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Transforming Growth Factor-β (TGF-β)-mediated Immunosuppression in the Tumor-bearing State: Enhanced Production of TGF-β and a Progressive Increase in TGF-β Susceptibility of Anti-tumor CD4$^+$ T Cell Function

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The present study deals with the effect of transforming growth factor-β (TGF-β) on anti-tumor immune responsiveness at various stages of the tumor-bearing state. 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 macrophage-activating factor (MAF)/interferon-γ (IFN-γ) 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 that had been pulsed with CSA1M tumor antigens *in vivo* in the tumor-bearing state. The IL-2-producing capacity of CD4$^+$ T cells reached the maximal level as early as one week after tumor implantation but decreased with the progress of tumor-bearing stages. In contrast, the capacity of CD4$^+$ T cells to produce MAF/IFN-γ was not affected but was maintained at high levels even late in the tumor-bearing state. The addition of recombinant TGF-β (rTGF-β) to cultures of spleen cells from various tumor-bearing stages resulted in the suppression of lymphokine production. However, the magnitude of the TGF-β-induced suppression varied depending on which tumor-bearing stages of splenic cells were tested as a responding cell population; it was slight in cells from early (1–3 wk) tumor-bearing stages but increased in cells from donor mice at later tumor-bearing stages. Thus, spleen cells from late tumor-bearing stages with weak but significant IL-2-producing and considerable MAF/IFN-γ producing capacities failed to produce these lymphokines when rTGF-β was present in cultures. A progressive increase in the TGF-β susceptibility was also observed for IL-4-producing Th2 as well as IL-2/MAF-producing Th1 cells. In addition, increased levels of TGF-β were detected in plasma from tumor-bearing mice at late stages. Taken together, these results indicate that tumor-bearing mice exhibit enhanced production of TGF-β as well as a progressive increase in the susceptibility of anti-tumor CD4$^+$ T cells to TGF-β-induced suppressive mechanisms.

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

A number of suppressive mechanisms come into operation and various types of immune dysfunction are manifested during the tumor-bearing state.[1-10] We have recently demonstrated that the self Ia-restricted CD4$^+$ helper T cell (Th[2]) 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 nominal antigens as well as cell-bound antigens such as tumor antigens.[13,14] Therefore, the functional impairment of the CD4$^+$ Th pathway represents a central aspect of immune dysfunction induced in the tumor-bearing state.

Two lines of studies from our laboratory have cast light on the molecular mechanisms of immunosuppression during the tumor-bearing state.[11,12,15] First, most tumor cell lines produce transforming growth factor-β (TGF-β) and the defect in CD4$^+$ Th function in immune responses to nominal antigens is due, mainly, to the activity of TGF-β produced by tumor cells in the tumor-bearing state.[12] Second, CD4$^+$ Th recognize processed forms of antigen in association with self Ia molecules expressed on antigen-presenting cells (APC).[16-18] Our recent studies illustrated that (i) APC in tumor-bearing hosts bind tumor antigens *in vivo* to construct an effective immunogenic unit[15,19]; and (ii) lymphokines (IL-2 and MAF/IFN-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.[15] 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.[15] Taken

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[2] Abbreviations: Th, helper T cells; APC, antigen-presenting cells; MAF, macrophage-activating factor; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; CTL, cytotoxic T lymphocyte; SN, supernatant.

together, these observations implied that the defect in $CD4^+$ Th can be ascribed to the progressive decline of responsiveness of $CD4^+$ Th themselves rather than the APC function. However, it has not been directly determined whether the two lines of observations are mutually related; namely, how the reactivity of anti-tumor $CD4^+$ Th, once activated, is influenced by TGF-β during progression of the tumor-bearing state.

In the present study, we investigated the effect of TGF-β on anti-tumor $CD4^+$ Th responsiveness in various tumor-bearing stages. The results demonstrate that anti-tumor $CD4^+$ Th from early tumor-bearing stages of mice can produce interleukin-2 (IL-2) and macrophage-activating factor (MAF)/interferon-γ (IFN-γ) *in vitro* through collaboration with APC binding and presenting tumor antigens. Such a capacity was slightly reduced by addition of rTGF-β to lymphokine-producing cultures. In contrast, late tumor-bearing stages of mice exhibited different patterns of anti-tumor $CD4^+$ Th responsiveness. IL-2-producing capacity was found to be markedly reduced when responding spleen cells from late stages of tumor-bearing mice were cultured without addition of exogenous rTGF-β. MAF/IFN-γ-producing capacity was maintained at high levels even at late tumor-bearing stages. However, such residual IL-2-producing and still potent MAF/IFN-γ-producing capacities were strikingly suppressed by addition of rTGF-β to cultures. Moreover, increased levels of TGF-β were found in plasma from tumor-bearing mice at late stages. These results indicate that tumor-bearing mice exhibit a progressive increase in TGF-β-susceptibility of anti-tumor $CD4^+$ Th responsiveness as well as increased levels of plasma TGF-β during progression of tumor-bearing stages.

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 be a nonproducer of RSV was kindly provided by Dr. T. O. Yoshida, Hamamatsu University School of Medicine, Hamamatsu. 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.

Cells and reagents Mv1Lu cells for TGF-β assays were obtained from the American Type Culture Collection, Rockville, MD. Recombinant TGF-β (rTGF-β1) and anti-TGF-β antibody were purchased from King Brewing Co. Ltd., Kakogawa. Normal rabbit IgG was obtained from Cappel Labs., West Chester, PA. Hybridoma cells producing anti-IL-2 antibody (S4B6)[21] anti-IL-4 antibody (11B11)[22] were kindly provided by Dr. T. Mosmann and Dr. W. E. Paul, respectively. Purified monoclonal antibodies were used.

