Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3373–3377, April 1993
Immunology

The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice

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Communicated by David W. Talmage, January 7, 1993

ABSTRACT **_In vitro_ studies have revealed several pathways by which T cells can respond to alloantigens, including CD4+ direct responses to allogeneic class II antigens, CD8+ direct responses to allogeneic class I antigens, and CD4+ "indirect" responses to peptides of alloantigens presented in association with responder class II molecules. _In vivo_ studies of skin graft rejection, however, have so far provided clear evidence for the contribution of only the two direct pathways and not for indirect recognition. We have used major histocompatibility complex class II-deficient mice as donors to test the role of indirect recognition in rejection of skin grafts. Class II-deficient skin was always rejected without delay by normal recipients. Removal of recipient CD8+ cells (to leave the animals dependent on CD4+ function) or depletion of recipient CD4+ cells revealed that CD4+ cells were usually involved and sometimes absolutely required in this rapid rejection. Since the donor grafts lacked class II antigens, the CD4+ cells must have recognized donor antigens presented in association with recipient class II molecules. These results therefore indicate that indirect recognition can initiate rapid skin graft rejection.**

Theories to explain the special importance of major histocompatibility complex (MHC) antigens in graft rejection and the extraordinary strength of the immune response to these antigens are all based on the capacity of T cells to recognize allogeneic MHC antigens directly without the usual requirement that their peptides be processed and presented by recipient antigen presenting cells (APCs) (1–3). As a result, studies of the mechanisms of graft rejection have been dominated by concern with the direct pathways of alloreactivity. A few investigators, however, have considered the possibility that graft rejection might also occur through more classical immunologic mechanisms in which peptides of donor antigens are presented in association with recipient MHC molecules. La Rosa and Talmage (4) described this possibility as "indirect" recognition, but the actual role of this pathway in graft rejection has been difficult to determine.

Recently, "knockout" mice lacking MHC class II antigens have been produced by the technique of targeted gene disruption by homologous recombination in embryonic stem cells (5, 6). Since these mice lack the MHC class II antigens responsible for stimulating CD4+ T cells, grafts from these mice can be used to examine rejection in the absence of direct stimulation of these helper T cells. The experiments described in this report suggest that in the absence of donor class II antigens, indirect recognition remains an effective pathway of alloreactivity leading to rapid graft rejection.

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MATERIALS AND METHODS

Class II-Deficient Mice. The development and initial characterization of the class II-deficient mice used in these experiments have been described (5). Briefly, the A_β^b gene was disrupted in the D3 embryonic stem cell line of 129/Sv origin by the technique of homologous recombination. Cloned embryonic stem cells expressing the mutant A_β^b gene were injected into B6 blastocysts and implanted in foster mothers. A chimeric male founder animal was bred with a normal C57BL/6 female to select for germ-line transmission of the defective gene. Their offspring were intercrossed and those of their offspring that were homozygous for the disrupted A_β gene were selected as founders for further breeding. Mice of the next (and subsequent) generations lack class II antigens and express a random assortment of B6 and 129 genes, some homozygous and others still heterozygous. These mice are referred to as F_3 class II-deficient mice (F_3 II^-).§

Previous characterization of the class II-deficient mice l as shown that no class II antigen expression can be detected in these animals (5). These animals also show a substantial depletion of CD4+ peripheral T cells, although 3–5% of peripheral Thy-1+ cells continue to express the CD4 antigen. The class II-deficient mice have no T-cell-dependent antibody response, but they have normal levels of B cells (44) and γδ cells (L.H.G., unpublished data) and a slightly increased level of CD8+ peripheral T cells.

Normal Animals. C57BL/6J (*H-2*^{b}) (B6), C3H.SW (*H-2*^{b}), and BALB/cByJ (*H-2*^{d}) mice were obtained from The Jackson Laboratory and BALB.B (*H-2*^{b}) mice were bred from animals obtained from Michael Potter (National Institutes of Health). Male mice were used as recipients of skin grafts.

