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Selective Suppression of the Generation of Anti-tumor L3T4$^+$ but Not of Lyt-2$^+$ T Cell-mediated Immunity in the Tumor-bearing State

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C3H/He mice hyperimmune against syngeneic MH134 hepatoma were prepared by intradermal (id) inoculation of viable tumor cells followed by surgical resection of the tumor and by repeated id challenges with viable tumor cells. Winn assays performed utilizing spleen cells from these mice have revealed that both Lyt-2$^+$ and L3T4$^+$ T cell subsets from MH134-hyperimmune mice produced complete tumor protection. The *in vivo* tumor-neutralizing activity was also found in spleen cells from tumor-bearing mice at various times after id implantation of MH134 tumor cells. However, in contrast to comparable tumor-neutralization by Lyt-2$^+$ and L3T4$^+$ T subsets from hyperimmune mice, only the Lyt-2$^+$ T cell subset from tumor-bearing mice was capable of mediating the *in vivo* protective immunity. L3T4$^+$ T cell-mediated immunity was not detectable in the tumor-bearing state irrespective of the length of the sensitization period with a primary growing tumor, but emerged in the mice which resisted the first tumor challenge after the resection of the primary tumor. These results indicate that the emergence of L3T4$^+$ T cell-mediated anti-tumor immunity is stage-dependent and the Lyt-2$^+$ T cells represent the main functional subset in the tumor-bearing state, although both subsets of T cells are potentially capable of effecting anti-tumor *in vivo* immunity. The results are discussed in relation to the selective suppression of the L3T4$^+$ but not of Lyt-2$^+$ T cell function in the tumor-bearing state.

Key words: Tumor-bearing state — Immunosuppression — Lyt-2$^+$ T subset — L3T4$^+$ T subset

The effect of tumors on the immune response of their hosts has been extensively studied. Tumor-bearing hosts fail to reject malignant cells even in the case of experimentally induced tumor cells which express immunogenicity. The numerous reports showing that various suppressive mechanisms[1–11] are generated in response to growth of immunogenic tumors have served to provide an explanation for the paradoxical growth of these tumors in immunocompetent syngeneic hosts.

While it has been generally found that T cell-mediated immunity is moderately or severely impaired in man and animals bearing solid tumors, we have recently determined more accurately the cellular site(s) of the immunosuppression observed during the tumor-bearing state.[12] The results demonstrated that C3H/He mice bearing a syngeneic MH134 hepatoma mass exhibited a selective inhibition of L3T4$^+$ but not of Lyt-2$^+$ T cell-mediated immunity against tumor-unrelated nominal antigens. Since tumor antigens of this MH134 hepatoma have been demonstrated to be capable of inducing both tumor-specific L3T4$^+$ and Lyt-2$^+$ T cell-mediated *in vivo* protective immunity,[13–15] the above observations raised the question of how the generation of each T subset-mediated immunity against tumor antigens is influenced by the tumor-bearing state.

The present study was undertaken to investigate the effect of tumor-bearing state on each subset of T cells responsible for tumor-protective immunity. The results demonstrate that anti-MH134 tumor-neutralizing activity emerged as early as one week after the tumor cell implantation and was detected through the 3-week tumor-bearing state. Importantly, such protective immunity was mediated exclusively by Lyt-2$^+$ T cell subset throughout the tumor-bearing stage tested. This contrasted with the fact that tumor neutralization was

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obtained in both Lyt-2$^+$ and L3T4$^+$ subsets of T cells when they were from hyperimmune mice which were prepared by the surgical resection of a primary tumor and by repeated tumor cell challenges. These results indicate that the tumor-bearing state imposes immunosuppression selectively upon the L3T4$^+$ T cells directed against tumor antigens as well as tumor-unrelated nominal antigens.

Materials and Methods

Mice and Tumor Female C3H/He mice, obtained from Charles River Laboratory, were used at 6–9 weeks of age. MH134 hepatoma, derived from C3H/He strain and maintained by serial intraperitoneal transplantation, was utilized.

