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Original Articles

Analysis of T Cell Responses to a Superantigen, Staphylococcal Enterotoxin-B

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T cell subsets that responded to a superantigen, staphylococcal enterotoxin-B (SEB), were analyzed using responder cells stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). It was shown that T cells dividing 5 times represented the major population 4 days after stimulation with SEB. When the proportion of each T cell subset was analyzed sequentially, Vβ8$^+$ T cells that represented the SEB-responding repertoire increased and reached more than 20% of viable cells 4 days after culture with SEB. Both CD4$^+$ and CD8$^+$ T cells in the Vβ8$^+$ population showed similar increases. By contrast, the proportion of SEB-non-reactive Vβ6$^+$ T cells showed no considerable increases. The Vβ6$^+$ T cells exhibited prominent apoptosis from an early phase in the culture, whereas Vβ8$^+$ T cells showed activation-induced cell death at the later stage. However, the proportion of dividing cells (CFSE^{low}) in living cells increased substantially in every T cell subset, including NK-T cells, suggesting that a bystander cell proliferation effect was involved. T cells stimulated with SEB produced both Th1 and Th2 type cytokines, although production of each cytokine followed different time courses.
Key words superantigen, T cell subsets, T cell receptor repertoire, apoptosis

INTRODUCTION

Superantigens polyclonally stimulate T cells, which are involved in various immune responses to infectious agents[1–6]. These superantigens are classified into two major subgroups: bacterium-derived and virus-derived in murine systems[7–12]. The superantigens stimulate T cells in a manner quite different from that of ordinary protein antigens[3–8]. Thus, the superantigens bridge the constant region of the major histocompatibility complex (MHC) molecule on the antigen presenting cell (APC) and the Vβ region of the T cell receptor (TCR), irrespective of the Dβ and Jβ regions and TCRα chain[13,14]. In this way, whole T cells that express a particular Vβ can respond to the respective superantigen, and an extraordinarily large population of T cells responding to the superantigen can be detected in vitro without priming[15].

A large number of monoclonal antibodies (mAb) to recognize each Vβ chain have been established, making it possible to trace the superantigen-reactive T cell clone that expresses the particular TCR. Thus far, taking advantage of these characteristic features of the superantigen-recognition system, mechanisms that underly T cell activation and T cell apoptosis by the superantigens have been pursued. Indeed, for instance, several reports have clearly demonstrated mechanisms by which the T cell repertoire specific for self-superantigen is eliminated during differentiation[16–18]. However, precise analyses of T cell subsets responding to superantigens and the time course of the response remain unclear.

Carboxyfluorescein diacetate succinimidyl ester (CFSF)-labeled lymphocytes have been recently used to trace the proliferating cells in each lymphocyte subset, both in vitro and in vivo[19,20]. Proliferating cells can be defined easily by flow cytometry because the CFSE intensity is reduced by half with every cell division.

In the present study, which used CFSE-labeled responding T cells and mAb specific for

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either TCR Vβ or constimulatory molecules, we analyzed T cell subsets that responded to staphylococcal enterotoxin-B (SEB), a superantigen, and the major histocompatibility complex (MHC) antigens that present SEB to the T cell subsets. We show that a T cell repertoire specific for SEB undergoes rapid proliferation and subsequent apoptosis, accompanied by proliferation of irrelevant T cell subsets and NK-T cells perhaps by a bystander mechanism.

MATERIALS AND METHODS

Responding cells

B10. BR mice (6–10 week old females) were purchased from Japan SLC (Hamamatsu, Japan) and maintained for 2 to 3 weeks before use in experiments. A single cell suspension was prepared, as described elsewhere[20]. These cells were suspended in RPMI-1640 medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) and 0.5% 2-mercaptoethanol (complete medium, CM).

Cell proliferation assay

Serially diluted SEB (Toxin Technology Inc., USA) were added with spleen cells (1×10^6/well) to a 96-well plate and cultured for 24 to 96 h. Eight h before harvest ^{3}H-thymidine (New England Nuclear, Boston, MA) was added and thymidine incorporation was assayed by a Direct Beta Counter Matrix-96 (Packard Co, USA)[21].

