Single Human T Cells Stimulated in the Absence of Feeder Cells Transcribe Interleukin-2 and Undergo Long-Term Clonal Growth in Response to Defined Monoclonal Antibodies and Cytokine Stimulation

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The two-signal model of T-cell activation postulates that T lymphocytes require at least two distinct signals for activation. This model has been established with bulk cultures of T cells in which T-cell–T-cell interaction can occur, possibly delivering further unrecognized costimulatory signals. The signal requirements of single T cells for the induction of clonal cell growth or the transcription of cytokines would best be studied in a cell cloning system in the absence of feeder cells; however, such an experimental system has not been reported so far. In this study, we report the long-term cloning of human resting peripheral blood CD4+CD45RO- T cells under feeder cell-free conditions in response to CD3 and CD28 stimulation in the presence of exogenous interleukin-2 (IL-2). Cloning efficiency ranged from 40% to 60% depending on the presence of additional cytokines IL-1 and IL-6. Single-cell polymerase chain reaction showed that transcription of IL-2 occurred in cells stimulated through CD3 plus CD28 alone. T cells grown in response to CD3 plus CD28 plus IL-2 stimulation produced both IL-4 and interferon-γ (IFN-γ) on restimulation (Th0 cells) and could be functionally differentiated into Th1- or Th2-type cells by the addition of IFN-γ or IL-4, respectively, during cell cloning. These data show on the single-cell level a two-signal model of T-cell activation for the transcription of IL-2. In addition, these experiments show that IFN-γ and IL-4 exert their T-cell-differentiating effects directly on the T cell without any further need for antigen-presenting cells. Together, our experiments show the feasibility of a defined long-term clonal cell culture system to study the growth and differentiation of human T lymphocytes.

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THE GROWTH AND differentiation of hematopoietic cells is regulated through soluble and cell-bound signals received through specific cell surface receptors. One of the best studied model systems within the hematopoietic system is the activation response of mature lymphocytes after in vitro stimulation. With these cells, it has been found that cell division and differentiation occur in response to and with strict dependence on clearly definable signals received from the microenvironment.

With T lymphocytes, classical theory postulates that these cells require at least two signals for activation. Signal one (antigen-specific) is elicited by peptide/major histocompatibility complex complexes presented by antigen-presenting cells (APCs) and received through the T-cell receptor (TCR)/CD3 complex. Signal two (antigen-nonspecific) is delivered by APCs, and among the candidate molecules for its receptor is the T-cell antigen CD28. Evidence for this model comes from experiments performed with bulk cultures of T cells, and the interpretation of these data is complicated because T-cell–T-cell interaction both directly as well as through cytokines can still occur in bulk cultures. Therefore, in this system, the formal question remains of whether two signals are sufficient to induce clonal growth of a single T cell or the transcription of interleukin-2 (IL-2).

Clonal growth of T lymphocytes has been studied in cell-cloning systems using feeder cell layers, mitogenic lectins, or monoclonal antibodies (MoAbs) and cytokines. Although such systems have allowed the establishment of T-cell clones of virtually any desired specificity, T-cell differentiation can be studied under these circumstances only to a limited extent, because feeder cell layer-derived signals have been found in the past to be essential for successful long-term clonal T-cell growth, yet were completely undefined. Feeder cell-free T-cell cloning has been attempted before by stimulating murine CD4+ or CD8+ T cells with immobilized CD3 MoAb and IL-2; however, only short-term cell growth has been reported using these conditions.

To test whether signals received through the T-cell antigen receptor and through CD28 are indeed sufficient on a single-cell level to induce activation including the transcription of IL-2 and the induction of long-term human T-cell growth, we established a feeder cell-free cloning system in which only defined stimulatory signals in the form of MoAbs and recombinant cytokines are presented. Using such a system, we report that single CD4+CD45RO– naive T cells grow in response to TCR/CD3 plus CD28 stimulation in the presence of IL-2 and transcribe IL-2 in response to CD3 plus CD28 stimulation alone. Moreover, cells grown under such conditions can functionally differentiate and develop into Th1 or Th2 cells in the presence of either interferon-γ (IFN-γ) or IL-4, respectively, during culture.

