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Suppression of Anti-tumor CD4$^+$ T Cell Responsiveness in the Tumor-bearing State and Its Recovery in *in vitro* Culture Free of Tumor Burden

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We investigated whether the responsiveness of anti-tumor CD4$^+$ T cells suppressed in the tumor-bearing state is reversed in conditions free of tumor burden. Spleen cells from BALB/c mice bearing a syngeneic tumor (CSA1M) 1–3 wk after inoculation with CSA1M cells produced interleukin-2 (IL-2) and IL-4 upon *in vitro* culture without addition of exogenous tumor antigens. This lymphokine production was achieved through collaboration between anti-CSA1M CD4$^+$ T cells and antigen-presenting cells (APC) that had been pulsed with CSA1M tumor antigens *in vivo* in the tumor-bearing state. However, spleen cells from late (8–10 wk) tumor-bearing stages produced reduced levels of lymphokine production despite the presence of comparable proportions of CD4$^+$ T cells. Because APC in these cell populations exhibited enhanced capacities to present tumor antigens, reduced responsiveness was ascribed to the dysfunction of CD4$^+$ T cells themselves. When spleen cells from early tumor-bearing mice were preincubated for 1–2 days and recultured in fresh medium, the magnitude of lymphokine production by these cells was not changed. In contrast, the same protocol of preincubation and reculture for cells from late tumor-bearing mice resulted in the recovery of anti-tumor lymphokine-producing capacity. The recovered capacity was comparable to or slightly higher than that expressed by cells from early tumor-bearing stages. Since the CD4$^+$ T cell content did not significantly differ before and after preincubation, enhanced lymphokine production was due to the recovered responsiveness of anti-tumor CD4$^+$ helper T cells. The recovery of anti-tumor responsiveness was also induced *in vivo* by tumor removal at the late tumor-bearing stage: spleen cells from mice 2–4 wk after tumor resection efficiently produced IL-2 and IL-4. These results indicate that the immunodysfunction of anti-tumor CD4$^+$ T cells induced in the tumor-bearing state is reversible because release from tumor burden either by preincubation *in vitro* or by tumor removal *in vivo* results in almost complete recovery of the potent anti-tumor responsiveness initially expressed.

Key words: Tumor-bearing state — Immunosuppression — Anti-tumor CD4$^+$ T cell — Transforming growth factor-β

Various types of immune dysfunction involving a number of suppressive mechanisms are induced during the tumor-bearing state.[1–10] We have recently demonstrated that the self Ia-restricted CD4$^+$ helper T cell (Th) function is more severely suppressed than the functions of CD8$^+$ cytotoxic T lymphocyte (CTL) precursors and CD8$^+$ allo-class I MHC-restricted Th subsets.[11, 12] The CD4$^+$ Th function is an absolute requirement for the induction of immune responses to tumor antigens.[13, 14] Therefore, the impairment of the CD4$^+$ Th function represents a central aspect of immune dysfunction induced in the tumor-bearing state.

CD4$^+$ Th recognize processed forms of antigen in association with self Ia molecules expressed on antigen-presenting cells (APC).[15–17] Our recent studies illustrated that: (i) APC in tumor-bearing hosts bind tumor antigens *in vivo* to construct an effective immunogenic unit.[18, 19]; and (ii) lymphokines (IL-2 and interferon-γ) can be produced upon culture of spleen cells from tumor-bearing mice through collaboration between anti-tumor sensitized CD4$^+$ Th and tumor antigen-binding APC.[18] The results further demonstrated that tumor-bearing mice exhibit a progressive increase in tumor antigen-presenting APC function and a reciprocal decrease in anti-tumor CD4$^+$ T cell reactivity.[18] Taken together, these observations implied that the defect in CD4$^+$ T cell function can be ascribed to the progressively declining responsiveness of CD4$^+$ Th themselves rather than the APC population. Although the CD4$^+$ T cell content was not significantly changed throughout the tumor-bearing stages,[18] the possibility that only tumor-specific CD4$^+$ T cell clones are eliminated in the CD4$^+$ T cell population remains to be examined. This possibility could be addressed by investigating whether the suppression of anti-tumor CD4$^+$ Th responsiveness is irreversible or whether the responsiveness is regained upon release from the tumor burden.

