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Modulation of Immune Response against Tumor Cells by the *in vivo* Administration of an Autoreactive Th Clone

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The immunization of C3H mice with irradiated syngeneic MM48 tumor cells induced specific tumor-neutralizing cells (TNC). The TNC activity was mediated by the L3T4$^+$, Ly-2$^-$ T cell population, and the generation of TNC coincided with the appearance of delayed-type hypersensitivity response against MM48 antigen. The administration of an auto-I-A^k reactive T helper cell clone MS202 to normal C3H mice resulted in the facilitation of growth of MM48 tumor due to the induction of T suppressor (Ts) cells. Splenic T cells from animals given this T cell clone inhibited the TNC activity of immunized mice resulting in the escape of MM48 from the TNC effect. The surface phenotype of the Ts cells was L3T4$^+$, Ly-2$^-$. The Ts cells induced by the clone MS202 were totally antigen-nonspecific, and were able to suppress both the effector and inductive processes of TNC. The results suggest the presence of a physiological MHC-restricted T cell circuit that regulates immune responses against the growing tumors.

Key words: Tumor-neutralizing cells — Autoreactive T cell clone — Major histocompatibility complex — T cell circuit

Developing tumors interact with the host's immune system to induce diverse types of responses, some of which are beneficial for the host and others for the tumor growth. The underlying factors which determine the final output of the responses to the growing tumors are not known.

It has been demonstrated that the immune response to external antigens is regulated by a complex series of cellular interactions the basis of which is programmed during the ontogeny of the immune system.[1,2] The concept of a regulatory circuit has been proposed from the observation that an antigenic input activates consecutive cellular processes where different subsets of T cells are sequentially activated to produce an output[3,4] that neutralizes the initial antigenic input.

We have reported[4] that the *in vivo* administration of cloned murine T helper (Th) cells to syngeneic animals induced a profound suppression of both primary and secondary antibody responses by interfering with the regulatory circuit, where these Th cells activated antigen-nonspecific T suppressor (Ts) cells. The circuit was strictly major histocompatibility complex (MHC)-restricted in that the MHC-restricted Th clone was able to induce Ts which in turn inhibits Th cells of the same MHC restriction specificity.

In order to study the immune mechanisms that inhibit or allow the tumor growth, we used the same experimental strategy where animals were treated with cloned Th cells with a known MHC-restriction specificity before immunization with MM48 tumor. We describe in this paper a class II-restricted Th clone that can activate an immunological circuit inducing antigen-nonspecific Ts cells that inhibit the tumor-neutralizing cells (TNC).

Abbreviations: CTL, cytotoxic T cells; DTH, delayed-type hypersensitivity; id intradermally; ip, intraperitoneal; iv, intravenously; mAb, monoclonal antibody; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; Th, T helper; TNC, tumor-neutralizing cells; Ts, T suppressor

MATERIALS AND METHODS

Animals Male C3H/HeN (8–12 weeks) mice were purchased from Ohmura Experimental Animal Co., Kanagawa.

Tumor cell lines A mouse mammary tumor cell line (MM48) of C3H origin was generously provided by Dr. T. Tachibana, Tohoku University, Sendai. The cell line was established from a spontaneous mammary tumor of a C3H mouse infected with murine mammary tumor virus.[5] MM48 tumor cells express class I major histocompatibility complex (MHC) but not serologically-defined MM-tumor antigen on their cell surface.[6] They grow as a solid tumor when 1×10^5 cells are inoculated subcutaneously (sc). X5563 plasmacytoma cells and MH134 hepatoma cells of C3H origin were kindly provided by Dr. H. Fujiwara, Osaka University, Osaka. These three tumor cell lines have been maintained *in vivo* by intraperitoneal (ip) injection of the cells into C3H mice.

The autoreactive Th clone (MS202) The T helper (Th) cell clone (MS202) of C3H origin with specificity for the self class II (I-A^k) antigen was established and maintained as previously described.[4] The clone can be stimulated by macrophages carrying the I-A^k molecule

without antigen, and can induce proliferation of unprimed B and T cells of I-A^k strains.[4] The surface phenotype of MS202 is Thy-1^+, L3T4^+, Ly-1^+2^- and I-J^+. The Th clone MS202 does not have a cytotoxic activity on normal lymphoid cells and MM48 tumor cells.

