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T Cells in G\textsubscript{1} Provide a Memory-Like Response to Secondary Stimulation

Ivana Munitic, Philip E. Ryan, and Jonathan D. Ashwell\textsuperscript{1}

The commitment of naive T cells to proliferate is a function of the strength and duration of stimuli mediated by the TCR and coreceptors. Ranges of 2–20 h of stimulation have been reported as necessary in vitro. Whether T cells actually experience uninterrupted stimulation for such long periods under physiological conditions is controversial. Here we ask whether commitment to proliferate requires continuous stimulation, or can T cells integrate intermittent periods of stimulation. T cells were stimulated for two short-term (subthreshold) periods (5–7 h) either sequentially or separated by an interval of rest. Naive lymph node T cells were able to integrate interrupted stimulation, even when the duration of rest was as long as 2 days. Furthermore, when short-term-stimulated T cells were separated by density, three populations were observed: low density blasts, intermediate density G\textsubscript{1} cells, and high density G\textsubscript{0} cells. Low density cells progressed to division without further stimulation, whereas G\textsubscript{0} and G\textsubscript{1} cells remained undivided. However, after a period of rest, a second subthreshold stimulation caused the G\textsubscript{1} but not the G\textsubscript{0} fraction to quickly proceed through the cell cycle. We conclude that noncycling T cells in the G\textsubscript{1} phase of the cell cycle remain in a state of readiness for prolonged periods of time, and may represent a population of memory-like effectors capable of responding rapidly to antigenic challenge. The Journal of Immunology, 2005, 174: 4010–4018.

During the adaptive immune response, T cell priming takes place in secondary lymphoid organs, where T cells encounter Ag-bearing dendritic cells (DC)\textsuperscript{2} that have migrated from peripheral tissues. T cells whose TCR recognizes its cognate MHC-peptide ligand become activated and proceed to proliferate and differentiate into effector and memory populations (1–3). The specificity of the immune response is ensured by TCR recognition of only cognate MHC peptide, but because the affinity of this interaction is very low (typically having a micromolar $K_D$) (4, 5), a variety of other molecules are necessary to stabilize and prolong the contact between the T cell and an APC (6). The area of contact has been extensively examined by visualizing T cells bound to APC or MHC-peptide complexes inserted in artificial lipid bilayers (7, 8). These studies have shown that stochastic T cell movement in tissue culture comes to a complete stop upon encountering a surface containing the appropriate MHC peptide (7, 9), and certain T cell molecules redistribute to the site of contact and form an organized structure called a supramolecular activation cluster (7) or immune synapse (10, 11).

The requirement for a highly organized molecular interface at the site of Ag encounter may in part explain the observation that activation of T cells requires a substantial period of contact with the APC. In studies using naive CD4\textsuperscript{+} TCR transgenic cells stimulated with plate-bound peptide-MHC II and anti-CD28 (12) or polyclonal activation with a mixture of anti-CD3 and anti-CD28 (13), at least 20 h was needed to cause T cells to commit to proliferate by 72 h. The required period of stimulation can be shortened using APC, in particular very efficient ones like mature DC or fibroblasts engineered to express high levels of I-E\textsuperscript{K} or ICAM-1, and LFA-1 (14). When pulsed with high concentrations of peptide, DC can induce naive TCR transgenic CD4\textsuperscript{+} T cells to commit to proliferate in 6 h (15). A recent report has shown that even 2 h is enough for DO11.10 TCR transgenic cells to commit after encountering APC pulsed with very high concentrations of antigenic peptide and then being adoptively transferred into congenic hosts or maintained on non-Ag-bearing APC in vitro (16). Naive CD8\textsuperscript{+} cells were found to commit to proliferate within 2–4 h of being stimulated with artificial APC expressing peptide-MHC I, resulting in six to seven cycles of division by 72 h (17). Once committed, T cells will eventually proliferate without further stimulation and multiply at a constant pace, dividing every 4–6 h (6).

