GENETIC CONTROL OF IMMUNE RESPONSES IN VITRO

VI. Experimental Conditions for the Development of Helper T-Cell Activity Specific for the Terpolymer L-Glutamic Acid\textsuperscript{60}-L-Alanine\textsuperscript{30}-L-Tyrosine\textsuperscript{10} (GAT) in Nonresponder Mice*

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The immune response of inbred strains of mice to the random terpolymer L-glutamic acid\textsuperscript{60}-L-alanine\textsuperscript{30}-L-tyrosine\textsuperscript{10} (GAT)\textsuperscript{1} is controlled by a specific autosomal dominant immune response (Ir) gene which maps in the Ir-IA, Ir-IB regions of the H-2 complex (1-3). In previous reports in this series, we have described the parameters for development of primary GAT-specific IgG plaque-forming cell (PFC) responses in mouse spleen cell cultures (4). Spleen cells from both responder (H-2a,b,d,k) and nonresponder (H-2p,q,s) mice develop GAT-specific IgG PFC responses after incubation with GAT complexed to methylated bovine serum albumin (GAT-MBSA) (4). However, only spleen cells from responder mice develop a GAT-specific PFC response when incubated with soluble GAT or macrophage-bound GAT (GAT-M\Phi) (5).

The immune responses to GAT and GAT-MBSA are dependent on the helper influence of T cells (6). Thus, spleen cells from GAT-primed, irradiated responder mice have GAT-specific helper T-cell function, while no GAT-specific helper cell function could be demonstrated in spleen cells from GAT-primed, irradiated nonresponder mice. B cells from nonresponder mice also differ from the B cells of responder animals in their susceptibility to suppression in culture by concentrations of soluble GAT, which are immunogenic for B cells of responder mice (7). In addition, GAT not only fails to elicit a GAT-specific response in nonresponder mice, but also specifically decreases the ability of nonresponder mice to develop a GAT-specific PFC response to a subsequent challenge with the normally immunogenic GAT-MBSA (7). This unresponsiveness is the result of an active suppression; spleen cells from GAT-primed nonresponder mice of the H-2p,q,s haplotypes could specifically suppress the anti-GAT PFC response of syngeneic spleen

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\textsuperscript{1}Abbreviations used in this paper: B cell, precursor of antibody-producing cell; C, complement; GAT, random terpolymer of L-glutamic acid\textsuperscript{60}-L-alanine\textsuperscript{30}-L-tyrosine\textsuperscript{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GAT-M\Phi, macrophage-bound GAT; GAT-SRBC, GAT coupled to sheep red blood cells; H-2, major histocompatibility complex in mice; Ig, Immunoglobulin; IgG refers to IgG\textsubscript{1}, and IgG\textsubscript{1a}, \textsubscript{2a}; Ir gene, specific immune response gene; M\Phi, macrophage; PFC, plaque-forming cells; (T,G)-A--L, Poly(L-tyrosine, L-glutamic acid-poly-L-alanine-poly-L-lysine); \textsubscript{θ}, Surface alloantigen on T cells.
cells from normal nonresponder mice incubated with GAT-MBSA (8; Kapp, unpublished data). The suppressor cells are identified as T cells by their sensitivity to treatment with anti-θ serum and C and by the demonstration that suppressor cells are not retained by an antimouse Ig column, but are eluted in the fraction containing θ-positive cells and a few nonimmunoglobulin-bearing cells, but no immunoglobulin-bearing cells. In contrast, the B cells eluted from the column by normal mouse serum do not have suppressor activity.

In their studies of the Ir gene-controlled responses to (T,G)-A-L, Hämmerling and McDevitt have observed (T,G)-A-L binding T cells in low responder, as well as high responder mice (9). In similar experiments, GAT-binding T cells have been detected in nonresponder mice by Kennedy et al. The observations that nonresponder mice have T cells capable of binding these antigens and that, in the case of the response to GAT, nonresponder mice develop GAT-specific suppressor T cells suggest that the Ir genes may behave as regulatory genes rather than as genes coding for the antigen receptors on T cells. It is critical to our understanding of the genetic control of immune responses to determine whether nonresponder mice are restricted to the development of only suppressor T cells after interaction with GAT, or whether conditions can be found which allow the development of GAT-specific helper T cells in nonresponder mice.