Skin Graft and Other *in Vivo* Techniques. Skin grafts were placed on mice according to the technique of Billingham and Medawar (7). Both trunk and tail skin grafts were used as indicated. Mice were anesthetized with chloral hydrate supplemented with ether. Grafts were placed on the lateral thoracic area and held in place with vaseline gauze and plaster bandages. The bandages were removed on the 7th or 8th day. Rejection was recorded when there was >90% destruction of the tissue. Thymectomies were performed on mice by the suction pipette technique with ether anesthesia. Statistical analysis comparing survival curves was performed with the logarithmic rank test.

Monoclonal Antibodies and *in Vivo* T-Cell Depletion. CD4+ T cells were depleted by using the GK1.5 (rat anti-mouse CD4) antibody (8), and CD8+ T cells were depleted by using the 2.43 (rat anti-mouse CD8) antibody (9) as described (10, 11). All treated mice were thymectomized before receiving 0.1 ml i.p. of unpurified ascites of monoclonal antibody

Abbreviations: MHC, major histocompatibility complex; APC, antigen presenting cell; F_3 II^-, F_3 class II-deficient mice.

§Homozygous B6 class II-deficient mice are now commercially available from GenPharm International (Mountainview, CA).

[roughly equivalent to 100 μg of purified antibody (10)] on days −6, −3, and −1 before skin transplantation.

RESULTS

Class II-Deficient Skin Transplanted to Normal Recipients. Over 50 skin grafts from mice lacking MHC class II antigens have been placed on normal recipients with antigenic disparities ranging from a few minor to widely divergent xenogeneic (rabbit) antigens. In every case, the class II-deficient skin was rejected without delay compared to control skin grafts. One example of this rapid rejection is shown in Fig. 1. These results indicate that class II antigen expression by the donor is not required for prompt skin graft rejection by normal mice.

Class II-Deficient Skin Transplanted to Recipients Depleted of CD4$^+$ Cells. The rapid rejection of class II-deficient skin by normal recipients might have occurred because CD8$^+$ cells can respond to alloantigens without CD4$^+$ helper cells [as several previous studies have suggested is possible (11–15)] or because CD4$^+$ helper cells can recognize donor antigens presented in association with recipient class II antigens, providing help for CD8$^+$ effectors. We therefore tested the role of CD4$^+$ T cells in the rejection of class II-deficient skin grafts by depleting these cells from recipients before transplantation. The results of these experiments showed that depending on the antigenic disparity and the type of graft used, survival was prolonged in some cases: if a class I disparity were present or if trunk skin were used, graft rejection occurred with little delay, but if only a few minor antigen disparities were present and tail skin was used, depletion of CD4$^+$ cells prolonged graft survival substantially (Fig. 2).

The rejection of class II-deficient skin in some of these cases is consistent with the notion that CD8$^+$ cells alone can reject murine skin grafts, functioning best when there is a class I antigen disparity (Fig. 2 *A* vs. *B*) and better when there are abundant donor APCs such as in trunk compared to tail skin (Fig. 2 *C* vs. *B*). On the other hand, the dramatically prolonged survival in the case of tail skin grafts with only a few minor antigen disparities (Fig. 2*D*) indicates that the rejection of these grafts by normal recipients depended completely on the function of the CD4$^+$ cells. Since class II antigens were lacking on the donor graft, the CD4$^+$ cells most likely responded to alloantigens presented in association with recipient class II antigens to initiate the rapid graft rejection.

Class II-Deficient Skin Transplanted to Recipients Depleted of CD8$^+$ Cells. Another way to examine the role of CD4$^+$ cells in the rejection of class II-deficient skin grafts was to deplete recipient CD8$^+$ cells, leaving the animals dependent on the function of the CD4$^+$ population. Fig. 3 shows the results of representative experiments in which class II-deficient skin grafts were placed on CD8$^+$-depleted mice. Graft survival

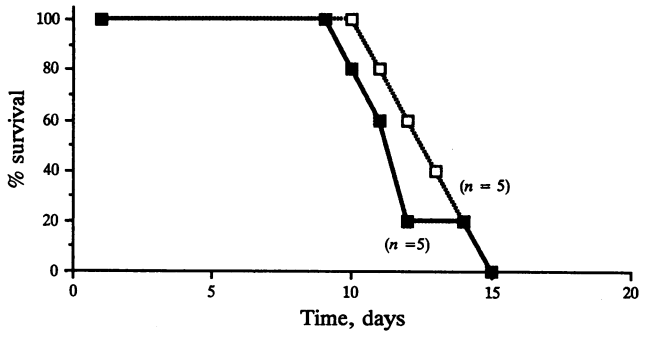

FIG. 1. Rejection of class II-deficient skin grafts by normal recipients. Trunk skin grafts from either F_3 II$^-$ (□) or from F_3 mice that express class II antigens (■) were transplanted to normal B6 recipients ($P > 0.5$).

