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Switching on the macrophage-mediated suppressor mechanism by tumor cells to evade host immune surveillance

(immunosuppression/T cell response/tumor cell/macrophage)

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ABSTRACT **The present study demonstrates a unique mechanism for tumor cell-induced immunosuppression. In the presence of a nonsuppressive dose of tumor cells, generation of cytotoxic T cells in the mixed lymphocyte culture (MLC) is completely suppressed by adding exogenous (peritoneal) macrophages (PMϕ) after the initiation of the MLC. This indicates that tumor cells can switch on a suppressor mechanism through host macrophages. It has further been determined that suppression can be induced only if resident (splenic) macrophages (SMϕ) are exposed to tumor cells prior to addition of PMϕ. If SMϕ and PMϕ are simultaneously present with the tumor cells, induction of suppression is completely precluded. These findings indicate that switching on of the suppressor mechanism by tumor cells has a critical requirement for the collaboration of two populations of macrophages, SMϕ and PMϕ, and their presence in a specific sequence (SMϕ preceding PMϕ). This may represent one of the mechanisms by which tumor cells evade host immune surveillance.**

Recent studies indicate that the existence of various "check and balance" mechanisms may be essential for ensuring a proper immune response to antigenic stimulation (1, 2). These mechanisms require the collaboration of various cellular and humoral compartments of the immune system. However, it may be the nature of some tumor cells to subvert this system by evoking reactions that offset these delicate check and balance mechanisms, thereby evading the host's immune surveillance, ensuring their survival. In this report, we present evidence to show that not only can tumor cells evade the host's immune defense system by directly suppressing the immune response but also they can activate a suppressor mechanism through the host's own immune surveillance network.

It has long been recognized that tumor cells or their products can be immunosuppressive (3). We and others have shown that some tumor cells or an immunosuppressive factor(s) obtained from tumor bearers can suppress T cell-mediated tumor immunity (4, 5). In previous studies we have shown that macrophages play an essential role in regulating the immune responses to tumor cells both in syngeneic (6, 7) and in allogeneic systems (8). Due to the immunosuppressive nature of some tumor cells, it is difficult to study the relationship between immunogenicity and immunosuppression in the syngeneic system—i.e., immune response to tumor-associated antigens. Use of an allogeneic system allows us to dissociate the immunosuppressive and immunogenic properties of the tumor cells (8), thereby making it possible to further examine the mechanisms for tumor cell suppression. In the present study we have investigated these mechanisms by studying the effect of four tumor lines, two virally induced leukemias (FBL-3 and HFL/d) and two chemically induced neoplasms (EL-4 and Meth A), on the induction of cytotoxic responses in the allogeneic mixed lymphocyte culture (MLC) reactions.

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MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice at 2 to 5 months of age were obtained from the Veterinary Resource Branch, Division of Research Services, National Institutes of Health, Bethesda, MD.

MLC Reactions. MLC were performed by a technique similar to that developed by Nabholz *et al.* (9). The culture medium was RPMI 1640 containing 5% fetal bovine serum (Flow Laboratories, Rockville, MD), 50 μM 2-mercaptoethanol (Sigma), and 20 mM Hepes buffer solution (Media Unit, National Institutes of Health, Bethesda, MD). The responding cells (responders) were spleen cells obtained from normal BALB/c (H-2^d) mice. The stimulating cells (stimulators) were C57BL/6 (H-2^b) spleen cells that had received 2000 roentgens (1 roentgen = 2.58×10^{-4} coulomb/kg) of x-irradiation. The spleen cells were pressed gently in Hanks' balanced salt solution containing 5% fetal bovine serum, and passed through two layers of cotton gauze to prepare single cell suspensions. They were treated with ACK lysing buffer (0.155 M NH_4Cl/0.1 mM Na_2EDTA/0.01 M $KHCO_3$) for 2 min at 4°C to remove erythrocytes. The responders were always suspended in culture medium at a final concentration of 2×10^6 cells per ml. They were either incubated alone (control culture) or incubated with stimulators at an appropriate responder-to-stimulator ratios (R/S). In some cultures, third-party 10,000 roentgen x-irradiated tumor cells were added at various responders-to-tumor cell ratios (R/T). Each flask usually contained a total of 5 ml of cultured cells. All cultures were established in 30-ml tissue culture flasks (Falcon) and incubated in an upright position at 37°C in a humidified atmosphere containing 5% CO_2. After 5 days of culturing, the cells were counted, washed once, and resuspended in 1640 medium/10% fetal bovine serum at appropriate concentrations to be tested in the [^{125}I]iododeoxyuridine (^{125}IdUrd) release assay for cell-mediated cytotoxicity.