Preparation of Tumor-bearing or Tumor-hyperimmune Mice One million MH134 tumor cells were inoculated id[*2] into C3H/He mice. At various times thereafter, these mice were used as tumor-bearers. MH134-hyperimmune mice were prepared by surgical resection of the primary tumor at the 7-day tumor-bearing stage and by repeated challenges with viable tumor cells at 7-day intervals.[14]

Monoclonal Antibodies Monoclonal antibodies to L3T4 and Lyt-2 antigens were used. The GK1.5 monoclonal antibody-producing hybridoma line, specific for the L3T4 molecule,[16] was a gift of Dr. F. Fitch, University of Chicago, IL, and the 3.155 monoclonal antibody-producing hyridoma line, specific for the Lyt-2 molecule was obtained from ATCC, Rockville, MD.

The GK1.5 hybridoma cells were inoculated into pristane-primed BALB/c *nu/nu* mice intraperitoneally and ascitic fluids were collected. Gamma globulin fractions of cell-free ascites were obtained by precipitation at 40% saturation with ammonium sulfate. Fluorescein isothiocyanate (FITC) was conjugated to GK1.5 monoclonal antibody purified from the GK1.5 hybridoma ascitic fluid by procedures previously described.[14] FITC-conjugated anti-Lyt-2 and anti-Thy-1.2 antibodies were the products of Becton Dickinson Immunocytometry Systems, Mountain View, CA and of Bio Yeda Ltd., Rehovot, Israel, respectively.

Treatment of Immune Spleen Cells with Antibody plus Complement Spleen cells (10^8) from immunized mice were incubated at room temperature for 30 min with ascitic form of anti-L3T4 or -Lyt-2 monoclonal antibody at a dilution of 1:50. Cells were washed and incubated at 37° 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 by flow microfluorometric analysis as shown in Fig. 1. The results of Fig. 1 illustrated that the treatment with anti-L3T4 or Lyt-2 antibody results in almost complete elimination of the Lyt-2$^+$ or L3T4$^+$ T cell subset, respectively, without damage to the other T cell subset.

Tumor-neutralization Test (Winn Assay) Spleen or lymph node cells (10^7, unless otherwise indicated) from normal or tumor-immunized mice were admixed with 10^5 viable tumor cells. The mixture was inoculated id in a volume of 0.1 ml into syngeneic C3H/He recipient mice, and tumor growth was expressed as the mean tumor diameter ± SE of five mice/group.

Immunofluorescence Staining and Flow Microfluorometry (FMF) The preparation and staining procedures were essentially the same as described previously.[17] Briefly, 1×10^6 spleen cells were incubated at 4° 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% bovine serum albumin (BSA) and 0.1% sodium azide. FMF analysis was performed by using a FACStar (Becton Dickinson Immunocytometry Systems). All data were collected by using log amplification, and dead cells were rejected from analysis on the basis of forward light scatter.

Results

We have confirmed the fact[14] that anti-MH134 tumor neutralization can be mediated by either L3T4$^+$ or Lyt-2$^+$ T cell subset from MH134-hyperimmune mice. Spleen cells from MH134-hyperimmune mice were treated with anti-L3T4 or -Lyt-2 antibody plus C, and fractionated into Lyt-2$^+$ or L3T4$^+$ T cell subset as shown in Fig. 1. The results of Table I demonstrate that either subset of T cells from hyperimmune mice is capable of producing complete protection at a higher spleen: tumor cell ratio (100:1) or partial but comparable tumor-neutralizing activity at a lower ratio (25:1).

We next investigated the ability of spleen cells from tumor-bearing mice to mediate tumor neutralization. Spleen cells from C3H/He mice at various times after inoculation of MH134 viable cells were used as effector cells in Winn assays. As shown in Fig. 2, tumor-neutralizing activity was generated in spleen cells from mice as early as one week after the

[*2] Abbreviations used in this paper: id, intradermal or intradermally; FITC, fluorescein isothiocyanate; FMF, flow microfluorometry; C, complement.