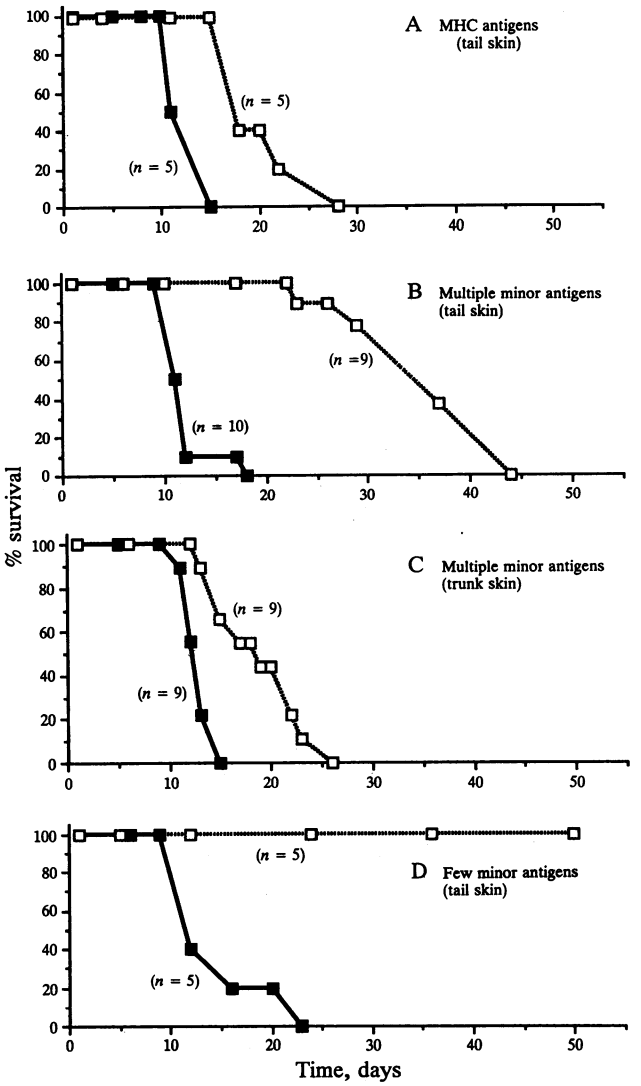

FIG. 2. Survival of class II-deficient skin grafts on recipients depleted of CD4$^+$ cells. Skin grafts from F_3 II$^-$ were transplanted to untreated recipients (■) or recipients that had been treated with anti-CD4 antibody after thymectomy (□). (*A*) F_3 II$^-$ tail skin to BALB/c recipients ($P < 0.01$). (*B*) F_3 II$^-$ tail skin to C3H.SW recipients ($P < 0.001$). (*C*) F_3 II$^-$ trunk skin to C3H.SW recipients ($P < 0.001$). (*D*) F_3 II$^-$ tail skin to B6 recipients ($P < 0.002$).

was hardly prolonged if there was a class I antigen disparity (Fig. 3*A*), prolonged slightly if there were many minor antigen disparities on trunk skin (Fig. 3*B*), and prolonged longer when there were few minor antigen disparities expressed on tail skin (Fig. 3*C*). The eventual rejection of all grafts after CD8$^+$ depletion suggests that CD4$^+$ cells played a role in each case despite the absence of donor class II antigens. That role was especially evident in the case involving a class I antigen disparity.

Class II-Deficient Skin Transplanted to Recipients Depleted of Both CD4$^+$ and CD8$^+$ Cells. To confirm the role of CD4$^+$ cells in the rejection of class II-deficient skin grafts by recipients depleted of CD8$^+$ cells, we performed experiments in which class II-deficient skin was placed on recipients depleted of both T-cell subsets (Fig. 4). Treatment of BALB/c mice with both antibodies substantially prolonged survival of the class II-deficient skin in this and several other experiments. Thus, CD4$^+$ T cells must indeed play an essential role in the rejection even of class I-disparate, class II-deficient skin grafts on recipients depleted of CD8$^+$ cells alone.

