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Recognition and lysis of altered-self cells by macrophages

I. MODIFICATION OF TARGET CELLS BY 2,4,6-TRINITROBENZENE SULPHONIC ACID

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Summary. Peritoneal exudate macrophages from normal, untreated or thioglycollate-elicited mice, lysed syngeneic fibroblasts and lymphoblasts modified by 2,4,6-trinitrobenzene sulphonic acid (TNBS) *in vitro*. Optimal lysis of the hapten-modified cells by elicited macrophages was usually seen after 18 hr of co-cultivation at E:T ratios of 10:1–30:1. Cytotoxicity was expressed by macrophages depleted of T cells, and was not potentiated by LPS. Allogeneic TNBS-modified cells were lysed by non-immune, non-activated macrophages to the same extent as syngeneic modified targets, indicating that genetic restriction does not appear to play a role in macrophage-mediated cytolysis of TNBS-modified cells.

INTRODUCTION

It is widely believed that the body eliminates autologous damaged and old cells resulting from inflammation, injury and ageing, primarily through the phagocytic activity of macrophages (Pearsall & Weiser, 1970). The alteration(s) occuring in self cell characteristics on the one hand, and the mechanism(s) underlying their recognition and destruction by macrophages on the other hand are still obscure. Studies examining T-cell-mediated lympholysis (CTL) of autologous cells modified with various, well characterized, haptenic groups such as trinitrophenyl hapten (TNP), showed that presensitized T lymphocytes can lyse hapten-modified autologous cells quite efficiently *in vitro* (Shearer, 1974; Forman, 1975; Schmitt-Verhulst & Shearer, 1975; Burakoff, Germain & Benacerraf, 1976; Teh, Phillips & Miller, 1978). The objective of the present study was to clarify whether macrophages (Mϕ), without any previous immunization or activation, have the capacity to recognize and destroy altered self cells. To that end, we investigated the interaction of murine macrophages and hapten-modified syngeneic and allogeneic cells. In our experimental system, fibroblasts or lymphoblasts were modified by 2,4,6-trinitrobenzene sulphonic acid which reacts with ε-amino groups of lysine residues (Ukuyama & Satake, 1960). The data indicate that these modified cells can, indeed, be lysed *in vitro* by syngeneic and allogeneic murine peritoneal macrophages.

Abbreviations: TNBS, 2,4,6-trinitrobenzene sulphonic acid; E:T, effector:target cell ratio; LPS, lipopolysaccharide; CTL, cytotoxic T lympholysis; TNP, trinitrophenyl hapten; Mϕ, macrophages; PEC, peritoneal exudate cells; FCS, foetal calf serum; [^{3}H]-TdR, [^{3}H]-thymidine; HBSS, Hanks's balanced salt solution; C, complement; TG, thioglycollate; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Animals

Inbred female mice, 2–4 months old, of BALB/c,

C57BL/6 and C3H/CRGL strains, were used in this study. The mice were obtained from the animal breeding farm, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Macrophages

Peritoneal exudate cells (PEC) were harvested from untreated mice or 4 days after an intraperitoneal injection of 1 ml thioglycollate medium (TG, Difco). After washing, the cells were suspended in either RPMI 1640 or M-199 medium (Gibco). Approximately 14×10^5 unelicited and $9–10 \times 10^5$ TG-elicited PEC were cultivated in 16 mm flat bottom plastic culture wells (Linbro). Following 1 hr of incubation at 37° the non-adherent cells were removed by intensive rinsing with a jet of phosphate-buffered saline (PBS). The adherent cells (approximately 8×10^5 per well) consisted of more than 95% macrophages as identified by morphological and phagocytic criteria (Schroit & Gallily, 1977). The number of adherent cells per well was determined by counting cell nuclei in representative wells with 0·15% erythrosin B following cell lysis by 0·5% non-ident (P-40, BDH) in PBS containing 5 mM EDTA.