Studies using two-photon and confocal technology for visualizing individual T cell:DC interactions in explanted lymph nodes (18-20) found that once the majority of T cells recognize peptide-loaded DC they stop their “random walks” and linger on the DC surface for prolonged periods, up to 15 h (19). However, there are other data indicating that activation and commitment to proliferate can occur even if a T cell encounters APC transiently but repeatedly (21, 22). For example, using a three-dimensional fibrillar collagen matrix system to mimic normal tissue topology, it was observed that TCRαβ transgenic CD4\textsuperscript{+} T cells did not stop moving once a peptide-loaded DC was encountered but continuously rolled on the DC surface, at times completely separating from it, later to interact with the same or another DC (22). In this case, T cell:DC contact was transient, being on average 6–12 min. Despite the fact that the cumulative time of interaction during the first 24 h was on average less than 2 h, and that long-term stable immune synapses were never attained, T cell proliferative capacity was preserved. Another study using two-photon microscopy in lymph nodes of anesthetized animals suggested that T cell priming occurs in three distinct phases (23). An initial phase of 8 h includes multiple repetitive short encounters between T cells and DC. During the subsequent 12 h, T cells slow their movements and form longer lasting contacts with DC. Finally, by the second day, most of the T cells...
resume their rapid motility and begin to proliferate. A recent study has addressed the effect of interrupted stimulation on immune synapse formation in human T cell clones (24). Stimulation was briefly interrupted by the addition of a Src family kinase inhibitor for 10 min intervals, and stimulation was restored by washing it away. Under these circumstances T cell clones could sum intermit- tency activation signals and respond with the same amount of IFN-γ production as cells that were continuously stimulated for the same period of time. The “serial encounter” model supported by these results holds that T cells can receive an activating stimulus even when the interaction with an APC is intermittent (25), and therefore, T cells must be able to “remember” previous transient APC contacts and sum up multiple encounters to proceed to proliferation.

To determine whether the duration of T cell “short-term mem- ory” can be observed in primary resting T cells, we assessed T cell proliferation under conditions of controlled activation. We find that T cells that were stimulated continuously for defined periods of time are comparable in their division potential to cells that were exposed twice to a subthreshold stimulus with an interval of rest, which can be remarkably long (days). The results demonstrate that T cells that progress to, but not beyond, the G1 phase of the cell cycle are rapidly induced to proliferate upon restimulation, and thus have acquired a memory-like functional phenotype.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from Frederick Cancer Research Facility. TCR-C57/7-IRα2g (5C.C7) mice, specific for pigeon/moth cytokeratin e (MCC) presented by I-Ek (26), were purchased from Taconic Farms. B10.A mice were obtained from The Jackson Laboratory.

Cell lines

P13.9 fibroblasts stably expressing L-E4, ICAM-1, and B7.1, and the parental cell line DAP.3 that lacks I-E4, were used as APC (14).

Reagents and Abs

CFSE and Hoescht 33424 were obtained from Molecular Probes. MCC peptide 88–103 was purchased from Macromolecular Resources. Purified anti-mouse CD3eNA/LE (145-2C11), anti-mouse CD28 NA/LE (37.51), and directly conjugated mAbs for CD4 (RM4.5), CD8α (53-6.7), TCRβ (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen. Mouse mAbs to p27Kip1 (F8) and cyclin D3 (H57-597), CD62L (MEL-14), CD44 (IM7), and IL-2 (JES6-5H4) were obtained from BD Pharmingen.

Cell stimulation and proliferation assays

Lymph node T cells were stained for surface markers for 45 min on ice in the presence of cyc/III/ block. Abs and analyzed either with a FACScan or FACSCalibur (BD Biosciences) in a buffer consisting of HBSS containing 0.1% sodium azide and 0.5% BSA (FACS buffer). In some of the experiments with unfixed cells, dead cells were excluded by staining with propidium iodide. DNA and RNA staining was performed with Hoechst 33342 and pyronin Y. All data were analyzed with FlowJo software (Tree Star). Doublet discrimination was applied for cell cycle analysis. For intracellular cytokine staining, cells were cultured alone or stimulated with plate-bound anti-CD3/anti-CD28 for 5 h in the presence of 2 μM monensin. Following surface staining, intracellular staining was performed using the BD CytoFLEX/Cytoperm kit according to the manufacturer’s instructions (BD Biosciences).