Antibodies Anti-Thy-1.2 monoclonal antibody (mAb) (30-H12)[7] and anti-L3T4 mAb (GK1.5)[8] were provided by Dr. L. A. Herzenberg, Stanford University, Stanford, CA, and Dr. F. W. Fitch, University of Chicago, Chicago, IL, respectively. Anti-Ly-2.1 (116-13.1) mAb was obtained from the American Type Culture Collection, Rockville, MD. Polyclonal anti-mouse immunoglobulin (anti-MIg) was prepared by repeated immunizations of rabbits with MIg followed by affinity chromatography.

Immunization C3H mice were immunized intradermally (id) with 10,000 rad irradiated tumor cells (1×10^7) twice with a one-week interval. Spleen cells were harvested one week after the last immunization.

Tumor neutralization test (Winn assay) TNC activity was determined by the Winn assay.[9] Briefly, 1×10^5 MM48 cells and 1×10^7 spleen cells were suspended in 50 μl of Eagle's minimal essential medium and were inoculated sc into C3H mice. Tumor size was expressed by measuring the area of the surface. Each point represents the arithmetic mean value of five mice. The data presented are representative of 3–5 indentical experiments.

Induction of Ts cells by MS202 C3H mice were injected ip with 1×10^7 MS202 twice with a one-week interval. Spleen cells were taken one week after the last injection. Splenic T cells were obtained by incubation with anti-MIg-coated plastic dishes according to the method of Mage *et al.*[10] The non-adherent cells contained more than 90% of Thy-1^+ cells and were used as a source of suppressor T (Ts) cells.

Delayed-type hypersensitivity (DTH) reaction A mixture of 1×10^6 irradiated tumor cells and immune spleen cells (1×10^7 or 3×10^7) was inoculated sc into the footpads of C3H mice. The footpad swelling 24 h after the inoculation was measured with a micrometer.

Statistical analyses The tumor size in the different groups at the last day of the experiment was compared by the use of Student's *t* test.

RESULTS

The immunization with MM48 induces L3T4^+, Ly-2^- TNC To study the immunogenicity of MM48, C3H mice were immunized with 10,000 rad irradiated MM48 tumor cells (1×10^7) id twice with a one-week interval. One week after the last immunization, spleen cells were harvested and were tested their TNC activity on the tumor cell growth by the Winn assay. A mixture of 1×10^5 MM48 and 1×10^7 immune spleen cells was inoculated SC into C3H mice. As shown in Fig. 1, MM48 tumor was measurable on day 5 and continuously increased in the group inoculated with MM48 alone. Tumor growth was inhibited in the group inoculated with MM48 mixed with immune spleen cells. Three out of five mice showed no growth of the tumor, and two others developed a growing tumor two to three weeks later. The TNC activity was not observed when normal spleen cells were inoculated with MM48 (Fig. 1).

