Rapid G0/1 transition and cell cycle progression in CD8+ T cells compared to CD4+ T cells following in vitro stimulation

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Original Article

Rapid G0/1 transition and cell cycle progression in $CD8^+$ T cells compared to $CD4^+$ T cells following in vitro stimulation

Takuya Mishima, Shotaro Fukaya, Shoko Toda, 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

*Correspondence:
Manabu Inobe, Laboratory of Human Molecular Genetics, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192 Japan; inobem@p.kanazawa-u.ac.jp; Phone: +81-76-264-6272; FAX: +81-76-234-4427.

Running title:
$CD4^+$ versus $CD8^+$ T cell proliferation

Subject Section : Immunology
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List of Abbreviations:
CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T-lymphocyte; FBS, fetal bovine serum; IL-2, Interleukin-2; LN, lymph node; MHC, major histocompatibility complex; mAb, monoclonal antibody; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SPL, spleen; TCR, T cell receptor; 7-AAD, 7-amino-actinomycin D

ABSTRACT

T cell population consists of two major subsets, $CD4^+$ T cells and $CD8^+$ T cells, which can be distinguished by the expression of CD4 or CD8 molecules, respectively. Although they play quite different roles in an immune system, many of their basic cellular processes such as proliferation following stimulation are presumably common. In this study, we have carefully analyzed time course of G0/1 transition as well as cell cycle progression in the two subsets of quiescent T cell population following *in vitro* growth stimulation. We found that $CD8^+$ T cells promote G0/1 transition more rapidly and drive their cell cycle progression faster compared to $CD4^+$ T cells. In addition, expression of CD25 and effects of its blockade revealed that IL-2 is implicated in the rapid progression, but not the earlier G0/1 transition, of $CD8^+$ T cells.

Keywords:

Cell cycle, G0/1 transition, IL-2, T cell proliferation

INTRODUCTION

T cells express a T cell receptor (TCR) by which they recognize an antigen. During development, each T cell progenitor rearranges the genome and expresses the TCR with distinct specificity. Although this diversity of T cells allows them to deal with a wide range of antigens, only a tiny population of T cells can respond to a certain antigen (1). To overcome this insufficiency, they must proliferate to form a bunch of effector T cells. T cells are divided into two major subsets, which are distinguished by the expression of either CD4 or CD8 molecules. Following activation, $CD4^+$ T cells function in producing cytokines, while $CD8^+$ T cells show cytotoxic effects, indicating their different roles in an immune system. $CD4^+$ and $CD8^+$ T cells develop from common progenitor cells and recognize the antigen by the TCR in the similar context of major histocompatibility complex (MHC). Accordingly, it is assumed that both $CD4^+$ and $CD8^+$ T cells employ common mechanisms to regulate most parts of cellular processes including proliferation. T cell proliferation has been traditionally examined by an incorporation of ^{3}H-TdR, which is a precursor of DNA and its usage reflects the DNA synthesis. This technique does not provide much information about the cell cycle progression. In addition, it is not applicable if $CD4^+$ and $CD8^+$ T cells are in the same culture and required to be analyzed separately. Using carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T

cells, Foulds et al. showed that $CD8^+$ T cells proliferate more extensively than $CD4^+$ T cells in both *in vitro* and *in vivo* (2). Thus, the ability to proliferate seems to be different between $CD4^+$ and $CD8^+$ T cells, but their cell cycle progressions remain to be carefully compared.

A certain TCR binds to the antigen MHC complex with intrinsic binding affinity and induces various responses in the activated T cells via distinct intra-cellular signaling both qualitatively and quantitatively (3). On the other hand, intra-cellular signaling cascades following the TCR engagement has been relatively clear (4) and the most part of these pathways can be artificially activated by chemical stimulants. One of such stimulants is a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (5), stimulating protein kinase C (PKC) and Ca^{2+} influx, respectively. Thus, PMA plus ionomycin activates both $CD4^+$ and $CD8^+$ T cells strongly in an antigen-independent manner. Furthermore, for the optimal proliferation, both $CD4^+$ and $CD8^+$ T cells require additional signals derived from costimulatory molecules (6). CD28 is expressed on both T cell subsets and is known to function as a major costimulator (7). A monoclonal antibody (mAb) against CD28 is available and it can provide the T cells sufficient costimulatory signal even under the limited condition of costimulatory ligands.