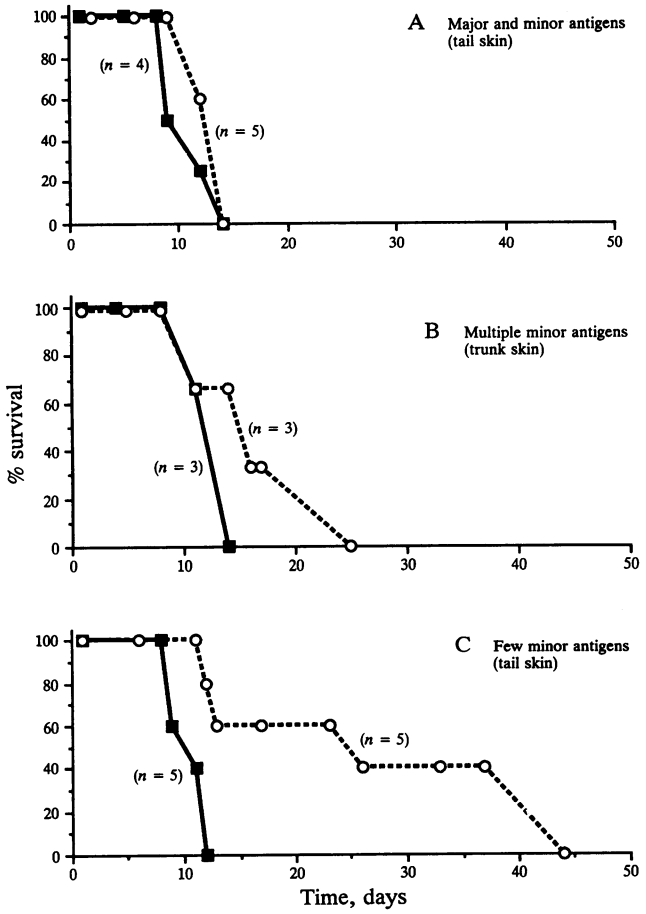

FIG. 3. Survival of class II-deficient skin grafts on recipients depleted of CD8$^+$ cells. Skin grafts from F_3 II$^-$ were transplanted to untreated recipients (■) or to recipients that had been treated with anti-CD8 antibody after thymectomy (○). (*A*) F_3 II$^-$ tail skin to BALB/c recipients ($P > 0.15$). (*B*) F_3 II$^-$ trunk skin to BALB.B recipients ($P > 0.2$). (*C*) F_3 II$^-$ tail skin to B6 recipients ($P < 0.01$).

DISCUSSION

The primary purpose of these experiments was to use the MHC class II-deficient mice to examine the contribution of indirect recognition to the process of graft rejection. Since these mice lack class II antigens, any rejection of their skin that can be shown to depend on CD4$^+$ cells would seem likely to involve recognition of donor antigens presented in association with recipient class II molecules. Rejection of the class II-deficient skin always occurred rapidly on normal recipients, and T-cell depletion experiments indicated that in some cases this occurred independent of CD4$^+$ function. In other cases, however, depletion demonstrated that CD4$^+$ cells were involved in the rejection process, and even when rejection depended absolutely on CD4$^+$ function it still occurred promptly. Thus, these results suggest that indirect recognition can provide a highly effective pathway to initiate graft rejection.

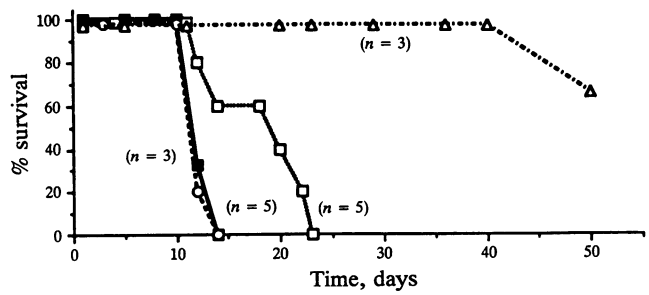

FIG. 4. Survival of class II-deficient skin grafts on recipients depleted of both T-cell subsets. Skin grafts from F_3 II$^-$ were transplanted to thymectomized BALB/c mice that had been treated with nothing (■), with anti-CD4 antibody (□) ($P > 0.05$), with anti-CD8 antibody (○) ($P > 0.5$), or with both antibodies (△) ($P < 0.05$).