Detection of TGF-β activity (Mv1Lu cell growth inhibition assay) The growth inhibition assay was performed, with slight modifications, according to the original method by Cheifetz *et al.*[23] as previously described.[12] Briefly, Mv1Lu cells (1×10^4) were cultured with diluted samples or rTGF-β in a 0.2-ml volume of PRMI 1640 medium containing 5% FCS in 96-well microplates (Corning #25860, Corning Glass Works, Corning, NY) for 24 h in a CO_2 incubator. Cells were pulse-labeled with 20 kBq of ^{3}H-thymidine (^{3}H-TdR) for the final 4 h, and the incorporated radioactivity was measured. Results are shown as the mean cpm $\pm$ SE of triplicate cultures.

Preparation of CSA1M tumor-bearing mice CSA1M tumor-bearing mice were prepared by inoculating i.d. 2×10^5 viable CSA1M cells.

Treatment of lymphoid cells with antibody plus complement (C) Spleen cells (10^8) were incubated at 4°C for 30 min with appropriate dilutions of anti-CD4 (GK1.5) or -CD8 (3.155) monoclonal antibody as previously described.[11] Cells were washed and incubated at 37°C for 45 min with rabbit complement (C) preabsorbed with syngeneic mouse spleen cells at a final dilution of 1/20. The efficacy of these antibody treatments was confirmed in flow microfluorometric analysis by demonstrating that treatment with anti-CD4 or -CD8 antibody results in almost complete elimination of the respective $CD8^+$ or $CD4^+$ T cell subset without damage to the other T cell subset.

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) 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 various times, 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 according to their ability to support the proliferation of the IL-2-dependent T cell line, CTLL-2.[24] The CTLL-2 line used here responded selectively to IL-2 but not to IL-4. 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 the uptake of ^{3}H-TdR during 4-h pulsing with 20 kBq of ^{3}H-TdR/well. IL-2-induced proliferation of CTLL-2 was not affected by addition of TGF-β.

Assay system for MAF activity Macrophage monolayers were prepared by seeding 10^5/well of thioglycolate-elicited peritoneal exudate cells in 0.2 ml into 96-well,

flat-bottomed microplates (Corning 25860). SNs were assayed for MAF activity according to their ability to induce nonspecific anti-tumor cytotoxic activity in thioglycolate-elicited macrophages as previously described.[15,25] Briefly, macrophage monolayers were exposed to lipopolysaccharide (LPS) at 0.1 μg/ml and recombinant IFN-γ (positive control) or SN samples at various concentrations. X5563 plasmacytoma cells (2×10^4) as targets were added to each well of the above macrophage monolayers, and these were incubated in a total volume of 0.2 ml for 24 h at 37°C. At 4 h before the end of culturing, 20 kBq of ^{3}H-TdR was added to each well. The labeled cells were collected on a glass filter, and the radioactivity trapped in the filter was counted in a liquid scintillation spectrometer. In some figures, MAF activity was expressed as the percentage of cytostasis according to the following formula:

% Cytostasis
= [1 − (cpm in culture containing sample-treated macrophages) / (cpm in culture containing control macrophages)] × 100,

where control macrophages represent macrophage monolayers preincubated with RPMI 1640 medium containing 5% FCS for 24 h instead of [LPS + test samples]. Radioactivity incorporated into macrophages was negligibly small, and the cpm incorporated by X5563 tumor target cells cultured with control macrophages was always more than or equal to the cpm taken up by tumor target cells incubated alone. The growth of X5563 tumor cells was not affected by TGF-β.

Assay system for IL-4 activity SNs were assayed for IL-4 activity according to 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 in terms of the uptake of ^{3}H-TdR during 6-h pulsing with 20 kBq of ^{3}H-TdR/well.

RESULTS

Capacities of spleen cells from CSA1M-bearing mice at various stages to produce IL-2 and MAF/IFN-γ upon *in vitro* culture We have confirmed the fact[15] that whole spleen cells from 2–3 wk CSA1M-bearing BALB/c mice produce IL-2 and MAF/IFN-γ upon *in vitro* culture without addition of exogenous CSA1M tumor antigens (Table I, line 3). Earlier studies from our laboratory further demonstrated that this lymphokine production was achieved through collaboration between CSA1M-specific CD4$^+$ T cells and APC that had been pulsed with CSA1M antigens *in vivo* in the tumor-bearing state.[15] The results of Table I also confirm that CD4$^+$ T cells from CSA1M-bearing mice are exclusively responsible for the production of both IL-2 and MAF/IFN-γ. This was the case irrespective of whether responding cell populations were obtained from tumor-bearing mice at early (1–3 wk) or late (8–10 wk) stages (data not shown). Because MAF activity detected in the present assay system was found to be completely neutralized by anti-murine IFN-γ antibody,[27] it has been regarded as representing IFN-γ activity. The term "MAF" is written as "MAF/IFN-γ" throughout this paper, considering that its detection was based on anti-tumor cytotoxic activity by macrophages.