Lymphoblasts

Suspended splenocytes were cultivated in tissue culture flasks (Falcon, 25 cm^2 area) at a concentration of 2×10^6 cells/ml in RPMI 1640 medium (Gibco) supplemented with 15% foetal calf serum (FCS, Gibco), 10^{-5} mM 2-mercaptoethanol (Sigma), 10 mM Hepes buffer (Sigma), 2 mM glutamine and antibiotics (combined antibiotics, Biolab). Con A (type III, Sigma) at a concentration of 2·5 μg/ml was added to the cultures which were incubated at 37° in a humidified atmosphere containing 5% CO_2. After 2 days in culture the lymphoblasts were labelled with 1 μCi/ml[^{3}H]-thymidine ([^{3}H]-TdR, specific activity 20 Ci/mM) for 18 hr. The cells were then washed and resuspended in Hanks's balanced salt solution (HBSS, Gibco) for trinitrophenylation.

Fibroblasts

Embryonic fibroblasts were obtained from 10 to 15 day old embryos. Decapitated, eviscerated, and skinned embryos were treated with versene-trypsin, (0·02% and 0·25%, respectively, Biolab) for 5 min at room temperature. The cell suspension was washed twice, suspended in M-199 medium (Gibco) supplemented with 10% FCS, 10 mM Hepes buffer, and antibiotics, and seeded at a concentration of 2×10^6/ml in tissue culture flasks (Nunc, 75 cm^2 area). After 3–4 days of cultivation, the fibroblasts were labelled for 18 hr with [^{3}H]-TdR (1 μCi/ml, specific activity 20 Ci/mM). The cells were then removed by trypsinization, washed, and resuspended in HBSS for trinitrophenylation. In some experiments, subcultures of the fibroblasts were used for trinitrophenylation.

Trinitrophenylation of target cells

2,4,6-Trinitrobenzene sulphonic acid (TNBS, Sigma), at the desired concentration (0·1–25 mM) was added to [^{3}H]-TdR-labelled lymphoblasts or fibroblasts suspended at pH 7·4 in HBSS at a concentration of 4×10^6 cells/ml. The mixture was stirred gently at room temperature for 20 min, washed once with HBSS, once with 10 mM glycylglycine solution in HBSS, and twice more with chilled HBSS (Sugimoto, Egashira, Pierres & Greene, 1980). Cell viability was determined by trypan blue dye exclusion.

Macrophages depleted of T lymphocytes

Suspensions of 10^7 washed PEC were incubated for 30 min at room temperature with anti-Thy 1,2 (F7D5 monoclonal IgM antibody—a generous gift of Dr Lake, University College, London), diluted 1:10,000. Following centrifugation, 5% non-toxic rabbit complement (C) was added, and incubation continued for 40 min at 37°. This procedure has been shown to eliminate all T cells (Lake, Clark, Khonshidi & Sunshine, 1979).

Effector target cell cultures

In most experiments 8×10^4 [^{3}H]-TdR-labelled TNBS-modified cells suspended in 1·5 ml medium were added to a monolayer of 8×10^5 macrophages cultivated in Linbro 16 mm flat bottom plastic wells (E:T ratio 10:1). The TNBS-modified lymphoblasts were cocultured with the macrophages in RPMI 1640 medium, whereas the TNBS-modified fibroblasts were co-cultured in M-199 medium. Both media were supplemented with 10% FCS, 10 mM Hepes and antibiotics.

Assay of target cell lysis

Supernatant aliquots of 150 μl were collected at desired intervals for determining the release of radioactive material and were counted in a Packard Tri-Carb Spectrometer. Percentage specific cytotoxicity was calculated as follows: % specific cytotoxicity $= [(E - SR)/(T)] \times 100$, where E signifies [^{3}H]-TdR release of the targets in the presence of effector cells; SR signifies spontaneous release of

[^{3}H]-TdR of the targets in the absence of the effector cells, and T signifies the total counts of the target cells determined by treating target cell samples with 1% SDS. Data from cytotoxicity assays are expressed as the mean percentage specific cytotoxicity of triplicate or quadruplicate cultures usually in a representative experiment. Each experiment was performed three–four times. Differences between experimental and control group values in individual experiments were analysed for significance by the one tail distribution free Mann-Whitney U test. Differences were considered significant when P was 0·05 or less.

RESULTS

Viability of TNBS-modified cells

The viability of TNBS-modified BALB/c fibroblasts and lymphoblasts following treatment with 1 mM and 10 mM TNBS was determined both by trypan blue dye exclusion and by assaying [^{3}H]-TdR incorporation of the modified cells. The results, shown in Table 1, indicate that following treatment with either concentration, about 90% of the fibroblasts and 83% of the lymphoblasts excluded the dye. The modified cells incorporated [^{3}H]-TdR during 14 and 18 hr of incubation, to about 80% of the control values.