Tumor Cells. Four tumor lines were used in these experiments. Two Friend virus-induced leukemias: FBL-3 (10) of H-2^b haplotype and HFL/d (11) of H-2^d haplotype; and two chemically induced tumors: a benzopyrene-induced leukosis EL-4 (12) of H-2^b haplotype and methylcholanthrene-induced sarcoma Meth A (13) of H-2^d haplotype. All tumor lines were adapted to grow in suspension culture and were maintained in 1640 medium/10% fetal bovine serum.

Cell-Mediated Cytotoxicity Assay. The ^{125}IdUrd release assay was used to measure cell-mediated cytotoxicity. The details of the technique have been described elsewhere (14). In brief, 0.05 ml of ^{125}IdUrd-labeled target cells at 1×10^5/ml and 0.15 ml of effector cells at appropriate concentration were

Abbreviations: MLC, mixed lymphocyte culture; R/S, responder-to-stimulator ratio; R/T responder-to-tumor cell ratio; E/T, effector-to-target cell ratio; PC, peritoneal cells; MLTC, mixed lymphocyte tumor cell culture; PMϕ, peritoneal macrophages; SMϕ, splenic macrophages.

Table 1. Suppression by syngeneic Meth A tumor cells of the MLC cytotoxic response through PC

Group	Responders*	Stimulators*	Meth A cells added at R/T†	PC‡	Net % lysis§
1	CS	XBS	—	−	47
2	CS	XBS	—	+	48
3	CS	XBS	10/1	−	2
4	CS	XBS	30/1	−	21
5	CS	XBS	100/1	−	45
6	CS	XBS	300/1	−	48
7	CS	XBS	1000/1	−	44
8	CS	XBS	100/1	+	−1
9	CS	XBS	300/1	+	5
10	CS	XBS	1000/1	+	9

* Standard MLC was performed by stimulation of BALB/c spleen cells (CS) with 2000-roentgen x-irradiated C57BL/6 spleen cells (XBS) at R/S of 10/1.

† Meth A cells x-irradiated with 10,000 roentgens were added to some cultures at the indicated R/T.

‡ At 1 day after initiating the MLC, syngeneic (BALB/c) peritoneal cells were added to some cultures at a final concentration of 10% of responders.

§ Cell-meduated cytotoxicity was tested at 5 days after MLC. The target cells were FBL-3. The E/T was 75/1.

added to the wells of Microtest II plates. All effector cells were adjusted to contain the same number of viable cells for each effector-to-target cell ratio (E/T). Incubation was carried out at 37°C in a 5% CO_2 atmosphere for 18–24 hr. Then the tests were harvested by MASH II (multiple automated sample harvester; Microbiological Associates, Bethesda, MD). The supernatants and cell pellets were collected separately and their radioactivity was determined in a well-type gamma scintillation counter. The results were expressed as total percentage of lysis and net percentage of lysis according to the following formulas:

$$\text{Total \% lysis} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant} + \text{cpm in cells}} \times 100$$

Net % lysis = (total % lysis obtained with responders incubated with stimulators) − (total % lysis obtained with responders incubated alone).

The SEMs obtained with total % lysis were usually between 0.5% and 3%.

The FBL-3 tissue culture line was used as target cells. This line was found to have a much lower level of spontaneous release of ^{125}IdUrd (between 5% and 15%) than some other tumors and provided more suitable target cells.

Preparation of Splenic Adherent Cells and Peritoneal Cells. The splenic adherent cells were obtained by the method described by Cowing *et al.* (15) with some modification. Spleen cells at 1×10^7/ml in 1640 medium/5% fetal bovine serum were incubated in plastic petri dishes at 37°C for 4 hr in a 5% CO_2 atmosphere. The nonadherent cells were removed by three exhaustive washings and the adherent cells (accessory cells) were removed by rubber policeman. The peritoneal cells (PC) were removed from normal mice by irrigating the peritoneal cavity with Hanks' balanced salt solution containing 5% fetal bovine serum.