In this study, we have tried to dissect the proliferation of $CD4^+$ and $CD8^+$ T cells into

the entry and the subsequent progression of the cell cycle and compare their ability following the same strong stimulation by PMA plus ionomycin and anti-CD28 mAb. We found that $CD8^+$ T cells initiate G0/1 transition earlier and drive cell cycle progression faster than $CD4^+$ T cells. This rapid proliferation of $CD8^+$ T cells was partly due to IL-2, since the blockade of IL-2 receptor signaling reduced the proliferation of $CD8^+$ T cells, but not $CD4^+$ T cells, without affecting the G0/1 transition. One of most important role of $CD8^+$ T cells is to deal with a serious threat of virulent viruses. $CD8^+$ T cells might be required to expand quickly compared to $CD4^+$ T cells to provide a rapid containment of viruses vigorously expanding following infection.

MATERIALS AND METHODS

T Cell Culture and CFSE analysis

Lymph nodes (LNs: 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 (8). LN cells were labeled for 15 min at 37˚C with 2 μM 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 LN cells were inoculated at 1.5 x 10^6 cells/ml along with the same density of non-labeled splenocytes 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). In some cases, T cells were stimulated by anti-TCRβ mAb (H57-597, 1 μg/ml, BioLegend) instead. To provide a costimulatory signal, anti-CD28 mAb from hybridoma PV1 (9) 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 (10) to reduce non-specific antibody binding. The cells were then stained with biotin-labeled

anti-CD4 mAb (GK1.5, BioLegend) or anti-CD8 mAb (53-6.7, BioLegend) followed by either PE-labeled (eBioscience) or APC-labeled (SouthernBiotech) streptavidin to distinguish two major T cell populations. The cells were finally treated with 7-amino-actinomycin D (7-AAD, BD Biosciences) or propidium iodide (PI, Sigma) to exclude dead cells from the analysis. The CFSE profile of each population was obtained via flow cytometry using FACSverse (Becton Dickinson) and the mean division number was determined by calculating percent contribution of the initial cohort in each division peak. Initiation of the second and the third cell cycle was estimated by the appearance of the cell division peak that was recognized by the CFSE dilution.

Estimation of the G0/1 transition of quiescent T cells using Pyronin Y staining

In order to determine the transition from G0 to G1, RNA transcription was detected using Pyronin Y (Sigma) staining. The cells were fixed with 4% paraformaldehyde for 15 min and then the cell membrane was permeabilized with 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.

Expression of IL-2 receptor and blockade of IL-2 signaling

Expression of high affinity IL-2 receptor was determined using an antibody against CD25. CD25 is a part of the IL-2 receptor complex and the one that is responsible to increase binding affinity for IL-2 (11). The cells harvested were stained with anti-mouse CD25 mAb (PC61, IMGENEX) followed by Alexa647-labeled anti-Rat IgG(H+L) Antibody (Invitrogen). CD4 or CD8 was also stained as described above and CD25 expression by those T cells was separately analyzed via FACS. This anti-mouse CD25 mAb has been shown to block binding of IL-2 to the receptor (12), thereby inhibits IL-2 signaling. For this purpose, 10 μg/ml of anti-mouse CD25 mAb was added to the culture at the time of inoculation.

Statistics

Statistical tests were performed using Student's independent *t* test. The results were considered to be significant at $p < 0.05$.