Table I. Production of IL-2 and MAF/IFN-γ by CD4$^+$ T Cells from CSA1M Tumor-bearing Mice

Responding cells[a] from mice	Treatment with	^{3}H-TdR uptake (cpm) by cells	
		CTLL-2 for IL-2 assay[b]	X5563 for MAF assay[c]
Bkg[d]		434 (1.07)	103825 (1.01)
Normal	−	549 (1.15)	123196 (1.03)
CSA1M-bearing	−	10820 (1.16)	5000 (1.20)
CSA1M-bearing	C	11813 (1.04)	19737 (1.03)
CSA1M-bearing	αCD4+C	1214 (1.10)	95931 (1.03)
CSA1M-bearing	αCD8+C	11864 (1.10)	10040 (1.14)

a) Spleen cells (5×10^6/well) from normal or 2–3 wk CSA1M-bearing (2–3 wk after CSA1M implantation) BALB/c mice were cultured in 24-well culture plates before or after treatment with anti-CD4 or anti-CD8 antibody plus C. SNs obtained 48 h after culturing were submitted to IL-2 and MAF assays.
b) CTLL-2 cells (10^4/well) were cultured with 50% SN for 20 h in 96-well microplates.
c) X5563 tumor cells (2×10^4/well) were cultured on macrophage monolayers which had been activated with 50% SN plus LPS for 24 h.
d) Bkg indicates the growth of CTLL-2 in the absence of SN or the growth of X5563 cells on macrophage monolayers not treated with SN.

We next asked how the capacities of CD4$^+$ T cells to produce IL-2 and MAF/IFN-γ are modulated during progression of the tumor-bearing state. Spleen cells from various stages of tumor-bearing mice were cultured, and SNs harvested were tested for IL-2 and MAF/IFN-γ activities. A representative result from among three experiments is shown in Fig. 1. Cells obtained from mice at early CSA1M-bearing stages (1–3 wk) exhibited high levels of IL-2 production. However, IL-2-producing capacity was reduced during progression of tumor-bearing stages, and cells from late (8–10 wk) tumor-bearing mice produced remarkably decreased amounts of IL-2 when compared to those from mice at early stages. In contrast, the MAF/IFN-γ production exhibited different time-related patterns from those of IL-2 production. Potent MAF/IFN-γ production was induced by cells from early tumor-bearing mice and such high levels of MAF/IFN-γ

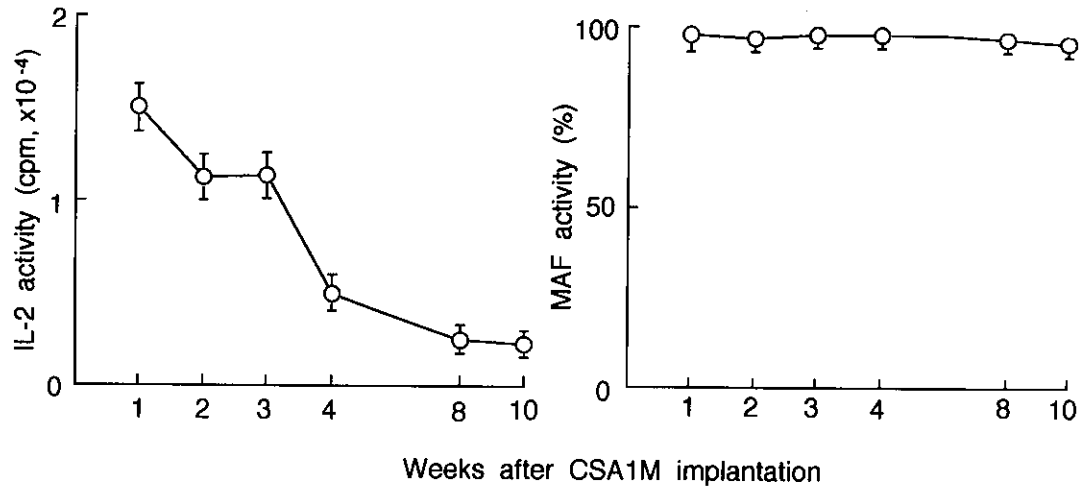

Fig. 1. Capacities of spleen cells from tumor-bearing mice at various stages to produce IL-2 and MAF/IFN-γ. Spleen cells (5×10^6/well) from BALB/c mice at various times (wk) after implantation with CSA1M tumor cells were cultured in 24-well culture plates for 48 h. Culture SNs were tested for their ability to support the proliferation of CTLL-2 cells (IL-2 activity) or to suppress the growth of X5563 plasmacytoma cells (MAF activity). MAF activity was expressed as % cytostasis of X5563 target cells as described in "Materials and Methods."