Killing of syngeneic cells by BALB/c macrophages

Macrophage-mediated lysis of fibroblasts and lymphoblasts modified by 1–25 mM TNBS was then assessed. In our assay system, normal unmodified syngeneic target lymphoblasts and fibroblasts were not lysed following up to 42 hr interaction with BALB/c macrophages (values of specific cytotoxicity were less than 9%). In these experiments, the spontaneous label release of unmodified targets was about 6%. Similarly, specific lysis of syngeneic TNBS-modified cells pretreated with 1–5 mM TNBS did not exceed 12% (Table 2). On the other hand, after modification with 10 mM TNBS ($TNBS_{10}$), 28% and 36% of the lymphoblasts and fibroblasts, respectively, were specifically lysed by macrophages in the 18 hr assay. Following co-cultivation for 42 hr, no significant increase in specific cytotoxicity against $TNBS_{10}$-modified cells was observed.

Kinetics of killing

Specific cytotoxicity of TG-elicited macrophages towards syngeneic $TNBS_{10}$-modified fibroblasts could not be detected after 5 or 10 hr of co-cultivation. At this time interval, the level of specific cytotoxicity ranged between 3·2%–12·2%. After 18 hr of incubation, 21%–48% specific cytotoxicity was observed. Specific cytotoxicity was only slightly, if at all, increased after 42 hr of incubation.

When macrophages and $TNBS_{10}$-modified fibroblasts were co-cultured at various E:T cell ratios, no linear relationship between E:T cell ratios and the level of cytotoxicity was exhibited (Table 3). Moreover, there seemed to be a range (10:1–30:1), rather than a particular value, of optimal E:T ratio, which gave rise to similar levels of cytotoxicity not differing significantly ($P > 0·05$) one from the other.

Killing of $TNBS_{10}$-modified cells by macrophages depleted of T lymphocytes

To exclude the possibility of T-cell participation in the killing of modified syngeneic cells, PEC were treated

Table 1. Viability of TNBS-modified cells

Cell type	Pretreatment with TNBS (mM)	Viability (%)*	Incorporation of† [^{3}H]-TdR (dpm)	Incorporation (%) of control
Fibroblasts	0	95	7982	
	1	93	6447	80·8
	10	89	6329	79·3
Lymphoblasts	0	82	13,716	
	1	83	11,530	84·1
	10	83	11,197	81·6

* Determined by trypan blue dye exclusion.

† [^{3}H]-TdR (0·05 μCi/well, specific activity 20 Ci/mM), was added to 4 and 0 hr of fibroblast and lymphoblast cultures, respectively. Radioactivity was determined 18 hr after onset on the experiment.

Table 2. TG macrophage-mediated cytotoxicity against syngeneic cells modified by various concentrations of TNBS

Pretreatment of cells with TNBS*	Specific cytotoxicity (%)†			
	fibroblasts		lymphoblasts	
	18 hr	42 hr	18 hr	42 hr
0	7·9	8·5	3·3	4·7
1·0	ND	ND	1·2	2·6
2·5	5·6	11·4	5·5	3·1
10·0	35·5	34·6	27·9	29·2
25·0	36·5	34·8	ND	ND

* Viability (%) of cells after TNBS treatment ranged between 88%–94% for fibroblasts, and 80%–85% for lymphoblasts.

† E:T cell ratio 10:1. Spontaneous release from both cell types modified with 1–5 mM TNBS after 18 and 42 hr of culturing was 2%–6% and 5%–12% respectively. That of cells modified with 10 and 25 mM TNBS was 5%–16% and 20%–25% after 18 and 42 hr, respectively. Spontaneous release from unmodified cells was 3% and 5%, respectively.

ND, not determined.