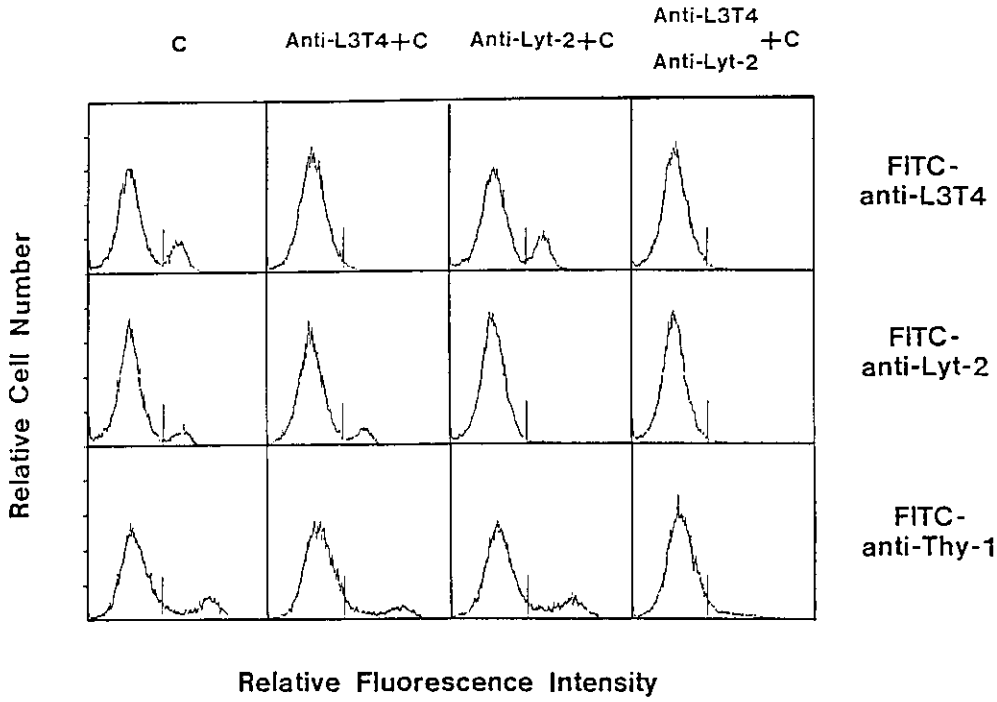

Fig. 1. Flow microfluorometric (FMF) study of spleen cells after treatment with anti-L3T4 or anti-Lyt-2 antibody plus complement (C). MH134-primed spleen cells were treated with various monoclonal antibodies (shown) plus C. Cells were stained with either anti-L3T4, anti-Lyt-2 or anti-Thy-1.2 monoclonal antibody conjugated with FITC.

Table I. Phenotype of Anti-tumor Effector T Cells from MH134-hyperimmune Mice

Effector cells used for Winn assay		E:T ratios[b]	Tumor diameter (mm) ±SE	
from mice	treatments[a]		day 7	day 10
Normal	−		6.9±1.1	8.6±1.3
Hyperimmune	C		<3.0	<3.0
	Anti-L3T4+C	100:1	<3.0	<3.0
	Anti-Lyt-2+C		<3.0	<3.0
	Anti-L3T4 & anti-Lyt-2+C		7.3±0.7	10.8±0.6
Normal	−		7.3±0.1	9.4±0.6
Hyperimmune	C		<3.0	<3.0
	Anti-L3T4+C	25:1	<3.0	7.0±1.8
	Anti-Lyt-2+C		<3.0	4.4±1.4
	Anti-L3T4 & anti-Lyt-2+C		7.9±0.6	11.5±0.7

a) Spleen cells from 5 MH134-hyperimmune C3H/He mice were treated with monoclonal antibodies (indicated) plus C.
b) Effector:tumor cell ratios (E:T ratio) are shown.