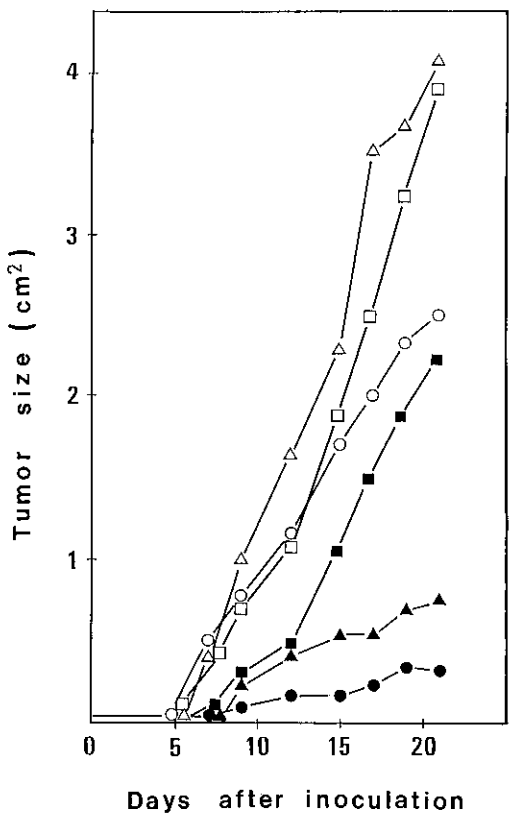

Fig. 1. The immunization with MM48 tumor cells induces L3T4^+, Ly-2^- TNC. C3H mice were immunized with 10,000 rad irradiated MM48 cells (1×10^7) id twice with a one-week interval. One week after the last immunization, spleen cells were harvested and were tested for their TNC activity on the tumor cell growth by means of the Winn assay. MM48 cells were inoculated either without (○), or with normal spleen cells (■) or MM48 immune spleen cells (●). MM48-immune spleen cells were cytotoxically treated with anti-Thy 1 (□), anti-L3T4 (△) or anti-Ly 2.1 (▲), and were inoculated with MM48 cells. Each point represents an arithmetic mean value of five mice (○ vs. ■; $P>0.2$, ○ vs. ●; $P<0.01$, ● vs. ▲; $P>0.2$).

The treatment of immune spleen cells with anti-Ly-2.1 and complement (C) did not affect the TNC activity. As shown in Fig. 1, the same cytotoxic treatment with anti-

Table I. Immunization with MM48 Induces MM48-specific TNC Population

Inoculated tumor cells	Immunized[a] with	Tumor size (cm^2)[b] on days after inoculation		
		7	15	22
MM48	——[c]	0.2±0.1	1.7±0.1	2.9±0.4
	MM48	0	0.2±0.2	0.8±0.5 ($P<0.02$)*
	MH134	0.2±0.1	1.6±0.4	2.7±0.6 ($P>0.2$)
MH134	——	0.4±0.1	1.3±0.2	0.7±0.2
	MM48	0.5±0.1	1.3±0.1	1.2±0.2 ($P>0.1$)
	MH134	0	0	0 ($P<0.02$)
X5563	——	0.6±0.1	2.4±0.2	4.0±0.3
	MM48	0.4±0.2	1.5±0.2	3.3±0.5 ($P>0.1$)
	MH134	0.5±0.1	1.9±0.4	3.6±0.4 ($P>0.1$)

a) C3H mice were immunized with 10,000 rad irradiated MM48 cells or MH134 cells (1×10^7) id twice with a one-week interval, and spleen cells were harvested at one week after the last immunization.
b) A mixture of tumor cells (1×10^7) and immune spleen cells (1×10^7) was inoculated sc into C3H. Values indicate arithmetic mean of 5 mice and standard errors.
c) Without immunization.
* For the difference from the mean value in the control group without immunization.

L3T4 and anti-Thy-1.2 completely eliminated the TNC activity. These results demonstrated that immunization with MM48 induced L3T4$^+$, Ly-2$^-$ T cells which inhibit the growth of MM48 *in vivo*. MM48-specific cytotoxic T cells (CTL) were not detectable by the *in vitro* chromium release assay (data not shown).

To determine the specificity of the TNC, 1×10^7 MM48-immune spleen cells were inoculated with X5563 plasmacytoma cells or MH134 hepatoma cells. As shown in Table I, the growth of MM48 was inhibited by MM48-immune spleen cells but not by MH134-immune spleen cells, while the growth of MH134 was inhibited by MH134-immune but not MM48-immune spleen cells.

We further studied whether the MM48-immune spleen cells can induce a DTH response against MM48 tumor cell antigen. A mixture of 10,000 rad irradiated MM48 (1×10^6) and the MM48-immune spleen cells (1×10^7 or 3×10^7) was inoculated sc into footpads of C3H mice. Footpad swelling 24 h after the inoculation was measured. As shown in Table II, MM48-immune spleen cells were able to induce a strong DTH response to MM48 when the immune spleen cells were inoculated with MM48. No DTH reaction was detected if MM48 cells or the MM48-immune spleen cells were injected alone.