Table 3. TG macrophage-mediated cytotoxicity against $TNBS_{10}$-modified syngeneic fibroblasts at various E:T ratios

E:T cell ratio*	Specific cytotoxicity (%) after†	
	18 hr	42 hr
3:1	23·8	29·7
10:1	42·8	45·5
30:1	42·2	41·4
60:1	24·1	25·8

* Macrophages (8×10^5) per well were cultivated with various numbers of $TNBS_{10}$-modified fibroblasts to obtain the required E:T ratio. Viability (%) of fibroblasts after $TNBS_{10}$ modification was 92%.

† Average of three experiments. Specific cytotoxicity against unmodified fibroblasts at the E:T ratios tested ranged between 4·9 and 14·7%.

with monoclonal anti-Thy 1·2 and complement before their interaction with $TNBS_{10}$-modified fibroblasts. As seen in Table 4, macrophage-mediated killing was unaffected by this treatment. Cultivation of modified syngeneic cells and TG-elicited macrophages in Ig-free FCS did not affect the extent of modified-self cell lysis.

Cytotoxicity by unelicited macrophages

To examine whether TG elicitation is a prerequisit for expression of macrophage cytotoxicity, $TNBS_{10}$-modified fibroblasts were also co-cultivated with syngenic unelicited macrophages. Killing of $TNBS_{10}$-modified fibroblasts by TG-elicited macrophages was slightly, but significantly higher ($P < 0.05$) than that expressed by unelicited macrophages (Table 4). In these experiments no difference in the number of adherent TG-elicited macrophages and unelicited macrophages was detected by nuclei counting in NP-40.

Effect of LPS

To find out whether non-specific activation of macrophages could potentiate their cytotoxic expression against syngeneic modified cells, LPS (5 μg/ml) was added to co-cultures of TG-elicited macrophages and $TNBS_{10}$-modified fibroblasts. As seen in Table 5, rather than an increase, a slight but significant decrease ($P < 0.05$) in TG macrophage-mediated cytotoxicity against $TNBS_{10}$-modified fibroblasts was detected in the presence of LPS. When tested in co-cultures of unelicited macrophages and $TNBS_{10}$-modified fibroblasts, no significant effect of LPS on cytotoxicity was noted (Table 5).

Killing of allogeneic TNBS modified cells

TG macrophages from BALB/c (syngeneic) and C57BL/6 (allogeneic) mice lysed both BALB/c fibroblasts and lymphoblasts modified by 10 mM TNBS. As was the case with syngeneic targets, allogeneic targets modified with up to 5 mM TNBS were not lysed (Table 6).

When syngeneic combinations of effector and target cells from three different strains were compared to six allogeneic combinations (Table 7), the specific cytotoxicity of $TNBS_1$-modified fibroblasts did not differ significantly from that of unmodified cells in any of the combinations tested. Macrophage-mediated cytotoxicity towards $TNBS_{10}$-modified fibroblasts reached,

Table 4. Cytotoxicity against syngeneic $TNBS_{10}$-modified fibroblasts, expressed by TG macrophages depleted of lymphocytes and by unelicited macrophages.

Exp. no.	Effector cells	Modification of target fibroblasts by 10 mM TNBS*	% specific cytotoxicity†
1	TG−Mϕ	−	2·9
	TG−Mϕ	+	40·4
	TG+Mϕ‡	+	34·0
	PEC+anti Thyl.2 +C	−	4·8
	PEC+anti Thyl.2 +C	+	43·9
2	TG−Mϕ	−	10·4
	TG−Mϕ	+	46·9
	unelicited Mϕ	−	8·7
	unelicited Mϕ	+	37·4

* Viability (%) following $TNBS_{10}$ modification was 94%.

† After 18 hr of cultivation E:T cell ratio 10:1. Spontaneous release from unmodified and $TNBS_{10}$-modified cells ranged between 8% and 14·1% in the different experimental groups.

‡ Cultivating in Ig-free FCS (done in separate experiment).

Table 5. Effect of LPS on macrophage-mediated cytotoxicity against $TNBS_{10}$-modified fibroblasts

$TNBS_{10}$ modification*	Presence of LPS (5 μg/ml)†	Specific cytotoxicity (%)‡ TG-Mϕ 18 hr	TG-Mϕ 42 hr	Unelicited Mϕ 18 hr	Unelicited Mϕ 42 hr
—	—	4·5	3·0	2·1	1·8
—	+	5·4	2·3	2·7	1·5
+	—	28·8	30·8	21·2	23·8
+	+	23·8	26·7	19·5	22·8

* Viability (%) of $TNBS_{10}$-modified fibroblasts was 96%.