RESULTS

The proliferation of $CD8^+$ T cells is greater than that of $CD4^+$ T cells

CFSE is a dye that is widely used to examine cell division. Since it is decreased by half in every cell division, it allows us to evaluate the proliferation by calculating the mean division number. Using this strategy, we examined the proliferation at 48 hr after stimulation *in vitro* to see whether any difference between $CD4^+$ and $CD8^+$ T cells exists (Fig.1). Both T cells were placed in the same culture and stimulated simultaneously, and yet CFSE profiles clearly demonstrated that more cell divisions occurred in $CD8^+$ T cells than in $CD4^+$ T cells (Fig.1a). The mean division number of $CD8^+$ T cells calculated using the CFSE profile was indeed 2-fold higher than that of $CD4^+$ T cells (Fig.1b). This greater proliferation of $CD8^+$ T cells was also observed when they were stimulated by anti-CD3 mAb (2) or anti-TCRβ mAb (Fig.1c), indicating that the stimulation with PMA plus ionomycin correctly reproduces T cell proliferation. Because the T cell population remaining in an undivided state affects the mean division number, we determined the sequential change of the undivided population and the mean division number as well (Fig.2). At 24 hr after stimulation, there was no cell division. Since then, the mean division number gradually increased in both $CD4^+$ and $CD8^+$ T cells and the latter kept higher than the former up to 60 hr after

stimulation (Fig.2a). In association with this increase, the undivided population was gradually decreased, but slightly larger population was always left behind in the $CD4^+$ T cells (Fig.2b). However, most of them were out of this state by 60 hr after stimulation, thus the undivided population was not the critical cause of the difference between $CD4^+$ and $CD8^+$ T cells. In fact, the mean division number determined in the proliferating cell population that excluded non-divided cells in $CD8^+$ T cells showed a greater elevation rate than that in $CD4^+$ T cells (Fig.2c). These results suggest that $CD8^+$ T cells have the ability to drive the cell cycle faster than $CD4^+$ T cells and this property is likely involved in their greater proliferation.

We have studied the G0/1 transition and the cell cycle progression of naive $CD4^+$ T cells following stimulation with PMA plus ionomycin and anti-CD28 mAb (13). Using the same approach, we further examined the difference between $CD4^+$ and $CD8^+$ T cell proliferation. Firstly, we evaluated the entry into G1 from G0 phase by RNA synthesis (Fig.3a and 3b). The cells synthesizing RNA were detected as Pyronin Y positive (Fig.3a) and the minimum time that the first population in $CD4^+$ or $CD8^+$ T cells entered into G1 phase was determined by the increase of this population (Fig.3b). $CD8^+$ T cells initiated RNA synthesis around 9 hr after stimulation, which is approximately one hour earlier than that of $CD4^+$ T cells. Thus, the earlier G0/1 transition is also a part of the

mechanism that causes better proliferation of $CD8^+$ T cells. Secondly, the onset of the second or the third cell cycle of $CD8^+$ T cells was estimated by the appearance of CFSE peak that had half or a quarter of the initial fluorescence intensity (Fig.3c and 3d). From this analysis, we determined the minimum time that $CD8^+$ T cells need to complete the first and second cell cycles and they were approximately 2 and 3 hr shorter than those of $CD4^+$ T cells, respectively. These results suggest that $CD8^+$ T cells are capable of driving each cell cycle in one hour less than the $CD4^+$ T cells (13). This finding is consistent with the result of the proliferation rate determined using the mean division number described above (Fig.2c).

IL-2 participates in the greater cell cycle rate, but not the earlier G0/1 transition, in $CD8^+$ T cells

Next, we investigated the role of IL-2 on the greater proliferation of $CD8^+$ T cells. We first compared expression of CD25, which is a part of a high-affinity IL-2 receptor complex, between $CD4^+$ and $CD8^+$ T cells at 48 hr after stimulation (Fig.4a). Both $CD4^+$ and $CD8^+$ T cells expressed CD25 and their levels were not clearly different. From the role of IL-2 on T cell proliferation (14-17), it should work early after stimulation. Therefore, we examined the expression of CD25 until 48 hr (Fig.4b). The