The present results demonstrate that spleen cells from early and late tumor-bearing mice produced large and small amounts of interleukin-2 (IL-2) and IL-4 in 1- to 3-

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day cultures, respectively. Preincubation of cells from late tumor-bearing mice and reculture of these cells in fresh medium resulted in enhanced levels of lymphokine production which are comparable to or slightly higher than those observed for cells from early tumor-bearing mice before or after preincubation. These results indicate that the suppression of anti-tumor $CD4^+$ T cells in late tumor-bearing stages is not due to elimination or irreversible attenuation, but their responsiveness is restored upon release from the tumor burden.

MATERIALS AND METHODS

Mice Male BALB/c mice were obtained from Shizuoka Experimental Animal Laboratory, Shizuoka, and used at 6–9 wk of age.

Tumors CSA1M fibrosarcoma[20] which was induced in BALB/c mouse with the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) and shown to produce no RSV virus was used. This tumor cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO_2.

Monoclonal antibodies Hybridoma cells producing anti-IL-2 antibody (S4B6)[21] and anti-IL-4 antibody (11B11)[22] were kindly provided by Dr. T. Mosmann and Dr. W. E. Paul, respectively. Purified monoclonal antibodies were used. The following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies were purchased: anti-Thy-1.2 (BioMakor, Rehovot, Israel) and anti-CD8 (Becton Dickinson, Mountain View, CA). FITC-anti-CD4 conjugate was prepared in our laboratory by using purified anti-CD4 (GK1.5) antibody.

Preparation of CSA1M tumor-bearing mice and tumor resection CSA1M tumor-bearing mice were prepared by inoculating i.d. 2×10^5 viable CSA1M cells. Tumor resection was performed at an 8 wk tumor-bearing stage.

Preparation of a T cell-enriched population T cells were separated from a splenic cell population by immunomagnetic negative selection as described.[23,24] Briefly, spleen cells were incubated with Advanced Magnetic Particles bound to goat anti-mouse IgG (Advanced Magnetic, Cambridge, MA). The magnetic particles attached to cells were then removed using a rare earth magnet (Advanced Magnetic) leaving behind surface IgG-negative cells. The purity of T cells was consistently >90% by flow microfluorometric (FMF) analysis.

Preparation of an APC-enriched population The procedure was the same as described.[18] Briefly, splenic accessory cells were enriched on a bovine serum albumin (BSA) gradient according to the method described by Glimcher *et al.*[25] The BSA gradient was prepared by layering 5 ml of 11% BSA over 5 ml of 35% BSA containing 5×10^8 spleen cells and centrifuging at 4°C for 30 min. Cells banding between 35% and 11% were found to be approximately 40% Mac-1-positive cells by FMF study. These fractionated cells were used as an APC-enriched population (APC population).

Preparation of lymphokine samples Fractionated or unfractionated spleen cells (5×10^6/well) were cultured in the absence or presence of an APC population (10^5 cells/well) in 24-well culture plates (Corning 25820, Corning Glass Works, Corning, NY) in a volume of 1 ml of RPMI 1640 medium supplemented with 10% FCS. After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h unless otherwise indicated, culture supernatants (SNs) were harvested by centrifugation and stored at −20°C until use.

Assay system for IL-2 activity SNs were assayed for IL-2 activity in terms of their ability to support the proliferation of the IL-2-dependent T cell line, CTLL-2. CTLL-2 cells (10^4/well) were cultured with the SN in a volume of 0.2 ml in 96-well flat-bottomed microplates (Corning 25860) for 24 h at 37°C. Proliferation was assessed in terms of uptake of ^{3}H-TdR during 4-h pulsing with 20 kBq ^{3}H-TdR/well.

Assay system for IL-4 activity SNs were assayed for IL-4 activity in terms of their ability to support the proliferation of the IL-4-dependent cell line, CT.4S[26] (a kind gift from Dr. W. E. Paul, NIH). CT.4S cells (1×10^4/well) were cultured with the SN in a volume of 0.2 ml in 96-well flat-bottomed microplates for 60 h at 37°C. Proliferation was assessed by measuring the uptake of ^{3}H-TdR during 6-h pulsing with 20 kBq ^{3}H-TdR/well.