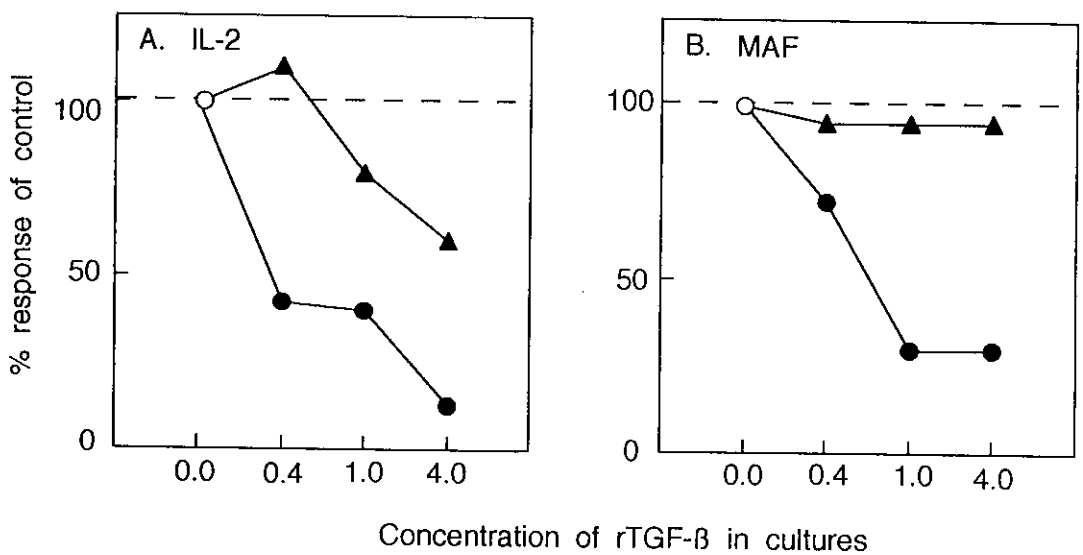

Fig. 2. Differential TGF-β susceptibility in IL-2/MAF-producing T cells from early vs late CSA1M-bearing mice. Spleen cells from early (2–3 wk) (▲) or late (8–10 wk) CSA1M-bearing mice (●) were cultured in the absence (○) or the presence of various concentrations of rTGF-β. Culture SNs were tested for IL-2 and MAF activities. Both activities were expressed as % response (lymphokine production) relative to the control (lymphokine activities produced in the absence of rTGF-β).

production were maintained even at late stages when reduced magnitudes of IL-2 were detected in the same culture SNs. When culture SN samples for MAF assays were titrated by serial dilution, no significant difference was found in the magnitude of MAF activity between samples from spleen cells of early and late tumor-bearing mice (data not listed). Thus, tumor-bearing mice are rendered responsive to tumor antigens, but a part of their responsiveness (IL-2-producing capacity) is reduced *in vivo* in the tumor-bearing state during stage progression.

Tumor-bearing stage-related increase in TGF-β susceptibility of lymphokine-producing capacities We previously reported that TGF-β produced by tumor cells in the tumor-bearing state induces deleterious effects on T cells, especially on the $CD4^+$ Th subset.[12] In considering this fact, we examined whether anti-tumor $CD4^+$ T cell responsiveness is influenced by the presence of TGF-β. Spleen cells from tumor-bearing mice at early (1–3 wk) or late (8–10 wk) stages were cultured in the presence of different concentrations of rTGF-β. The results (Fig. 2) illustrate that the capacity of spleen cells from early vs. late tumor-bearing stages to produce IL-2 and MAF/IFN-γ is influenced by TGF-β in a different way; the addition of 1–4 ng/ml of rTGF-β to cultures of spleen cells from mice at early stages resulted in slight to mild (20–40%) and only marginal reduction of IL-2 and MAF/IFN-γ production, respectively. In contrast, the same concentrations of rTGF-β exerted more potent suppressive effects on the lymphokine production by spleen cells from tumor-bearing mice at late stages. However, the IL-2-producing capacity was already reduced *in vivo* in the tumor-bearing state. Therefore, the TGF-β effect was further examined under conditions in which an increased number (8.0×10^6/well) of responding spleen cells were cultured to obtain increased levels of IL-2 production. The IL-2-producing capacity that was observed in these cultures containing no rTGF-β, although it was appreciably increased but still weak compared to that exhibited by cells from early tumor-bearing mice, was severely suppressed (Table II).

We examined the time course of lymphokine production to confirm that the suppression of the lymphokine production by rTGF-β is not limited to the specific timing for harvesting cultures. Fig. 3 illustrates that TGF-β exhibits its suppressive effects irrespective of when cultures are harvested. We investigated in more detail the relationship between the tumor-bearing stages and the TGF-β susceptibility. Spleen cells from tumor-bearing mice at various stages were cultured in the absence or presence of 4 ng/ml rTGF-β, and SNs were tested for lymphokine activities (Fig. 4). The results (i) confirm that IL-2-producing capacity is already reduced *in vivo* and the residual capacity to produce IL-2 upon *in vitro* culture is suppressed in the presence of rTGF-β and

Table II. Increased TGF-β-susceptibility of MAF/IFN-γ-producing Capacity in Spleen Cells from Late Tumor-bearing Mice

Responding cell No.[a]	Concentration of rTGF-β (ng/ml)	IL-2 activity (cpm) produced by cells from tumor-bearing mice: early [% response][b]	late [% response]
5×10^6	0.0	37590 (1.01) [100.0]	3623 (1.02) [100.0]
	0.4	30542 (1.03) [80.9]	1655 (1.05) [45.7]
	4.0	20533 (1.03) [54.6]	489 (1.03) [13.5]
8×10^6	0.0	33401 (1.03) [100.0]	5751 (1.04) [100.0]
	0.4	26491 (1.02) [79.9]	4376 (1.09) [76.1]
	4.0	16312 (1.07) [49.4]	1148 (1.10) [20.2]

a) Spleen cells (5 or 8×10^6/well) from early (2–3 wk) or late (8–10 wk) CSA1M-bearing mice were cultured in the presence of various concentrations of rTGF-β.
b) The % response is the ratio of cpm in the presence of rTGF-β to cpm in the absence of rTGF-β (control).