CD25 expression reached its maximum around 24 hr after stimulation and was significantly higher on $CD8^+$ T cells than on $CD4^+$ T cells at this time point. The higher expression of CD25 on $CD8^+$ T cells was also observed at 12 hr after stimulation, but beyond 24 hr, there was no significant difference between $CD4^+$ and $CD8^+$ T cells. Since $CD8^+$ T cells showed higher expressions of CD25 at earlier time points, we further investigated whether $CD8^+$ T cells begin to express CD25 earlier than $CD4^+$ T cells (Fig.4c). Both $CD4^+$ and $CD8^+$ T cells began to express CD25 as early as 2 hr after stimulation and there was no significant difference between their onsets, while the expression level on $CD8^+$ T cells became clearly higher around 10 hr after stimulation (Fig.4d). Given the fact that T cells enter into the cell cycle around 10 hr after stimulation (Fig.3b), these results suggest that IL-2 seems to participate in the cell cycle progression rather than the earlier G0/1 transition in $CD8^+$ T cells. In order to challenge this hypothesis, we took advantage of anti-CD25 mAb (PC61), which is capable to block binding of IL-2 to the receptor (12). It was added to the culture and the effect on the G0/1 transition of $CD4^+$ or $CD8^+$ T cells was examined (Fig.5). The time of the G0/1 transition was not changed in both T cells, which is consistent with the idea that IL-2 is not responsible for the earlier entry of $CD8^+$ T cells into G1 phase. In contrast, 10 μg/ml of anti-CD25 mAb reduced proliferation of $CD8^+$ T cells, but not $CD4^+$ T

cells, significantly at 48 hr (Fig.6). These results suggest that the cell cycle progression of $CD8^+$ T cells is at least partly dependent on IL-2.

DISCUSSION

In this study, we have characterized the cell cycle progression to determine the cellular basis in the greater proliferation of $CD8^+$ T cells compared to $CD4^+$ T cells. We have found that $CD8^+$ T cells not only drive the cell cycle progression faster, but also enter into the cell cycle state earlier than $CD4^+$ T cells. Foulds et al. suggested that the intrinsic mechanism is involved in the difference between $CD4^+$ and $CD8^+$ T cell proliferation (2). In this regard, the G0/1 transition was not affected by the IL-2 blockade, while it reduced subsequent proliferation in $CD8^+$ T cells. Moreover, these findings also suggest that the T cell proliferation is regulated at multiple levels.

There are memory T cells as well as naive T cells in the LN cells. In this study, we determined the minimum times that T cells initiate the G0/1 transition and drive the first and second cell cycle. Veiga-Fernandes et al. reported that the memory T cells exhibit greater proliferation than the naive T cells and CDK6 is involved in this difference (18). The CDK6 is a regulator of G1 phase progression and already preactivated in the resting memory T cells. Thus, we might compare the responses of memory T cells rather than naive T cells. In addition, CDK6 could be the key molecule involved in the difference between $CD4^+$ and $CD8^+$ T cells. Moreover, in the combined culture of the LN and SPL cells, regulatory cells, which regulate T cell proliferation, are also present. Such

regulatory cells are not just antigen presenting cells, but also Treg cells. Therefore, the difference in the proliferation between $CD4^+$ and $CD8^+$ T cells reported here is under the influence of these regulatory cells.

In the presence of the antibody against the IL-2 receptor, which can block its binding to the IL-2, the proliferation of $CD8^+$ T cells was partially inhibited. In contrast, the same antibody did not affect the proliferation of $CD4^+$ T cells in our experiment. Lowenthal et al. have shown that 20 nM of this antibody almost completely inhibits IL-2 binding, therefore 10 μg/ml (~67 nM) of the antibody used in this study is thought to be sufficient to eliminate the effects of IL-2 (12). Although it has been shown that IL-2 promotes the proliferation of $CD4^+$ T cells (14, 17) as well as $CD8^+$ T cells (14-16), it might not be very important for the $CD4^+$ T cell proliferation (19, 20). Thus, $CD8^+$ T cells are strictly dependent on the IL-2, which is presumably produced by the $CD4^+$ T cells activated in close proximity.