Immunofluorescence staining and FMF The preparation and staining procedures were essentially the same as described previously.[27] Briefly, 1×10^6 spleen cells were incubated at 4°C for 30 min with FITC-conjugated antibodies, washed twice, resuspended, and analyzed for fluorescence. These procedures were performed in Hanks' balanced salt solution (without phenol red) containing 0.1% BSA and 0.1% sodium azide. FMF analysis was performed by using a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). All data were collected by using log amplification, and dead cells were rejected from analysis on the basis of forward light scatter.

RESULTS

Suppression of anti-tumor $CD4^+$ Th function at late tumor-bearing stages and its recovery after tumor removal BALB/c mice were inoculated i.d. with 2×10^5 viable CSA1M tumor cells. These mice bore a solid tumor approximately 10 mm in diameter 2–3 wk after the tumor cell inoculation. The tumor continued to grow gradually and reached about 20–25 mm diameter at around the 7–8 wk tumor-bearing stage. Most of the

animals died more than 15 wk after the tumor initiation. Spleen cells (5×10^6/well) from normal (tumor-free) mice or early (2–3 wk) or late (8–10 wk) CSA1M-bearing mice were cultured for 1–3 days without addition of exogenous tumor antigens. Culture SNs were tested for IL-2 and IL-4 activities, which were detected as the capacity to support the growth of IL-2 or IL-4-dependent CTLL-2 or CT.4S cells, respectively. Significant levels of IL-2 and IL-4 activities were not detected in culture SNs from normal spleen cells (data not shown). Spleen cells from early CSA1M-bearing mice produced high levels of IL-2 and IL-4 (Fig. 1, upper panels), which confirmed our previous results.[18] This lymphokine production was previously demonstrated to be achieved by collaboration between anti-CSA1M $CD4^+$ T cells and APC binding CSA1M tumor antigens, both of which are contained in spleen cells from early tumor-bearing mice.[18] In contrast to the potent lymphokine-producing capacity of cells from early tumor-bearing mice, spleen cells from late tumor-bearing mice exhibited reduced levels of IL-2 and IL-4 production (Fig. 1, lower panels). The decreased lymphokine activity in culture SNs from the latter type of cells was not due to the coexistence of inhibiting substances, because the addition of these culture SNs to rIL-2 or rIL-4-stimulated CTLL-2 or CT.4S cultures (lymphokine bioassay) did not result in suppression of the proliferation of responding cell lines (data not shown).

To analyze cellular mechanisms underlying $CD4^+$ Th function at late tumor-bearing stages, we examined whether the tumor-bearing state influences the number of

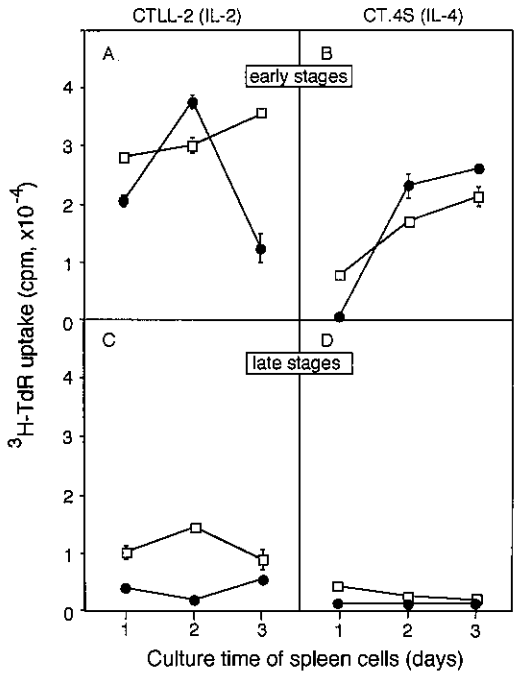

Fig. 1. Time course of lymphokine production by spleen cells from early vs. late tumor-bearing stages. Spleen cells were obtained from two pools (● or □) of 2–3 tumor-bearing mice at the early (upper panels) or late (lower panels) stage. Cells (5×10^6/well) were cultured *in vitro* for 1–3 days without stimulation with exogenous tumor antigens. SNs were tested for their ability to support the growth of CTLL-2 (left panels: IL-2 assay) or CT.4S (right panels: IL-4 assay) cell lines. Backgrounds of ^{3}H-TdR uptake by CTLL-2 or CT.4S were less than 1000.