Percoll gradient fractionation

Percoll gradient separation was performed as described (30, 31). Briefly, a discontinuous 50:60:70% Percoll gradient was assembled by underlaying. Activated or resting CFSE-labeled cells were resuspended in HBSS and layered onto the gradient. Centrifugation was performed at 3000 rpm for 12 min at 6°C. After centrifugation, cells were retrieved from three interfaces: low density (above 50% step), intermediate density (50–60% interface), and high density (60–70% interface).

SDS-PAGE and Western blotting

Cell lysates were prepared in Tris-Cl 500 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM Na3EDTA, 2 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 10 μM leupeptin) and loaded (15 μg protein/lane) on 15% SDS-PAGE gels. Immunoblotting was performed in TBS (pH 8) with 1% milk and washed in TBS with 0.1% Tween 20. Blots were incubated with HRP-labeled secondary anti-mouse IgG and developed with SuperSignal West Dura Enhancer (Pierce).

Results

Discontinuous stimulation is similar to continuous stimulation in driving naive T cell proliferation

We assessed the minimum time needed in our hands to cause rest- ing murine T cells to proliferate when stimulated with plate-bound anti-CD3 and anti-CD28 Abs. Stimulation was stopped by transferring cells to uncoated plates (12, 27, 32), and proliferation was assessed 2–4 days later by CFSE dilution (33). In general, 10–14 h of continuous stimulation resulted in most of the cells being equally distributed into four to five CFSE peaks by 3 days (below and data not shown). Having established minimum continuous

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stimulation periods required for proliferation, discontinuous periods of stimulation were used to test whether T cells can be integrate interrupted signals over time. Cells were stimulated for 7 h in Ab-coated wells, and then either transferred to another set of Ab-coated wells (S7/S7) or to uncoated wells for 7 h (depicted in Fig. 1A). One set of cells in uncoated wells was incubated without further stimulation until analysis (S7), while another was subjected to one more 7-h period of stimulation before finally being returned to uncoated wells (S7/07/S7). To allow the comparison of groups whose final stimulation ended at different times, proliferation was assessed at several analyses that were each offset by 7 h. Sorted naive CD4+ (CD44low) cells (Fig. 1B) were analyzed after 62 h (Fig. 1C, upper row). Naive CD4+ cells stimulated for just 7 h showed only a small amount of proliferation, whereas cells stimulated for two immediately consecutive 7-h periods (S7/S7) proliferated well. Strikingly, cells that were rested for 7 h between the two 7-h periods of stimulation proliferated to a similar extent as those whose stimulation was continuous. CD4+ T cells that were restimulated after 14 h of rest had divided to a lesser extent at 62 h, but if analyzed 7 h later (69 h) proliferated as well as the cells that received their final stimulation earlier (compare S7/07/S7 at 62 h to S7/014/S7 at 69 h). When the time after the final stimulation is taken into account, the percentage of cells from the initial population that underwent one or more divisions was comparable between cells receiving continuous and those receiving discontinuous stimulation (Fig. 1D). Sorted naive CD8+ cells proliferated more poorly than CD4+ cells due to the lower levels of IL-2 they produce. Nevertheless discontinuously stimulated cells proliferated as well as continuously stimulated cells (data not shown). Therefore, naive T cells can recall a subthreshold period of stimulation hours after its termination.

