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RAPID PROLIFERATION OF ACTIVATED LYMPH NODE CD4+ T CELLS IS ACHIEVED BY GREATLY CURTAILING THE DURATION OF GAP PHASES IN CELL CYCLE PROGRESSION

TAKUYA MISHIMA[1], SHOKO TODA[1], YOSHIAKI ANDO, TSUKASA MATSUNAGA and MANABU INOBE*

Laboratory of Human Molecular Genetics, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192 Japan

Abstract: Peripheral T cells are in G0 phase and do not proliferate. When they encounter an antigen, they enter the cell cycle and proliferate in order to initiate an active immune response. Here, we have determined the first two cell cycle times of the leading population in CD4+ T cells stimulated by PMA plus ionomycin in vitro. The first cell cycle began around 10 h after stimulation and took approximately 16 h. Surprisingly, the second cell cycle was extremely rapid and required only 6 h. T cells might have a unique regulatory mechanism to compensate for the shortage of the gap phases in cell cycle progression. This unique feature might constitute a basis for a quick immune response against pathogens by maximizing the rate of proliferation.

Keywords: CD4+ T cells, Cell cycle, G0/G1 transition, Gap phase, In vitro, PMA plus ionomycin, Proliferation, T cell activation

*Author for correspondence: Email: inobem@p.kanazawa-u.ac.jp; phone: +81-76-264-6272; fax: +81-76-234-4427.

[1]These authors contributed equally to this work.

INTRODUCTION

Peripheral T cells exist in a quiescent state [1]. They are capable of proliferation once they encounter an antigen and initiate the cell cycle. There are two major populations of T cells differentiated by the expression of either CD4 or CD8 molecules. Following proliferation, both types of T cell differentiate into effector cells, but acquire different abilities. CD4+ effector T cells produce cytokines, while CD8+ effector T cells show cytotoxicity and play a distinct role in the

immune system. CD4$^+$ and CD8$^+$ T cells are developed from common precursor cells, which express both CD4 and CD8 molecules, and recognize the antigen via a T cell receptor (TCR) in the similar context of the major histocompatibility complex (MHC) molecule. Therefore, they are likely to have a similar regulatory mechanism to drive the cell cycle. Each T cell expresses the TCR specific for a distinct antigen, thereby ensuring that proliferation occurs in the corresponding T cells. The T cells that can respond to a single antigen are very limited [2], so rapid proliferation is required to enable a quick immune response. Several reports have indicated that CD4$^+$ T cells could duplicate as quickly as every 5 to 6 h in vivo [3–5]. Thus, the speed with which T cells complete their cell cycle is extremely rapid. However, the nature of this cycle has yet to be well characterized.

T cell activation and subsequent proliferation are also induced by mitogens such as phorbol 12-myristate 13-acetate (PMA) plus ionomycin in an antigen-independent manner. PMA and ionomycin respectively stimulate protein kinase C (PKC) and Ca^{2+} influx, activating downstream TCR-initiated signaling. Thus, PMA and ionomycin together induce polyclonal T cell activation regardless of its TCR specificity. This solves the difficulty of measuring T cell proliferation due to the small precursor frequency for a certain antigen and allows us to track cell division. However, polyclonal stimulation induces diverse T cell proliferation and does not provide a suitable model to investigate cell cycle progression.

In this study, we focused on the CD4$^+$ T cells, which are the first to enter into each cell cycle phase, and determined minimum times to accomplish a G1, S, G2 and M phase during the first two cell cycles. CD4$^+$ T cells needed approximately 16 h to finish their first cell cycle, but only around 6 h for their second cell cycle. Surprisingly, the G1 and possibly G2 phases for the second cell cycle were almost completely absent, which enabled particularly rapid proliferation. CD4$^+$ T cells might have a unique regulatory mechanism to compensate for the shortage of their gap phases and maximize their rate of proliferation.

