with a geometric mean of 1.78x/÷1.20 for the HET and 2.19x/÷1.06 for the WT. These means were not significantly different in a Student's *t*-test. Therefore, under optimal stimulation conditions the number of cells that could produce IL-2 (approximately half) was equivalent for both the WT and the HET mice.

Table 1. The precursor frequency of IL-2-producing cells is equivalent for IL-2 WT and IL-2 HET in an LDA

Experiment no.	Ag	1/Frequency (95 % confidence levels)[a]	
	(μM)	WT	HET
1	1	2.80 (2.33–3.51)	2.01 (1.52–2.32)
	1	1.94 (1.40–2.32)	
2	1	1.79 (1.32–2.12)	1.76 (1.27–2.19)
	1		1.33 (1.09–1.51)
	1		1.70 (1.26–2.07)
3	1	1.95 (1.62–2.18)	1.95 (1.64–2.16)
4	1	2.30 (1.83–2.65)	2.38 (1.88–2.76)
	1	2.20 (1.75–2.52)	
5	1	2.10 (1.7 –2.38)	1.96 (1.62–2.19)
	1		1.15 (0.96–1.27)
6	3	2.33 (1.92–2.64)	1.72 (1.36–1.98)
	0.3	1.91 (1.53–2.20)	2.08 (1.69–2.37)
	0.01	2.00 (1.64–2.25)	2.22 (1.77–2.56)
7	10	2.50 (2.08–2.73)	2.19 (1.84–2.43)
	0.1	2.50 (2.08–2.79)	2.80 (2.34–3.12)

a) LN T cells were stimulated in varying numbers with 10^3 DC and Ag for 60 h. Experiments 1–5 used Ag pulsing and experiments 6 and 7 used soluble Ag.

2.2 LN cells from the HET mice show a kinetic delay in IL-2 production

Our LDA results differ from those of Holländer et al. [7] who found that the frequencies of responding Con A-stimulated cells from the HET mice were 1/3 to 1/2 of those observed for the WT. One difference between our assay systems is the length of time that the cells were stimulated in culture. We routinely assayed the supernatants at 60 h while they assayed them at 30 h. To determine whether this was a critical variable, we froze the plates at different times after stimulation. For these experiments we had individual $CD4^+$ T cells placed in single wells by flow cytometry sorting. As shown in Table 2, plates with HET cells assayed at 24 or 30 h after stimulation gave lower numbers of responding wells than plates with WT cells. This difference greatly diminished at 48 h and completely disappeared by 60 h. Thus, the T cells from the HET mice take longer to generate detect-

Table 2. Production of detectable amounts of IL-2 is delayed for some cells from the HET

	Experiment 1		Experiment 2		Experiment 3	
Time (h)	WT	HET	WT	HET	WT	HET
24–30[a)]	26	10	12	4	9	2
48	37	32	35	36	24	33
60	39	37	40	38	27	32

a) The first time point in experiment 3 was taken at 30 h, while in experiments 1 and 2 it was at 24 h. The number of wells (out of 50) scoring for IL-2 production is shown.

able amounts of IL-2 in this assay. This gives the impression at early time points that the frequency of responding HET cells is lower than it actually is.

2.3 Cells from WT mice generate more IL-2 per cell than cells from HET mice at maximum Ag stimulation

If both alleles at the IL-2 locus are available for transcription, then under nonlimiting conditions, the WT cells should make twice as much IL-2 as cells from the HET, because they can potentially transcribe both alleles, while the HET can only fully transcribe its one good allele. To test this we quantitated the amount of IL-2 produced in each well using proliferation of the IL-2-dependent cell line CTLL as a sensitive bioassay. To ensure that we were comparing the two populations equally, we had single cells from either WT or HET LN sorted and placed into 96-well plates with 10^3 DC and different amounts of peptide Ag.