MATERIALS AND METHODS

Cell cloning. CD4+CD45RO– T cells obtained from the peripheral blood of healthy volunteers by density gradient centrifugation, nylon wool purification, and negative depletion of CD14+, CD16+, CD19+, CD8+, and CD45RO+ cells (Dynabeads; Dynal Inc, Fort Lee, NJ) were diluted to 20 cells/mL, and 30–50 µL aliquots of this suspension were distributed into 96-well U-bottomed microtiter culture plates. Plates were centrifuged for 10 minutes at 100g, and each well was thoroughly examined with a microscope for the presence of a single cell. Wells containing no cells or more than one cell

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were excluded. Typically, between 18 to 25 wells per 60 wells seeded were appropriate and were stimulated with OKT3-coated (Ortho Diagnostic Systems Inc., Raritan, NJ) rabbit-antimouse Ig (H+L) beads ("CD3beads," 2 to $5 \times 10^4$ beads/well; Bio-Rad, Richmond, CA), with soluble CD28 MoAb Anti-Leu28 (0.5 $\mu$g/mL; Becton Dickinson Immunocytometry Systems, Mountain View, CA), and with IL-1, IL-2, and IL-6 (100 U/mL each) as indicated in RPMI 1640 plus 5% pooled human serum (final volume, 200 $\mu$L). Depending on their growth rate, clones were split between days 14 and 21 and were always maintained in 96-well plates. Usually, the propagation of the clones necessitated splitting and addition of fresh antibody stimulation once a week, whereas fresh medium containing cytokines was supplied also in between every 3 to 4 days. In some experiments, the TCR$\alpha\beta$-specific MoAb BMA031 (Behring, Vienna, Austria) was used instead of OKT3 with similar results. Similarly, we found that Dynal magnetic beads could be used instead of Bio-Rad beads.

Single-cell polymerase chain reaction (PCR). Total RNA from single cells was prepared by adding 200 $\mu$L of 4 mol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate, 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol (2-ME), 0.2 mol/L NaAc (pH 4.2), and 2 $\mu$g carrier tRNA to the wells. Homogenates were extracted with phenol/chloroform. For reverse transcription (RT), dried RNA pellets were incubated with 10 $\mu$L of 2 U Moloney murine leukemia virus (M-MuLV; Boehringer Mannheim, FRG), 1X enzyme buffer, 400 $\mu$mol/L deoxynucleotide triphosphates (dNTPs), and 100 ng each of IL-2-specific primers IL-2A (5' ATGTACAGGATGCAACTCCTGTCTTT3') and IL-2B (5' GTTAGTGATGAGTGTGCTTGGGAC3') for 45 minutes at 37°C. After RT, a two-step PCR with nested primers was performed by adding 90 $\mu$L of a solution containing 10 $\mu$L of DynaZyme buffer, 5 $\mu$L of a mixture of dNTPs (2 mmol/L each), 1 U DynaZyme (Finnzymes Oy, Espoo, Finland), and 100 ng each of outer primers IL-2A and IL-2B and amplification for 20 cycles (1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C (extended 1 second per cycle, final incubation step at 72°C for 1 min.). Three microliters of the first PCR product was added to 47 $\mu$L of 0.5 U DynaZyme, 1X buffer, 200 $\mu$mol/L dNTPs, and 500 ng each of inner primers IL-2C (5' CAGTGCACCTACAGTTCACACAGC3') and IL-2D (5' ATGGTTGCTGTCTCATCAGC3') and was amplified for 35 cycles under the same conditions. TCR typing. Total RNA from individual clones (1x 10$^3$ cells) was isolated, and first-strand cDNA was synthesized from 0.5 pg of RNA with the GeneAmp RNA PCR Kit (Perkin Elmer-Cetus, Norwalk, CT). For each clone, 20 individual PCR amplifications were performed using each of 20 different V$\beta$ primers with a single C$\beta$
primer\textsuperscript{a} for 30 cycles (1 minute at 94°C, 1.5 minutes at 55°C, and 2 minutes at 72°C). The quality of the cDNAs was controlled by amplifying TCR \textit{C}e sequences in parallel.