MATERIALS AND METHODS

T cell culture and FACS analysis

Lymph nodes (cervical, axillary, brachial, inguinal, mesenteric, periaortic and pancreatic) were isolated from ddY mice aged 8–10 weeks. The mice had been euthanized according to the guidelines of the Animal Care and Use Committee in Kanazawa University. The lymph nodes were crushed to make a single cell suspension [6]. Lymph node cells were labeled for 15 min at 37ºC with 2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) to track mitotic divisions of T cells following stimulation. After 3 washes with PBS containing 5% heat-inactivated fetal bovine serum (FBS), CFSE-labeled lymph node cells were inoculated at 1.5 x 10^6 cells/ml along with the same density of non-labeled spleen cells prepared from the same mice. The cells were co-cultured in RPMI1640 containing 10% FBS supplemented with

gentamycin (10 µg/ml) and stimulated with PMA (2 ng/ml, Sigma-Aldrich) plus ionomycin (20 ng/ml, Sigma-Aldrich). To provide a co-stimulatory signal, anti-CD28 mAb from hybridoma PV1 [7] was added to the culture at a concentration of 2 µg/ml. The cells were harvested from the culture at various times after stimulation and first treated with anti-FcγR II/III mAb from hybridoma 2.4G2 [8] to reduce non-specific Ab binding. $CD4^+$ T cells were then stained with biotin-labeled anti-CD4 mAb (GK1.5, BioLegend) followed by either PE-labeled (eBioscience) or APC-labeled (SouthernBiotech) streptavidin. The CFSE profiles were examined via flow cytometry using FACSverse and FACSuite software (Becton Dickinson).

Detection of G1, S, G2 and M phase initiation

For the transition from G0 to G1, RNA transcription was detected using Pyronin Y (Sigma) staining. The cells harvested were fixed with 4% paraformaldehyde for 15 min and then permeabilized a cell membrane by 0.02% Saponin (ICN Biomedicals) for 20 min. After that, the cells were treated with 100 µg/ml Pyronin Y for 10 min before FACS analysis.

Initiation of the second and third G1 phases was estimated as the appearance of a cell division peak that is recognized by the CFSE dilution. For the S phase, 5-ethynyl-2'-deoxyuridine (EdU, 25 µM) was added to the culture for the last 15 min. It is one of the nucleoside analogs and it can be incorporated into newly synthesized DNA. The cells were then harvested and treated with the Click-iT EdU Flow Cytometory Assay Kit (Invitrogen) according to the manufacturer's instructions.

For G2, EdU was added at the beginning of culture and its concentration was reduced to 25 nM in order to identify a position where the cells have completed DNA replication in the FACS plots. The start of G2 phase was estimated as the time when the cells began to accumulate in this position.

Finally, M phase was evaluated via phosphorylation of histone H3 at a position S28, which occurs from prophase to telophase during mitosis [9, 10]. The cells were fixed with 70% ethanol for 20 min following antibody staining against CD4 and 4% paraformaldehyde crosslinking. The phosphorylated H3 was then stained with Alexa Fluor 647-labeled anti-histone H3 (pS28) mAb (BD Biosciences) before FACS analysis.

RESULTS

Estimation of the transition time from G0 to G1 phase

First, we tried to evaluate the transition from the quiescent state into the active cell cycle. We examined RNA transcription, since it begins after mitogenic stimulation [11]. Peripheral T cells have poorly developed cytoplasm, indicating minimal levels of intra-cellular biosynthesis, so RNA transcription is considered one of the indications of entry into the active cell cycle. RNA transcription is easily detected via FACS analysis using the fluorescent dye, Pyronin Y [12]. $CD4^+$