The results of a typical experiment are shown in Fig. 1. The first thing to note is that approximately one third of the wells failed to show a significant IL-2 response. This is consistent with our LDA results above which showed that approximately 1 in every 2 T cells respond. Similar observations have been made by others in bulk culture [19, 20]. The second observation is the wide range in the level of the response by the wells displaying detectable IL-2 production. This heterogeneity in response spans 30-fold even under optimum stimulation conditions. This suggests that the magnitude of the response of each cell is greatly influenced by other factors such as effects on mRNA stability [6]. Nonetheless, it is possible to calculate the mean level of response for each population.

In the data shown in Fig. 1, at 10 and 3 μM of Ag the geometric mean of the IL-2 U produced by responding cells

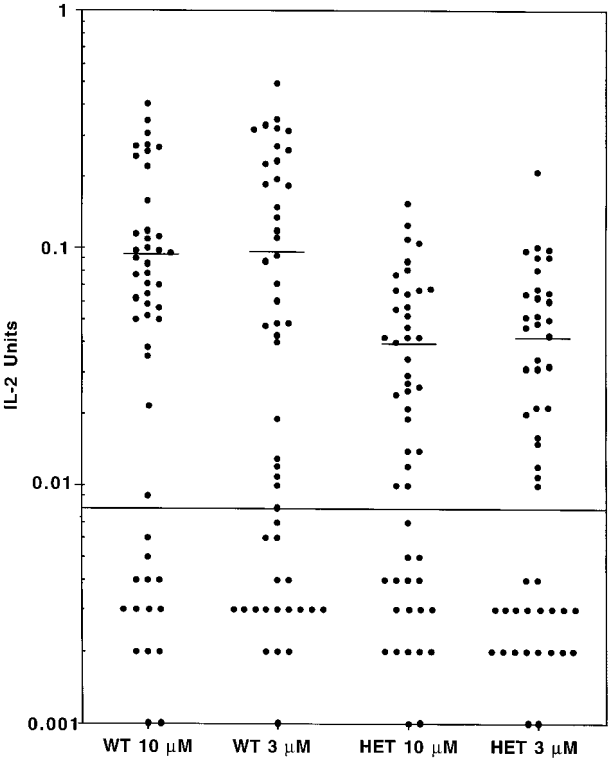

Fig. 1. $CD4^+$ T cells from Tg IL-2 WT and Tg IL-2 HET mice have equal frequencies of IL-2 producers but the latter make only half as much IL-2. Measurement of IL-2 produced by single sorted T cells was carried out as described in Sect. 4.4. Each dot represents the IL-2 produced in a single well. The background cutoff was 0.008 U (the straight line across the graph). The geometric means of the positive wells are shown by the short lines in each column.

of the WT is 0.093x/÷1.19 and 0.096x/÷1.24 U while for the responding HET cells it is 0.040x/÷1.16 and 0.042x/÷1.18 U. In this and other experiments, these concentrations of Ag are on plateau (Fig. 1, data not shown). For six experiments (two done by LDA and four by cell sorting) the amount of IL-2 produced at the plateau values for one cell per well was always significantly greater for the WT than for the HET. The geometric means for all these experiments were 0.148 U for the WT and 0.072 U for the HET, and these were statistically significantly different at $p = 0.002$. Thus, the number of IL-2 producers was equivalent for both the WT and the HET, but the amount of IL-2 made by the WT cells was twice that of the HET at optimal Ag stimulation.

Since the sample size is small in our single-cell analyses and the difference we expected to detect was only two-fold between distributions ranging in values over 30-fold, we decided to verify our observations using another method with a larger sampling size, namely, intracellular