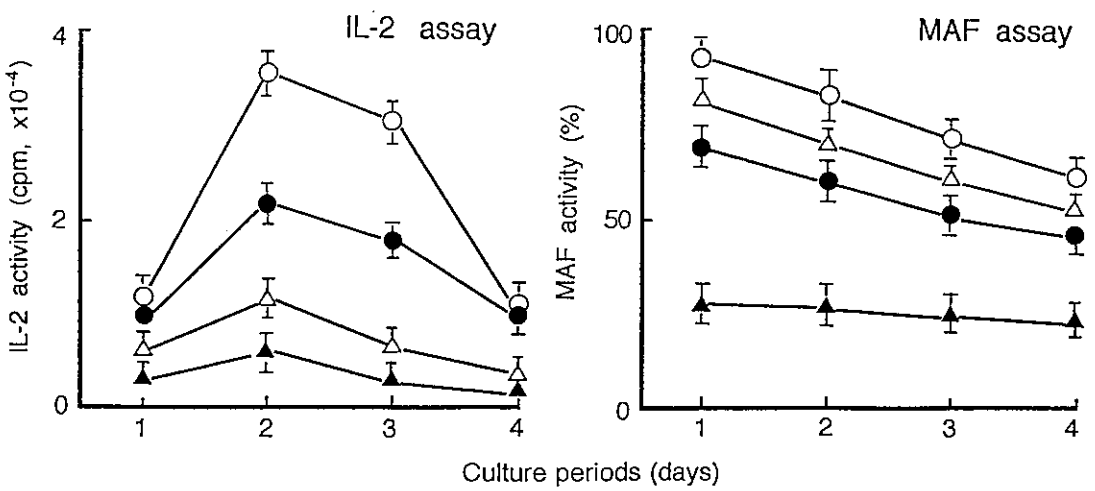

Fig. 3. Time course of lymphokine production by cells from early (○, ●) or late tumor-bearing mice (△, ▲) in the absence (○, △) or presence of rTGF-β (●, ▲). Spleen cells from early or late CSA1M-bearing mice were cultured for various times (days) in the absence or presence of 4.0 ng/ml rTGF-β.

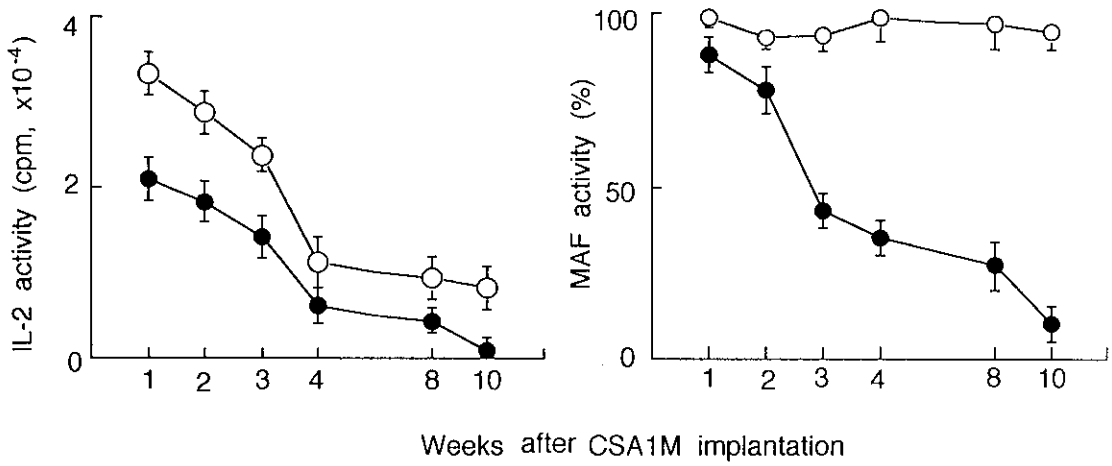

Fig. 4. Effect of rTGF-β on lymphokine production by spleen cells from tumor-bearing mice at various stages. Spleen cells from various stages of CSA1M-bearing mice were cultured in the absence (○) or presence of 4.0 ng/ml rTGF-β (●).

Table III. Capacity of Spleen Cells from Various Stages of Tumor-bearing Mice to Produce IL-4

Responding cells[a] from tumor-bearing mice	^{3}H-TdR uptake (cpm) of CT.4S (IL-4 activity)[b]	
	Exp. 1	Exp. 2
1 wk	48673 (1.02)	43996 (1.22)
2 wk	24934 (1.01)	56207 (1.00)
3 wk	41125 (1.03)	22269 (1.10)
4 wk	22382 (1.25)	12400 (1.08)
8 wk	1738 (1.07)	2579 (1.09)
10 wk	698 (1.13)	4292 (1.06)

a) Spleen cells (5×10^6/well) from mice at the indicated tumor-bearing stages were cultured for 2 days. Culture SNs were tested for their IL-4 activity using the IL-4-dependent CT.4S cell line.

b) ^{3}H-TdR uptake in the absence of culture SNs (Bkg) or ^{3}H-TdR uptake in the presence of 50 U/ml IL-4 were 807 (1.12) or 82682 (1.05) for Exp. 1 and 356 (1.15) or 109395 (1.05) for Exp. 2, respectively.

(ii) demonstrate that MAF-producing capacity that is maintained until late stages is rendered more susceptible to TGF-β during progression of the tumor-bearing stage. Taken together, the results of Figs. 2 and 3 and Table II indicate a tumor-bearing stage-related increase in TGF-β susceptibility of lymphokine-producing capacities.