Inhibition of Src family kinases during rest does not prevent recollection of previous subthreshold stimulation

To ensure that a low level of T cell signaling did not persist during the interval of rest, proximal TCR-mediated signaling was interrupted during this period with the Src family kinase inhibitor PP2 (34, 35). PP2 was added to the cells at the time of their transfer from Ab-coated to uncoated wells, and was removed by washing when the cells were returned to Ab-coated plates. As expected, T cells stimulated for as long as 26 h in the presence of PP2 did not proliferate when analyzed 41 h after removing PP2 (Fig. 2A). CD4+ T cells stimulated for 5 h only (S5) did not proliferate (Fig. 2B). Cells that were stimulated for 5 h, rested for 20 h, and then restimulated for 5 h, in contrast, proliferated extensively by 67 h. The inclusion of PP2 during the 20-h rest period had no effect on the extent to which the restimulated cells proliferated. This result confirms the finding that subthreshold stimulation is “remembered” by T cells after prolonged periods in the absence of TCR signaling.

T cells integrate discontinuous stimulation with physiologic ligand

To determine whether integrated responses to discontinuous stimulation occur with a physiologic ligand, APC were used to present cognate MHC-peptide to T cells of known Ag-specificity. T cells were obtained from 5C.C7 mice, which are transgenic for an αβ TCR recognizing MCC_{88,103} in the context of I-Ek (26). The mice were also deficient in RAG-2, ensuring that all of the T cells, the large majority of which are CD4+, bore the transgenic TCR. The fibroblast cell line P13.9, which stably expresses high levels of I-Ek and the costimulatory molecules ICAM-1 and B7.1 (14, 36), or its I-Ek-negative parental cell line DAP.3, was used as APC. The APC were allowed to phagocytose magnetic beads before their use so that they could be efficiently removed from the T cells by magnetic separation. MCC_{88,103} peptide-pulsed control DAP.3 fibroblasts were cultured with 5C.C7 T cells for up to 18 h, resulting in only a small number of cells that had divided by 67–73 h, thereby providing background values (Fig. 3). Stimulation with Ag-pulsed P13.9 cells for 6 h resulted in negligible T cell proliferation, but continuous stimulation for 12 h was very effective in inducing T cell proliferation. When the two periods of stimulation

**FIGURE 1.** Sorted naive CD4+ cells are able to integrate discontinuous stimulation. A, Schematic time-line of stimulation/restimulation experiments. B, Purified naive (CD44low) CD4+ lymph node T cells were obtained by sorting. The CD44 expression profile of total (unfilled histogram) and sorted (filled histogram) CD4+ cells is shown. C, Cells were stimulated on anti-CD3/anti-CD28-coated plates for the indicated periods of time (A) and analyzed by flow cytometry for CFSE dilution at 62 and 69 h after the beginning of stimulation. Cells were continuously stimulated (S7 and S7/S7) or restimulated after 7- and 14-h periods of rest (S7/07/S7 and S7/014/S7). The diagonal lines indicate cells that were analyzed at the same time after the last stimulation ended. D, The percent of cells from the original population that had divided was calculated with FlowJo software. The data show the fraction of cells in the original population that had divided by 48 (□) or 55 h (■) after stimulation had ceased, and were derived from the data obtained 62, 69, and 76 h (the first two shown in C) after the experiment had begun. Representative data from one of three experiments is shown.
some low density cells (blasts) were recovered, as well as 18 h of rest were reflected in their forward vs side scatter after Percoll fractionation (or 63 h from the beginning of the experiment). All populations were analyzed 40 h after subthreshold stimulation separated by periods of rest.

Intermediate but not high density cells are responsive to restimulation after rest

The progression of T cells from a resting to a proliferative state requires the movement from the G0 phase of the cell cycle to G1, and then through S and G2/M. Cell cycle progression involves blast transformation, characterized by an increase in new RNA and protein synthesis and cell size, and a corresponding decrease in cell density. To determine the state of the T cells after subthreshold periods of stimulation, CFSE-labeled resting T cells were either incubated in uncoated wells or stimulated for 5 h in anti-CD3/anti-CD28-coated wells and then transferred to uncoated wells. Both groups were cultured for a total of 23 h, after which time the cells were separated on a discontinuous density gradient consisting of 50, 60, and 70% Percoll; steps that have previously been extensively used to separate resting from activated B and T cells (30, 31). When unstimulated cells were separated after 23 h of culture virtually no viable cells were recovered from the low density layer, the large majority being of high density (Fig. 4A, upper row). There were some apoptotic cells (low forward scatter, high side scatter) present in all fractions after the overnight culture. The densities of the different fractions of cells after 5 h of stimulation and 18 h of rest were reflected in their forward vs side scatter profiles. Some low density cells (blasts) were recovered, as well as a large number of cells with high and intermediate density (Fig. 4A, lower row).