Injections of an auto-reactive Th clone (MS202) suppress the induction of TNC A previous publication from this laboratory indicated that the administration of an auto-reactive Th clone (MS202) to syngeneic mice induced Ts cells which inhibited Th cells with the same MHC-restriction specificity.[4] We studied whether an *in vivo* injection of the Th clone MS202 can modulate tumor immunity by affecting the induction of TNC. Ten million

Table II. MM48-immune Spleen Cells Mediate DTH Response

MM48-immune[a] spleen cells added	Inoculation of MM48 cells	DTH response[b] ($\times10^{-2}$ mm)
1×10^7	−	10.3±0.9
3×10^7	−	9.5±6.1
——[c]	+	11.0±5.0
1×10^7	+	29.3±5.8 ($P<0.05$)*
3×10^7	+	66.3±1.2 ($P<0.001$)

a) See Table I.
b) A mixture of MM48 cells (1×10^6) and immune spleen cells (1×10^7 or 3×10^7) was inoculated sc into footpads of C3H mice. Values indicate arithmetic means of 3 mice and standard errors.
c) Not added.
* For the difference from the mean value in the control group without MM48-immune spleen cells.

MS202 cells were injected ip twice into C3H mice simultaneously with immunizations with irradiated MM48 at a one-week interval. Spleen cells were harvested one week after the last immunization, and were tested for their TNC activity by the Winn assay (Fig. 2). Immune spleen cells induced by MM48 inhibited the tumor growth. This TNC activity was greatly reduced if the donor of immune spleen cells had been injected simultaneously with MS202 and MM48 cells.

MS202-induced suppression of TNC is antigen-non-specific MS202 cells (1×10^7) were injected ip into C3H mice twice simultaneously with irradiated X5563 cells or

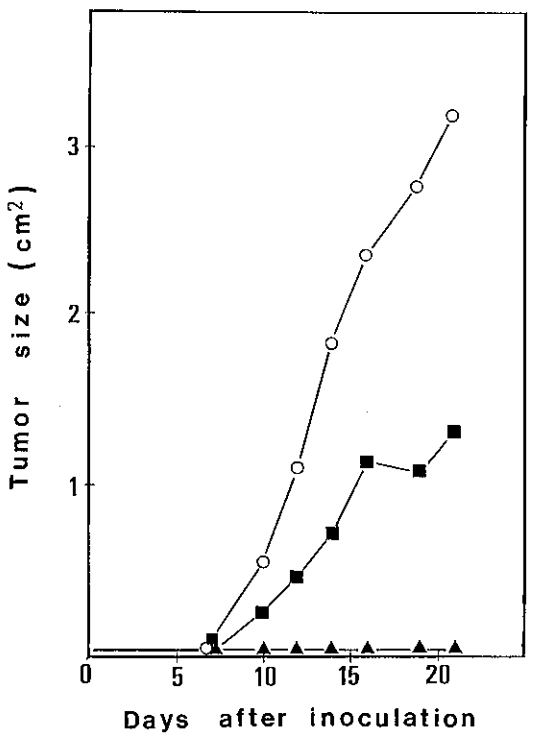

Fig. 2. Suppression of the induction of TNC activity by MS202. C3H mice were immunized with irradiated MM48 cells id either with (■) or without (▲) the ip injection of 1×10^7 MS202 twice with a one-week interval. At one week after the last immunization, spleen cells were harvested and were tested for their TNC activity on the tumor cell growth by the Winn assay. MM48 cells only were inoculated as a control (○). Each point represents an arithmetic mean value of five mice (○ vs. ▲; $P<0.001$, ○ vs. ■; $P<0.05$, ■ vs. ▲; $P<0.02$).

MH134 cells. Spleen cells were harvested one week after the last injection, and were tested for their TNC activity by the Winn assay. As shown in Table III, the injection of MS202 cells inhibited the generation of TNC activity against X5563 and MH134 although the spleen cells from X5563 or MH134-immunized mice were able to neutralize the growth of the respective tumors.