Two arguments might be made against this conclusion. First, perhaps the class II-deficient mice actually express some class II antigens. Previously published data, however, have revealed no detectable class II antigen expression in these mice, even when tested with antibodies that should detect A_α/E_β heterodimers (5, 6). Furthermore, the class II-deficient mice are profoundly deficient in CD4$^+$ T-cell function and thus have demonstrated the functional loss of their class II antigens. Second, perhaps the CD4$^+$ cells responsible for initiating rejection in these experiments responded to donor class I antigens directly, without a requirement for antigen processing and presentation by recipient class II antigens. However, CD4$^+$ helper responses to class I antigens have not been detected *in vitro* except in response to class I peptides presented by class II antigens (16), and CD4$^+$ cytotoxic cells responding to class I alloantigens have been detected at only 1/10th the frequency of CD8$^+$ cytotoxic cells (17, 18). It is therefore unlikely that either residual donor class II expression or recipient CD4$^+$ stimulation directly by donor class I antigens is responsible for the rapid graft rejection in our experiments. We conclude instead that indirect recognition of donor antigens is probably involved.

The capacity of donor antigens to be presented by recipient APCs and to generate an immune response has been known for a long time. First demonstrated in the form of "cross priming" (19, 20), recipient presentation has been repeatedly demonstrated in more recent experiments (21–24). In addition, experiments have shown that peptides of donor MHC antigens can be recognized in association with recipient MHC antigens (25–28). But while a recipient response to processed donor antigens clearly occurs, it has not been clear that this response can initiate skin graft rejection. Indeed, Rosenberg and Singer (29) concluded from their experiments that indirect recognition did not contribute to skin graft destruction in the case of a particular class I-only disparity. They showed that while B6 CD4$^+$ cells could generate a helper response *in vitro* to peptides of the bm6 antigen presented in association with I-A^b molecules, this helper response was unable to initiate rejection of bm6 grafts *in vivo*. The effectiveness of indirect recognition shown in our experiments, but not in the case of the mutant class I antigen disparity, might reflect particular limitations on the processing of donor class I antigens by recipient APCs or simply quantitative effects from the much larger number of donor peptides available in our experiments.

Prior to our experiments, several other investigators have reported results suggesting that indirect recognition can contribute to graft rejection. Most of these experiments, however, used endocrine tissues or xenogeneic grafts that may be susceptible to different mechanisms of graft destruction. La Rosa and Talmage suggested the terminology and considered the contribution of indirect recognition from their and others' experiments with endocrine grafts depleted of APCs (4, 30–32). More recently, Gill (33) showed that rejection of xenogeneic islets was dependent on the function of CD4$^+$ cells, even though no direct recognition of the xenogeneic cells by CD4$^+$ T cells could be demonstrated *in vitro*. Experiments involving xenogeneic skin graft rejection reached the same conclusion (34). In the case of allogeneic organ rejection, two studies from Fabre's laboratory have suggested, first, that rejection of allogeneic rat kidneys could be blocked by an antibody specific for recipient MHC antigens (35) and, second, that immunization with donor peptides

seemed to speed the rejection of subsequent donor grafts (22). The experiments reported here with the class II-deficient mice provide strong evidence that indirect recognition of alloantigens can initiate allograft rejection even of skin grafts and that it can do so very effectively.

If indirect recognition can play such an important role in graft rejection, why is depletion of donor APCs so effective in prolonging the survival of allogeneic grafts (36–38)? Donor APC depletion would clearly eliminate the direct pathways of alloreactivity but might not be expected to abrogate indirect recognition, which uses recipient APCs. Even indirect recognition, however, may depend on the presence of donor APCs, acting not as stimulating cells but as the vehicles to carry donor antigens to recipient lymph nodes where indirect presentation and T-cell stimulation may actually occur. If so, then donor APC depletion would prevent indirect recognition at least until the graft was slowly repopulated by recipient APCs. Furthermore, such grafts would depend entirely on indirect mechanisms of alloreactivity, even for the effector mechanism causing their destruction. Whether indirect effector mechanisms exist for all types of tissue rejection remains unclear.