To determine the proliferative capacity of the different density populations, one-half of each fraction was left untreated and the other was stimulated for 5 h. All populations were analyzed 40 h after Percoll fractionation (or 63 h from the beginning of the experiment). T cells that had never been stimulated (0/0) or that had received only the second 5 h stimulation (0/S) never divided (insufficient numbers of low density cells were recovered under these conditions for analysis) (Fig. 4B). A substantial number of the low density blasts from the S5/0 group proliferated even when no second stimulus was applied, and virtually all underwent division after receiving a second 5-h period of activation. The high density cells did not commit to proliferate whether or not they received a second period of activation, and thus reflect the population that did not remember the initial subthreshold stimulation. The most interesting group was that of the intermediate density cells. If they received only one 5-h period of stimulation the cells did not progress into division. However, a second 5-h stimulation caused the majority of them to proceed through the cell cycle by 40 h. Therefore, the intermediate density population comprises cells that have not been adequately stimulated to divide after one stimulus, but can sum two discontinuous periods of stimulation and respond with vigorous proliferation.

To determine to what extent the rapid and robust response of intermediate density cells to restimulation might be due to the presence of preexisting memory T cells, similar experiments were performed with CD4+ cells that had been depleted of CD44high (i.e., memory) T cells. As above, cells that had been rested for 23 h before Percoll density separation did not proceed to proliferate, whether or not they received a 6-h period of stimulation before analysis (Fig. 4C). As opposed to unfractionated CD4+ T cells, naive (CD44low) CD4+ cells stimulated for 6 h and then recovered in the low density fraction underwent only a small amount of division, although they proliferated robustly when given a second
percentage of cells recovered in the different Percoll fractions. The Table indicates the separated Percoll density gradient. Forward and side-scatter of unseparated fractionation, cells were left untreated or subjected to a second 5-h period of stimulation, and proliferation was assessed 40 h after fractionation. Only a few high density T cells proliferated after a second stimulation, which may represent a small number of contaminating intermediate density cells. In any case, the results clearly show that naive T cells of intermediate density can integrate two subthreshold periods of stimulation separated by at least 18 h.

Intermediate density cells are in the $G_1$ phase and rapidly progress through the cell cycle upon restimulation

The density of T cells after activation decreases as they enter the cell cycle, with cells in $G_0$ being the densest. One can assign a cell to a particular phase of the cell cycle based upon its RNA and DNA content. DNA content is in direct proportion to cell’s metabolic potential, as the largest contributor is ribosomal RNA (37). RNA levels increase in a continuum from the $G_0$ phase to dividing cells. $G_0$ cells have 2N DNA (where N = haploid) and the lowest RNA levels, $G_1$ cells have 2N DNA, $S$ cells have between 2N and 4N DNA, and $G_2/M$ cells have 4N DNA and high RNA levels (28). DNA and RNA levels can be determined simultaneously by using Hoechst 33342 and pyronin Y, respectively (28).