† Lipolysaccharide W, E. coli 055:B5 (Difco).

‡ E:T cell ratio of 10:1. Spontaneous release (%) from unmodified and $TNBS_{10}$-modified fibroblasts ranged between 5·9% and 13·3%.

Table 6. TG macrophage-mediated cytotoxicity against allogeneic TNBS-modified cells

Pretreatment of target cells with TNBS (mM)*	Specific cytotoxicity† Fibroblasts 18 hr	Fibroblasts 42 hr	Lymphoblasts 18 hr	Lymphoblasts 42 hr
0	9·2	2·8	9·0	−3·9
0·1	ND	ND	−5·8	−4·1
1·0	ND	ND	1·0	−5·1
2·5	6·2	0·6	7·5	−4·6
5·0	6·4	−8·1	6·9	−6·0
10·0	31·1	37·5	22·0	28·8

* Viability (%) of $TNBS_{10}$-modified fibroblasts and lymphoblasts was 88%–94% and 80%–85%, respectively.

† E:T cell ratio 10:1. Spontaneous release from both cell types modified with 1–5 mM TNBS after 18 and 42 hr of culturing was 2%–6% and 5%–12%, respectively. Following modification with 10 mM TNBS, spontaneous release after culturing for 18 and 42 hr was 17·1% and 31·0% respectively for fibroblasts, and 19·5% and 17·6% respectively for lymphoblasts.

more or less, the same level of cytotoxicity in all of the syngeneic combinations tested and did not significantly exceed the levels expressed towards allogeneic $TNBS_{10}$-modified targets.

DISCUSSION

The present study demonstrates that macrophages from unsensitized, non-activated mice expressed *in vitro* cytotoxicity against TNBS-modified syngeneic cells. It was observed that these macrophages did not lyse cells modified by 0·1–5 mM TNBS, whereas they did lyse lymphoblasts and fibroblasts after modification with 10 mM TNBS. Following co-cultivation of

Table 7. TG macrophage-mediated cytotoxicity against allogeneic TNBS-modified fibroblasts

Origin of target fibroblasts modified by TNBS*		Specific cytotoxicity (%) by effector Mϕ derived from†					
		BALB/c		C3H/CRGL		C57BL/6	
1 mM	10 mM	18 hr	42 hr	18 hr	42 hr	18 hr	42 hr
BALB/c		1·5	3·8	2·4	3·4	5·5	5·1
C3H/CRGL		4·3	3·7	3·9	6·2	8·2	9·0
C57BL/6		2·2	2·6	0·6	0	6·0	2·6
	BALB/c	27·1	25·2	22·3	20·1	31·8	28·4
	C3H/CRGL	26·8	25·6	20·7	20·8	26·3	25·7
	C57BL/6	25·2	20·7	22·3	18·5	25·5	22·8

* Viability (%) of TNBS-modified cells ranged between 95–98% in the different experimental groups.

† E:T cell ratio of 10:1. Spontaneous release in the different experimental groups ranged between 5·1%–9·7% and 7·7%–13·2% after 18 and 42 hr, respectively. Cytotoxicity of Mϕ towards unmodified syngeneic and allogeneic fibroblasts derived from the different mice strains, ranged between 0·9% and 9·8% and 0% and 6·6% after 18 and 42 hr, respectively.

macrophages and $TNBS_{10}$-modified cells for 18 hr, optimal killing was usually observed at E:T ratios of 10:1–30:1. Longer incubation (42 hr) did not significantly increase, in most of the experiments, the percentage of specific target lysis by macrophages. This may be due to the higher spontaneous release of [^{3}H]-TdR by $TNBS_{10}$-modified cells obtained after extended cultivation, which might mask any increment in specific lysis. It should be pointed out, that following modification with 10 mM TNBS, 85%–93% of the lymphoblasts and fibroblasts were viable, respectively. Similarly, during the first 18 hr of cultivation these modified cells incorporated [^{3}H]-TdR to about 80% of the control values. Moreover, after this period of time, the spontaneous release of the labelled $TNBS_{10}$-modified lymphoblasts and fibroblasts did not exceed 13% above the values of the unmodified cells. Thus, the killing of $TNBS_{10}$-modified cells could not be attributed to recognition and destruction of dying cells, but rather to recognition of modified-self cells by the macrophages.