Table 3. Characterization of the PC responsible for suppression

PC	Treatment*	X-irradiated Meth A†	Net % lysis
−	—	−	46
+	—	−	47
−	—	+	48
+	—	+	3
+	Adherent	+	4
+	Nonadherent	+	42
+	X-irradiated	+	0
+	Anti-Thy 1.2	+	−3
+	Carbonyl iron and magnet	+	47

The standard MLC reaction was performed as described in Table 1. PC addition and measurement of net % lysis were as in Table 1.

* The PC were treated by petri dish separation (adherent cells and nonadherent cells), 750 roentgens of x-irradiation, anti-Thy 1.2 antibody lysis, or removal of phagocytes by the carbonyl iron and magnet technique.

† Third-party 10,000-roentgen x-irradiated Meth A tumor cells added at R/T of 300/1.

Sephadex G-10 Column Treatment. Macrophages (or accessory cells) were removed from normal spleen cells by the method of Ly and Mishell (16) with some modification. The starting spleen cells usually contained 7–12% macrophages as tested by latex bead ingestion. After one passage through the Sephadex G-10 column, the separated spleen cells contained less than 1% macrophages. After two consecutive passages through Sephadex G-10 column, there was usually less than 0.5% macrophages. Most experiments were performed with one passage through the Sephadex G-10 column.

Treatment with Anti-Thy 1.2 Antibody. This antibody was purchased from Litton Bionetics (Kensington, MD). The specificity of the antibody has already been well characterized.

Removal of Phagocytes by Carbonyl Iron and Magnet Technique. Between 1 and 3×10^7 PC were preincubated with carbonyl iron particles (Technicon) for 1 hr at 37°C, and the phagocytes were then removed by magnet. This treatment could remove over 99% of the phagocytes as shown by latex bead ingestion.

RESULTS

Suppression by Tumor Cells of the MLC Cytotoxic Response Through Macrophages. In a previous study, it had been shown that peritoneal macrophages (PMϕ) added at the onset of the mixed lymphocyte tumor cell culture (MLTC) reactions can reverse the immunosuppressive effect of tumor cells (8). However, adding (PMϕ) at a latter time—e.g., 1 day after the initiation of MLTC—fails to reverse the tumor cell suppression;

Table 2. Suppression by tumor cells of the MLC cytotoxic response through macrophages

	Net % lysis obtained with MLC containing tumor cells at various R/T												
	None	HFL/d				EL-4				FBL-3			
PC	0	10/1	100/1	300/1	1000/1	10/1	100/1	300/1	1000/1	10/1	100/1	300/1	1000/1
−	47	19	47	54	45	16	48	51	49	0	28	44	47
+	48	ND	0	3	48	ND	1	5	43	ND	−3	−3	13

The standard MLC reaction was performed as described in Table 1. Some cultures contained 10,000-roentgen x-irradiated tumor cells as third-party cells added as indicated (none indicates that no tumor cells were added). ND, not done.

furthermore, it suppresses the induction of cell-mediated cytotoxic response to some immunogenic tumor cells (17). Because tumor cells were used as the source of immunogen in these experiments, it was still not clear whether this phenomenon was restricted to the immune response to tumor cells. The present study was designed to investigate whether such a suppressor mechanism could also be extended to the immune response to other antigenic sources—e.g., alloantigen on normal spleen cells. Experiments were performed by determining the effect of adding peritoneal cells and third-party, x-irradiated tumor cells on the cytotoxic responses in standard MLC. As shown in Table 1, standard MLC reactions were performed by stimulation of BALB/c (H-2^d) spleen cells (responders) with 2000-roentgen x-irradiated allogeneic C57BL/6 (H-2^b) spleen cells. In some cultures, x-irradiated, syngeneic Meth A tumor cells were added as third-party cells at various R/Ts. Normal syngeneic (BALB/c) PC were added to some cultures 1 day after the initiation of MLC and their effect on the generation of cytotoxic response was determined. It was found that addition of large amounts of third-party x-irradiated tumor cells (R/T at 10/1 to 30/1, groups 3 and 4) directly suppressed the generation of cytotoxic T cells. This direct suppressive effect was not seen upon addition of small amounts of tumor cells (from R/T at 100/1 to 1000/1 groups 5–7). However, if syngeneic peritoneal cells were also added after MLC had been initiated—e.g., 1 day after MLC—the cytotoxic responses were completely suppressed in the presence of these nonsuppressive doses of tumor cells (groups 8–10), whereas addition of peritoneal cells without tumor cells had no such effect (group 2). Further study showed that other tumor cells also produced similar effects (Table 2). The tumor cells added can be either syngeneic (HFL/d) or allogeneic (EL-4 or FBL-3) to the responders (BALB/c). These findings indicate that tumor cells can activate the suppressor mechanism through PC.