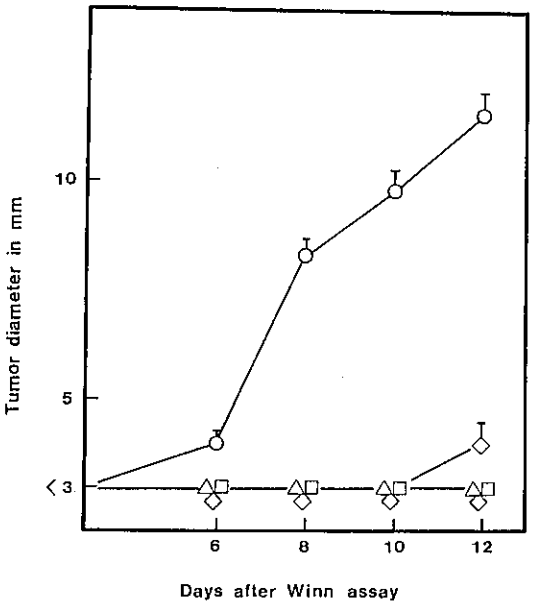

Fig. 2. Tumor-neutralizing activity of spleen cells from tumor-bearing mice. Spleen cells (10^7) from normal C3H/He mice (○) or 1-week (△), 2-week (□) or 3-week (◇) tumor-bearing mice (each 2/group) were used as effector cells in the Winn assay.

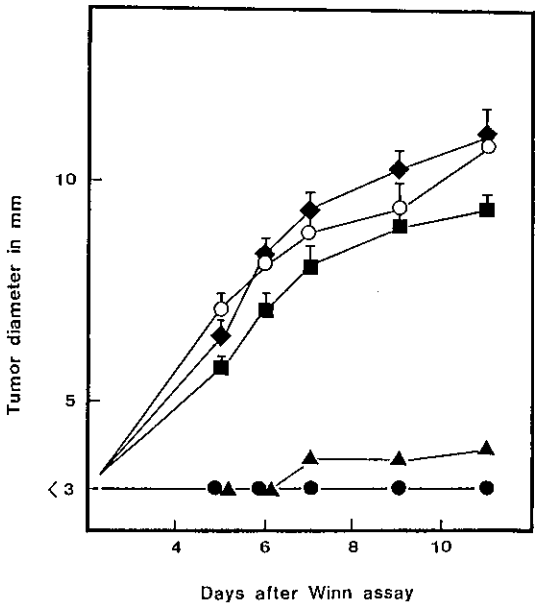

Fig. 3. Phenotype of anti-tumor effector T cells from mice one week after tumor inoculation. Spleen cells from five one-week tumor-bearing mice were treated with either C (●), anti-Lyt-2+C (■), anti-L3T4+C (▲) or anti-Lyt-2 and -L3T4 +C (◆). These fractionated spleen cells (10^7) or normal untreated spleen cells (○) were mixed with 10^5 viable MH134 tumor cells.

tumor implantation. Such protection was also found in spleen cells from mice two or three weeks after the tumor implantation. When the phenotype of the above effectors from tumor-bearing mice was analyzed, it was revealed that tumor-neutralizing activity was mediated exclusively by Lyt-2^+ T cell subset. This was the case 1) irrespective of when splenic effector cells were obtained (Fig. 3 for one week, Fig. 4 for two weeks and data not shown for three weeks after the tumor cell implantation) and 2) irrespective of whether spleen cells or lymph node cells are used as an effector cell source (Fig. 4). Moreover, such a preferential mediation of tumor protection by Lyt-2^+ but not by L3T4$^+$ T cell subset was also observed in spleen cells from mice whose primary tumor was surgically resected one week after the tumor implantation (Table II).