We have found that GAT-specific helper T-cell function can develop in nonresponder mice and in this communication we will describe the conditions for eliciting and demonstrating GAT-specific helper T cells in nonresponder mice.

Materials and Methods

Mice. C57BL/6 (H-2b) and DBA/1 (H-2b) mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. The F, hybrid mice of (C57BL/6 × DBA/1) matings (H-2,14) were produced in our animal facilities. Mice used in these studies were 2- to 8-mo old and were maintained on acidified-chlorinated drinking water and laboratory chow ad libitum.

Antigens. The synthetic linear random terpolymer GAT, mol wt 32,000 daltons, used in these studies was synthesized by Miles Laboratories, Kankakee, Ill. Preparation of GAT, GAT-MBSA, and GAT bound to peritoneal macrophages (GAT-MΦ) for addition to cultures was described in detail previously (4,6). 5 μg of soluble GAT, GAT-MBSA containing 5 μg GAT, or 7 × 10⁶ macrophages bearing 1-2 ng of GAT were added to cultures according to the experimental protocol.

Immunization of Mice. Mice were injected intraperitoneally with 0.01 to 10.0 μg GAT, GAT-MBSA containing 10 μg GAT, or 50 μg MBSA in a mixture of magnesium-aluminum hydroxide gel (Maalox, W.H. Rorer, Inc., Ft. Washington, Pa.) and pertussis vaccine (Eli Lilly, Indianapolis, Ind.) as previously described (1). Other mice were injected intraperitoneally with 2 × 10⁶ syngeneic macrophages bearing 15-20 ng GAT.

Preparation of B Cells. θ-bearing T cells were eliminated from normal spleen cell suspensions by treatment with AKR anti-θ C3H serum and C before culture initiation (6). After treatment, 8 × 10⁶ cells in MEM were added to cultures with or without additional cells and with GAT, GAT-MBSA, or GAT-MΦ according to the experimental protocol.

Preparation of T Cells. Since antigen-primed, helper T-cell function is radioresistant, mice primed 1-4 mo earlier with GAT, GAT-MBSA, or GAT-MΦ were X-irradiated with 700-800 R delivered by a General Electric Maximag 250 Type III X-Ray therapy unit (250 kvp, 15mA) (General Electric, Medical Systems Div., Milwaukee, Wis.) as described previously (5,6,8). Within 3 h after X-irradiation, the mice were sacrificed and single cell suspensions prepared from their spleens. 8 × 10⁶ cells were added to cultures with or without additional cells and with antigen.

Spleen Cell Cultures and PFC Assay. Techniques of spleen cell culture and the hemolytic plaque assay using GAT-SRBC as indicator cells have been described previously (4,6). The data in these
experiments are expressed as the geometric means of the number of GAT-specific IgG PFC per culture from 3 to 13 separate experiments. To compare data from all experiments, the data from each experiment were first normalized. Since each experiment contained cultures of normal spleen cells stimulated by GAT-MBSA and since the purpose of each experiment was to assess the effect of various populations of antigen-primed irradiated cells (T cells) on one population of normal spleen cells treated with anti-μ serum (B cells), we determined the geometric mean of the responses by 13 separate cultures of DBA/1 or F1 spleen cells to GAT-MBSA (733 and 594 GAT-specific IgG PFC per culture, respectively). The data in each experiment were then normalized as follows:

\[
\frac{\text{No. of PFC in spleen cell culture containing GAT-MBSA}}{733 \text{ or } 574} \times \frac{\text{No. of PFC in experimental group}}{\text{normalized PFC/culture}}.
\]

**Results**

**Failure of Nonresponder (H-2^q) Mice to Develop GAT-Specific Helper T Cells after Immunization with GAT.** We have previously demonstrated that nonresponder B10.S (H-2^q) mice not only fail to develop a primary PFC response to GAT, but also fail to develop radioresistant GAT-specific helper T-cell function after injection with GAT (6). In contrast, (responder × nonresponder)F1 (H-2^\text{b/b}) mice injected with GAT develop radioresistant GAT-specific helper T cells which efficiently cooperate with F1 B cells and responder (H-2^b) parental B cells in responses to GAT, GAT-MBSA, and GAT-MΦ. However, efficient cooperative interaction with nonresponder (H-2^q) parental B cells only occurs in cultures stimulated with GAT-MBSA or GAT-MΦ, but not those stimulated with soluble GAT (5).