Th2 (IL-4-producing) function in spleen cells from tumor-bearing mice at various stages It has been reported that the Th1 function is suppressed by the lymphokine (IL-10) that is produced by the Th2 type of cells.[28] Therefore, we examined whether the Th2 activity is generated during the tumor-bearing state, and if this is the case, whether such an activity increases reciprocally to the decrease of Th1 activity. Table III summarizes the capacity of spleen cells from CSA1M-bearing mice at various stages to produce the representative Th2-type lymphokine, IL-4. The results demonstrate that cells from tumor-bearing mice at early stages produced large amounts of IL-4, whereas markedly reduced levels of IL-4 production were observed in cells from mice at late stages. This pattern of lymphokine production was similar to that seen for IL-2 production in Fig. 1. The results of Table IV confirm that lymphokine activities assessed by using CTLL-2 and CT.4S cell lines are due to IL-2 and IL-4, respectively; the proliferation of CTLL-2 induced by 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.

We have further examined whether spleen cells from late tumor-bearing mice produce a factor capable of suppressing the Th1 function of cells from early tumor-bearing mice. The results of Table V demonstrate that addition of rTGF-β to cultures of cells from early tumor-bearing mice resulted in comparable levels of suppression of IL-2/MAF production to those observed in Fig. 4. In contrast, the addition of culture SNs from cells of late tumor-bearing mice had only a marginal influence on the lymphokine-producing capacity of cells from early tumor-bearing mice. This also excluded the possibility that the reduced lymphokine activities in the SN from late tumor-bearing mice resulted from the inclusion of substances that interfere with detection in the assay systems. Taken collectively, these results indicate that (i) the Th2 function as assessed by IL-4 activity is also suppressed late in the tumor-bearing state; and (ii) the

Table IV. Effect of Anti-IL-2 and -IL-4 Antibodies on Proliferation of CTLL-2 and CT.4S Cell Lines

Sample[a]	^{3}H-TdR uptake (cpm, $\times 10^{-3}$) by cell line in the presence of mAb					
	CTLL-2			CT.4S		
	−	αIL-2	αIL-4	−	αIL-2	αIL-4
−	0.3 (1.21)	ND	ND	0.6 (1.25)	ND	ND
rIL-2	40.0 (1.02)	2.9 (1.12)	44.9 (1.04)	1.0 (1.04)	ND	ND
rIL-4	1.2 (1.14)	ND	ND	34.7 (1.05)	35.3 (1.04)	1.3 (1.09)
SN	18.2 (1.08)	0.3 (1.06)	20.2 (1.06)	28.4 (1.01)	25.5 (1.08)	0.7 (1.21)

a) CTLL-2 or CT.4S cells were stimulated with rIL-2 (25 U/ml), rIL-4 (25 U/ml) or culture SN (25%) from spleen cells of 2–3 wk CSA1M-bearing mice in the absence or presence of anti-IL-2 or anti-IL-4 monoclonal antibody (5 μg/ml).
ND, not detected.

Table V. Failure of Culture SNs from Cells of Late Tumor-bearing Mice to Influence Lymphokine Production by Cells from Early Tumor-bearing Mice

Samples[a] added to cultures	IL-2 activity (cpm)	MAF activity (cpm)
Bkg[b]	258 (1.15)	124779 (1.00)
None	26744 (1.10)	5874 (1.74)
rTGF-β (4 ng/ml)	17051 (1.05)	13026 (1.19)
SN[c] (50%)	26270 (1.02)	7241 (1.38)
SN (25%)	27398 (1.05)	4380 (1.81)

a) Various samples at indicated concentrations were added to cultures of cells from early (2–3 wk) tumor-bearing mice.
b) Bkg shows ^{3}H-TdR uptake of CTLL-2 cells alone for IL-2 assay and ^{3}H-TdR uptake of X5563 cells alone for MAF assay.
c) Culture SNs were obtained 24 h after cultures of cells (5×10^6/well) from late (10 wk) tumor-bearing mice.

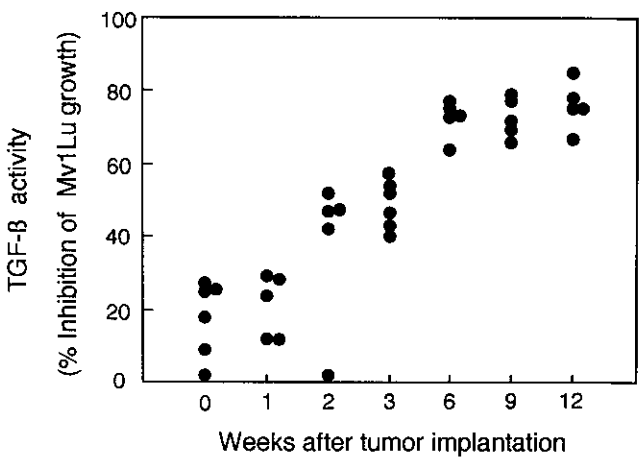

Fig. 5. Increased levels of TGF-β activity in plasma from tumor-bearing mice at late stages. Plasma was obtained from normal BALB/c mice or tumor-bearing mice at various stages. After treatment of each plasma sample with acid, the sample was included in the Mv1Lu cell growth inhibition assay at the final concentration of 5%. TGF-β activity was expressed as % inhibition of Mv1Lu cell growth and individually plotted. The % inhibition was calculated as follows: $100\times[1-$(cpm in culture with plasma sample/cpm in culture without plasma sample)].

tumor-bearing stage-related suppression of Th1 function is not due to either enhanced function of Th2 cells or humoral products derived from spleen cells of late tumor-bearing mice.