In order to determine when L3T4$^+$ T cell-mediated protective immunity emerges during the period from the tumor initiation to the acquisition of tumor resistance, Winn assays were performed with the use of spleen cells from mice at various immunizing stages. The results of Fig. 5 show that L3T4$^+$ splenic T cells from 3-week tumor-bearing mice again failed to exhibit tumor-neutralizing activity. In contrast, L3T4$^+$ T cells from mice which resisted the first tumor challenge after the tumor resection produced complete protection. These mice were prepared at the same timing as 3-week tumor-bearing mice after the tumor cell implantation. Therefore, these results indicate that the failure of splenic L3T4$^+$ T cells from 3-week tumor-bearing mice to generate tumor-neutralizing activity is not due to an insufficient tumor-sensitizing period.

Additional experiments were performed to examine the content of L3T4$^+$ T cells in spleen cells from mice at various stages. The results of Table III demonstrate that the number of L3T4$^+$ T cells is not affected by the tumor-bearing state, but is maintained at a level comparable to that observed in normal

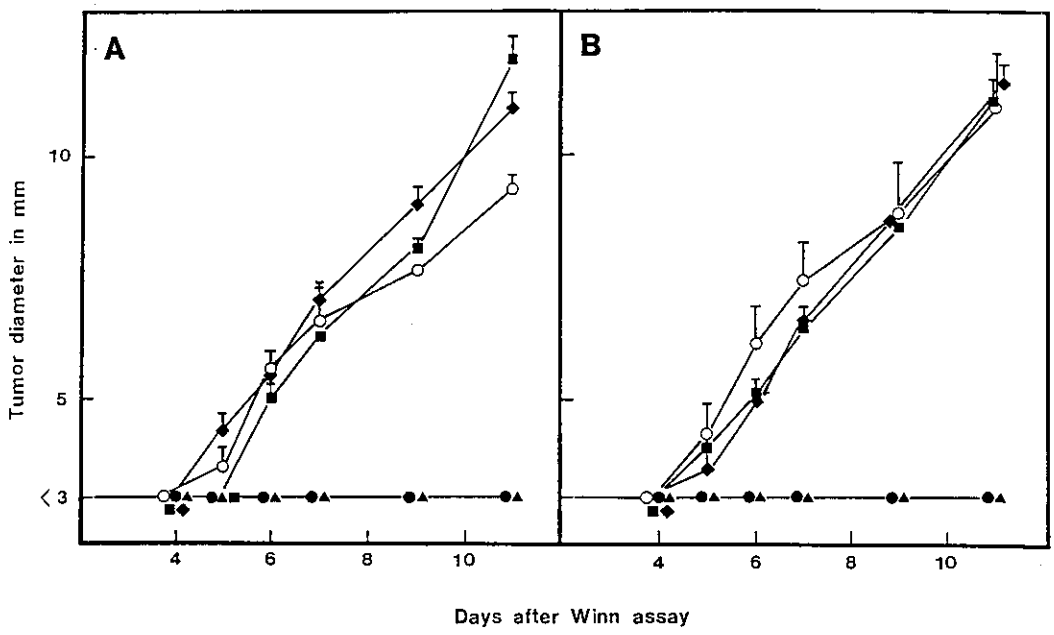

Fig. 4. Phenotype of anti-tumor effector T cells from mice two weeks after tumor inoculation. Spleen cells (panel A) or lymph node cells (panel B) from five two-week tumor-bearing mice were treated with either C (●), anti-Lyt-2+C (■), anti-L3T4+C (▲), or anti-Lyt-2 and -L3T4+C (◆). These fractionated cells (10^7) or normal untreated cells (○) were mixed with 10^5 viable MH134 tumor cells.