CFSE staining and FACS analysis

Spleen cells were stained with 1.25 μm CFSE (Molecular Probes, Eugene, OR) for 10 min, washed with FCS once, and then with CM twice. These cells (1×10^6 well) were cultured with SEB (2.5 μg/ml) in a 96-well plate for 24 to 96 h. Cells collected from the culture were stained with biotinylated anti-Vβ6 or anti-Vβ8 mAb (PharMingen, San Diego, CA) followed by anti-mouse CD4, CD8 or NK1.1 mAb (PharMingen) and Streptavidine-Cy-chrome (PharMingen). These cells were then analyzed using a FACScan (Becton Dickinson, Mountain View, CA), as described previously[20].

Purification of CD4$^+$ or CD8$^+$ T cells

Spleen cells were incubated with fluorescein isothiocyanate (FITC)- conjugated anti-CD4 or anti-CD8 mAb and labeled further with anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, the CD4$^+$ or CD8$^+$ T cells were depleted by magnet-activated cell sorting (MACS) (Miltenyi Biotec), according to the manufacture's protocol.

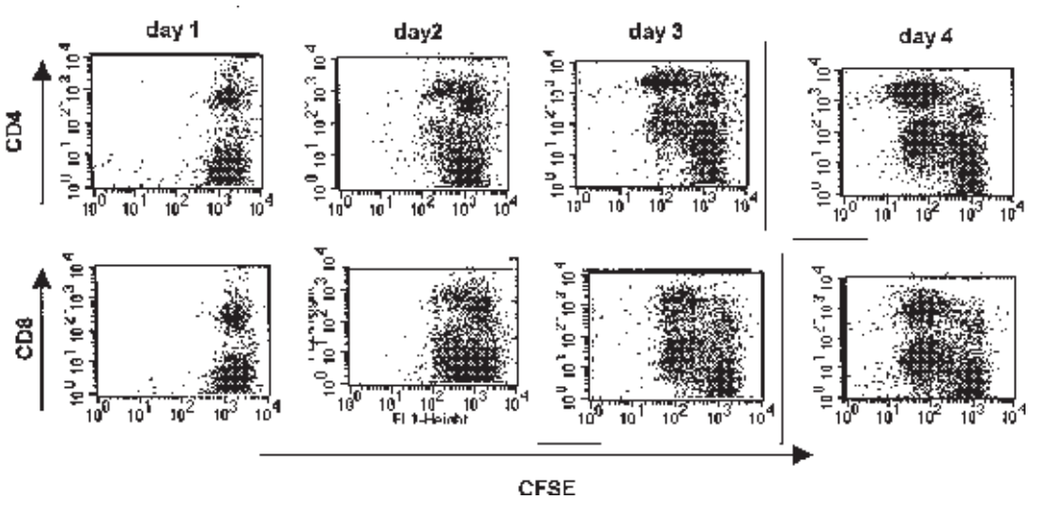


Fig. 1. Response of CFSE-labeled T cells to SEB. CFSE-labeled T cells were cultured with SEB (2.5 μg/ml) for 1 to 4 days. These cells were analyzed for expression of CD4 or CD8 and CFSE. A representative result from 10 independent experiments.

Apoptosis assay

Spleen cells (1×10^6/well) were cultured with SEB (2.5 μg/ml) for 1 to 7 days. Then cells collected were stained with biotinylated anti-Vβ6 or anti-Vβ8 mAb and Annexin V (Roche Molecular Biochemicals, Germany). These cells were analyzed by a FACScan.

Cytokine assay

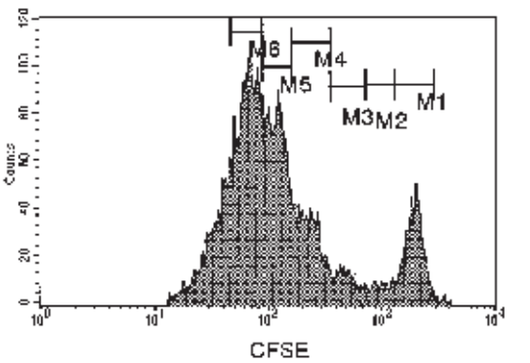
Spleen cells (1×10^6/well) were cultured with SEB for 4 days, as described above. The supernatants collected were analyzed for IL-2, IL-4, IL-10 and IFN-γ production by enzyme -linked immunosorbent assay (ELISA) using the Immunoassay Kit (BioSource International, USA) according to the manufacturer's protocol. The extinction rate was measured by an ImmunoMini microplate reader (Inter Med, Japan). Intracytoplasmic cytokine (IL-4 and INF-γ) was stained as described elsewhere[22]. Briefly, SEB-stimulated T cells were treated with monensin (Sigma Chemical) and stained with various mAb. Thereafter, the cells were fixed with paraformaldehyde, treated with saponine (Sigma) and then stained with either FITC-labeled anti-INF-γ or phycoerythrin (PE)- labeled anti-IL-4 mAb (PharMingen).