\textit{Measurement of cytokines}. A total of $2 \times 10^6$ cloned cells was stimulated in 200 \textmu L in triplicates with $2 \times 10^4$ CD3 beads plus IL-2 (100 U/mL) for 2 days. Supernatants were assayed with enzyme-linked immunosorbent assays specific for IL-4 and IFN-\gamma.\textsuperscript{a}

\section*{RESULTS}

\textbf{Single-cell cloning of human T cells under feeder-free culture conditions.} To test whether the combined stimulation of the TCR/CD3 and the CD28 antigen, which results in the efficient stimulation of bulk culture T cells,\textsuperscript{6,11} is also sufficient on the single-cell level to induce clonal growth of resting peripheral blood human T cells, purified CD4\textsuperscript{+}CD45RO\textsuperscript{-} cells were distributed at a frequency of one cell per well into microtiter culture plates and were stimulated with CD3 MoAb-coated beads, a soluble CD28 MoAb, the costimulatory cytokines IL-1 and IL-6, and the T-cell growth factor IL-2 alone or in combinations (Fig 1a). Cell growth lasting for at least 20 days occurred only in wells containing CD3 and CD28 MoAbs and IL-2, suggesting that these signals are sufficient to trigger long-lasting mitogenesis in about 40% of freshly isolated naive human T cells. The addition of IL-1 plus IL-6 increased the cloning efficiency to 62.5%; however, both cytokines could not substitute for CD3, CD28, or IL-2 stimulation (Fig 1a). Figure 1b shows photographs taken from the same well of a culture plate on days 0, 1, 10, and 20 of stimulation illustrating typical appearance of clonal growth under feeder-free cloning conditions. Most of the growing clones could be expanded easily and could be cultured for several months with periodical restimulation using the same agents.

\textbf{Single T cells transcribe IL-2 in response to CD3 plus CD28 stimulation.} Because CD3 plus CD28 stimulation under the same conditions as those used for cloning was found to result in high-level IL-2 production in bulk culture of purified T cells (our unpublished observations), the requirement for additional IL-2 to allow growth of CD3 plus CD28-stimulated single T cells suggested that IL-2 was produced by stimulated cells but rapidly diluted to concentrations too low to allow its autocrine usage. Alternatively, the induction of IL-2 transcription might require further signals, which could be provided in bulk cultures by T-cell--T-cell contact. To distinguish between both possibilities, single CD4\textsuperscript{+}CD45RO\textsuperscript{-} T cells were stimulated through CD3 plus CD28 and IL-2--specific RT, and nested primer PCR was performed after 24 hours. In this experiment 4 of 12 stimulated cells (33%) but none of 12 unstimulated cells transcribed IL-2--specific mRNA ($P < .02$, \textit{\chi\textsuperscript{2}} test; Fig 2). This experiment shows that, at the single cell level, the two signals used are sufficient for the induction of IL-2 transcription in a substantial fraction of freshly isolated T cells. However, it is important to note that the PCR method used allows no quantitative estimate regarding the amount of mRNA produced. Therefore, it is possible that, in the presence of additional costimulatory signals, further enhanced IL-2 transcription would be the result. Nevertheless, our data support the hypothesis that IL-2 is produced by CD3 plus CD28-stimulated cells, but, possibly because of the low cell concentration (1 cell/200 \textmu L) at the beginning of the cloning culture, it becomes too diluted to allow saturation of high-affinity IL-2 receptors.