Following primary stimulation of purified CD4+ T cells by plate-bound anti-CD3/anti-CD28 and 18 h rest (S5/018), cell cycle status of the different populations recovered from the Percoll density gradient was determined by measuring RNA/DNA content. Unfractionated unstimulated cells were almost entirely in $G_0$, as evidenced by 2N DNA and few cells with more than baseline RNA levels (Fig. 5A). To determine the kinetics of cell cycle recruitment, S5/018 cells were either analyzed immediately after fractionation or subjected to continuous stimulation for 15 or 25 h (S5/018-Percoll/S15 and S5/018-Percoll/S25). Immediately after fractionation, the low density population was composed almost entirely of cells that had progressed past $G_0$ (Fig. 5B), with cells of intermediate to high RNA content and >2N DNA (i.e., in $S$ or $G_2/M$). In contrast, there were almost no cells in the intermediate or high density populations that had >2N DNA. What distinguished the intermediate from the high density cells was their RNA content, which was elevated in the former. Therefore, we conclude that the intermediate density cells are largely in $G_1$, whereas the high density cells are in $G_0$. After 15 or 25 h of continuous secondary stimulation, the large majority of cells in the low density fraction were actively cycling. In contrast, after 15 h of continuous restimulation high density cells had just begun to enter $G_1$. Only after 25 h of restimulation did a substantial number of high density cells progress into $G_1$ (Fig. 5B) and go on to divide by 48 h (data not shown), demonstrating that these cells are not a “dead-end” population. Importantly, intermediate density cells accumulated large amounts of RNA much earlier, and by 25 h many cells were in $S + G_2/M$.

Cell cycle analysis was performed on the different density fractions that had received one or two subthreshold (5 h) periods of stimulation (Fig. 5C). Cells in all three density populations retained their cell cycle phenotypes when simply cultured for 25 h after Percoll separation, although the low density cells showed some evidence of becoming less active (compare with Fig. 5B). Notably, the majority of intermediate density $G_1$ cells that received a second subthreshold period of stimulation moved further into the cell cycle, with many cells in late $G_1$, $S$, and $G_2/M$. The same treatment caused a small percentage of high density $G_0$ cells to progress into early $G_1$, but the majority remained in $G_0$. Therefore, both intermediate and high density fractions were able to respond to a secondary stimulus, but only the intermediate density fraction was able to respond to 5 h of restimulation by passing through the whole cell cycle.

FIGURE 4. Intermediate but not high density CD4+ cells are responsive to restimulation after rest. A, Purified lymph node T cells were left untreated or stimulated for 5 h on anti-CD3/anti-CD28-coated wells, followed by 18 h of rest. Gated CD4+ cells are shown. At 23 h, cells were separated Percoll density gradient. Forward and side-scatter of unseparated and Percoll density separated cells are shown. The Table indicates the percentage of cells recovered in the different Percoll fractions. B, Following fractionation, cells were left untreated or subjected to a second 5-h period of stimulation, and proliferation was assessed 40 h after fractionation. C, As in B, except that naïve (CD44low) CD4+ cells were used and the period of stimulation/restimulation was 6 h. CFSE dilution is shown 45 h after fractionation. One representative experiment of three is shown.
T cells can remain in G1 and respond to subthreshold stimulation for at least 2 days.

Having observed that T cells were able to recall previous stimulation at least for 20 h, experiments were performed to test the maximum duration of reset permissible (Fig. 6). After a 5-h subthreshold stimulation, T cells were rested for up to 46 h and re-stimulated another 5 h. Cells that received only one period of stimulation divided very little by 74–92 h. Samples that were rested for 20–25 h before restimulation, had divided extensively by 74 h, with a similar division profile observed at 92 h, indicating that by 72 h they had divided to their full potential. Notably, cells that were rested by periods as long as 46 h underwent some division by 74 h, but had “caught up” with the S5/020/S5 cells by 92 h. It was not possible to analyze cells rested for >2 days because of the increasing amounts of cell death observed in prolonged in vitro culture. Therefore, cells can remain in a responsive state for at least 2 days after the initial subthreshold stimulation is withdrawn.