In the experiments reported here, nonresponder DBA/1 (H-2^q) and the responder (C57BL/6 × DBA/1)F1 (H-2^\text{b/b}) mice were compared for the presence of radioresistant, GAT-specific helper T-cell function 1-4 mo after injection of 10 μg GAT in Maalox-pertussis. The results (Fig. 1) verify that mice with another nonresponder H-2 haplotype (H-2^q) fail to develop GAT-specific helper T cells after injection of GAT. Groups A and B illustrate the GAT-specific IgG PFC response by normal spleen cells from DBA/1 and (C57BL/6 × DBA/1)F1 incubated with GAT or GAT-MBSA. Control groups C, D, E, F, and J demonstrate that normal B cells and antigen-primed, irradiated T cells fail to develop GAT-specific PFC responses when cultured separately. Spleen cells from DBA/1 mice primed with GAT 1-4 mo before X-irradiation and culture initiation do not provide helper T-cell function for a response by DBA/1 B cells (group G) or for F1 B cells (group H) incubated with GAT or GAT-MBSA. The functional capacity of F1 B cells is verified by the observation that GAT-primed irradiated F1 T cells provided helper cell function for PFC responses to GAT and GAT-MBSA (group I). The failure of GAT-primed DBA/1 T cells to support a response was not due to an unresponsive DBA/1 B-cell population; T cells from GAT-MBSA-primed, irradiated DBA/1 mice when added to cultures of B cells, the same as those in groups C and G, supported development of a GAT-specific PFC response to GAT-MBSA. Significantly, data in Fig. 1 also demonstrate that GAT-MBSA-primed DBA/1 T cells can cooperate with F1 B cells in developing a good response to GAT-MBSA and a much smaller but significant response to GAT (group L). This response was observed in five out of eight experiments. Although not shown, cultures of normal, irradiated DBA/1 spleen cells and F1 B
Fig. 1. Failure of nonresponder DBA/1 mice (H-2<sup>+</sup>) to develop GAT-specific helper T cells after immunization with GAT. GAT-specific IgG PFC response is expressed as the geometric means of pooled normalized data. B cells were prepared by treating spleen cells from normal DBA/1 or (C57BL/6 x DBA/1)<sub>F</sub><sub>1</sub> mice with anti-<sub>C</sub>H serum plus C. T cells were obtained from DBA/1 or <sub>F</sub><sub>1</sub> mice immunized 30 days or more before irradiation and culture initiation.

cells did not respond to GAT or GAT-MBSA. This experiment demonstrates that GAT-specific helper T cells cannot be stimulated in nonresponder mice by GAT using an immunization protocol which stimulates GAT-specific helper T cells in responder animals. However, immunization with GAT-MBSA results in development of weak but detectable GAT-specific T-cell helper activity, demonstrable, however, only on <sub>F</sub><sub>1</sub> B cells and not on nonresponder B cells incubated with GAT. It is important to note that the failure to detect GAT-specific helper T cells in DBA/1 mice injected with GAT is not due to masking of helper activity by the simultaneous presence of suppressor T cells because: (a) spleen cells were taken from GAT-primed mice a month or more after priming at a time when suppressor T cells can no longer be detected and the animals are again able to respond to GAT-MBSA (Kapp, unpublished data); and (b) the spleen cells were taken from X-irradiated mice and we have been unable to detect radioresistant suppressor activity in this system.

**Development of GAT-Specific Helper T Cells in DBA/1 Mice After Immunization with GAT-MBSA or GAT-MΦ.** To further examine the antigen-specificity
of helper T cells in GAT-MBSA primed DBA/1, and because of the known suppressive activity of soluble GAT for nonresponder B cells, these T cells were added to cultures of DBA/1 or F₁ B cells and incubated with macrophage-bound GAT (Fig. 2). Spleen cells from DBA/1 mice, like other nonresponder mice, did not develop GAT-specific PFC responses when incubated with GAT bound to syngeneic macrophages (5). Furthermore, cultures containing GAT-MBSA-primed DBA/1 T cells, DBA/1 B cells, and GAT bound to DBA/1 macrophages (GAT-MΦ) did not develop GAT-specific PFC responses (group A), whereas these cells developed normal responses to GAT-MBSA. However, when GAT-MBSA primed DBA/1 T cells were incubated with F₁ B cells and GAT-MΦ, a