MS202 induces L3T4$^+$, Ly-2$^-$ Ts cells for TNC To determine whether the observed reduction of TNC activity is due to the induction of Ts cells by MS202, cell transfer experiments were carried out. C3H mice were injected with 1×10^7 MS202 twice with a one-week interval, and their splenic T cells were obtained one week after the last injection. The splenic T cells (2×10^6) separated by panning with anti-MIg-coated dishes were injected intravenously (iv) into syngeneic mice that were immunized twice MM48. Spleen cells from the recipient mice were tested for their TNC activity by the Winn assay. As shown in Fig. 3, immune spleen cells of mice immunized with MM48 alone were able to inhibit the tumor growth, whereas the cells from mice receiving MS202-treated splenic T cells failed to neutralize the tumor growth. Normal untreated T cells had no ability to inhibit the generation of TNC upon transfer into MM48-immunized mice (data not shown).

The Ts cells were treated with anti-L3T4 or anti-Ly-2.1 and C before transfer to the MM48-immunized mice. The Ts activity was eliminated by cytotoxic treatment with anti-L3T4 but not with anti-Ly-2.1 (Fig. 3), indicating that the injection of MS202 into normal recipients induced the L3T4$^+$, Ly-2 Ts cells. The Ts cells thus generated, in turn, suppressed the induction of L3T4$^+$, Ly-2$^-$ TNC in the recipient.

Table III. MS202-induced Suppression of TNC Activity is Tumor-nonspecific

Inoculated tumor cells	Preparation of TNC by immunization[a] with	Preparation of TNC injection of MS202[b]	Tumor size (cm²)[c] on days after inoculation 8	15	21
X5563	——	−	0.7±0.1	1.8±0.3	2.6±0.3
	X5563	−	0	0.8±0.2 ($P<0.05$)*	2.0±0.4 ($P>0.2$)
	X5563	+	1.1±0.1	2.3±0.3 ($P>0.2$)	3.9±1.1 ($P>0.2$)
MH134	——	−	1.0±0.2	1.4±0.2	1.0±0.3
	MH134	−	0	0 ($P<0.01$)	0 ($P<0.02$)
	MH134	+	0.5±0.1	1.4±0.2 ($P>0.2$)	2.8±0.3 ($P<0.01$)

a) C3H mice were immunized id with 10,000 rad irradiated X5563 cells or MH134 cells (1×10^7) twice with a one-week interval, and spleen cells were harvested one week after the last immunization.
b) MS202 (1×10^7) was injected ip into C3H twice simultaneously with the immunization with X5563 cells or MH134 cells.
c) See Table I.
* For the difference from the mean value in the control group.

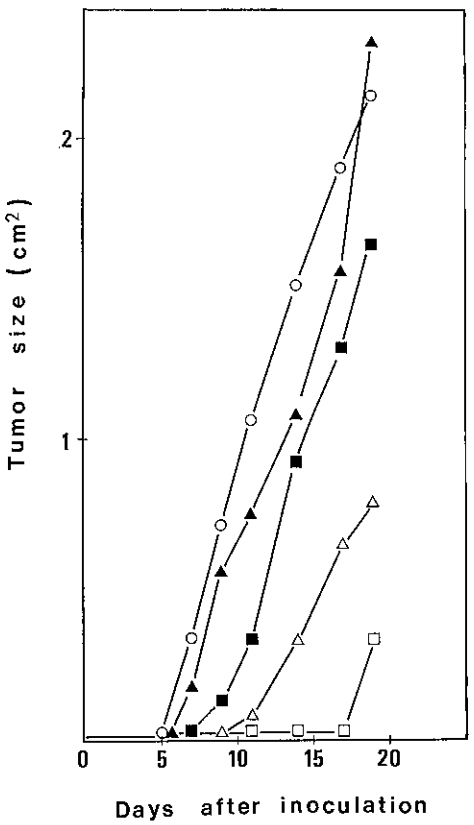

Fig. 3. MS202 induces L3T4$^+$, Ly-2$^-$ Ts cells for TNC. C3H mice were injected with 1×10^7 MS202 twice with a one-week interval, and their splenic T cells were obtained one week after the last injection. Then 2×10^6 of these splenic T cells were injected iv either untreated (▲) or after treatment with anti-L3T4 and C (□) or anti-Ly-2.1 and C (■) into syngeneic mice which had been immunized previously (as described in "Materials and Methods") with MM48 cells. Spleen cells from these recipient mice (▲ □ ■) and untreated mice (△) were used in Winn assays. As a control, MM48 cells were injected alone (○). Each point represents an arithmetic mean value of five mice (○ vs. □; $P<0.001$, ○ vs. △; $P<0.001$, ○ vs. ■; $P>0.2$, ○ vs. ▲; $P>0.2$).