Table 3 shows that the PC that trigger suppression are likely to be macrophages: they are adherent to plastic dishes, phagocytic, radioresistant, and resistant to anti-Thy 1.2 antibody lysis.

Mechanism for Suppression. In order to determine how tumor cells induce suppression through the host's macrophages, a different experimental scheme was used.

Because the spleen contains a sufficient amount of resident (splenic) macrophages (SMϕ), it appears that PMϕ must be functionally distinct from SMϕ. In order to investigate the in-

Table 4. Mechanism for tumor cell-triggered macrophage-mediated immunosuppression

Responder	Group	Addition to MLC on* Day 0	Day 1	Day 2	Net % lysis at E/T† 75/1	15/1
Unseparated spleen cells	1	S			47	25
	2	S PMϕ			49	28
	3	S	PMϕ		48	26
	4	S T			45	39
	5	S T PMϕ			47	41
	6	S T	PMϕ		−1	0
	7	S T	SMϕ		48	27
	8	S T PMϕ	PMϕ		49	38
	9	S	T		42	21
	10	S	T PMϕ		47	26
	11	S	T	PMϕ	0	−1
	12		S		38	24
	13	T	S		34	29
	14	T	S PMϕ		3	2
	15		S T		37	27
	16		S T PMϕ		39	30
	17		S T	PMϕ	−2	0
Sephadex G-10-separated spleen cells	18	S			2	−1
	19	S T			0	−1
	20	S PMϕ			49	15
	21	S SMϕ			43	11
	22	S	PMϕ		45	18
	23	S	SMϕ		35	14
	24	S T PMϕ			56	20
	25	S T SMϕ			43	20
	26	S T	PMϕ		53	30
	27	S T	SMϕ		34	10
	28	S T PMϕ	PMϕ		53	34
	29	S T SMϕ	SMϕ		42	14
	30	S T PMϕ	SMϕ		50	25
	31	S T SMϕ	PMϕ		4	−1
	32	S T PMϕ SMϕ	PMϕ		52	28

The MLC reaction was performed as was that described in Table 1. Groups 1–17 had unseparated BALB/c spleen cells as responders and groups 18–32 had Sephadex G-10 column-separated spleen cells as responders.

* S, 2000-roentgen x-irradiated C57/BL6 spleen cells at R/S = 10/1. T, 10,000-roentgen x-irradiated Meth A tumor cells at R/T = 200/1. PMϕ and SMϕ, BALB/c peritoneal cells or splenic adherent cells, respectively, added at a final concentration of 10% of responders. The splenic adherent cells were prepared by incubating spleen cells in plastic petri dish at 1×10^7/ml for 4 hr. The nonadherent cells were removed by three exhaustive washings and the adherent cells (simply designated as SMϕ) were removed by rubber policeman. The PMϕ or SMϕ were added at onset (day 0) or at 1 or 2 days after MLC.

† The cytotoxicity was tested at 5 days after MLC at E/T of 75/1 and 15/1; the target cells were FBL-3.

terrelationship of the different macrophage populations and their involvement in the generation of T cell-mediated cytotoxic response and in the tumor cell-induced immunosuppression, the following experiment was performed (the results are summarized in Table 4). Two responding groups were used, a normal unseparated splenic responder (groups 1–17) and a spleen population separated by a Sephadex G-10 column (groups 18–32), which was depleted of resident (splenic) macrophages. In these experiments allogeneic stimulators and syngeneic tumor cells, PC (PMϕ), or splenic adherent cells (SMϕ) were added at the onset (day 0) or at 1 or 2 days after initiation of MLC. Their effects on the generation of a cytotoxic response were then determined on day 5 of MLC. The findings obtained with unseparated spleen cells as responders (groups 1–17) can be summarized as follows: (*i*) Suppression could only be induced when PMϕ, not SMϕ, were present 1 day after addition of tumor cells to MLC, regardless of whether the stimulators were added on day 0 (groups 6 and 11) or day 1 (group 17); that is, the responders (spleen cells) have to be preexposed to tumor cells prior to addition of PMϕ. (*ii*) Addition of PMϕ and tumor cells simultaneously not only gave no suppression (groups 5, 10, and 16), it precluded the induction of suppression by further addition of PMϕ on day 1 (group 8). It should be noted that addition of either PMϕ or tumor cells (Meth A at R/T of 200/1) alone to MLC gave no suppression. The failure of SMϕ to induce suppression (group 7) also suggests that only exogenous (peritoneal) macrophages (with respect to splenic responders) can produce such effect.