| GROUP | B CELLS PRIMED WITH | DBA/1 T CELLS PRIMED WITH |
|-------|---------------------|---------------------------|
| A     | DBA/1               | GAT-MBSA                  |
| B     | F₁                  | GAT-MBSA                  |
| C     | F₁                  | MBSA                      |
| D     | DBA/1               | GAT-MΦ                    |
| E     | F₁                  | GAT-MΦ                    |

Fig. 2. Development of GAT-specific helper T cells in DBA/1 mice immunized with GAT-MBSA, MBSA, or GAT-MΦ. See Fig. 1 for (a) preparation of T and B cells and (b) control PFC responses for cultures of T and B cells incubated separately with antigen. Data for responses to GAT and GAT-MBSA in groups A and B are the same as in Fig. 1, groups K and L.

This demonstrated again that nonresponder mice immunized with GAT-MBSA develop GAT-specific helper T cells. GAT-specific helper T cells can be distinguished from MBSA-specific helper T cells because T cells from DBA/1 mice immunized with the carrier, MBSA, alone cooperate with F₁ B cells incubated with GAT-MBSA, but not with GAT or GAT-MΦ (group C).

Since GAT itself does not stimulate the development of helper T cells in DBA/1, two mechanisms could account for the development of GAT-specific helper T cells in DBA/1 mice immunized with GAT-MBSA. The first is that GAT must be bound to a carrier which is, itself, immunogenic to stimulate the development of GAT-specific helper T cells. Alternatively, GAT can stimulate helper T cells in nonresponder mice if it is presented in a physical form which does not elicit GAT-specific suppressor T cells.

To differentiate between these two alternatives, DBA/1 mice were injected
with 2 to 4 × 10⁶ peptone-induced DBA/1 peritoneal MΦ bearing 15-20 ng of GAT. After 5 wk, these mice were irradiated and examined for helper T-cell activity specific for GAT. Cultures containing GAT-MΦ-primed DBA/1 T cells and DBA/1 B cells developed a GAT-specific response only when incubated with GAT-MBSA, and not when incubated with soluble GAT or GAT-MΦ (group D). The failure of these cultures to develop a response to soluble GAT can be attributed to the sensitivity of nonresponder B cells to suppression by GAT in vitro (7). Since macrophage-bound GAT did not cause suppression of the GAT-specific response to GAT-MBSA in vitro (5), another explanation must be sought for the failure of GAT-specific DBA/1 helper T cells and DBA/1 B cells to develop a response to GAT-MΦ compared to their ability to respond to GAT-MBSA. One possibility is that the concentration of GAT on macrophages is insufficient to stimulate nonresponder B cells. Alternatively, GAT-MBSA overcomes a still to be defined defect in nonresponder B cells.

X-irradiated spleen cells from DBA/1 mice primed with GAT-MΦ did cooperate with F₁ B cells incubated with GAT-MBSA and GAT-MΦ, but not soluble GAT (group E). Presently, we do not understand the variation in the ability of GAT-specific DBA/1 T cells to support the F₁ B cell response to soluble GAT (compare Fig. 1, group L and Fig. 2, group B to Fig. 2, group E). It could, however, be related to the differences in the immunization protocols used to stimulate the GAT-specific helper T cells, i.e., immunization with GAT-MBSA vs. GAT-MΦ. Although the GAT-specific helper T-cell activity of primed, irradiated DBA/1 spleen cells is more readily demonstrated in cultures containing responder B cells than in those containing nonresponder B cells, both sets of data support the conclusion that GAT need not be bound to a carrier which is itself immunogenic to stimulate GAT-specific helper T cells in nonresponder mice.