\textbf{TCR\textsubscript{\textit{\beta}}-chain typing of T-cell clones grown under feeder-free conditions.} To assure monoclonality of our cell cultures, TCR\textsubscript{\textit{\beta}}-chain typing of a randomly selected sample of 13 clones was performed by PCR with a set of 20 different \textit{\textit{\beta}}-specific primers, which together were calculated to cover approximately 95% of the postulated TCR\textsubscript{\textit{\beta}}-chain repertoire.\textsuperscript{5} Unambiguous results showing only one \textit{\textit{\beta}}-family--specific transcript were obtained for 9 clones, and three cell lines were positive with two different \textit{\textit{\beta}}-chain--specific primers (Table 1). Although these results might imply that the latter cultures consisted of a mixture of two cell clones, another possibility might be that T cells can transcribe simultaneously more than one \textit{\textit{\beta}}-chain mRNA, in a manner similar to TCR\textsubscript{\textit{\alpha}} chains.\textsuperscript{12} Finally, one cell clone was negative by PCR \textit{\textit{\beta}}-chain typing, although a TCR\textsubscript{\textit{\alpha}}-chain--specific signal was obtained (not shown), suggesting that it had rearranged a TCR\textsubscript{\textit{\beta}}-chain specificity not recognized by our set of primers.

\begin{table}[h]
\centering
\small
\begin{tabular}{|c|c|}
\hline
\textbf{Clones} & \textbf{\textit{\textit{\beta}}} Family \\
\hline
AS-A2 & V/6  \\
AS-4.8 & V/8  \\
CH-F1 & V/17  \\
RL-A4 & V/6  \\
WH-C1 & V/7  \\
WH-C2 & V/8  \\
WH-C4 & V/7  \\
WH-4.2 & V/18  \\
WH-4.3 & V/1  \\
WH-4.4 & V/4, V/13.1  \\
WH-4.5 & V/5, V/6  \\
AS-4.1 & V/9, V/17  \\
WH-4.1 & NR  \\
\hline
\end{tabular}
\caption{TCR\textsubscript{\textit{\beta}}-chain Typing of 13 Clones by PCR}
\end{table}

Abbreviation: NR, negative results with all \textit{\textit{\beta}} primers.
Table 2. IL-4 and IFN-\(\gamma\) Production by T-Cell Clones Generated With CD3 Plus CD28 Stimulation Plus IL-1, IL-2, and IL-6

<table>
<thead>
<tr>
<th>Clone</th>
<th>IL-4 (pg/mL)</th>
<th>IFN-(\gamma) (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-A2</td>
<td>(158 \pm 145)</td>
<td>(1,870 \pm 853)</td>
</tr>
<tr>
<td>HC-H3</td>
<td>(45 \pm 5)</td>
<td>(170 \pm 93)</td>
</tr>
<tr>
<td>EP-E1</td>
<td>(89 \pm 4)</td>
<td>(178 \pm 19)</td>
</tr>
<tr>
<td>CH-F1</td>
<td>(632 \pm 34)</td>
<td>(2,010 \pm 195)</td>
</tr>
<tr>
<td>RL-A4</td>
<td>(691 \pm 141)</td>
<td>(1,750 \pm 82)</td>
</tr>
<tr>
<td>WH-C1</td>
<td>(353 \pm 9)</td>
<td>(5,750 \pm 1,310)</td>
</tr>
<tr>
<td>WH-C2</td>
<td>(66 \pm 42)</td>
<td>(1,490 \pm 705)</td>
</tr>
<tr>
<td>WH-C3</td>
<td>(251 \pm 66)</td>
<td>(3,940 \pm 1,600)</td>
</tr>
<tr>
<td>WH-C4</td>
<td>(52 \pm 42)</td>
<td>(2,620 \pm 850)</td>
</tr>
<tr>
<td>WH-E2</td>
<td>(107 \pm 76)</td>
<td>(1,790 \pm 188)</td>
</tr>
</tbody>
</table>

Data are shown as the mean \(\pm\) SE of two independent restimulations. Clones were generated and propagated as detailed in Materials and Methods. Restimulation experiments for cytokine production were performed 1 week after the last antibody stimulation of the maintenance cultures as soon as enough cells were available (usually within 6 to 8 weeks after cloning) and 2 to 3 weeks later. Cloned cells were restimulated through CD3 in the presence of IL-2 for 2 days, and supernatants were then analyzed by enzyme-linked immunosorbent assay.