To further elucidate these points, we have carried out experiments using spleen cells separated by a Sephadex G-10 column as responders; they were first depleted of resident (splenic) macrophages (16), then reconstituted with PMϕ or SMϕ to determine the effects of these macrophages on MLC with or without tumor cells (groups 18–32). This approach should allow us to directly examine the functional diversity between SMϕ and PMϕ in tumor cell-induced immunosuppression. In these experiments, the stimulators and third-party tumor cells were added on day 0. Some different and unexpected results were obtained after reconstituting with PMϕ or SMϕ on day 0, day 1, or both. As expected, responders depleted of SMϕ gave no cytotoxic response (group 18), and the response could be fully restored by reconstituting with PMϕ or SMϕ (groups 20–23). It was unexpected to find that addition of either PMϕ or SMϕ at the same time or at 1 day after addition of tumor cells did not induce suppression (groups 24–27). Furthermore, if macrophages of the same anatomic site (PMϕ in group 28, SMϕ in group 29) were added at both days 0 and 1, or if PMϕ were added prior to SMϕ (group 30), no suppression was seen. These findings appeared to be contradictory to the above observation that suppression could be induced if responders were preexposed to tumor cells prior to addition of PMϕ. However, this puzzle was quickly resolved by the finding that suppression could be induced only if the components of the culture were added in the right sequence after addition of tumor cells: SMϕ must precede PMϕ (group 31), and addition of PMϕ and SMϕ simultaneously with the tumor cells prevented the induction of suppression by further addition of PMϕ on day 1 (group 32). These results clearly indicate that the induction of suppression not only requires the presence of tumor cells prior to PMϕ, but it also strictly requires the collaboration between two populations of macrophages (PMϕ and SMϕ) and their presence in the correct sequence (SMϕ prior to PMϕ), indicating a critical requirement of preexposure of SMϕ to tumor cells. Simultaneous presence of PMϕ and SMϕ with tumor cells precludes the induction of suppression. It should be noted that tests of the splenic adherent cells (SMϕ) are also consistent with their being macrophages: they are adherent to plastic dishes, radioresistant, and resistant to anti-Thy 1.2 antibody lysis (not shown).

DISCUSSION

Immunosuppressive macrophages have been described in several systems (18–20), and there is also evidence for phenotypic and functional heterogeneity of macrophages (15, 21–23). In the present study, it is clearly shown that tumor cells at a nonsuppressive dose can induce suppression of immune response through the host's macrophages (Tables 1–3). However, in order to achieve this purpose, the tumor cells have to evade several levels of the host's immune network.

All the experiments presented in this paper have been repeated at least 3–10 times and the results are reproducible. The behavior of the active component for inducing suppression in the splenic adherent cells and peritoneal cells is consistent with its being macrophages. They are adherent to plastic dishes, radioresistant, and resistant to anti-Thy 1.2 antibody lysis. The failure of unrestored Sephadex G-10 column-separated cells