Following activation, $CD8^+$ T cells differentiate into effector cells, namely cytotoxic T-lymphocytes (CTLs), which destroy cells infected with viruses. It has been known that IL-2 has multiple effects on $CD8^+$ T cells during anti-viral immunity. It not only enhances $CD8^+$ T cell proliferation (14-16), but also promotes differentiation toward the CTL (15, 21-23). In order to minimize the collateral damage in our own cells, rapid

expansion of the $CD8^+$ T cell clone is extremely important. Thus, $CD8^+$ T cells enter into the cell cycle earlier and drive the subsequent progression faster than $CD4^+$ T cells in the IL-2 independent and dependent mechanisms, respectively.

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DISCLOSURE

The authors have no conflicts of interest to disclose.

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Figure legends

Fig. 1. $CD8^+$ T cells proliferate better than $CD4^+$ T cells *in vitro*. LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated with PMA (2 ng/ml) plus ionomycin (20 ng/ml) (a,b) or anti-TCRβ mAb (1 μg/ml) (c) in the presence of anti-CD28 mAb (2 μg/ml) for 48 hr. (a) The CFSE profile of $CD4^+$ or $CD8^+$ T cells was separately analyzed via FACS and the representative profile is shown. The number indicated above each peak shows the division number being determined by the intensity of the CFSE fluorescence, which is decreased by half in every cell division. The dotted histogram represents the peak of unstimulated cells. (b) The mean division number was determined as described in the Materials and Methods and the average ± SEM from six independent experiments is shown. (c) The mean division number was determined as in (b) and the average ± SEM from three independent experiments is shown. $*p < 0.05$, significantly different.

Fig. 2. $CD8^+$ T cells leave undivided state earlier and proliferate faster than $CD4^+$ T cells. LN cells were labeled with CFSE and combined with SPL 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). At various times after stimulation, the cells were harvested and the

CFSE fluorescence of $CD4^+$ (■) or $CD8^+$ (○) T cells was separately analyzed via FACS. (a) The mean division number was determined as in Fig.1 and the average ± SEM from five independent experiments is shown. *$p < 0.05$, significantly different. (b) The population remaining in the undivided state was determined and the average ± SEM from five independent experiments except for data at 30 hr, which was performed once, is shown. *$p < 0.05$, significantly different. (c) The mean division number in the population excluding non-divided cells was determined. The average ± SEM from five independent experiments is plotted against time and the slope, which was calculated by fitting points to a linear regression curve, is shown.

Fig. 3. $CD8^+$ T cells initiate G0/1 transition earlier and drive subsequent cell cycle faster than $CD4^+$ T cells. LN cells were labeled with CFSE and combined with SPL 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). (a) The cells were stained by Pyronin Y at 6 or 14 hr after stimulation and $CD4^+$ and $CD8^+$ T cells were analyzed separately. The representative dot plots are shown. The cells in the gate presented in the dot plots are Pyronin Y positive. (b) The Pyronin Y positive population in $CD8^+$ T cells (□) was determined at various time points via FACS as in $CD4^+$ T cells (■) (13) and the

average ± SEM from three independent experiments is plotted against time. (c, d) The population in $CD8^+$ T cells experienced cell division once (○) or twice (◇) was determined at various time points via FACS as in $CD4^+$ T cells (●, ◆) (13) and the average ± SEM from three independent experiments is plotted against time.

Fig. 4. $CD4^+$ and $CD8^+$ T cells begin to express CD25 with the same kinetics, but its amount becomes larger on the latter cells since when they enter into the cell cycle. LN cells were labeled with CFSE and combined with SPL 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). The cells were harvested at 48 hr (a) or various times after stimulation (b-d) and stained with anti-CD25 mAb. CD25 expression on $CD4^+$ or $CD8^+$ T cells was separately analyzed via FACS. (a) The representative FACS profile is shown. The dotted histogram represents the staining of unstimulated cells. (b-d) The MFI (b, d) or the positive population (c) of CD25 was determined in $CD4^+$ (■) or $CD8^+$ (○) T cells and the average ± SEM from three independent experiments is shown. *$p < 0.05$, significantly different.