RESULTS

Visualization of T cell proliferative response to SEB

To determine the optimal concentration of SEB, spleen cells were stimulated with serially diluted SEB and ^{3}H-thymidine uptake was measured. It was shown that 2.5 μg/ml SEB induced maximum proliferation. When the time course of ^{3}H-thymidine uptake was analyzed, the peak response was observed 48 h after culture (data not shown).

In the next step CFSE labeled spleen cells were used as responders. These cells were cultured with SEB (2.5 μg/ml) for 1 to 4 days. Fig. 1 shows a representative result. After being stimulated with SEB (2.5 μg/ml), CFSE^{low} population (dividing cells) appeared in both CD4$^+$ and CD8$^+$ T cells. On day 4, the proportion of CFSE^{low} cells was greater than CFSE^{high} (non-dividing) cells in either CD4$^+$ or CD8$^+$ subset. This finding demonstrates that the majority of lymphocytes undergo cell division in the viable cell population. When the CFSE intensity of the responding cells was analyzed in a histogram at day 4, cells that divided 5 times (M6) represented the major proportion in the population (Fig. 2).



	M6	M5	M4	M3	M2	M1
Mean	73.8	128.9	244.3	512.1	1039.6	1902
%	32.14	20.91	11.99	3.56	2.35	9.72

Fig. 2. CFSE expression on responding T cells. Splenic T cells were labeled with CFSE and cultured with SEB (2.5 μg/ml) for 4 days. Compared to the original population (M1), dividing cells express one-half CFSE per division. Note that cells dividing 5 times (M6) represent the major population.

Responsiveness of purified CD4$^+$ or CD8$^+$ T cells

We then prepared spleen cell populations depleted of CD4$^+$ or CD8$^+$ T cells by MACS. These CD4$^-$ or CD8$^-$ cells that contained 17–22% CD4$^+$ or CD8$^+$ T cells, respectively, were labeled with CFSE and cultured with SEB for 2 to 4 days. The total cell number recovered was greater in the CD4$^+$ T cell culture than that in the CD8$^+$ T cell culture during the whole period of observation (Fig. 3A). Then, the response of Vβ8$^+$ cells that represent an SEB-responding population was analyzed. When CFSE^{low} (dividing) cells were compared between CD4$^+$ and CD8$^+$ T cell subsets, the number of CD8$^+$Vβ8$^+$ cells was greater than that of CD4$^+$ Vβ8$^+$ cells (Fig. 3B). Vβ6$^+$ T cells showed a negligible proliferation. These findings demonstrated that both CD4$^+$ and CD8$^+$ cells can respond to SEB independently of each other.

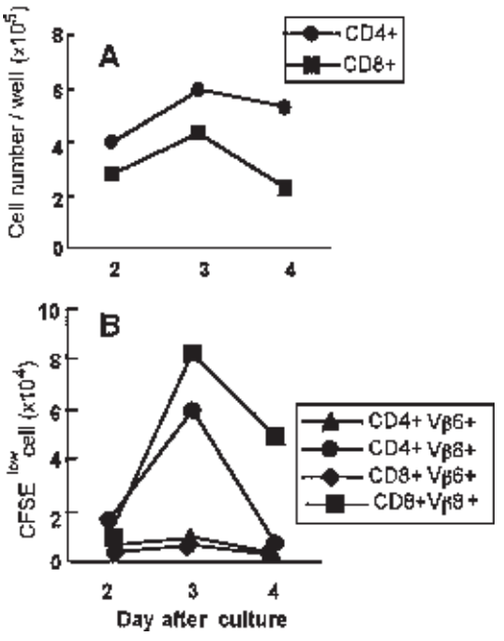


Fig. 3. Response of $CD4^+$ or $CD8^+$ T cells to SEB. $CD4^+$ or $CD8^+$ T cells were removed from splenic cells by MACS. These CD4-depleted ($CD8^+$) and CD8-depleted ($CD4^+$) populations were cultured with SEB for 2 to 4 days. A. The cell number of $CD4^+$ or $CD8^+$ T cells. B. The number of $CFSE^{low}$ cells in each T cell subset.