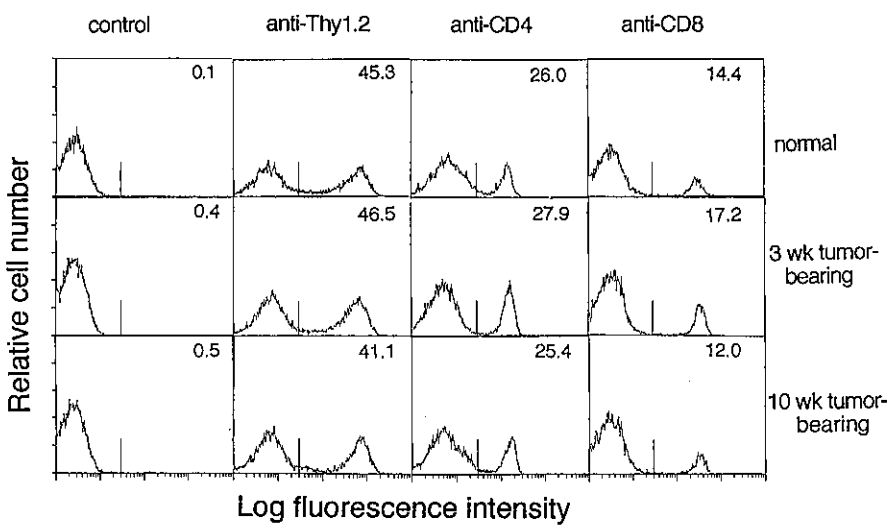

Fig. 2. Content of $CD4^+$ T cells in spleen cells from mice at various tumor-bearing stages. Thy-1.2-, CD4- or CD8-positivity was determined by FMF study with the use of FITC-conjugated anti-Thy1.2, anti-CD4 or anti-CD8 antibody. The numbers on the figures are the percentages of cells stained by each reagent.

T cells, especially that of $CD4^+$ T cells. The results of Fig. 2 illustrate that the percentages of total T cells and $CD4^+$ T cells from mice at the 2 wk tumor-bearing stage are slightly decreased. This was due to a relative increase in the numbers of non-T cells. However, the absolute number of $CD4^+$ T cells was not decreased (data not shown). Moreover, the proportion of $CD4^+$ T cells observed at this early tumor-bearing stage was not significantly changed even at the 12 wk tumor-bearing stage.

We next examined whether anti-tumor $CD4^+$ Th responsiveness, once suppressed in the tumor-bearing state, recovers after release from tumor burden by removal of the tumor. The results in Table I show that spleen cells from mice 2 wk after tumor removal performed at a late (8 wk) tumor-bearing stage had regained high responsiveness of anti-tumor $CD4^+$ Th, comparable to that of cells from early tumor-bearing mice. Taken collectively, these results indicate that anti-tumor $CD4^+$ Th function is suppressed at late tumor-bearing stages but its suppression is reversed following removal of the tumor.

Recovery of anti-tumor $CD4^+$ Th responsiveness following preincubation *in vitro* We next investigated whether reduced anti-tumor responsiveness of $CD4^+$ Th at late tumor-bearing stages is reversed by preincubating these $CD4^+$ Th *in vitro*. Spleen cells from late tumor-bearing mice were preincubated for one or two days. Cells harvested were extensively washed with medium 3 times, adjusted to 5×10^6/well, and cultured for an additional one or two days in fresh medium. The results are summarized in Figs. 3–5. Spleen cells from early tumor-bearing mice did not exhibit enhanced levels of lymphokine production after preincubation for 1–2 days (Fig. 3). In contrast, the preincubation of spleen cells from late tumor-bearing mice induced a remarkable improvement of their lymphokine-producing capacities (Figs. 4 and 5). The results demonstrate that (i) spleen cells from late tumor-bearing mice continued to produce reduced amounts of IL-2 and IL-4 irrespective of culture period without the preincubation; and (ii) portions of the same spleen cells could produce enhanced amounts of lymphokines after preincubation, especially after the 2-day preincubation.