To exclude the possibility that any residual T cells in the macrophage monolayers assist in the lysis of syngenic $TNBS_{10}$-modified cells, PEC were pretreated with monoclonal anti-Thy 1.2 and complement. These T-depleted macrophage populations expressed the same level of cytotoxicity as untreated macrophage monolayers which may have contained a small percentage of lymphocytes. It is unlikely that target cell lysis is due to natural antibodies that cross-react with TNP, as $TNPS_{10}$-modified cells were destroyed to the same extent when they were interacted with macrophages in Ig-free FCS (Sigma). Thus, antibody-dependent cellular cytotoxicity (ADCC) which might be involved in cytotoxic expression of macrophages (Lohmann-Matthes, Donzig & Roder, 1979), does not seem to operate in self-modified cell killing. It was interesting to learn that unelicited (resident) macrophages, which differ in various characteristics from elicited macrophages (Soberman & Karnovsky, 1981), lyse $TNBS_{10}$-modified syngeneic fibroblasts to a slightly, but significantly lesser extent that TG macrophages. It was also observed that *in vitro* activation of macrophages by LPS did not potentiate killing by either TG or unelicited macrophages, and even decreased the capacity of TG macrophages to lyse $TNBS_{10}$-modified fibroblasts. These data support the idea that destruction of chemically altered-self cells by macrophages differs from macrophage tumouricidal potential which is dependent on an activation signal for its expression (Hibbs, Weinberg & Chapman, 1980). Yet, at present, we cannot exclude the possibi-

lity that TNBS in itself activates macrophages to some degree.

Hapten-specific cell-mediated cytotoxicity has been used by several investigators, as an experimental model in studying lymphocyte-mediated cytotoxic activity against altered-self targets (Shearer, 1974; Forman, 1975; Schmitt-Verhulst & Shearer, 1975; Burakoff *et al.*, 1976; Teh *et al.*, 1978; Rehn, Shearer, Koren & Inman, 1976; Sherman, Burakoff & Benacerraf, 1978; Pohlit, Hass & Van Boehmer, 1979; Levy, Henkart & Shearer, 1981; Gilheany, Aora, Levy & Shearer, 1981). It has been concluded that TNP molecules conjugated to H-2 moieties, as well as to non-H-2 cell surface proteins, are involved in the *in vitro* presensitization needed for the generation of specific CTL (Chiavarra & Forman, 1981; Levy, Shearer, Richardson & Henkark, 1981). It was shown that major histocompatibility complex (MHC) gene products influence the CTL activity against TNBS-modified targets in several aspects: CTL effector cells from mice expressing the K^k haplotype were found to be high genetic responders to TNP-self, as compared with those of mice expressing the $D^{d,b,k}$ (Levy & Shearer, 1979). There was preferential lysis of TNBS-modified syngeneic targets, over that of TNP-modified allogeneic targets, by the CTL effector cells generated by TNP-self cells; strain differences have also been observed in the extent of lysis of CTL effectors, sensitized to TNP-self and assayed on TNBS-modified allogenic targets (Billings, Burakoff, Dorf & Benacerraf, 1978). Strain differences in the response to TNP-self, were found when the hapten was present on the stimulating or target cells in limiting concentrations (Shearer, Schmitt-Verhulst, Petinelli, Miller & Gilhearny, 1979).

Our study clearly demonstrates that macrophages are capable of recognizing and lysing altered-self cells without lymphocyte assistance. This killing differs from CTL activity in not requiring presensitization and in being expressed towards allogeneic $TNBS_{10}$-modified fibroblasts of three different haplotypes to the same extent as towards syngeneic, similarly modified cells. Further experiments will clarify whether other hapten or enzyme-modified syngeneic cells can also be recognized and lysed by macrophages. It will also be interesting to find out whether the lysis of altered-self cells by macrophages differs or resembles, the killing of xenogeneic cells by non-activated, non-immune macrophages, which we previously described (Cabilly & Gallily, 1981a, 1981b). We believe that the approach described here will be useful in elucidating the mechanism(s) of altered-self recognition by macrophages.

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