T cells stimulated by PMA plus ionomycin along with anti-CD28 mAb for 6 h showed a single sharp peak in this assay (Fig. 1A). The cells containing more RNA appeared around 10 h after stimulation and their number gradually increased thereafter (Fig. 1B).
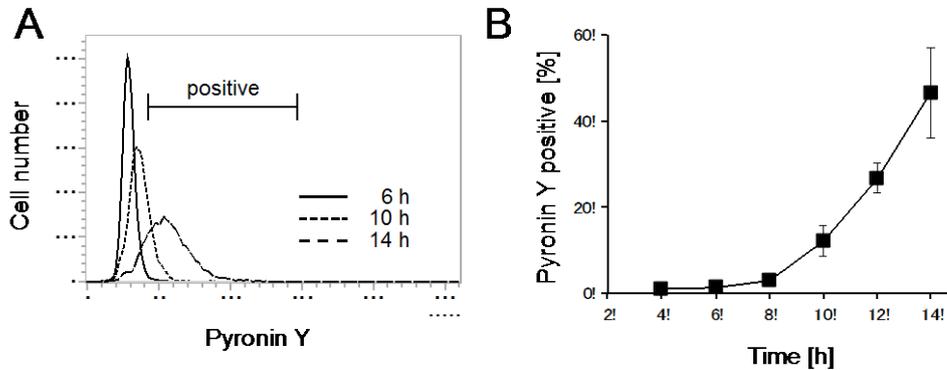
Fig. 1. Activated CD4$^+$ T cells initiate RNA transcription around 10 h after stimulation. A – Lymph node cells were labeled with CFSE and combined with spleen cells. The cells were stimulated by PMA (2 ng/ml) plus ionomycin (20 ng/ml) in the presence of anti-CD28 mAb (2 µg/ml). After 6, 10 or 14 h stimulation, the cells were harvested and stained with Pyronin Y. Representative histograms of the Pyronin Y fluorescence in the CD4$^+$ T cells are shown. B – The mixture of CFSE-labeled lymph node cells and non-labeled spleen cells was stimulated as described in A and harvested at the indicated time points before FACS analysis. Pyronin Y-positive populations were determined and the average ± SEM from three independent experiments are plotted against time.

Estimation of first and second cell cycle times

We next determined the cell cycle time through an evaluation of cell division identified by the CFSE dilution. When a cell undergoes cell division, CFSE distributes two daughter cells equally, resulting in 50% reduction of fluorescence. In fact, an additional peak with nearly half the fluorescence intensity was detected 28 h after stimulation (Fig. 2A). Moreover, the third peak, which was expected to have 25% CFSE, was further detected at 36 h (Fig. 2A). By quantifying the ratio of those second and third peaks, we could estimate the shortest times at which the cells entered into second and third cell cycles – after roughly 26 and 32 h, respectively (Fig. 2B). CD4$^+$ T cells needed approximately 16 h for the first cell cycle. By contrast, just around 6 h was sufficient to go through the second cell cycle. These results suggest that CD4$^+$ T cells dramatically change their cell cycle pattern during the second cell cycle.
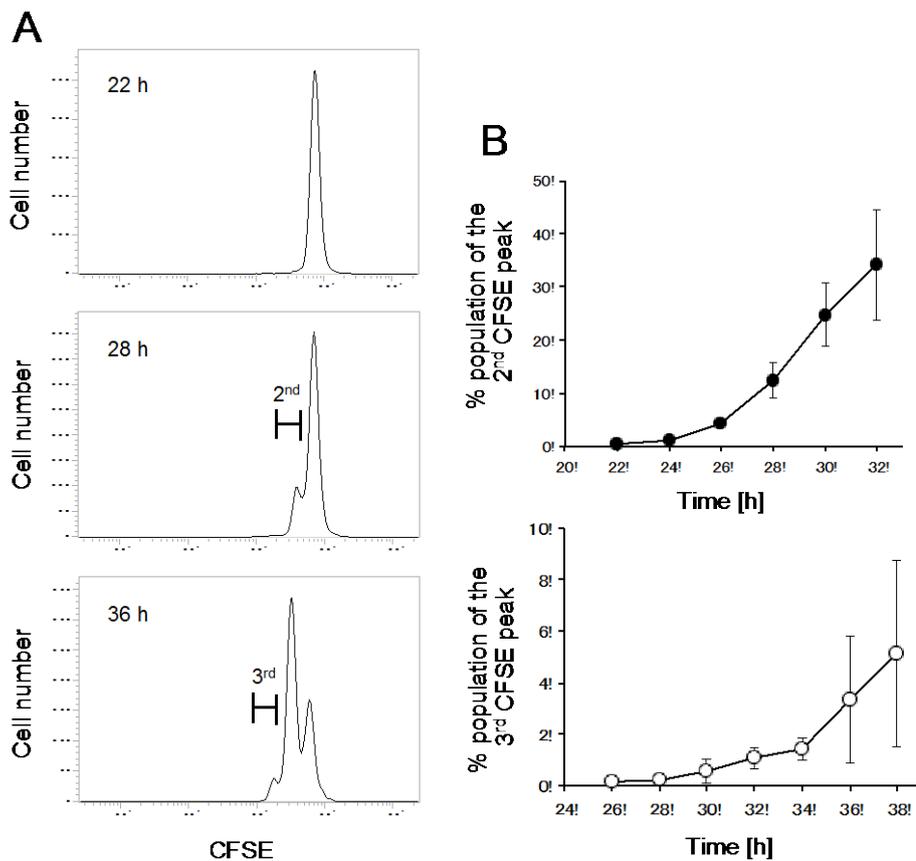
Fig. 2. The first and second division peaks for the CD4$^+$ T cells appear at around 26 and 32 h, respectively. A – Lymph node cells were labeled with CFSE and combined with spleen cells. The cells were stimulated with PMA (2 ng/ml) plus ionomycin (20 ng/ml) in the presence of anti-CD28 mAb (2 µg/ml). After 22, 28 or 36 h stimulation, the CFSE fluorescence of CD4$^+$ T cells was analyzed via FACS and the representative profiles are shown. B – The mixture of CFSE-labeled lymph node cells and non-labeled spleen cells was stimulated as described in A and harvested at the indicated time points before FACS analysis. The numbers of cells in the second or third cell cycles were determined and the averages ± SEM from three independent experiments are plotted against time.