Increased levels of TGF-β activity in plasma from tumor-bearing mice at late stages CSA1M tumor cells are known to produce large amounts of TGF-β in culture SN when cultured *in vitro*.[12] We finally examined whether increased levels of TGF-β activity can be detected in plasma from tumor-bearing mice. Plasma from tumor-bearing mice at various stages was obtained through careful bleeding with a heparinized syringe. An active form of TGF-β activity was not found in any sample of plasma (data not shown). Plasma samples were treated with acid to detect latent form of TGF-β. Acid treatment was performed by dialysis against two changes of 1 *M* acetic acid and then against phosphate-buffered saline (pH 7.4). TGF-β activity in these acid-treated plasma samples is individually plotted in Fig. 5. The results illustrate that increased levels of TGF-β activity are detected in plasma from tumor-bearing mice and that such an increase is related to the progress of the tumor-bearing state.

Additional experiments were performed to determine whether the TGF-β activity detected in the growth inhibition assay using Mv1Lu cells is actually due to TGF-β. This was done by examining the blocking of such TGF-β activity by anti-TGF-β antibody. The results of Table VI demonstrate that plasma TGF-β activity can be suppressed almost completely by addition of anti-TGF-β antibody, confirming that the activities that are detected by the growth inhibition assay are those of TGF-β itself.

Table VI. Blocking of TGF-β Activity in Plasma by Anti-TGF-β Antibody

Sample	Concentration	^{3}H-TdR uptake of Mv1Lu cells[a] (cpm)	
		Normal IgG	αTGF-β Ab
Medium	—	10727 (1.03)	—
rTGF-β	1 ng/ml	1401 (1.25)	10735 (1.11)
Plasma #1[b]	5%	3985 (1.10)	12212 (1.04)
Plasma #2[b]	5%	1531 (1.08)	11442 (1.02)

a) Mv1Lu cells were cultured with various samples in the presence of normal (control) chicken IgG or anti-TGF-β antibody (50 μg/ml).
b) Plasma #1 and #2 samples were obtained from 6 and 12 wk CSA1M-bearing mice (3 mice/group).

DISCUSSION

Tumor-bearing hosts fail to reject malignant cells even in experimentally induced animal tumor models in which tumor cells express detectable levels of immunogenicity. Numerous reports showing that a number of suppressive mechanisms[1-10] are generated during the growth of immunogenic tumors have served to explain the paradoxical growth of these tumors in immunocompetent syngeneic hosts. Previous studies from our laboratory have investigated immune dysfunction induced in the tumor-bearing state.[11, 15] The results have demonstrated that (i) the function of the CD4$^+$ T cell subset, especially of CD4$^+$ Th, whose activation requires self APC, is more severely suppressed than that of the CD8$^+$ T cell subset[11]; (ii) the functional impairment of CD4$^+$ Th is due to CD4$^+$ Th themselves rather than the APC function[11]; and (iii) the immunodysfunction of anti-tumor CD4$^+$ Th forms a part of the generalized CD4$^+$ T cell defect, and although anti-tumor CD4$^+$ Th are activated at early tumor-bearing stages, the activity is depressed with the progress of tumor-bearing stages.[15]

Another line of our work has revealed that most tumor cell lines produce TGF-β and that this cytokine induces deleterious effects especially on CD4$^+$ Th directed against a nominal antigen.[12] Although these two lines of work[11, 15, 12] implied that TGF-β produced in the tumor-bearing state induced the suppression of anti-tumor CD4$^+$ Th function, this was not directly demonstrated in the previous studies. Since anti-tumor CD4$^+$ Th are activated in tumor-bearing mice at the early stages, the present study was undertaken to investigate whether TGF-β actually suppresses the function of the anti-tumor CD4$^+$ Th once activated. This study demonstrated that (i) CD4$^+$ T cells from tumor-bearing mice at early stages produced IL-2 and MAF/IFN-γ upon *in vitro* culture and this lymphokine production was slightly (IL-2) or marginally (MAF/IFN-γ) suppressed by addition of rTGF-β to the culture; (ii) the IL-2-producing capacity was markedly reduced in CD4$^+$ T cells from tumor-bearing mice at late stages whereas the same CD4$^+$ T cells retained high levels of MAF/IFN-γ-producing capacity; (iii) CD4$^+$ T cells at late tumor-bearing stages exhibited higher susceptibility to TGF-β-induced immunosuppression compared to those at early stages; and (iv) increased levels of plasma TGF-β were observed at late tumor-bearing stages.

Suppressive mechanisms generated in the tumor-bearing state could be mediated by tumor cell-derived factors and by interactions between different types of host lymphoid cells. TGF-β is a hormonally active polypeptide that is produced by many types of cells, irrespective of whether they are nontransformed or transformed.[29, 30] This unique growth factor was originally discovered as one of the molecules with transforming activities able to support the anchorage-independent growth of normal cells.[31-33] However, it has been reported that TGF-β exerts multiple actions on both normal and transformed cells,[29] including a negative growth-modulatory effect.[34] 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.[35-39] We have also investigated host immune reactivity in the tumor-bearing state.[11, 12, 15] Thus, our previous[12, 15] and present studies have delineated the role of a tumor cell-derived factor, TGF-β, in inducing immunosuppression in the tumor-bearing state.

We have found that the level of TGF-β in blood (plasma) increases during progression of the tumor-bearing state. TGF-β is present in serum as a latent form (the form secreted) and an inactive form (a complex with serum α_2-macroglobulin),[40] both of which can be detected after acidification. Although growing tumor cells are at least partly responsible for the production of elevated levels of TGF-β, host-derived components such as platelets are also expected to produce TGF-β at the tumor site and thereby contribute to the enhanced production in the tumor-bearing state. Irrespective of this, increased amounts of latent TGF-β produced would be converted into an active form through various physiological mechanisms,[41, 42] consequently providing enhanced immunoregulatory activity.