Fig. 5. IL-2 blockade does not change the time of the G0/1 transition in both $CD4^+$ and

$CD8^+$ T cells. LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated with PMA (2 ng/ml) plus ionomycin (20 ng/ml) along with anti-CD28 mAb (2 μg/ml) in the presence (○, □) or absence (●, ■) of anti-CD25 mAb (10 μg/ml). The Pyronin Y positive population in $CD4^+$ or $CD8^+$ T cells was separately determined at various time points via FACS as in Fig.3a and the average ± SEM from three independent experiments is shown.

Fig. 6. IL-2 blockade attenuates the proliferation of $CD8^+$ T cells, but not $CD4^+$ T cells. LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated with PMA (2 ng/ml) plus ionomycin (20 ng/ml) along with anti-CD28 mAb (2 μg/ml) in the presence or absence of anti-CD25 mAb (10 μg/ml) for 48 hr. The mean division number was determined as in Fig.1 and the average ± SEM from four independent experiments is shown. *$p < 0.05$, significantly different.

Fig.1

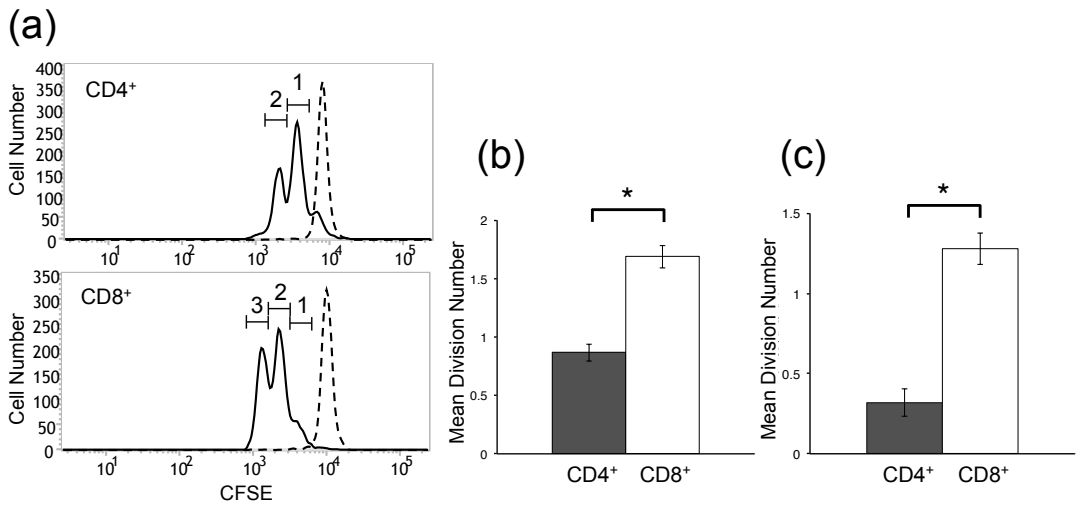
(a)
CD4+
CD8+
Cell Number
CFSE
1
2
3
(b)
(c)
Mean Division Number
CD4+
CD8+
*