Although our experiments did not involve APC depletion, they still raise interesting questions regarding the subsequent rejection mechanisms after initiation by the indirect pathway. In the case of class I-disparate, class II-deficient grafts, for example, the rapid rejection following $CD8^+$ depletion suggests that $CD4^+$ cells alone may have rejected these grafts despite their lack of donor class II antigens. A delayed-type hypersensitivity or some other noncytotoxic effector mechanism may have been responsible. On the other hand, Rosenberg and Singer and colleagues (39, 40) have shown that $CD8^+$-depleted mice do have a population of cells (derived from the CD8 lineage) that require help from $CD4^+$ cells and also *in vivo* sensitization to class I antigens for their expression. Thus, their data would suggest that the requirement for $CD4^+$ cells in our experiments might reflect the need for $CD4^+$ cells to generate CD8-derived cytotoxic cells that were then responsible for graft rejection. Our preliminary *in vitro* studies (R.L. and H.A., unpublished data) confirm that CD8-derived cytotoxic cells can be identified in the CD8-depleted recipients of class II-deficient grafts.

Whether or not the CD8-derived cytotoxic cells caused rejection of the class I-disparate, class II-deficient grafts, it is still a problem to explain how these cytotoxic cells were generated. Since the donor class I antigens were not matched with those expressed by the recipient, it would seem that the cytotoxic cells may have been sensitized by contact with cells from the donor while receiving help from $CD4^+$ cells sensitized by contact with cells from the recipient. Thus, these experiments suggest that helper cells can assist cytotoxic cells even when the two cell types are sensitized by contact with different APCs. This suggestion, however, is in violation of the principle suggested by Mitchison (41) that T cells, B cells, and the cells that stimulate them must interact together in a "three-cell cluster." Furthermore, Rosenberg and Singer demonstrated that under some circumstances this three-cell requirement might also apply to the subpopulations of T cells involved in graft rejection. They showed that the class II helper determinant on bm12 antigens had to be expressed on the same graft as the bm6 class I cytotoxic determinant to initiate graft rejection (13). The difference between our studies and those of Rosenberg and Singer is that in their case the two populations of APCs came from two different donor grafts, while in our case one set of APCs came from the recipient. Perhaps the greater number or different anatomic distribution of recipient APCs in draining lymph nodes allows for an effective four-cell cluster among cells of two T-cell subpopulations and two populations of APCs.

While our studies support the conclusion that indirect recognition can initiate graft rejection, the question that remains is how important is this pathway compared to others? At least three different mechanisms might generate a helper response to allografts *in vivo*: (*i*) a $CD4^+$ direct response to allogeneic class II antigens, (*ii*) a $CD8^+$ direct response to allogeneic class I antigens, and (*iii*) a $CD4^+$ indirect response to donor antigens presented by recipient class II antigens. It is likely that the importance of these different mechanisms may vary depending on the type of graft, the species, and the time after transplantation. For example, $CD8^+$ helper mechanisms do not seem to be effective in the rejection of many vascularized grafts, allowing the prolongation of graft survival in these cases by $CD4^+$ depletion alone (42, 43). In addition, replacement of donor APCs by recipient cells over time may leave only the indirect pathway of alloreactivity available to initiate rejection late after transplantation. It is also possible, however, that indirect recognition of alloantigens by $CD4^+$ cells is the primary *in vivo* pathway of alloreactivity, even early after transplantation, and that $CD4^+$ direct activation, so long the focus of transplantation immunology, is of secondary importance.

We thank Karla Stenger for her preparation of antibody reagents and supervision of the laboratory; Christine Shea for her expert secretarial support; David Schoenfeld for his help with the statistical analysis; and David Sachs, Amy Rosenberg, Al Singer, Megan Sykes, and Henry Winn for their suggestions and critical reading of the manuscript. This work was supported in part by U.S. Public Health Service Grants HL36372 (H.A.), HL18646 (H.A.), AI21569 (L.H.G.), and AI31541 (L.H.G.), and by support from the Mather Foundation (L.H.G.). M.J.G. is supported by the Leukemia Society of America and the Arthritis Foundation.

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