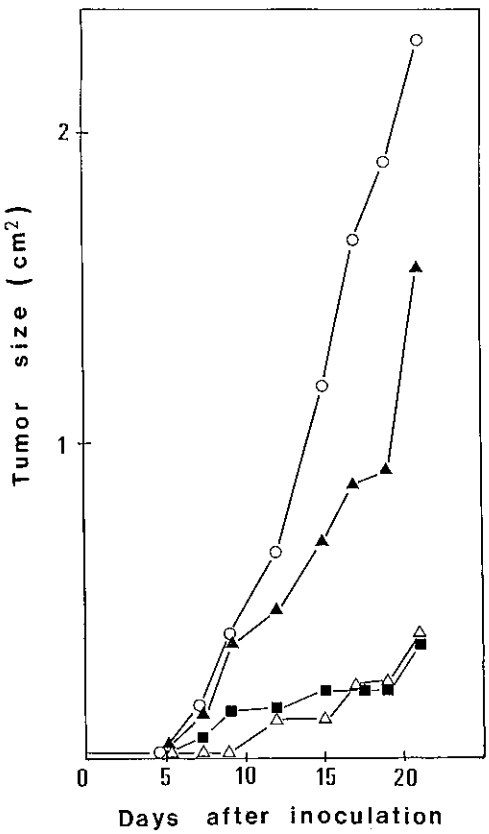

Fig. 4. MS202-induced Ts cells suppress the effector function of TNC as well. MM48-immune spleen cells (1×10^7) were mixed either without (△) or with 2×10^6 MS202-induced Ts cells (▲) or 1×10^6 MS202 (■). The TNC activity of these mixtures was studied by inoculating them with 1×10^5 MM48 sc into C3H mice. As the control, 1×10^5 MM48 cells were injected alone (○). Each point represents an arithmetic mean value of five mice (○ vs. △; $P<0.01$, ○ vs. ▲; $P>0.2$, ○ vs. ■; $P<0.05$).

MS202-induced Ts cells suppress also the effector function of TNC We studied whether or not the MS202-induced Ts cells can suppress the effector function of TNC. Two million Ts cells obtained from MS202-injected mice were mixed with 1×10^7 MM48-immune spleen cells. The TNC activity of this mixture was studied by inoculating these cells with 1×10^5 MM48 sc into C3H mice. As shown in Fig. 4, 1×10^5 tumor cells injected without lymphocytes grew progressively. When the same number of tumor cells was injected together with 1×10^7 MM48-immune spleen cells, the tumor growth was greatly inhibited. The same inhibition was observed when 1×10^5 MM48 cells were injected together with 1×10^7 MM48-immune spleen cells and 1×10^6 MS202 Th cells. However, when 1×10^5 of MM48 cells were injected together with 1×10^7 MM48-immune spleen cells and 2×10^6 T cells taken from animals which had previously been injected with MS202 cells, the tumor progressively grew nearly as well as in the controls (tumor cells alone).

These results indicated that MS202-induced Ts cells suppress both the effector (Fig. 4) and inductive processes (Fig. 3) of TNC. This inhibition of TNC activity was not due to the effect of clone Th cell MS202, as MS202 itself was unable to inhibit TNC activity of MM48-immune spleen cells (Fig. 4).

DISCUSSION

MM48 is a rapidly growing transplantable mouse mammary tumor, which was established from a spontaneous mammary tumor (SMC XXIII) of a murine mammary tumor virus-positive C3H mouse.[5] Serological specificities of this tumor have been well analyzed with alloantibodies.[6] MM48 has been found to be moderately immunogenic in syngeneic animals, as seen by the induction of Ly-1^-2^+ CTL and Ly-1^+2^- regulatory T cells.[11]

We have described here the modulation of the immune response against tumor cells by the administration of an autoreactive Th clone MS202. MM48-specific TNC activity was found in spleen cells of C3H mice immunized with irradiated syngeneic MM48 tumor cells. The TNC activity was mediated by the L3T4$^+$, Ly-2^- T cell population and coincided with the appearance of DTH response against MM48. The generation of MM48-specific TNC activity was suppressed by Ts cells induced by the *in vivo* administration of the autoreactive Th clone MS202. The MS202-induced suppression was effective to inhibit the anti-tumor responses to other syngeneic tumors. The Ts cell was found to be L3T4$^+$, Ly-2^-, and was able to suppress the effector as well as the inductive process of TNC.