We further examined whether enhanced lymphokine production following preincubation was due to the enrichment of $CD4^+$ T cells in the cell population that was prepared by readjusting preincubated spleen cells. The

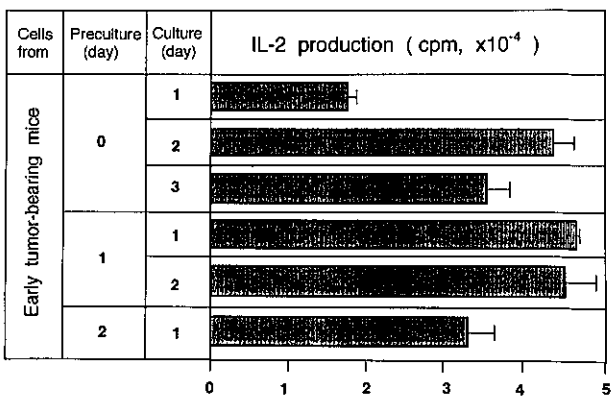

Fig. 3. Effect of preincubation on the capacity of spleen cells from early tumor-bearing mice to produce lymphokine. Spleen cells (5×10^6/ml/well) from early stages of tumor-bearing mice were incubated for 24 h in 24-well culture plates without any stimulation (preculture). Cells harvested were extensively washed with medium, readjusted to the original cell concentration and cultured in fresh medium for an additional period (1–2 days). SNs of recultured cells were tested for IL-2 activity.

Table I. Suppression of Anti-tumor $CD4^+$ Th Function at Late Tumor-bearing Stages and Its Recovery Following Tumor Resection

Responding spleen cells from mice[a]	Lymphokine production[b]: ^{3}H-TdR uptake (cpm)	
	IL-2	IL-4
Normal	3592 (1.13)	1595 (1.07)
Early CSA1M-bearing	36063 (1.09)	21022 (1.03)
Late CSA1M-bearing	9591 (1.02)	4937 (1.02)
Post tumor resection	32979 (1.09)	26540 (1.02)

a) Spleen cells (5×10^6/well) from early (2–3 wk) or late (8–10 wk) CSA1M-bearing mice or mice 2 wk after tumor resection at an 8 wk CSA1M-bearing stage (post tumor resection) were cultured in 24-well culture plates for 48 h.

b) Culture SNs were assayed for IL-2 or IL-4 activity according to their ability to support the proliferation of the respective IL-2 or IL-4-dependent T cell lines, CTLL-2 or CT.4S. Backgrounds of ^{3}H-TdR uptake by CTLL-2 or CT.4S were less than 1000 in all experiments.

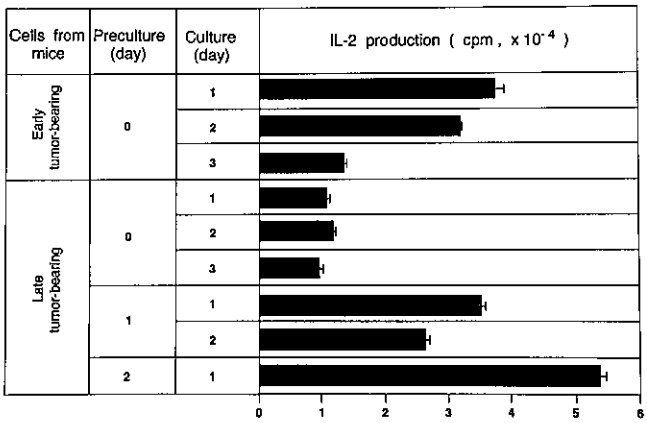

Fig. 4. Recovery of IL-2-producing capacity of spleen cells from late tumor-bearing mice following *in vitro* pre-cultures. Spleen cells (5×10^6/ml/well) were obtained from early or late stages of tumor-bearing mice, and cultured for 1–3 days. A portion of the latter type of cells was preincubated for 1 or 2 days, and recultured in fresh medium for an additional 1–2 days. SNs of recultured cells were tested for IL-2 activity.