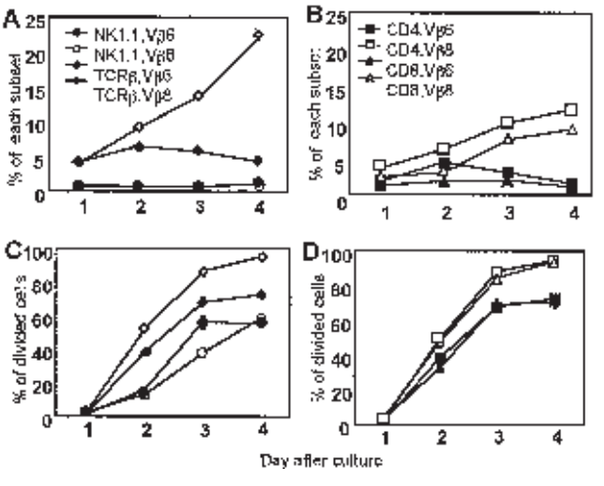


Fig. 4. Proportion of T cell subsets in cultures with SEB. A. Proportion of $V\beta6^+$ or $V\beta8^+$ cells in mainstream T cells and NK-T cells. B. Proportion of $V\beta6^+$ or $V\beta8^+$ cells in $CD4^+$ and $CD8^+$ T cells. C. Proportion of $CFSE^{low}$ (dividing) $V\beta6^+$ or $V\beta8^+$ cells in T cells and NK-T cells. D. Proportion of $CFSE^{low}$ $V\beta6^+$ or $V\beta8^+$ cells in $CD4^+$ and $CD8^+$ T cells.

Further analysis of TCR Vβ expression in T cells responding to SEB

Whole spleen cells were labeled with CFSE and stimulated with SEB. Thereafter, the TCR $V\beta$ expression on the responding T cells was analyzed. The population of $V\beta8^+$ T cells increased gradually and reached more than 20% of viable cells recovered from the culture at 4 days after stimulation (Fig. 4A). Both $CD4^+$ and $CD8^+$ cells among the $V\beta8^+$ cells showed similar increases (Fig. 4B). NK-T cells bearing $V\beta8$ showed no increase at all (Fig. 4A). On the other hand, the proportion of $V\beta6^+$ T cells maintained a constant value during the culture period.

When the proportion of $CFSE^{low}$ cells in each T cell subset was evaluated, almost 100% of $V\beta8^+$ T cells in the $CD4^+$ or $CD8^+$ subsets underwent cell division at day 4 (Fig. 4C and 4D). Interestingly, surviving $V\beta6^+$ T cells and NK-T cells also showed substantial proliferation, although the number of viable cells in these sub-

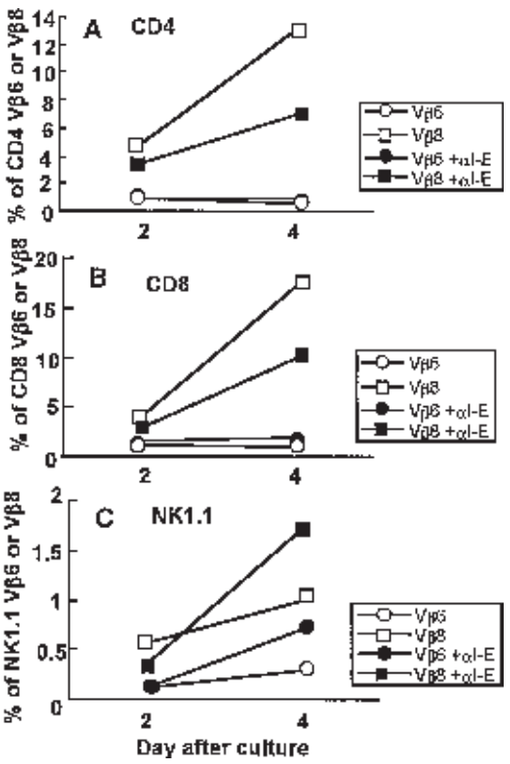


Fig. 5. Proportion of T cell subsets in cultures with SEB in the presence or absence of anti-I-E mAb. A. Proportion of $V\beta6^+$ $CD4^+$ or $V\beta8^+$ $CD4^+$ T cells. B. Proportion of $V\beta6^+$ $CD8^+$ or $V\beta8^+$ $CD8^+$ T cells. C. Proportion of NK-T cells bearing $V\beta6$ or $V\beta8$ TCR.