The suppression of anti-tumor Th1 function in the tumor-bearing state should be considered from the following aspect. Differential suppression was observed for the capacity of Th1 cells from late tumor-bearing mice to produce IL-2 vs. IFN-γ. Thus, Th1 cells from these mice exhibited a reduced capacity to produce IL-2 upon *in vitro* culture under conditions in which the same responding cells produced comparable (unaffected) levels of

IFN-γ to those produced by cells from mice at early stages. Our earlier study revealed that impairment of $CD4^+$ T cell immunity is not due to either loss of $CD4^+$ T cells[11] or induction of suppressor cell activity.[15] The fact that comparable magnitudes of MAF/IFN-γ activity can be generated by cells from tumor-bearing mice at early and late stages supports this notion. Thus, it is implied that the tumor-bearing state imposes deleterious suppressive effects on $CD4^+$ T cells. This study directly demonstrated that TGF-β, which would be produced *in vivo* by tumor cells, reduces the reactivity of anti-tumor $CD4^+$ Th once activated at early tumor-bearing stages. Moreover, detailed analyses revealed a progressive increase in the susceptibility of Th1 function (both IL-2- and MAF-producing capacities) to TGF-β-induced immunosuppressive effects during progression of tumor-bearing stages. These observations, however, raise the question of why differential suppression was observed for IL-2- vs. MAF/IFN-γ-producing capacities when responding cells from late tumor-bearing mice were cultured in the absence of exogenous TGF-β. This might be explained by considering that there exists a difference in susceptibility to a given concentration of TGF-β between IL-2- and MAF-producing capacities of $CD4^+$ T cells. For example, the results of Fig. 2 that responding cells even from early tumor-bearing mice exhibit different sensitivity to TGF-β (1–4 ng/ml) may be compatible with the above postulation. More accurate analyses will be required to confirm this by using a variety of cloned Th1 cell lines with the capacity to produce both IL-2 and MAF/IFN-γ.

Mosmann *et al.* isolated two distinct groups of murine Th clones.[43–46] One type of clones, designated Th1, produces IL-2 and IFN-γ but not IL-4, IL-6 or IL-10. The second type of clones, designated Th2, produces IL-4, IL-6 and IL-10 but not IL-2 or IFN-γ. It appears that the activation of these two types of Th cells is reciprocally controlled and the induction of a Th1-mediated response can lead to suppression of Th2 function, and *vice versa*.[28,47] IFN-γ produced by Th1 clones inhibits the cytokine production by Th2 clones, and IL-10 produced by Th2 clones down-regulates the cytokine production by Th1 clones.[28,47] Thus, it is increasingly evident that both Th1 and Th2 cross-regulate the other types of cells, providing an immunoregulatory mechanism distinct from that mediated by tumor-derived factors.

In the CSA1M tumor model studied here, it was found that IL-4-producing Th (Th2) cells are activated in parallel with the activation of IL-2/MAF-producing Th1 cells at early tumor-bearing stages. The function of Th2 cells once activated was also reduced at late tumor-bearing stages. It may be postulated that a concurrent decline in both Th1 and Th2 activities is due to their cross-regulation, involving the operation of their products, IFN-γ (MAF) and IL-10. However, even at late stages when IL-2 production by anti-tumor Th1 cells was reduced, the capacity to produce MAF/IFN-γ was not suppressed. Considering that IL-10 exerts a more potent suppressive effect on the MAF/IFN-γ production than on the IL-2 production,[28,47] it is unlikely that the suppression of the Th1 function is due mainly to the Th2 product, IL-10. Conversely, it is likely that MAF/IFN-γ produced at high levels by Th1 cells even at late tumor-bearing stages contributes to the suppression of the Th2 function.

We have previously reported that, in contrast to the decline in IL-4 production, increased levels of IL-6 were continuously detected in serum from CSA1M-bearing mice at late stages.[48] However, IL-6 can be produced by various types of cells including $CD4^+$ and $CD8^+$ T cells and macrophages. We have also found that the IL-6 production was mediated not by the $CD4^+$ T cell subset (Th2 cells) but by $CD8^+$ T cells and/or macrophages at late tumor-bearing stages.[48] This contrasts with the fact that enhanced production of IL-6 was mediated exclusively by $CD4^+$ T cells in type II collagen-induced arthritic mice,[49] and is compatible with the notion that the activation of Th2 is suppressed at late tumor-bearing stages. Although the possibility is not excluded that the limited amount of IL-10 produced by Th2 cells functioned to suppress the Th1 function, the suppression in the tumor-bearing state is considered to be due mostly to mechanisms other than Th1/Th2-derived factor-mediated regulation.

Our present results show that anti-tumor $CD4^+$ T cells are rendered less responsive during progression of tumor-bearing stages and that such reduced responsiveness is associated with a progressive increase in TGF-β susceptibility. Further studies will be required to investigate which mechanisms and/or factors are responsible for the increase in TGF-β susceptibility of $CD4^+$ T cells in the tumor-bearing state, and in particular to examine the possibility that such an increase is based on enhanced expression of TGF-β receptors. These analyses could contribute to a better understanding of the reduction of anti-tumor $CD4^+$ T cell responsiveness during progression of tumor-bearing stages and also provide an important insight into tumor immunology from the viewpoint of the host's defence mechanisms.

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