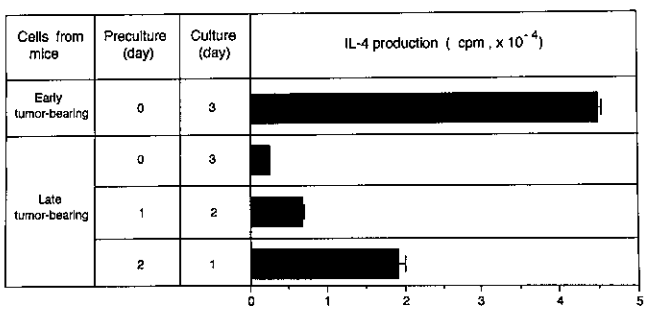

Fig. 5. Recovery of IL-4-producing capacity of spleen cells from late tumor-bearing mice following *in vitro* precultures. The experimental protocol was the same as that in Fig. 4. SNs of recultured cells were tested for IL-4 activity.

results of Table II illustrate that this was not the case: the $CD4^+$ T cell percentage was similar in spleen cell populations before and after 2-day preincubation. Moreover, it was previously found that increasing the number of fresh spleen cells from late tumor-bearing mice did not lead to enhanced production of lymphokines in cultures[28] in such a way as observed for cells after preincubation. These results indicate that the suppression of responsiveness of anti-tumor $CD4^+$ Th at late tumor-bearing stages is reversible in nature and that the capacity is recovered by preincubating the cells in conditions free of tumor burden.

Table II. Content of $CD4^+$ T Cells in Spleen Cells from Late Tumor-bearing Mice before and after 2-Day Preincubation

Spleen cells[a] from mice		Percentages of cells stained with		
tumor-bearing	preincubation	anti-Thy-1.2	anti-CD4	anti-CD8
Early	−	36.0	25.7	12.7
	+	30.1	25.7	14.0
Late	−	33.0	22.5	11.7
	+	39.6	22.1	13.6

a) Spleen cells were obtained from each pool of 2–3 tumor-bearing mice at early and late stages. Portions of cells were incubated alone for 48 h. The freshly prepared and cultured cells were tested for Thy-1.2, CD4 and CD8 antigens.

Additional experiments were performed to test the specificity of the two cell lines, CTLL-2 and CT.4S, used for the detection of IL-2 and IL-4 activities, respectively, in spleen cell culture SNs. Our previous study demonstrated that IL-2 and IL-4 activities in culture SNs of spleen cells from tumor-bearing mice were neutralized by anti-IL-2 and anti-IL-4 antibodies, respectively.[28] SNs obtained after preincubating spleen cells from late tumor-bearing mice were tested for IL-2 or IL-4 activity using a similar protocol to that in our previous study (Table III). The proliferation of CTLL-2 induced by both types of culture SNs was inhibited by anti-IL-2 but not by anti-IL-4 antibody, and likewise the proliferation of CT.4S was blocked only by anti-IL-4 antibody. These results confirm that the activities that have been detected by using these CTLL-2 and CT.4S cell lines can be ascribed to IL-2 and IL-4, respectively.

Transforming growth factor-β (TGF-β) susceptibility of lymphokine-producing capacities of spleen cells from late tumor-bearing mice following preincubation We previously reported that anti-tumor $CD4^+$ T cells from tumor-bearing mice exhibit a progressive increase in the susceptibility to TGF-β-induced suppression of lymphokine-producing capacities.[28] We finally examined how TGF-β susceptibility of spleen cells from late tumor-bearing mice is influenced by preincubation. The results in Table IV demonstrate that (i) compared to cells from early tumor-bearing mice, cells from late tumor-bearing mice exhibit enhanced susceptibility to TGF-β-induced suppression; (ii) recovered capacity of the latter type of cells for IL-2 production is rendered resistant to TGF-β; and (iii) in contrast, recovered capacity for IL-4 production is still susceptible to TGF-β. Thus, the recovery of anti-tumor $CD4^+$ Th responsiveness as induced by preincubation *in vitro* is associated with enhanced resistance of IL-2- but not of IL-4-producing capacity to TGF-β-mediated suppression.