In the present experiments, we were unable to detect MM48-specific Ly-2^+ CTL in the *in vitro* ^{51}Cr release assay, but we consistently found TNC activity in the spleen of mice that had been immunized with irradiated MM48. Unlike the previous reports, which indicated that Ly-2^+ CTL cells are effector cells for *in vivo* anti-tumor immunity,[11-15] the TNC activity of MM48-immune mice were found to be mediated by the L3T4$^+$, Ly-2^- T cell population. The activity coincided with the appearance of DTH response against MM48. The results are in agreement with those of Greenberg *et al.*[16,17] and Fujiwara *et al.*,[18,19] who demonstrated that Ly-1^+2^- T cells were major effector cells responsible for *in vivo* tumor regression, at least for the tumors studied.

The L3T4$^+$ TNC was found to be the direct target of Ts cells induced by the Th clone. We have previously reported that an injection of a cloned MHC-restricted Th cell MS202 into syngeneic mice resulted in the generation of Ts cells that nonspecifically inhibited normal heterogeneous Th cells having the same MHC restriction specificity. All the cells in this circuit were L3T4$^+$, Ly-2^- T cells.[4] Since the observed TNC was L3T4$^+$, we used the same strategy to induce Ts with the Th clone.

The results presented above indicate that the ultimate effect of the injection of the Th clone MS202 was the inhibition of the generation of TNC activity. Spleen cells from mice treated with MS202 were able to inhibit the TNC activity of MM48-immune spleen cells. This indicates that the injection of Th clone had generated Ts cells in the recipient. The surface phenotype of the Ts cells thus induced was L3T4$^+$, Ly-2^-. Ts cells were found to be effective not only in the facilitation of the tumor growth by inhibiting the preactivated TNC but also in the induction/initiation of MM48-specific TNC. It was found that the transfer of splenic T cells from MS202-treated mice into a normal recipient prevented the induction of TNC caused by a subsequent immunization with irradiated MM48.

The function of Ts thus induced was nonspecific. Ts inhibited the response to other syngeneic tumors, e.g., X5563 and MH134. The situation is remarkably similar to that observed in the suppression of humoral immune response where MS202-induced Ts was able to inhibit the responses to various T cell-dependent antigens.[4]

The previous publication indicated that the regulatory circuit activated by MS202 was linked by the MHC-restriction specificities of the participating T cells: T cells induced by the I-A^k-restricted clone MS202 were able to inhibit the same I-A^k restricted Th cells.[4] Thus, the class II-restricted Th clone MS202 selected the Ts cells that had been preprogrammed to recognize the MHC restriction site present on the TcR of MS202. Since the L3T4$^+$ TNC generated by MM48 might recognize the tumor antigen with self class II antigen, the Ts might directly interfere with the class II restriction site of TNC. Thus, the L3T4$^+$ Ts cells inhibit the L3T4$^+$ TNC cells having the same class II restriction site as that of MS202.

These results indicate that a physiological MHC-restricted circuit is operating to regulate not only the immune responses to a foreign antigen but also those to endogenously derived growing tumors. When a foreign antigen is introduced on class II MHC to disturb the circuit, a counter singnal is produced to maintain the steady state of the system (suppression). In the case of developing tumors, tumor antigens in association with class II antigen can stimulate the L3T4$^+$ Th cell population. Once Th cells are activated, Ts cells recognizing the MHC restriction site of the activated Th cells are generated, and in turn inhibit the tumor-specific Th. The dominant unresponsiveness in the tumor-bearing host may be due to the over-activation of such antigen-nonspecific Ts cells in the circuit.

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