**Comparison of Suppressor and Helper T-Cell Activity in Mice Immunized with Low Doses of GAT.** We examined spleen cells from DBA/1 mice 7 days after immunization with GAT-MΦ (15-20 ng GAT) for the development of GAT-specific PFC responses and found no detectable response. This finding correlated with the failure of spleen cells from DBA/1 mice to respond to GAT-MΦ in vitro, but was inconsistent with the observation that DBA/1 mice immunized with GAT-MΦ develop radioresistant helper T cells. We therefore examined spleen cells from mice primed 4 days earlier with GAT-MΦ for the presence of GAT-specific suppressor T cells. When co-cultured with spleen cells from normal DBA/1 mice, these cells did not suppress the response of GAT-MBSA (Fig. 3, group A). In fact, the response of cultures containing normal and GAT-MΦ-primed spleen cells was equal to the sum of the responses to the spleen cell populations incubated separately. In contrast, mice immunized with 1.0 to 10 μg GAT had developed suppressor T cells by 4 days after immunization (groups B and C). Spleen cells from mice immunized with less than 1.0 μg GAT did not suppress the response to GAT-MBSA of normal DBA/1 spleen cells (group D).

The amount of GAT (15-20 ng) which, when bound to macrophages, stimulated the development of GAT-specific helper T cells (group A), was less than the minimum (1.0 μg) amount of GAT required to induce significant suppressor T cells (group C). In the event that the development of helper T cells
in nonresponder mice requires only very low doses of GAT, we examined DBA/1 mice immunized with low doses of GAT for (a) development of GAT-specific PFC responses in vivo and (b) presence of GAT-specific helper T cells after immunization. Mice immunized with 10, 1.0, 0.1, or 0.01 µg GAT did not develop a response to GAT in vivo (data not shown) nor did GAT-specific helper cells arise which would cooperate with normal DBA/1 B cells incubated with GAT-MBSA (groups B, C, D, E).

We can conclude (a) that the failure of nonresponder mice to develop a GAT-specific PFC response to GAT-MΦ is not due to a lack of helper T cells, nor is it due to simultaneous stimulation of suppressor T cells; and (b) mice immunized with soluble GAT in doses too low to elicit suppressor T cells do not develop helper T cells.

**Discussion**

The data presented in these studies demonstrate that GAT-specific helper T cells can be elicited in nonresponder mice by administering GAT in the appropriate form, i.e., GAT-MBSA or GAT-MΦ. However, this helper T-cell activity generated in nonresponder mice immunized with GAT-MBSA or GAT-MΦ may be less effective than that generated in responder mice, since it is incapable of providing helper T-cell function for nonresponder B cell, incubated with GAT-MΦ. However, these nonresponder B cells can be stimulated by GAT-MΦ in the presence of GAT-primed (responder × nonresponder)F₁ T cells (5). It is important to emphasize that GAT-specific helper T cells cannot be identified in nonresponder mice by studying the GAT-specific antibody of intact
mice. In this circumstance, antibody is produced only when the mice are immunized with GAT bound to a carrier such as MBSA or RBC, and the influence of carrier-specific helper T cells obscures presence of GAT-specific helper T cells.

Before discussing the relevance of these experiments to our understanding of Ir gene-controlled antibody responses, we would like to summarize these findings and those of earlier studies. (a) Injection of nonresponder mice with doses of GAT (1–100 μg) in Maalox and pertussis which are immunogenic for responder mice (i) fails to elicit a GAT-specific antibody response; (ii) elicits the development of radiosensitive, GAT-specific suppressor T cells; and (iii) fails to elicit radioresistant GAT-specific helper T cells. (b) Injection of nonresponder mice with syngeneic macrophages bearing 15–20 ng of GAT (i) fails to elicit a GAT-specific antibody response; (ii) fails to elicit GAT-specific suppressor T cells; but (iii) stimulates the development of radioresistant GAT-specific helper T cells. (c) Injection of nonresponder mice with GAT-MBSA in Maalox-pertussis (i) stimulates the formation of GAT-specific antibody responses; and (ii) stimulates the development of radioresistant helper T cells, but not detectable suppressor T cells.

An interpretation of these observations is that the Ir gene for GAT is expressed in both B cells and T cells and that the process(es) controlled by this gene is very sensitive to both the physical form and concentration of the antigen. Thus, with regard to nonresponder T cells, soluble GAT preferentially stimulates suppressor T-cell activity, whereas GAT bound to MBSA or to macrophages fails to stimulate demonstrable suppressor T-cell activity, but does stimulate the development of GAT-specific helper T cells. With regard to nonresponder B cells, in contrast to responder B cells, soluble GAT has never elicited antibody production in cultures of these B cells containing either DBA/1 or F1 GAT-specific helper T cells.