Table III. Effects of Anti-IL-2 and Anti-IL-4 Antibodies on the Proliferation of CTLL-2 and CT.4S Cell Lines

Target cell line	^{3}H-TdR uptake (cpm) by cell line[a]			
	SN[b]		Addition of mAb	
	−	+	anti-IL-2	anti-IL-4
CTLL-2	180 (1.10)	28494 (1.14)	57 (1.02)	31839 (1.04)
CT.4S	1794 (1.01)	10896 (1.07)	11746 (1.03)	1168 (1.14)

a) CTLL-2 or CT.4S cells were stimulated with culture SNs (25%) in the absence or presence of anti-IL-2 or anti-IL-4 monoclonal antibody (5 μg/ml).
b) SN was prepared from one-day cultures subsequent to two-day preincubation of spleen cells from 8 wk tumor-bearing mice.

Table IV. TGF-β Susceptibility of Lymphokine-producing Capacities Recovered after Preincubation

Cells from tumor-bearing mice[a]	Preculture day	Culture		Lymphokine production (^{3}H-TdR uptake; cpm)	
		day	TGF-β	IL-2	IL-4[b]
Early	0	1	−	43352 (1.05)	717 (1.15)
	0	1	+	29793 (1.08)	399 (1.20)
Late	0	1	−	10526 (1.02)	98 (1.32)
	0	1	+	4158 (1.00)	75 (1.25)
	1	1	−	40440 (1.03)	4121 (1.14)
	1	1	+	19474 (1.07)	107 (1.22)
	2	1	−	49944 (1.02)	11357 (1.29)
	2	1	+	43889 (1.02)	510 (1.00)

a) Spleen cells from early or late stage of tumor-bearing mice were cultured for one day with or without 1–2 day preincubation. Four ng/ml of rTGF-β1 was included in half of the cultures/group.
b) The activities of IL-4 produced after 2- or 3-day cultures without TGF-β by cells from early tumor-bearing mice were 23819 (1.09) or 26764 (1.02), respectively.

DISCUSSION

T cell-mediated immunity is, in general, impaired in the tumor-bearing state due to various suppressive mechanisms.[1-10] We have also found that the function of the CD4$^+$ T cell subset is remarkably suppressed compared to phenotypically and/or functionally different subsets of T cells[11, 12] and that anti-tumor CD4$^+$ Th are activated at early tumor-bearing stages, but the responsiveness declines with the progress of the tumor-bearing state.[18] Therefore, molecular mechanisms underlying the immunodysfunction of CD4$^+$ T cells as well as their fate in the context of the functional elimination vs. recovery of anti-tumor CD4$^+$ Th clones represent important aspects of immune dysfunction induced in the tumor-bearing state.

In the present study, we investigated whether anti-tumor CD4$^+$ Th once activated are irreversibly attenuated/functionally eliminated or whether their activity is recovered when they are free of tumor burden. The results demonstrated that (i) anti-tumor CD4$^+$ Th reactivity that is suppressed at late tumor-bearing stages is recovered *in vivo* after tumor removal; (ii) the recovery of reactivity of anti-tumor CD4$^+$ Th is induced *in vitro* by preincubating and reculturing in fresh medium; (iii) the CD4$^+$ T cell contents are comparable in the spleen cell populations from early vs. late tumor-bearing mice as well as in the cell populations from late tumor-bearing mice before vs. after preincubation. These results indicate that the tumor-bearing state imposes potent immunosuppression upon CD4$^+$ Th themselves, but this suppression can be reversed by release from the tumor burden.

It is well established that the T cell-mediated immune defects in patients with acquired immunodeficiency syndrome (AIDS) are associated with a severe reduction in the number of CD4$^+$ T cell as the result of infection with HIV.[29] However, before severe depletion of CD4$^+$ T cells, a long period without apparent syndrome progression can ensue between the time of infection and development of AIDS symptoms.[30-32] This period is characterized by near normal numbers of CD4$^+$ T cells and a selective loss of CD4$^+$ Th function to recall antigens but not T cell mitogens or alloantigens.[33] It is evident that the defect of CD4$^+$ Th function at late tumor-bearing

stages is not due to the loss or reduction of $CD4^+$ T cells (ref. 11 and this study). Thus, the immune dysfunction observed in the tumor-bearing state resembles the impairment of T cell-mediated immunity in early asymptomatic AIDS.