Cytokine pattern of human T-cell clones established in response to CD3 plus CD28 stimulation. Cord blood T cells and naive peripheral blood T cells produce IL-2 and acquire the ability to produce further cytokines only after an activation and IL-2–dependent differentiation step. To test whether our T-cell clones had acquired the ability to produce cytokines typical for differentiated effector cells, cloned cells were restimulated, and the culture supernatant was assayed for IL-4 and IFN-\(\gamma\). Restimulation was performed by stimulating cells through CD3 in the presence of IL-2 without a CD28 MoAb, because earlier data had shown a strict IL-2 dependency of IL-4 and IFN-\(\gamma\) production (Holler et al\(^a\) and data not shown); however, CD28 costimulation had little effect in these restimulation assays (unpublished observations), possibly because of desensitization of the CD28 pathway caused by prolonged incubation with a CD28 MoAb during cell cloning.

Table 2 shows that both IL-4 and IFN-\(\gamma\) were produced, indicating that T-cell differentiation had occurred and that the T-cell clones generated resembled Th0 cells, capable of producing both Th1 and Th2 cytokines. To test whether the presence of additional cytokines during cell cloning would result in functional subdifferentiation, 36 clones were generated in response to CD3 plus CD28 stimulation either in the presence of IL-1, IL-2, and IL-6 alone ("control") or in the presence of additionally added IL-4 or IFN-\(\gamma\). The cytokine pattern of these clones was determined after restimulation, and the ratio of IL-4/IFN-\(\gamma\) was calculated. Figure 3 shows that the addition of either cytokine alone led to a shift in the resulting IL-4/IFN-\(\gamma\) ratio, indicating that T-cell differentiation had taken place, although it is unclear whether this shift reflects changes in relative cytokine production levels within individual cells within clones or whether the proportion of individual cells within the clones producing either cytokine alone was influenced.

Possibly because of the relatively small number of cell clones studied, statistical significance in cytokine ratios was not demonstrable between control clones and clones grown with either cytokine, yet the level of significance was reached between the ratios from clones grown in IL-4 as compared with clones grown in IFN-\(\gamma\), indicating that these sets of cell clones were indeed functionally different (Fig 3). In these experiments, we noted a clear growth inhibitory effect of added IFN-\(\gamma\), resulting in the outgrowth of only a relatively small number of T cells (Fig 3).

DISCUSSION

This study reports the successful long-term cloning of single naive human T cells under feeder cell-free conditions in response to defined MoAb and cytokine stimuli. The present data define minimal signal requirements of naive T cells for the induction of cell growth and for the transcription of IL-2 under well-defined experimental conditions in the absence of uncharacterized conditioned medium or feeder cell layers.

Feeder cell-free T-cell cloning has been attempted before by stimulating murine CD4\(^+\) or CD8\(^+\) T cells with immobilized CD3 MoAb and IL-2 in short-term feeder cell-free limiting dilution assays. In these systems, costimulation with