Fig.2

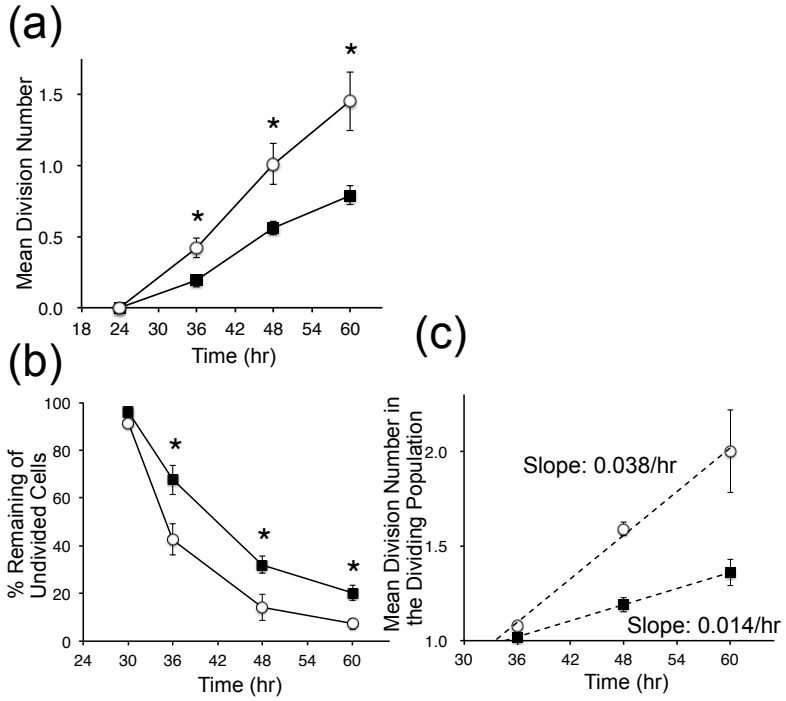
(a)
Mean Division Number
0.0
0.5
1.0
1.5
18 24 30 36 42 48 54 60
Time (hr)
*
*
*
(b)
% Remaining of Undivided Cells
0
20
40
60
80
100
24 30 36 42 48 54 60
Time (hr)
*
*
*
(c)
Mean Division Number in the Dividing Population
1.0
1.5
2.0
30 36 42 48 54 60
Time (hr)
Slope: 0.038/hr
Slope: 0.014/hr

Fig.3

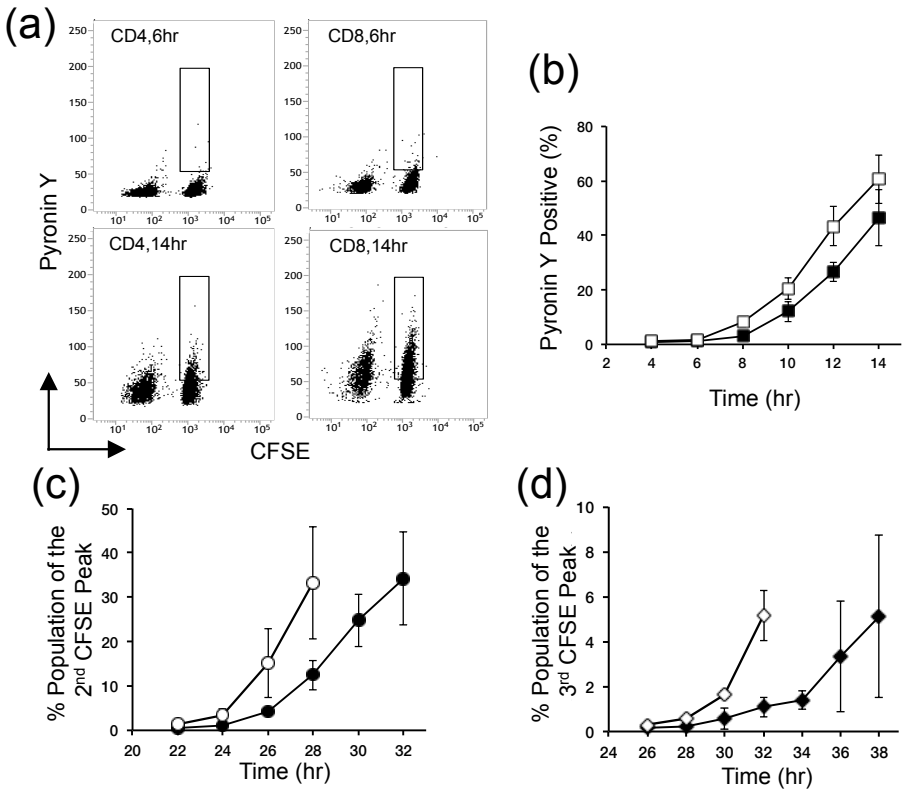
(a)
CD4,6hr
CD8,6hr
CD4,14hr
CD8,14hr
Pyronin Y
CFSE
(b)
Pyronin Y Positive (%)
Time (hr)
(c)
% Population of the 2nd CFSE Peak
Time (hr)
(d)
% Population of the 3rd CFSE Peak
Time (hr)