Table II. Selective Mediation of Tumor-neutralization by Lyt-2^+ T Cell Subset from Primary Tumor-resected Mice

Effector cells		Tumor diameter (mm) ±SE			
		Exp. 1		Exp. 2	
from mice	treatments	day 7	day 10	day 7	day 10
Normal	−	5.5±0.5	9.3±0.3	ND	ND
Tumor-resected[a]	C	<3.0	<3.0	<3.0	<3.0
	Anti-L3T4+C	<3.0	<3.0	<3.0	<3.0
	Anti-Lyt-2+C	7.5±0.8	9.8±0.3	6.0±0.8	10.6±0.4

a) MH134 tumors which had been initiated in 5 C3H/He mice one week before were resected and these mice were used an additional week later as the tumor-resected mice.

C3H/He mice. Taken collectively, these results indicate that the generation and/or implementation of L3T4$^+$ T cell-mediated immunity is suppressed during the tumor-bearing stage.

Discussion

A central issue in tumor immunology is to explain why immunogenic tumors grow progressively in their syngeneic immunocompetent hosts. Several attempts have been made to explain the above paradox. These include suggestions that (a) the weakness of tumor antigens allows tumor cells to escape immune surveillance[18]; (b) tumor cells are capable of hiding their tumor antigens from immune recognition and effector mechanisms by a process known as antigenic modulation[19, 20]; (c) tumors favor the generation of various immunosuppressive mechanisms.[1-11]

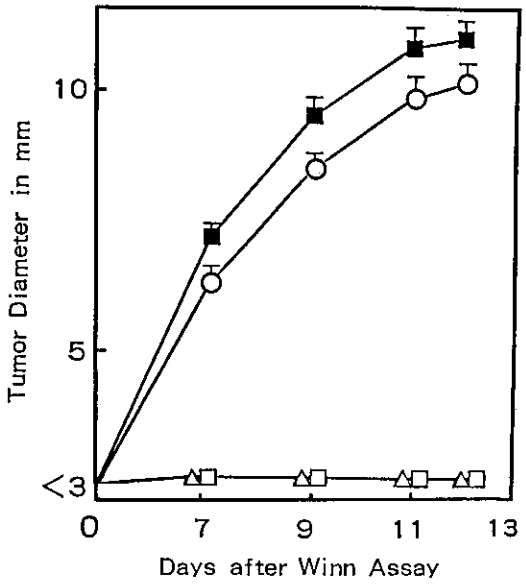

Fig. 5. Emergence of tumor-neutralizing activity in L3T4$^+$ T cell subset from mice after the acquisition of resistance. L3T4$^+$ T cell subsets were isolated by anti-Lyt-2 antibody treatment of the following spleen cells from 3 donor mice/group: normal (○), 3-week MH134 tumor-bearing mice (■), mice which had acquired resistance to the first (△) or second (□) challenge with 10^5 viable MH134 tumor cells.

Table III. Content of L3T4$^+$ T Cells in Spleen Cells from Mice at Various Immunizing Stages

Spleen cells from mice	No. of spleen cells ($\times 10^7$)			
	Exp. 1		Exp. 2	
	total[a]	L3T4$^+$[b]	total	L3T4$^+$
Normal	6.5	1.2	7.5	0.9
One-week tumor-bearing	8.6	1.1	10.8	1.1
Two-week tumor-bearing	8.6	1.0	11.3	1.0
Three-week tumor-bearing	8.0	1.4	13.3	1.0

a) Mean of 3 mice/group.
b) L3T4-positivity was determined by FMF study, and expressed as percent. The number of L3T4$^+$ T cells was obtained by multiplying total spleen cell number by L3T4-positivity.

Ample evidence has been accumulated to suggest the generation of cellular[1-6] or humoral[7-11] components that function as suppressor cells or immunosuppressive factors. In addition to the existence of suppressive mechanisms specific for anti-tumor immune responses,[2-4, 8-11] it has been documented that the tumor-bearing state induces a generalized immunosuppression as reflected by humoral and cell-mediated immune responses against tumor-unrelated nominal antigens.[1, 2, 5-7] Such an antigen-nonspecific suppression is a widely-observed phenomenon that is also seen in acquired immune deficiency syndrome (AIDS)[21-23] and acute graft vs. host (GVH) disease.[24-26] Recent analyses concerning cellular site(s) affected in these immunosuppressive states have, however, revealed that not all types of T cell-mediated immunities are suppressed, but the selective depletion of CD4$^+$ T helper function is observed in the developmental stages of AIDS[27] or GVH diseases.[28] These observations led us to re-examine cellular mechanisms by which immunosuppression is induced in the tumor-bearing state.