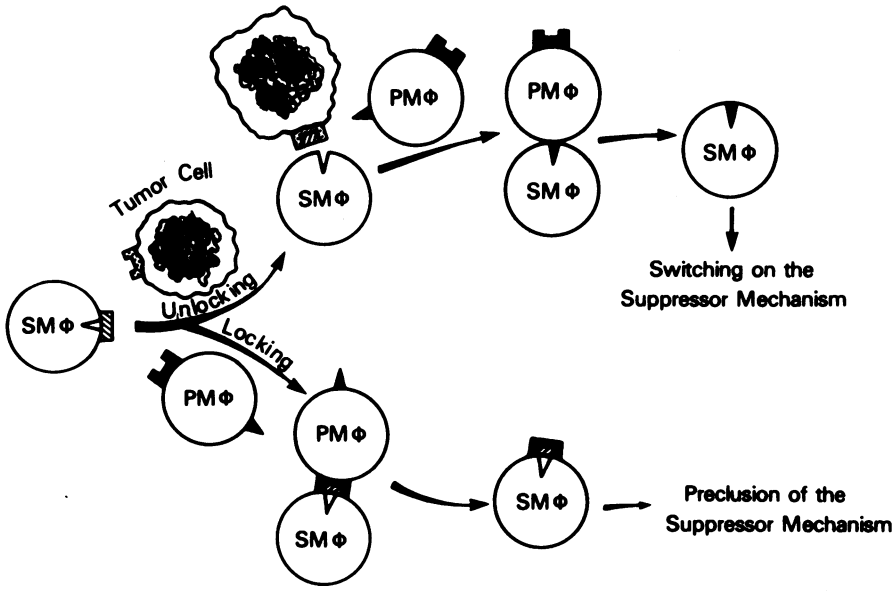

FIG. 1. Hypothetical model of the macrophage-mediated suppressor mechanism.

to induce suppression upon further addition of peritoneal macrophages indicates that preexposure of tumor cells to T cells (group 26, Table 4) or to a mixture of T cells and (peritoneal) macrophages (group 28, Table 4) is not sufficient for inducing suppression, because these Sephadex G-10 column-separated spleen cells contain sufficient amounts of T cells. The tumor cell-triggered macrophage-mediated suppression has the critical requirement for both the SMϕ and PMϕ (group 31, Table 4).

The critical requirement for tumor cells and the collaboration of two populations of macrophages to induce suppression denotes the possible existence of a unique mechanism for tumor cell suppression. In this pathway, there is also a requirement for a specific sequence of addition of tumor cells and macrophages—the resident (splenic) macrophages (SMϕ) have to be preexposed to tumor cells prior to the addition of exogenous (peritoneal) macrophages (PMϕ) (Table 4). These findings are in complete agreement with our previous report, which involved using tumor cells as stimulators in the MLTC reactions (17). Furthermore, the simultaneous presence of peritoneal macrophages and tumor cells prevents the induction of suppression (Table 4, groups 8 and 32). These results indicate that despite the fact that PMϕ are needed to induce suppression, the SMϕ hold the key for suppression. It is possible that such mechanisms may also operate in normal immune response reactions as part of host's check and balance mechanisms. That is, after the immune response is initiated, then at a proper time the exogenous macrophages are recruited to trigger the resident macrophages to generate a negative signal to terminate the immune response at a proper level. The presence of tumor cells may complicate the situation by switching on this suppressor mechanism prematurely. To safeguard against this possibility, if the host can recruit exogenous macrophages at the time of tumor cell presence, such suppression can be prevented. Thus the tumor cells must evade this checking mechanism before they can escape the host's immune surveillance network.

A hypothetical model for suppression is proposed to illustrate this intriguing phenomenon (Fig. 1). The model is similar to the suggestion by Monroy and Rosati (24) that the cell–cell communication system has evolved from membrane structures originally meant for cell recognition in the mating process (25); in our experiments, the SMϕ, which hold the key for inducing suppression, behave like (−) cells possessing the receptors for the (+) cells (PMϕ). The receptors on SMϕ are masked or semilocked in an unstimulated state. After the immune response has been initiated and generated to a certain level, the receptors in SMϕ are unmasked or unlocked, then the exogenous PMϕ are recruited to "mate" with the SMϕ by inserting some essential factors that will trigger the SMϕ to switch on the suppressor mechanism and to terminate the immune response at a proper level. However, tumor cells have the ability to unlock the SMϕ's receptors at an earlier stage, allowing them to "mate" with PMϕ, thereby prematurely switching on the suppressor mechanism that results in an abortive T-cell response. On the other hand, if PMϕ and SMϕ are exposed to each other prior to tumor cells, because PMϕ have another cell surface component that can keep the SMϕ's receptors firmly locked, thus preventing the unlocking process and precluding the induction of suppression. Alternatively, there may be two subsets of PMϕ that can either "mate" with SMϕ or keep the receptors of SMϕ firmly locked.

Not only does there appear to be a remarkable resemblance between host–tumor and host–parasite relationships, but also the methods that the tumor cells use to evade the host's immune surveillance are strongly reminiscent of the tactics of parasites (26). They can jam the immune response by direct suppression, or they can subvert the immune system by switching on the host's own suppressor mechanism.

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