Fig.4

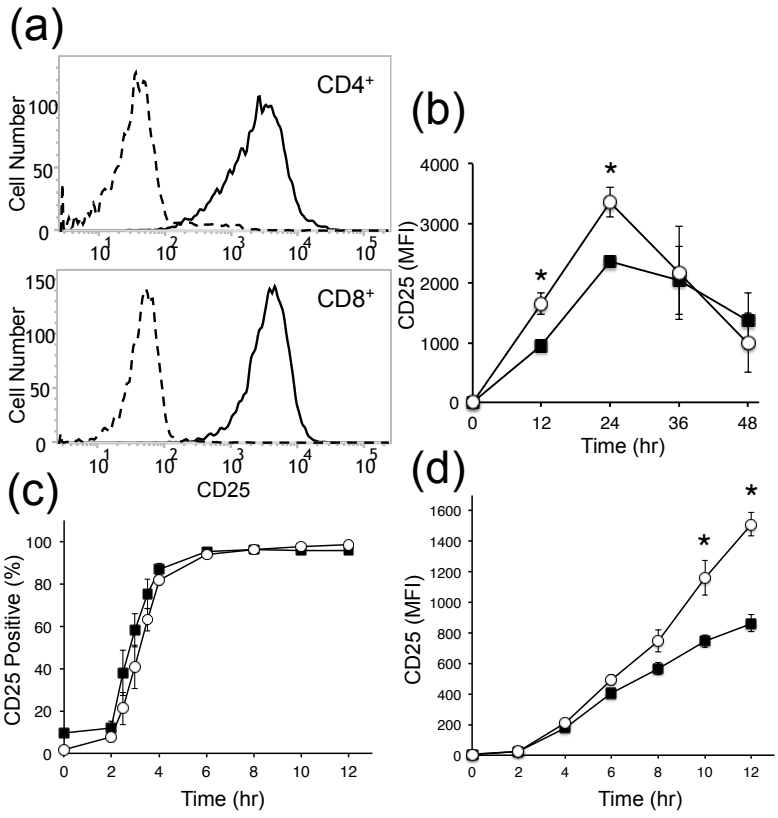
(a)
CD4+
Cell Number
CD8+
Cell Number
CD25
(b)
CD25 (MFI)
Time (hr)
(c)
CD25 Positive (%)
Time (hr)
(d)
CD25 (MFI)
Time (hr)

Fig.5

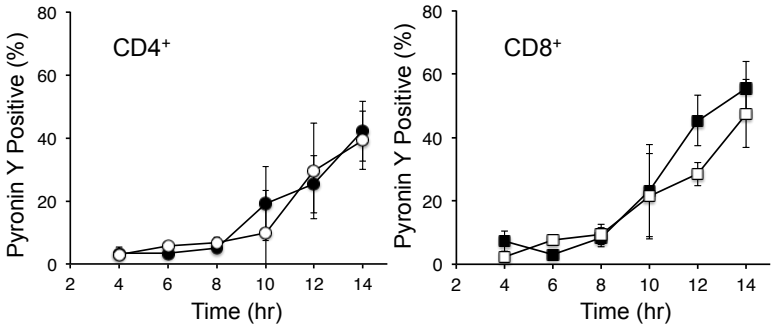

Fig.6