G1 but not G0 T cells produce IL-2 rapidly upon restimulation

To determine whether other activation-induced T cell responses are enhanced in G1 T cells, IL-2 production was measured. As a control, CD4 T cells were cultured for 23 h without stimulation and then stimulated for 5 h in the presence of monensin (to prevent cytokine secretion) and stained for intracellular IL-2 (38); almost no IL-2 cells were detectable (Fig. 7). In the experimental group, cells were stimulated for 4 h (S4) and then rested for 19 h, at which time they were Percoll-fractionated and rested or restimulated for 5 h in the presence of monensin. Cells that did not receive the second round of stimulation were IL-2 . In contrast, after 5 h of restimulation all fractions contained some IL-2 cells. Notably, the number of IL-2 cells in the intermediate density G1 population was similar to that in low density cells, and 3- to 4-fold greater than in the high density G0 fraction. Thus, G1 T cells can rapidly respond to secondary stimulation not just by proliferating, but also by producing IL-2.

Rapidly responsive G1 T cells are CD44intCD62Lhigh and have high cyclin D3 and low p27Kip1 expression

The expression of activation/memory markers on CD4 T cell populations of different density following a subthreshold period of stimulation was assessed. Purified CD4 T cells were cultured without any stimulation for 46 h had high L-selectin (CD62L) and low CD44 expression (Fig. 8A, upper row). Cells that received 46 h of continuous stimulation with immobilized anti-CD3/anti-CD28 down-regulated CD62L and up-regulated CD44. The same initial CD4 T cells were subjected to 5 h of subthreshold stimulation, rested for 18 h, density fractionated, and incubated for additional 23 h in the absence of further stimulation (a total culture time of 46 h). Unlike continuously activated cells, CD62L levels remained high in all fractions, although it was slightly lower in the low density cells (blasts). CD44 levels were more heterogeneous,
being high on low density cells (although less than that for continuously activated cells; compare with Fig. 8, upper row), intermediate on the intermediate density (G1) fraction, and only slightly increased above unstimulated levels on high density (G0) cells (Fig. 8A, lower row). Therefore, the rapidly responsive G1 T cell population is CD44\textsuperscript{low}CD62L\textsuperscript{high}.

The rapid recruitment of memory T cells to divide has been ascribed to faster advancement through the G1/S-phase transition due to high cyclin D3 (G1/S progression factor) and low p27\textsuperscript{kip1} (G1 cell cycle inhibitor) expression (39, 40). We compared cyclin D3 levels in unstimulated and continuously stimulated purified native T cells at 23 h to subthreshold-stimulated fractions (S4) obtained by Percoll gradient at 23 h (Fig. 8B). Cyclin D3 expression was undetectable in unstimulated cells, and rose dramatically after 23 h of continuous stimulation. Notably, the level of cyclin D3 in intermediate density G1 cells was also quite elevated, and it was undetectable in high density G0 cells. Analysis of the cell cycle inhibitor p27\textsuperscript{kip1} levels gave a reciprocal result. As expected, p27\textsuperscript{kip1} levels were high in unstimulated cells and low in continuously stimulated cells (Fig. 8B). Among the experimental groups, intermediate density cells had unmeasurable levels of p27\textsuperscript{kip1} whereas high density cells had levels comparable to those of unstimulated cells. Thus, intermediate density cells were “relieved” of cell cycle inhibition and had accumulated the cell cycle progression factor cyclin D3, while high density cells had not reached that stage.

Discussion

Upon finding the appropriate peptide-MHC on DC, T cells do not necessarily form a long-term sedentary interaction but can continue to “scan” other APC (21–23). Such observations have led to the notion that T cells can interrogate intermittent and short term periods of stimulation. Indeed, our results show that the recollection of previous stimulation can last remarkably long (up to 2 days in vitro). Moreover, upon subthreshold stimulation we can isolate a population of T cells in G1 that are endowed with the capacity to respond rapidly upon restimulation, although they cannot be classified as traditional memory cells.

Memory T cells emerge during the adaptive immune response to provide a more rapid response to secondary Ag encounter (41). They outlast the majority of cells that perish upon Ag clearance, and are thought to arise after multiple cycles of division, long after the effector population has reached its peak (42–45). Different populations of memory T cells have several traits in common: relatively high TCR affinities for peptide-MHC (46), low activation threshold, and less stringent dependence on costimulation (12), improved survival (47–49), and a high capacity to expand (50, 51). Unambiguous identification of memory cells in mice is difficult because the widely used marker, high levels of CD44, is also found on both activated and homeostatically proliferating cells (52, 53).