Evaluation of S phase using EdU (a nucleoside analog)

DNA replication during S phase is detected by an incorporation of its precursor analog. EdU is one of the nucleoside analogs used for such purpose [13]. When EdU is added to the culture for a short enough period of time, it was possible to distinguish the CD4$^+$ T cells that were synthesizing DNA (Fig. 3A and C). In combination with the CFSE fluorescence, CD4$^+$ T cells that were in either the first or second S phase could be separately detected. By determining those populations sequentially, we could estimate the time of S phase initiation (Fig. 3B and D). At 14 h, none of the CD4$^+$ T cells had yet initiated DNA synthesis, while those that incorporated EdU began to appear at around 18 h. Similarly, we could also determine the start of the second S phase at around 26 h.
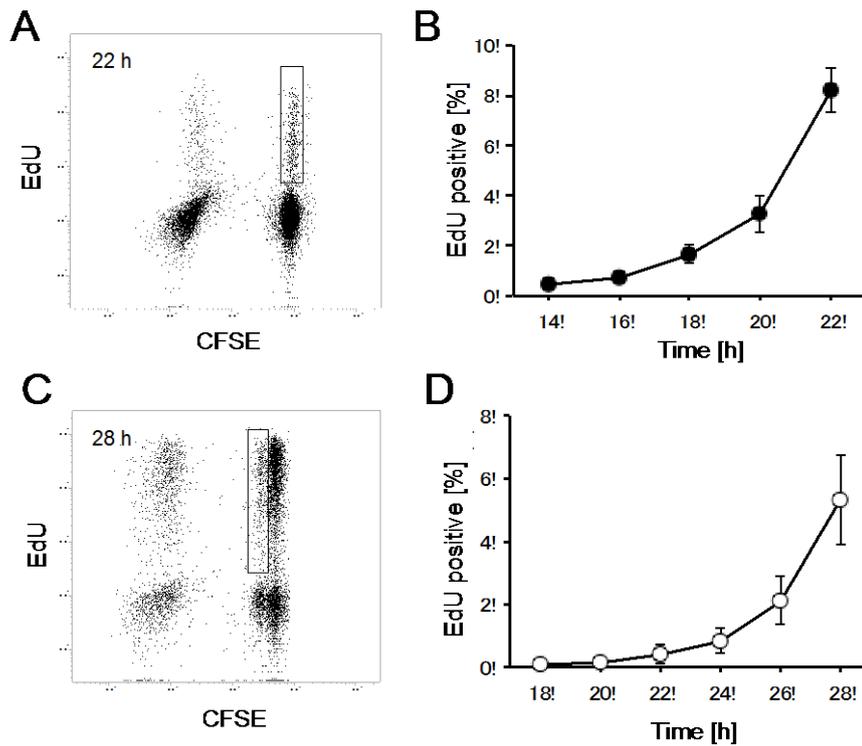
Fig. 3. The first and second S phases of CD4^{+} T cells begin at around 18 and 26 h, respectively. Lymph node cells were labeled with CFSE and combined with spleen cells. The cells were stimulated by PMA (2 ng/ml) plus ionomycin (20 ng/ml) in the presence of anti-CD28 mAb (2 µg/ml). After 22 (A) or 28 h (C) stimulation, the cells were harvested from the culture following an EdU (25 µg/ml) pulse treatment and stained as described in the Materials and Methods section for FACS analysis. The representative dot plots are shown. The cells identified by the gate indicated in the dot plots were EdU-positive and therefore replicating DNA during the last 15 min of culture. EdU-positive populations in either the first (B) or second (D) cell cycle were determined and the averages ± SEM from three independent experiments are plotted against time.