staining to detect IL-2 production. While this technique is less sensitive than LDA, we could accurately determine the percentage of IL-2 producers in response to optimal anti-CD3 and anti-CD28 stimulation as well as the mean fluorescence intensity (MFI), which is proportional to the amount of IL-2 the cells produce. We looked at more than 35 000 cells for each population. At a maximum stimulating dose (20 μg/ml of anti-CD3 in Fig. 2 A), the percent of positive cells was equal for both the WT and the HET (71.2 % *vs.* 72.7 %), although the range of fluorescence intensity is quite broad. The MFI of the positive cells was approximately 1.62 times higher for cells from the WT than cells from the HET (983 *vs.* 607). For four experiments the mean ratio was 1.7. These results confirm our data with the single-cell analysis, that an equal number of cells are capable of making IL-2 in both the WT and the HET, and they are consistent with the activation of both alleles in many of the WT cells. We think that the less than twofold difference in the MFI results from suboptimal activation conditions for T cells in bulk culture. The frequency histogram of the WT responder cells is not symmetrical and appears skewed toward a lower MFI (Fig. 2 A). This can be interpreted as a bimodal distribution containing a small proportion of cells that have activated only one IL-2 allele, while all the other cells have turned on both alleles. When lower amounts of anti-CD3 were used as a stimulus (Fig. 2 B and C), both the percent of positive cells and the amount of IL-2 produced per cell decreased in both the WT and the HET. Under these less than optimal stimulation conditions, the frequency of cells capable of producing IL-2 in the HET was 83 % of that for the WT using 2.5 μg/ml anti-CD3 (Fig. 2 B) and only 74 % at 1.25 μg/ml anti-CD3 (Fig. 2 C). Thus, suboptimal stimulatory conditions in bulk culture can lead to the false impression that the HET has a lower frequency of IL-2-producing cells.

3 Discussion

Several years ago we performed a quantitative analysis of IL-2 production by single cloned Th1 cells stimulated with DC and Ag [6]. A frequency histogram of the responses showed three overlapping peaks. One consisted of cells that had not responded. The other two positive peaks had mean response values of 0.04 and 0.1 U/ml IL-2 per cell. We proposed at that time that the two peaks represented transcription of either one IL-2 allele or both. The data presented in this report extend those observations and argue strongly that the IL-2 gene can be biallelically expressed. We have shown by single-cell analysis that the precursor frequencies of IL-2 production are equivalent for cells from either WT mice or HET mice possessing one defective allele at the IL-2 locus. Furthermore, the amount of IL-2 generated was always less for cells from the HET. When we maximized the stimulation of the T cells with an excess of DC and Ag, the difference was approximately twofold. The data from intracellular staining experiments following Ab activation were in agreement with these results. These observations suggest that the HET cells can only generate IL-2 from their single intact allele, while the WT cells can utilize both alleles.

In the work by Holländer et al. [7], the T cells from the WT also produced twice as much IL-2 as the cells from the HET; however, this was attributed to a difference in the precursor frequencies of IL-2-producing cells. Their fre-