sets were markedly few.

Response of T cell subsets to SEB in the presence of anti-I-E mAb

Spleen cells were stimulated with SEB in the presence or absence of anti-I-E mAb. Anti-I-E mAb decreased the proportion of Vβ8$^+$, but not Vβ6$^+$ T cells, in both the CD4$^+$ and CD8$^+$ subsets 4 days after culture, compared to that in the control culture (without anti-I-E mAb) (Fig. 5). No influence of anti-I-E mAb was detected with the response of NK-T cells (Fig. 5C). When the proportion of CFSE^{low} cells in cells collected from the culture at day 2 was analyzed and compared, it was shown clearly that anti-I-E mAb considerably decreased the proportion of CFSE^{low} Vβ8$^+$, but not CFSE^{low} Vβ6$^+$ T cells, in either the CD4$^+$ or CD8$^+$ subset (Table 1).

Induction of apoptosis in SEB-stimulated T cell subsets

It has been reported that T cells undergo apoptosis by activation-induced cell death (AICD) following stimulation with superantigens[23,24]. We then analyzed apoptosis in SEB-stimulated Vβ6$^+$ and Vβ8$^+$ T cells by Annexin V staining. Spleen cells were cultured with SEB (2.5 μg/ml) for 1 to 7 days. As was shown in prior experiments, the proportion of Vβ8$^+$ T cells increased, reached a plateau at day 4 and decreased at day 7 after culture (Fig. 6A). Only a gradual decrease was observed in Vβ6$^+$ T cells during the culture period. Fig. 6B shows a representative result of Annexin V staining of cells

Table 1. Dividing T cell subsets after stimulation with SEB

T cell subsets	αI-E antibody	Dividing cells (%)
CD4, Vβ6	−	9.6+4.0
CD4, Vβ6	+	6.1+1.6
CD4, Vβ8	−	43.0+6.8
CD4, Vβ8	+	15.8+4.6
CD8, Vβ6	−	12.4+7.0
CD8, Vβ6	+	7.0+2.8
CD8, Vβ8	−	41.3+14.6
CD8, Vβ8	+	15.5+4.8

Mean of dividing cells (%) after 2 days culture ±SD (n=3)