In contrast to the $CD4^+$ Th defect in AIDS, the anti-tumor $CD4^+$ Th immunodysfunction in the tumor-bearing state can be reversed by release from tumor burden. This point is of importance when one considers the prospect for treatments of individuals with clinical tumors. If the tumor-bearing state induces functional elimination or irreversible attenuation of anti-tumor clones, attempts to improve tumor-specific immunity at either tumor-bearig or post-operative stages would be theoretically difficult. However, the fact that removal of a tumor enables anti-tumor $CD4^+$ Th once suppressed to regain their high responsiveness would make such an attempt realistic. Nevertheless, the recovery of anti-tumor $CD4^+$ Th responsiveness in post-operative mice may not necessarily imply the functional improvement of $CD4^+$ Th themselves. It is also possible that anti-tumor $CD4^+$ Th existing at sites other than the tumor mass are recruited into the spleen after tumor resection. In this context, the present results that the responsiveness of spleen cells from late tumor-bearing mice can be improved by preicubating harvested cells *in vitro* and reculturing them in fresh medium strongly support the idea of functional improvement of $CD4^+$ Th themselves.

Although the recovery of anti-tumor $CD4^+$ Th responsiveness reduced at late tumor-bearing stages was observed *in vivo* and *in vitro*, there was a difference in the period required for the recovery between *in vivo* (1–2 wk after tumor resection) and *in vitro* (1–2 days of preincubation) conditions. This may be explained by postulating that mechanisms or factors responsible for inducing the $CD4^+$ Th dysfunction in the tumor-bearing state would be eliminated from post-operative mice not immediately but gradually after tumor resection. Indeed, both observations strengthen the case for the reversible nature of immunosuppression in the tumor-bearing state.

There was also some difference in the time course and TGF-β susceptibility between the recovery of IL-2 and IL-4-producing capacities of anti-tumor $CD4^+$ Th. The recovery of IL-2-producing capacity was observed after one day of preincubation, whereas that of IL-4-producing capacity required two days or preincubation. Moreover, the IL-4-producing capacity recovered after 2-day preincubation was still highly susceptible to TGF-β when compared to the TGF-β resistance of the recovered IL-2-producing capacity. These results may be related to the difference in the time courses of production of IL-2 vs. IL-4 as observed in fresh cells from early tumor-bearing mice (without preincubation).

The most important goal of our research, including the present study, is identification of the molecular mechanisms underlying the tumor-bearing state-induced dysfunction of T cells, especially of the $CD4^+$ T cells. The reduced responsiveness of spleen cells from late tumor-bearing mice has been repeatedly demonstrated.[18, 28] This is not due to the inclusion of some suppressive substance in culture SNs of these cells that would otherwise interfere with the bioassays for lymphokine activities, because (i) the addition of such SN to the bioassay of rIL-2-stimulated CTLL-2 or rIL-4-stimulated CT.4S did not inhibit proliferation of these responding cells (our unpublished observation) and (ii) its addition to cultures of cells from early tumor-bearing mice did not interfere with their responsiveness.[28] Moreover, our recent analyses have revealed that decreased anti-tumor responsiveness of $CD4^+$ Th cells from late tumor-bearing mice was also detected as a reduction in the transcription of lymphokine in the mRNAs (manuscript in preparation). Taken together with the results on the recovery of responsiveness, these results reinforce the view that the responsiveness of $CD4^+$ Th themselves is suppressed in a reversible way by mechanisms and/or factors generated in tumor-bearing hosts.

TGF-β is a hormonally active polypeptide that has been demonstrated to exert multiple actions on both normal and transformed cells.[34, 35] Among a variety of TGF-β actions, recent studies have defined various immunoregulatory properties of TGF-β including inhibition of T and B cell proliferation and lymphokine production.[36–40] A series of studies from our laboratory has delineated the role of a tumor cell-derived factor, TGF-β, in inducing the $CD4^+$ Th-selective immunosuppression in the tumor-bearing state.[11, 12] Moreover, we have found that the level of TGF-β in blood (plasma) increases with the progress of the tumor-bearing state.[28] It remains to be determined whether increased amounts of TGF-β or other factors are responsible for reducing the responsiveness of anti-tumor $CD4^+$ Th at late tumor-bearing stages. In this context, the present system consisting of preincubation and reculture in fresh medium may provide us with a model for investigating this by addition of various factors to the preincubation cultures. Such a study is in progress in our laboratory.

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