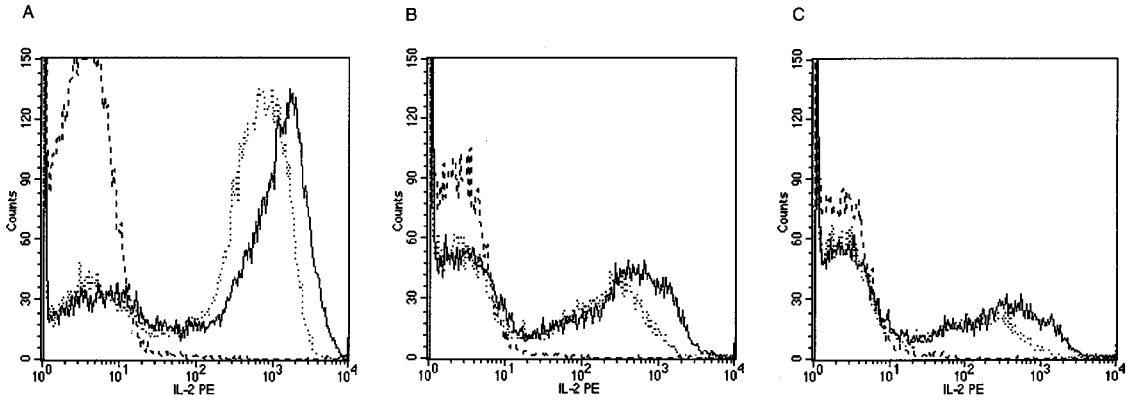

Fig. 2. At optimal doses of Ab, the IL-2 HET T cells make less IL-2 per cell than IL-2 WT T cells, although the frequency of IL-2 producers is the same, as measured by intracellular staining. LN T cells were activated with anti-CD3 (20 μg/ml in A, 2.5 μg/ml in B and 1.25 μg/ml in C) plus anti-CD28. Intracellular staining for IL-2 was done as described in Sect. 4.5. The number of cells displaying a particular amount of IL-2 are represented as a solid line for the WT, a dotted line for the HET and a dashed line for the isotype control.

quencies for cells from the HET were only one third to one half of that from the WT cells. The authors suggested this was due to monoallelic expression of the IL-2 gene, *i.e.* the HET was only able to transcribe from its one good allele in half the cells, whereas the WT could transcribe from either allele no matter which one was active. We also observed a difference in the number of responding wells when we sampled the T cells at 24–30 h. This difference disappears, however, if the HET's cells are given sufficient time to produce IL-2. We think this delay in the kinetics may be due to the structural nature of the KO allele. The inserted *neo* gene only disrupts exon 3 of the IL-2 gene [17]. Thus, despite the fact that IL-2 protein is detected in IL-2 KO mice, it is likely that transcription factors assemble on the intact promoter of the defective allele, allowing it to compete with the intact allele. The kinetic shift suggests that some of the cells are delayed in their IL-2 production because they have initially selected the KO allele, but then they eventually start transcribing the intact allele. Such a situation could arise if the initial concentrations of critical transcription factors (such as AP-1) were limiting [8].

Our observations are consistent with those of Naramura et al. [14] in which IL-2-GFP^{ki} HET mice showed biallelic expression at the IL-2 locus following activation. After stimulation with anti-CD3 and anti-CD28, intracellular staining demonstrated that both IL-2 and GFP were expressed in 32 % of the cells, which represented about half of the responding population. Similarly, it has been shown that expression of the IL-4 gene can be biallelic, although the frequency of biallelism was dependent on the strength of the TCR signal [21]. Increasing Ag stimulation through the TCR resulted in increased production of IL-4 and more cells expressing both alleles. Our data indicate that this is also true for the IL-2 gene. For the intracellular staining, both the percentage of positive cells and the amount of IL-2 made per cell were affected by the strength of signal through the TCR (Fig. 2). We found that it was difficult to activate both IL-2 alleles maximally *in vitro*. We had to use an excess of DC for the single-cell assays. Furthermore, even under optimum conditions, our bulk cultures did not yield a twofold difference in mean levels of IL-2 produced by the WT and the HET cells. Thus, suboptimal TCR stimulation can lead to underestimating the potential of a T cell to produce IL-2 or the amount that it can produce. This might explain the observed use of only one allele per cell in the study by Holländer et al. [7] for *M. musculus* × *M. spretus* F_1 mice [7]. In addition, the responding cells were assayed at 10 h after activation, which might have been too soon to observe transcription from both alleles, because of limitations in the amounts of transcription factors produced. Once an allele is turned on, however, it appears to be stably active even following cell division, possibly because of changes in chromatin structure [20, 22]. Thus, cells suboptimally activated to express only one allele could continue to do so following expansion under the same conditions [23].

We think that the selection of the IL-2 allele that is first utilized occurs by a random mechanism, *i.e.* there is no imprinting or inactivation of one of the alleles during development. What would be the biological purpose of silencing one of the two alleles? This would only give a twofold difference in the total amount of IL-2 made by the cell. It seems that there are other levels of control capable of varying the IL-2 response by a far greater magnitude. In our experiments, the amount of IL-2 made by individual cells varied over 30-fold, even under optimal conditions for transcriptional initiation. Thus, the major way for the T cell to control its level of IL-2 production is through other mechanisms such as post-transcriptional regulation [6].