When EdU was added at the start of culture, CD4^{+} T cells continued to take up EdU until the end of the S phase. Thus, we could also estimate the entry into G2 phase using EdU. From sequential analysis, we identified positions where CD4^{+} T cells finish either the first or second round of DNA replication in the FACS plots (Fig. 4). The populations included in these positions were carefully determined, thus providing the estimated times of the first and second G2 phases as approximately 24 and 32 h, respectively (Fig. 4).
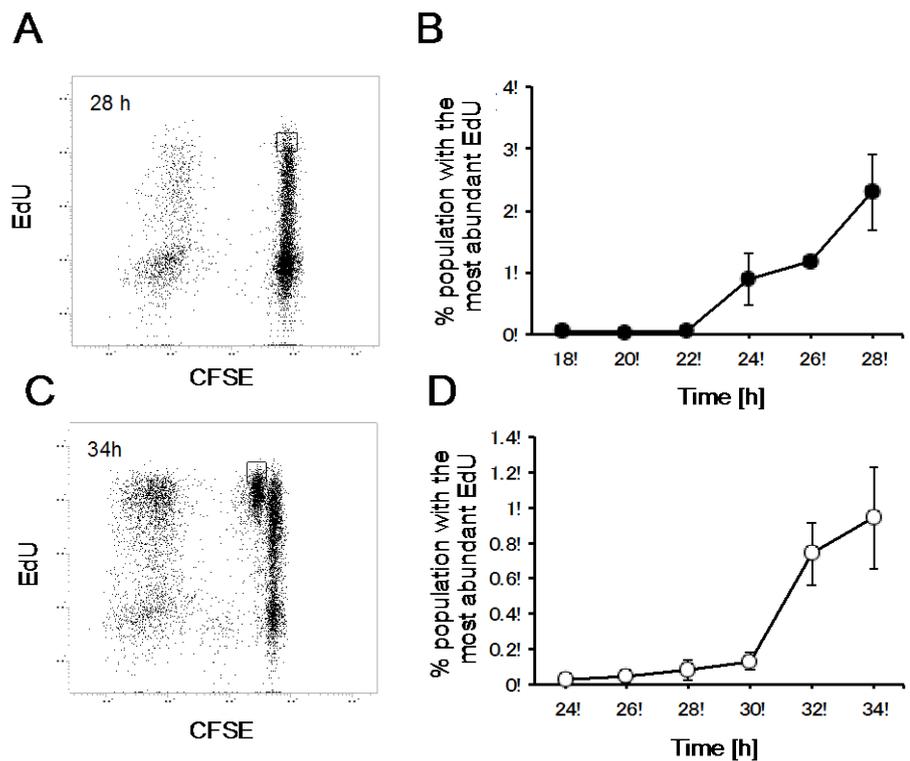
Fig. 4. The first and second G2 phases of CD4^{+} T cells begin at around 24 and 32 h, respectively. Lymph node cells were labeled with CFSE and combined with spleen cells. The cells were stimulated by PMA (2 ng/ml) plus ionomycin (20 ng/ml) in the presence of anti-CD28 mAb (2 µg/ml). EdU (25 ng/ml) was added at the start of culture in this experiment. After 28 (A) or 34 h (C) stimulation, the cells were harvested following an EdU treatment and stained as described in the Materials and Methods section for FACS analysis. The representative dot plots are shown. The cells identified by the gate in the dot plots contained the most abundant EdU and were therefore considered to have finished DNA replication but not yet finished cytokinesis. These populations in either the first (B) or second (D) cell cycle were determined and the averages ± SEM from three independent experiments were plotted against time.