Memory T cells can be divided into two broad groups, effector and central memory cells, which are CD62L low and high, respectively (54). Although all memory populations continuously migrate (55), effector memory cells preferentially accumulate in peripheral nonlymphoid tissues, where if rechallenged they rapidly release cytokines and/or exert direct cytotoxicity (51, 54). Central memory T cells, in contrast, express the lymph node homing receptor CCR7 and largely reside in secondary lymphoid organs (51). Provided with greater survival and proliferative capacities than both naive and effector memory cells, central memory cells can rapidly expand and seed the periphery with new generations of effector cells. One of the requisites for a quicker response is rapid entry into the cell cycle, which has been described for memory T cells (39, 40). For example, memory cells are different from the majority of resting lymphocytes in that they have preactivated cyclinD/CDK6 complexes in the cytoplasm and decreased levels of the cell cycle progression inhibitor p27\textsuperscript{kip1} (39). Although there are many nuances between different subsets of CD8\textsuperscript{+} and CD4\textsuperscript{+} memory cells, their single indisputable hallmark compared with
their naive counterparts is a rapid response to secondary Ag encounter.

By providing a limited (subthreshold) period of stimulation, we have identified a population of cells that are ready to rapidly commit to proliferate following a second subthreshold stimulus, meeting the fundamental requirement of classical memory cells. These cells, although they have not committed to DNA synthesis or division, are characterized by a change in density coupled with increased RNA content (indicating higher metabolic potential), high levels of cyclin D3, and low p27Kip1 expression. Moreover, they can rapidly secrete cytokine upon restimulation. These results are consistent with the observation that T cells undergo a G0 commitment step after 3–5 h of stimulation with anti-CD3/anti-CD28 (56). However, these G1 cells cannot be classified as either central or effector memory cells because they remain CD62Lhigh and only CD44int. Moreover, memory T cells are thought to develop only after multiple cycles of division, and the G1 cells arising during the course of subthreshold stimulation have not even commenced S phase. It should be emphasized that although the brief (4–7) h periods of stimulation used in this study can be considered “suboptimal” because they resulted in little proliferation, this is not the same suboptimal stimulation that has been described for cells activated by altered peptide ligands or low levels of co-stimulation (57, 58), because in those cases the initial stimulation leads to tolerance/anergy rather than readiness to proliferate upon restimulation. Hence, the processes initiated in cells that stopped at G1 must have been qualitatively similar to those of continuously stimulated cells.

How Ag-responsive G1 T cells might arise in vivo and contribute to an adaptive immune response remains to be determined. Repeated intermittent contacts could be a way to avoid activation-induced cell death by preventing a sustained and thus lethal stimulation. Cells have developed adapting mechanisms to control excessive stimulation at many levels, from TCR down-regulation and degradation to down-modulation of various signaling molecules (59–63), and expression of inhibitory receptors like CTLA-4 and lymphocyte activation gene-3 (64–66). Therefore, by transient dissociation from APCs, cells might escape both activation-induced cell death and cell senescence as the consequences of vigorous division (67, 68).

There is some evidence that T cell activation occurs after serial encounters with different APCs. For example, after s.c. Ag injection the first APC to present the foreign Ag are DC residing in the lymph nodes, and a second wave of Ag-delivering cells comes from the inflamed tissues hours later (69). If the second set of DC was denied access to lymph nodes, delayed hypersensitivity could no longer be observed. Therefore, recollection of previous Ag encounter by G1 T cells could be a way to integrate two different and/or similar stimuli generated by different DC subpopulations.

The data in the present report demonstrate that Ag-responsive G1 T cells represent a nonclassical source of memory-like responsiveness to secondary stimulation. Studies in which these cells are adoptively transferred and then restimulated in host animals may provide insights into how they function in vivo.

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References