Fig 3. Cytokine production of 36 T-cell clones grown under different conditions expressed as ratio of IL-4/IFN-\(\gamma\) (picograms per milliliter/picograms per milliliter). Cloning was performed with CD3 plus CD28 stimulation in the presence of IL-1, IL-2, and IL-6 alone (control) or together with IFN-\(\gamma\) (5 ng/mL) or IL-4 (10 ng/mL). Values are the mean of the ratios of two independently performed restimulation experiments. For statistical analysis the Student's t-test was used.
CD4 or CD8 MoAb6 or with IL-1 and IL-67 strongly enhanced the responsiveness of the cells to IL-2. Our data show that TCR/CD3 plus CD28 signaling is sufficient to induce transcription of IL-2 in about 30% of human naive peripheral blood T cells and that, in the presence of exogenously added IL-2, both signals are sufficient for inducing long-lasting clonal cell growth with a cloning efficiency of greater than 40%. This figure corresponds well to the previously estimated 26% of CD4+ cells responding to a stimulation by the immobilized CD3 MoAb 64.1 in the presence of IL-2 in a limiting dilution assay system.16 However, it is important to note that in our culture system CD28 costimulation was an absolute requirement for successful clonal growth, whereas in the previous study no requirement for CD28 costimulation was reported. It is possible that differences in experimental set-up (single cell cloning versus limiting dilution culture) or different reagents (OKT-3 versus 64.1 MoAb) might account for this difference.

TCR/CD3 stimulation results in a well-understood cascade of signal transduction leading to the activation of tyrosine kinases and protein kinase C and the mobilization of free cytoplasmic Ca2+17,18. It has been shown earlier, in the context of MoAb stimulation, that the timing and the amount of CD3 cross-linking is important for the resulting functional response.7,19 Furthermore, different CD3 MoAbs appear to differ in their potential to induce cell cycle progression in a bulk culture system of purified CD4+ T cells in the absence of further costimulation.20 For our stimulation experiments CD3-coated beads were monitored for the amount of loaded OKT3 antibody, and efficient clonal stimulation was observed only if the beads showed a high level of MoAb coating (data not shown). It is possible that maximal CD3 stimulation is necessary in the presence of minimal further costimulation, such as that in the present study. It would be interesting to study whether suboptimal CD3 stimulation (in the presence of CD28 costimulation) could lead to clonal growth if further costimulatory signals were supplied.20

The signal transduction pathway of CD28 seems to involve tyrosine kinases as well and results in an increase in stability of the IL-2 mRNA together with a direct stimulation of IL-2 transcription.21-23 Our data that CD28 costimulation is necessary even in the presence of high amounts of IL-2 for inducing cell growth (Fig 1a) indicate that CD28 stimulation must have further effects on the commitment of stimulated T cells to undergo clonal expansion. In this context, it might be of importance that, in different experimental systems, CD28 stimulation has been found to counteract activation-induced apoptosis in T cells.24,25

For a complete understanding of the principles of lymphocyte activation, it will also be interesting to test whether other signals can substitute for either TCR/CD3 or CD28 signaling under feeder cell-free cloning conditions. In particular, costimulation of T-lymphocyte growth has been reported through an extensive number of other cell surface receptors apart from CD3/TCR and CD28,26 which will be interesting to study under defined cloning conditions. Furthermore, using this experimental approach, one can also test if defined lymphocyte subpopulations differ in their activation threshold on a single-cell basis. Finally, although in our study the feasibility of single-cell PCR as a readout for cytokine transcription is shown only for IL-2, it is likely that the same approach can be extended to most other inducible genes of interest.

With regard to the effects of IL-4 and IFN-γ on T-cell differentiation, earlier data had been obtained in APC-containing cloning systems or bulk cultures of purified T cells or by experiments performed in vivo.29,30 The question of whether the cytokines acted directly on the T-cell or indirectly through accessory cells such as dendritic cells, monocytes, or B cells, on which both IL-4 and IFN-γ have multiple regulatory effects, was formally not resolved by these experiments. The data presented here show that IL-4 and IFN-γ influence T-cell differentiation by acting directly on the T-cell. Similarly, it will be interesting to investigate the effect of other cytokines as well as of purified cell surface molecules on the differentiation of single T cells under similar conditions.

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Single human T cells stimulated in the absence of feeder cells transcribe interleukin-2 and undergo long-term clonal growth in response to defined monoclonal antibodies and cytokine stimulation

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