Evaluation of M phase via phosphorylation of histone H3

Histone H3 is phosphorylated at position S28 during M phase [9, 10]. An available mAb binds to only the phosphorylated form of histone H3 at this position, making it a good marker to estimate the time to enter M phase. Simultaneous detection of cell division via CFSE fluorescence identified the populations in either the first or second M phase (Fig. 5). The time course analysis of those populations provided the times of M phase initiation as approximately 26 h for the first and 32 h for the second cell cycle (Fig. 5).
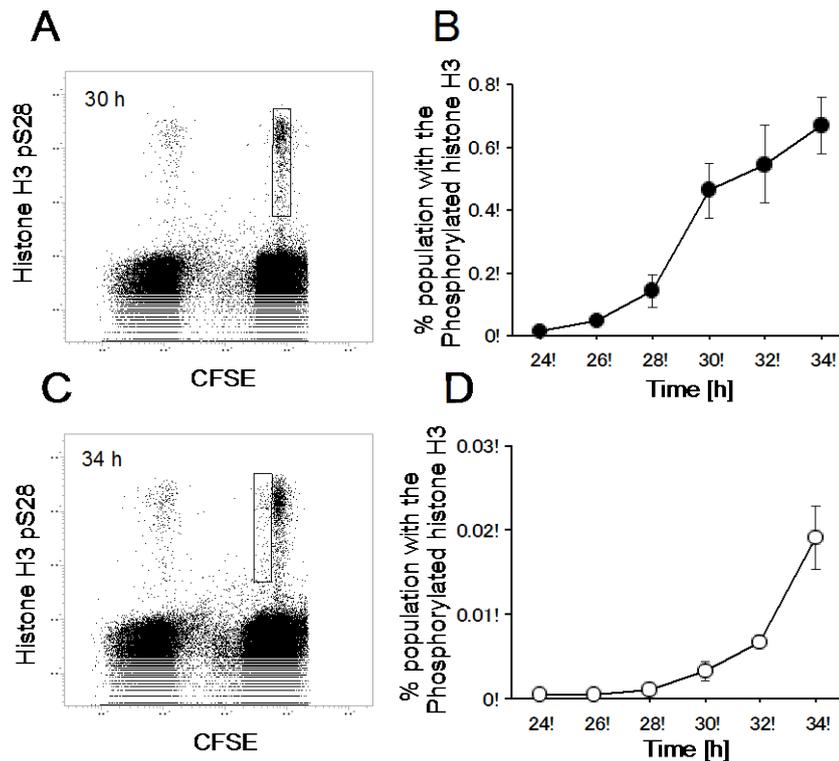
Fig. 5. The first and second M phases of CD4$^+$ T cells begin at around 26 h and 32 h, respectively. Lymph node cells were labeled with CFSE and combined with spleen cells. The cells were stimulated by PMA (2 ng/ml) plus ionomycin (20 ng/ml) in the presence of anti-CD28 mAb (2 µg/ml). After 30 (A) or 34 h (C) stimulation, the cells were harvested from the culture and stained with anti-phosphorylated histone H3 at position S28 mAb for FACS analysis. The representative dot plots are shown. The cells identified by the gate in the dot plots contained the phosphorylated histone H3. These populations in either the first (B) or second (D) cell cycle were determined and the averages ± SEM from four independent experiments were plotted against time.