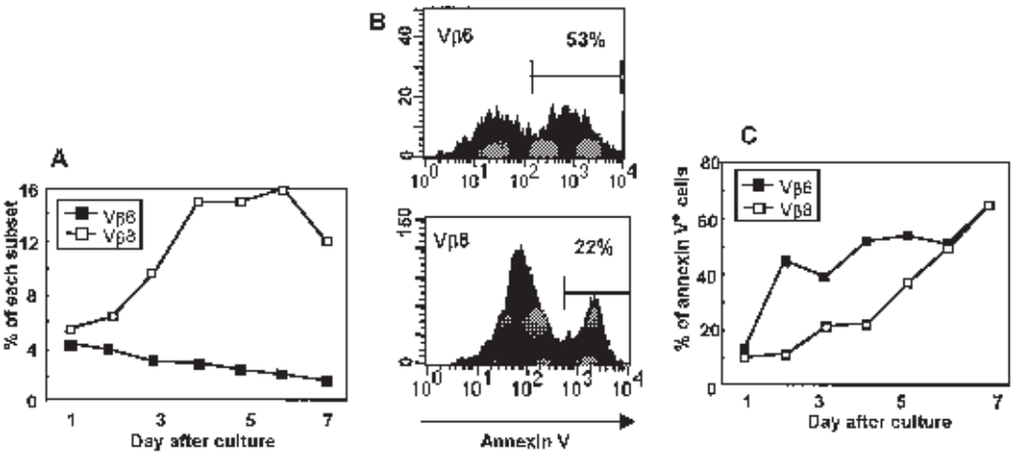


Fig. 6. Apoptosis of T cell subsets after culture with SEB. Splenic T cells were cultured with SEB (2.5 μg/ml) for 1 to 7 days. These cells were stained with anti-Vβ6 or anti-Vβ8 mAb and Annexin V. A. Proportion of Vβ6$^+$ or Vβ8$^+$ T cells. B. A representative FACS pattern of Annexin V straining of Vβ6$^+$ or Vβ8$^+$ T cells 3 days after culture. C. Time course of Annexin V$^+$ cells in the culture with SEB. A representative result from 3 separate experiments.

obtained from the culture at day 3. 53% of Vβ6$^+$ cells and 22% of Vβ8$^+$ cells were Annexin V-positive at this time point. When the proportion of apoptotic cells was analyzed sequentially, the proportion of apoptotic Vβ8$^+$ T cells showed an increase from day 5 (Fig. 6C). On the other hand, the proportion of apoptotic cells in the Vβ6$^+$ subset increased after day 2. These findings demonstrated that Vβ8$^+$ T cells underwent apoptosis by AICD, whereas most Vβ6$^+$ T cells followed a natural course of death.

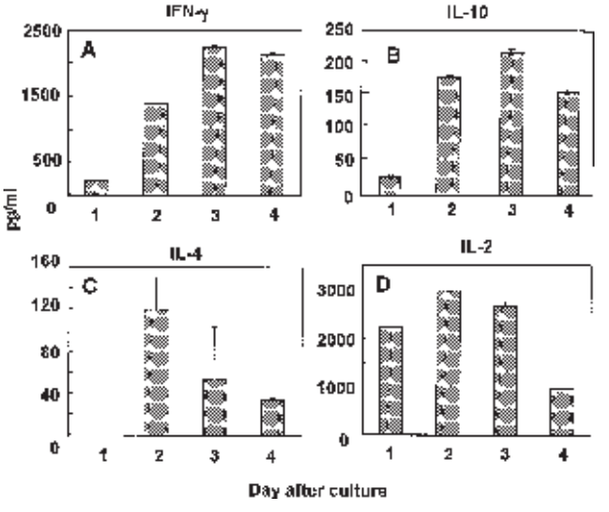


Fig. 7. Cytokine production by splenic cells stimulated with SEB. Splenic cells were cultured with SEB (2.5μg/ml) for 1 to 4 days. Amounts of IFN-γ (A), IL-10 (B), IL-4 (C) and IL-2 (D) in the supernatants were sequentially quantitated by ELISA.

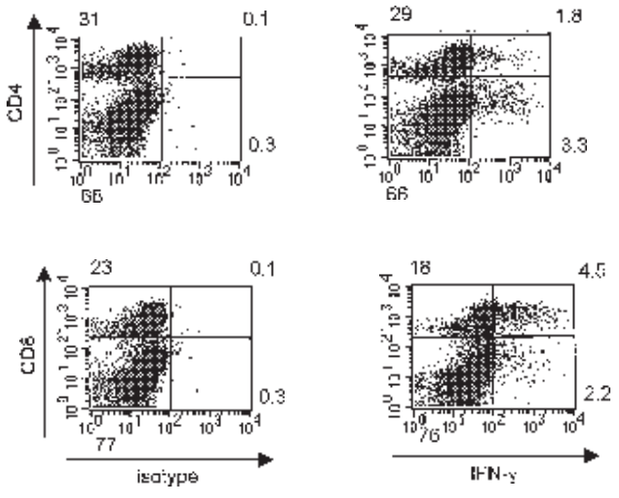


Fig. 8. Intracytoplasmic IFN-γ in CD4$^+$ or CD8$^+$ T cells stimulated with SEB. Splenic T cells were cultured with SEB (2.5 μg/ml) for 3 days. These cells were stained with anti-CD4 or anti-CD8 and anti-IFN-γ. A representative result from 3 separate experiments.