In a separate paper, we have dealt with cellular site(s) at which T cell-mediated immunity in tumor-bearing hosts is affected.[12] The results demonstrated that anti-trinitrophenyl (TNP) cytotoxic T lymphocyte (CTL) responses are almost completely suppressed in lymphoid cells from tumor-bearing hosts, whereas anti-alloantigen CTL responses generated in portions of the same lymphoid cells are almost comparable to those obtained with normal lymphoid cells. These results were similar to those observed in the developmental stages of AIDS[27] and a class II H-2-disparate GVH disease.[28] It has been established that the participation of CD4$^+$ (L3T4$^+$) helper T cells is an absolute requirement for anti-TNP CTL responses whereas anti-allo-CTL responses can utilize an alternate CD4$^-$(Lyt-2$^+$) T helper pathway.[29-31] Therefore, the above observations are interpreted as follows: there is a selective deficiency of L3T4$^+$ helper T cell function, but the function of Lyt-2$^+$ subset of T cells is maintained as intact irrespective of whether the activities represent CTL or helper T cell function.

In considering the selective deficiency of L3T4$^+$ T cell function in the tumor-bearing state, we investigated in the present study whether the generation of tumor-specific im-

munity in tumor-bearing hosts is also influenced by the above L3T4$^+$-selective immunosuppressive potential. In a MH134 hepatoma model, both L3T4$^+$ and Lyt-2$^+$ T cell-mediated immunities were detected in anti-MH134 hyperimmune mice.[14] It was also demonstrated that these subsets of T cells produce lymphokine(s) capable of activating other types of anti-tumor effectors such as macrophages, and function as tumor immunity-inducer T cells rather than as direct attacking T cells in this tumor model.[15] When the generation of each subset of anti-MH134 inducer T cells was examined at various tumor-bearing stages, it was revealed that the Lyt-2$^+$ subset of inducer T cells is exclusively generated throughout the 3-week tumor-bearing period. The induction of anti-MH134 L3T4$^+$ T subset was not detected until tumor-resected mice exhibited the rejection response against the subsequent viable tumor cell challenge. Thus, these results are compatible with the notion that the tumor-bearing state results in a selective inhibition of L3T4$^+$ but not of Lyt-2$^+$ T cell-mediated immunity.

It should also be noted that while the cellular pattern of immunosuppression in the tumor-bearing state is similar to that in AIDS or acute GVH disease, there is an essential difference in the defect of L3T4$^+$ T cell function between these immunosuppressive states and the tumor-bearing state. In contrast to a selective loss or decrease in the number of L3T4$^+$ T cells in AIDS or GVH disease,[27,28] the number of this T cell subset was not altered even at the time of severe defect of L3T4$^+$ T cell function in the tumor-bearing state. This implies that the loss of L3T4$^+$ T cell activity in tumor-bearing hosts is ascribed to the functional impairment of this T cell subset.

The mechanisms by which L3T4$^+$ T cell function is impaired in the tumor-bearing state have not been determined in the present study. It is possible that the defect of the L3T4$^+$ T cell function is due to functional impairment of L3T4$^+$ T cells themselves or antigen-presenting cells (APC) that are required for activating L3T4$^+$ T subset. The nature of the cellular or humoral components generated during the tumor-bearing state that affects the L3T4$^+$ T cell function also remains to be investigated. Further studies are in progress to identify more accurately the cellular mechanisms underlying L3T4$^+$ T-selective immunodeficiency as well as to analyze cellular or humoral factors responsible for such an immunosuppression specific to the tumor-bearing state.

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