DISCUSSION

In this study, we have characterized the cell cycle progression of CD4$^+$ T cells during the first two cell divisions after mitotic stimulation. Although T cells show substantial diversity in the proliferation, the sequential analysis of each phase has provided considerable details of the actual cell cycle pattern of this vigorously proliferating population (Fig. 6). T cells need approximately 26 h to accomplish the first cell division, but T cells are in G0 phase initially and considered to be out of the cell cycle for at least 3 to 5 h after stimulation. Lea et al. reported that there is a commitment point in this period and it controls the transition from G0 to G1 phase [1]. Therefore, we wanted to identify the point of entry into the active cell cycle from the quiescent state in order to estimate the duration and timing of the first cell cycle. For this purpose, we chose RNA transcription, which starts around 10 h after stimulation. Several genes, including IL-2, are

important for T cell proliferation. They are directly regulated by the TCR signal. Indeed, IL-2 mRNA transcription begins less than 2 h after stimulation in vivo [14]. Similarly rapid production of IL-2 was also observed in vitro under our own culture conditions (data not shown). Thus, the RNA transcription, which begins around 10 h after stimulation, indicates a second wave of gene expressions and reflects the conversion of the cell state. Based on this criterion, the first cell cycle begins around 10 h after stimulation and takes approximately 16 h. By contrast, the second cell cycle needs only about 6 h, which is consistent with the observations in vivo [3–5]. The evidence indicates that the T cells are able to greatly curtail the duration of gap phases to maximize the rate of proliferation.

In this study, PMA plus ionomycin was used to stimulate T cells to determine the minimal cell cycle time. This polyclonal stimulation might induce an excess amount of growth factor such as IL-2, which may have an intense effect on the gap phases. Since the second cell cycle time seems to be very close to the doubling time estimated from in vivo expansion of the limited population of T cells [3–5], it will be important to determine whether those T cells show a similar cell cycle pattern.
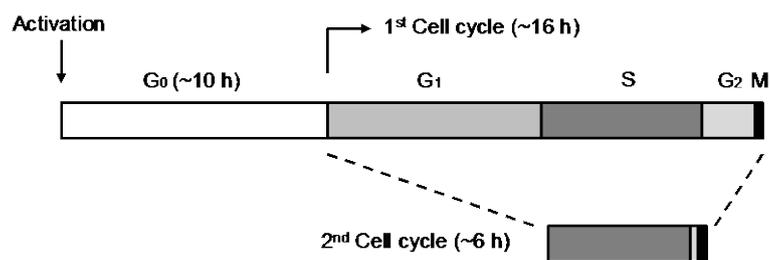
Fig. 6. CD4$^+$ T cells dramatically shorten the cell cycle time by curtailing the duration of G1 and G2 phase in the second cell cycle. The first and second cell cycle times of the leading population in CD4$^+$ T cells following stimulation are shown. The length of each bar reflects the time of the corresponding cell cycle phase.

This cell cycle pattern consisting of only S and M phases resembles "cleavage division", which is the behavior of fertilized eggs during their early cell divisions [15]. Amphibian eggs accumulate a large stockpile during their maturation process. These abundant resources may enable gap phase-free cell cycle progression. Instead, their cell size becomes smaller and smaller every cell division until midblastula transition.

Furthermore, there is no RNA synthesis. Since T cells transcribe RNA and grow in size even during this rapid cell cycle, our data suggest that the T cells are capable of synthesizing sufficient cellular components without gap phases. Like *Xenopus* eggs, T cells are also used as a model for study of cell cycle regulation [16–18]. Thus, our findings about cell cycle entry and progression after T cell activation not only provide the basis of T cell expansion during a course of immune

response, but also suggest a unique regulatory mechanism in cell cycle progression.

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