Cytokine production by SEB-stimulated T cells

Cytokine production by SEB-stimulated cells were analyzed sequentially by ELISA. Spleen cells produced IFN-γ, IL-10, IL-4 and IL-2 following culture with SEB (2.5 μg/ml). IL-4 and IL-2 production showed a peak at day 2 after culture, whereas IFN-γ and IL-10 productions demonstrated a peak at day 3 (Fig. 7). These time courses are consistent with the report of Rajagopalan *et al.*[25]. Then, we analyzed intracytoplasmic cytokines by flow cytometry analysis. Fig. 8 shows a representative FACS analysis of IFN-γ producing cells. Respectively, 20% and 6% of CD8$^+$ and CD4$^+$ T cells were intracytoplasmic IFN-γ^+ at 3 days after culture. Although the proportion was relatively low, approximately 2% of cells were IL-4$^+$ in either the CD4$^+$ or CD8$^+$ T cell subsets at day 3 of culture (data not shown).

DISCUSSION

In this study, we used CFSE-labeled T cells to visualize responses of T cell subsets to SEB, a superantigen. The proliferating cells were clearly visualized as the CFSE^{low} population. Four days after culture with SEB, T cells that underwent cell division 5 times represented the largest population. This finding suggests that most of the SEB-responding cells begin proliferating immediately after stimulation with SEB.

We could show that purified CD4$^+$ or CD8$^+$ T cells responded to SEB independently. Thus, it appears that T cells bearing SEB-reactive TCR (i.e. Vβ8) respond to SEB irrespective of CD4 or CD8 expression. In addition, it was shown that both the Vβ8$^+$ CD4$^+$ and Vβ8$^+$ CD8$^+$ T cells recognized SEB in the context of I-E antigens. However, among CFSE^{low} Vβ8$^+$ T cells (proliferating cells), the proportion of CD8$^+$ T cells was higher than that of CD4$^+$ T cells. It seems that T cells bearing SEB-reactive Vβ other thanVβ8 (i.e. Vβ7), are involved more frequently in the response of CD4$^+$ T cells than the response of CD8$^+$ T cells.

It should be noted that substantial proportions of Vβ6$^+$ T cells and NKT cells were CFSE^{low} in the viable cell population. Anti-I-E mAb exerted no significant influences on these proliferations. Thus, although most of the Vβ6$^+$

T cells and NKT cells appeared to follow the natural course of death and the cell numbers were markedly reduced, the remaining viable cells proliferated perhaps through a bystander mechanism under the influence of cytokines produced by T cells specific for SEB. Indeed, the SEB-specific Vβ8$^+$ T cells produced both Th1 and Th2-type cytokines and thereafter underwent apoptosis by an AICD mechanism[16,18].

In characterizing the types of cytokines produced by SEB-stimulated T cells, we reported previously that ovalbumin-specific and I-A^d-restricted CD4$^+$ T cells produced mainly IFN-γ in the presence of high dose antigens, but produced mainly IL-4 in the presence of low dose antigens[22]. Thus, cytokine production by T cell subsets should be analyzed in the presence of various concentrations of SEB in future studies. In another system, we observed that IL-7 was involved in homeostatic and perhaps bystander proliferation (Manuscript submitted). Further, in our previous study[20], it was suggested that IL-2 was involved in bystander proliferation. In the present study, we attempted to analyze the involvement of IL-2. However, addition of anti-IL-2 mAb to the culture exerted no significant influence on the response to SEB.

Interestingly, among CFSE^{low} NKT cells no differences were observed between the proportion of Vβ6$^+$ and Vβ8$^+$ cells cultured with SEB. This finding again suggests that Vβ8$^+$ NKT cells hardly evoke specific responses to SEB, and the CFSE^{low} NK-T cells are generated by a non-specific mechanism. T cells and NK-T cells showed different responsiveness to MHC antigens and superantigens[26]. Recently, we observed marked differences in the responsiveness to the specific antigens (CD1d or OVA plus I-A^d) between T cells and NKT cells bearing the same TCR (Iwabuchi C *et al*. unpublished observation). Further, we have reported in our previous study that proliferation of NKT cells in syngeneic mixed lymphocyte reaction (SMLR) is mainly attributable to the bystander effect[20].

In the present study, using CFSE-labeled responding T cells, we showed that T cell responses to certain antigens *in vitro* involved a markedly complex cell proliferation. Thus, it seems important to define the initial response that induces subsequent complex cell proliferation to elucidate the essential responsiveness of T cells to the particular antigen.

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