4 Materials and methods

4.1 Mice

B10.A/SgSn (H-2^a) mice were used as a source of APC in all experiments. T cells were from TCR-5CC7 Tg mice that specifically recognize pigeon cytochrome *c* (PCC) peptide 81–104 bound to I-E$^{a/k}$ [24]. These mice were bred onto a B10.A RAG-2$^{-/-}$ background (5CC7$^{+/+}$, RAG-2$^{-/-}$, IL-2$^{+/+}$). From this strain we derived mice that were heterozygous for a gene-targeted mutation of the IL-2 locus by crossing them with B10.A IL-2$^{+/-}$ (N19) mice carrying a KO allele [17]. We selected mice from the intracross that were 5CC7$^+$, RAG-2$^{-/-}$ and either IL-2$^{+/+}$ (WT) or IL-2$^{+/-}$ (HET). Each mouse was tested for Tg and RAG-2 expression by FCM and for IL-2 genotype by PCR [17]. In a few experiments, Tg homozygotes and heterozygotes were identified by test crosses to outbred mice. Since no correlation was observed between TCR transgene copy number and the pattern of response for IL-2 WT or HET phenotype, the data from all experiments have been pooled for analysis. Mice were used between 2 and 8 months of age with similar results. LN cells from these mice were 92–96 % CD4$^+$.

4.2 Reagents

Click's EHAA : RPMI medium (Biofluids, Rockville, MD) with 10 % heat-inactivated FCS was prepared as described previously [18]. The 81–104 peptide of PCC was synthesized and HPLC purified by the NIAID core facility (NIH, Rockville, MD). Other reagents used were mouse IL-2 (BioSource, Camarillo, CA), [^{3}H] dThd (ICN, Irvine, CA), ionomycin and PMA (Calbiochem-Behring Diagnostics, LaJolla, CA).

4.3 DC preparation

DC were prepared from B10.A splenocytes by an adherence/nonadherence protocol and recovered on a 50 % Percoll gradient [18]. DC were either pulsed with 1 μM Ag during the overnight incubation or Ag was added at the beginning of the culture with the T cells. DC purity was greater than 90 % by staining for CD11c.

4.4 LDA

LN cells were titrated (0.5–32 cells per well) into 96-well round-bottom plates (Falcon #3799) with 10^3 DC and varying concentrations of the PCC peptide in a total of 100 μl. Replicates of 20–100 wells were done at each T cell density and ten negative wells lacking Ag were set up on each plate. In some experiments, single cells from WT or HET LN cells were sorted with a FACStar® (Becton Dickinson, Mountain View, CA) into the plates with DC and Ag. All plates were frozen after 24–60 h of culture. After thawing, 10^3 CTLL-2 cells (ATCC, Rockville, MD) were added to each well. The CTLL cultures were pulsed with 0.1 μCi [^{3}H] dThd (6.7 Ci/mmol) for the last 6 h of a 24-h culture. IL-2 U were determined by comparison to an IL-2 standard. The background was determined from the negative wells on all the plates. Wells were scored positive if the readout was greater than the mean of the negative wells plus 3 SD. The precursor frequencies were estimated by χ^2 minimization [25]. Statistical significance between results obtained with WT and HET cells was determined by a paired two-tailed Student's *t*-test.

4.5 IL-2 intracellular staining

LN cells were cultured with anti-CD3 coated on a plate and soluble anti-CD28 [26]. After 41–43 h, 2 μM ionomycin and 10 ng/ml PMA were added to each culture. Monensin was added 2 h later at 2 μM to prevent the secretion of IL-2. After an additional 2 h of culture, the cells were harvested. FcR were blocked with the mAb 2.4G2 (PharMingen, San Diego, CA) and surface staining was done with TriColor-conjugated anti-Thy 1.2 antibody (Caltag Laboratories, Burlingame, CA). Cells were then fixed with paraformaldehyde and permeabilized using a buffer containing saponin. Intracellular staining was done with PE-conjugated anti-IL-2 or an isotype control (PharMingen, San Diego, CA) and cells were examined on a FACSCan® and analyzed with CellQuest software.

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