In contrast to the behavior of nonresponder mice, responder mice develop not only GAT-specific antibody responses, but also develop radioresistant GAT-specific helper T cells after stimulation with GAT, GAT-MBSA, and GAT-MΦ. It remains to be determined whether responder mice can develop GAT-specific suppressor T cells.

From these observations, we must conclude that the difference between responder and nonresponder mice is not in the absolute capacity to generate GAT-specific helper T cells, nor in the capacity of B cells to produce GAT-specific antibody. Based on the fact that both responder and nonresponder T cells bind GAT and that both have the ability to develop into helper T cells, we propose that some event occurs after antigen recognition which governs the development of helper or suppressor T cells. Therefore, it is possible that, in the case of the response to GAT, genetic control is not determined by the specificity of antigen receptors on T cells, but is mediated by regulatory genes which function subsequent to antigen recognition. The possibility must be considered, however, that the relatively complex antigens used to detect responses under H-linked Ir gene control such as the terpolymer GAT may possess several determinants capable of stimulating T cells and that different determinants stimulate the development of suppressor vs. helper functions. This possibility is given
some support by the finding that the copolymer of L-glutamic acid and L-alanine (GA) is immunogenic in H-2a mice (10), whereas GAT with 10% tyrosine stimulates the development of suppressor T cells in these mice. The suppressor T cells in H-2a mice could therefore be specific for poly L-tyrosine sequence. This possibility is currently being investigated.

Together with experiments of Mozes and collaborators (11-13) with the branched copolymers, our studies on the process(es) controlled by Irgenes in the GAT system indicate that the genetic defect can be expressed in T cells and B cells or only in B cells. In their studies of the response to (T,G)-A--L, Mozes et al., (11,12) found that thymocytes from low responder C3H/HeJ (H-2a) mice and those from high responder C3H.SW (H-2b) mice educated to (T,G)-A--L produce an antigen-specific factor capable of cooperating with responder (H-2a) but not nonresponder (H-2b) bone marrow cells in vivo. This suggests that the low responder C3H/HeJ mice fail to produce high levels of antibody to (T,G)-A--L because of a defect in the capacity of B cells to receive a T-cell signal rather than a failure of their T cells to respond to (T,G)-A--L. In contrast, thymocytes from the low responder SJL (H-2a) mice did not elaborate a (T,G)-A--L specific factor nor did their B cells produce (T,G)-A--L-specific antibody when mixed with a specific responder thymocyte factor, indicating a defect in both cell types (13).

The H-linked Irgenes have only been demonstrated for antibody responses to T-cell-dependent antigens and their action is concerned either with the generation of helper or suppressor T-cell function, or with the effectiveness of helper T-cell function for B cells. It is therefore reasonable to consider that the process(es) controlled by these genes is concerned with T cell-T cell and/or T cell-B-cell interactions in the regulation of immune responses. Further, it is not unexpected that the genes concerned with the control of these crucial interactions have been mapped in the I region of the H-2 complex in precisely the same region where H-linked Irgenes are located (14), and that the murine immune responses to T-cell-dependent antigens can be inhibited in vitro by the addition of alloantisera directed against the K and I regions of the H-2 gene complex (15, footnote 3). The recent discovery that factors from activated T cells with helper or suppressor activity are both antigen-specific (16,17) and may possess determinants coded for in the H-2 complex (16) is consistent with this interpretation.

Summary

Mice which are genetic nonresponders to the random terpolymer of L-glutamic acid^6-L-alanine^8-L-tyrosine^10 (GAT) not only fail to develop GAT-specific antibody responses when stimulated with soluble GAT either in vivo or in vitro, but develop GAT-specific T cells which suppress the GAT-specific plaque-forming cell response of normal nonresponder mice stimulated with GAT complexed to methylated bovine serum albumin (MBSA). Thus, both responder and nonresponder mice have T cells which recognize GAT. However, nonresponder mice can develop GAT-specific helper T cells if immunized with GAT bound to

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MBSA or to macrophages. The relevance of Ir gene-controlled responses is discussed.

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