Brief Definitive Report

Cytotoxic T Cells Deficient in Both Functional Fas Ligand and Perforin Show Residual Cytolytic Activity yet Lose Their Capacity to Induce Lethal Acute Graft-Versus-Host Disease

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Summary

Graft-versus-host disease (GVHD) is the main complication after allogeneic bone marrow transplantation. Although the tissue damage and subsequent patient mortality are clearly dependent on T lymphocytes present in the grafted inoculum, the lethal effector molecules are unknown. Here, we show that acute lethal GVHD, induced by the transfer of splenocytes from C57BL/6 mice into sensitive BALB/c recipients, is dependent on both perforin and Fas ligand (FasL)–mediated lytic pathways. When spleen cells from mutant mice lacking both effector molecules were transferred to sublethally irradiated allogeneic recipients, mice survived. Delayed mortality was observed with grafted cells deficient in only one lytic mediator. In contrast, protection from lethal acute GVHD in resistant mice was exclusively perforin dependent. Perforin–FasL–deficient T cells failed to lyse most target cells in vitro. However, they still efficiently killed tumor necrosis factor α–sensitive fibroblasts, demonstrating that cytotoxic T cells possess a third lytic pathway.

CTL are important cytolytic effector cells of the immune defense system against viruses and tumors (1). $CD4^+$ and $CD8^+$ CTL are also involved in autoimmunity and transplant rejection. The recent development of mice in which the gene for perforin is disrupted (2–4) has provided evidence for the existence of two separate lytic pathways: first, the formation of membrane pores by perforin released from intracellular stores (5) followed by the proteolytic action of granzymes that are coreleased with perforin and that induce apoptosis in the target cell (6); second, the triggering of the Fas (Apo-1)–induced apoptotic pathway by Fas ligand (FasL) exposed on the CTL surface (7). At present it appears that the two pathways account for all cytolytic activity of CTL. However, only a restricted number of target cells could be analyzed, since functional inactivation of the Fas pathway is achieved by the use of target cells from *lpr* mice, which express little or no Fas (8). Thus, it cannot be excluded that other lytic molecules such as TNF-α, which is detected on the surface of activated T lymphocytes (9), contribute to the cytotoxicity of CTL. For this reason we generated mice in which both perforin and FasL are nonfunctional, by crossing perforin knock-out mice with *gld* mice, which bear a mutation in the FasL gene leading to its functional inactivation (10). Our study now shows that CTL derived from these mice still display lytic activity on transformed fibroblast cell lines.

These double-mutant mice also offered the unique possibility to evaluate the role of perforin and FasL in GVHD. GVHD continues to be a major complication after allogeneic bone marrow transplantation (BMT) (11). An acute lethal form of GVHD is caused by activation of the host-reactive donor effector T cells (12). The major organs affected in GVHD are skin, liver, intestine, and lymphoid tissues (13, 14), and symptoms range from varying degrees of erythroderma, wasting, diarrhea, splenomegaly, and lymphadenopathy to death (14). The lytic mechanisms leading to lesion formation are only poorly understood and might involve, for acute GVHD, direct interactions between donor CTL and host target cells, or more indirect mechanisms mediated by interleukins released by the donor T cells (15). We therefore explored the possibility that perforin or FasL may play a role in the etiology of the lesions seen in GVHD.

Materials and Methods

Animals. Perforin-deficient ($P^{-/-}$) *gld/gld* mice were generated by crossing perforin knock-out (C57BL/6 × 129)F3 mice (16) with C57BL/6 *gld/gld* mice (The Jackson Laboratory, Bar Harbor, ME) and by subsequent intercrossing of heterozygous F1 animals. To detect the FasL-*gld* mutation, mouse tail DNA was

The first two authors contributed equally to this work.

PCR amplified using the following primers: 5′-CACTCAAGGTCCATCCCTCTG-3′ and 5′-ATATTCCTGGTGCCCATGAT-3′. The amplification product was sequenced to detect the *gld* homozygotes (17, 18). Mice were screened for the defective perforin gene as described (16) .

Flow Cytometric Analysis. Splenocytes from 6- and 16-wk-old mice were isolated and freed from red blood cells by 15-min incubation in an ice cold buffer containing 13 mM sodium bicarbonate, 156 mM ammonium chloride, and 127 μM EDTA, and then resuspended in PBS supplemented with 5% FCS and 0.02% NaN_3. Splenocytes were stained with Tri-Color–conjugated anti-B220 antibodies and FITC-labeled anti-CD3 antibodies (CALTAG Laboratories, South San Francisco, CA) and analyzed using a FACScan® flow cytometer (Becton Dickinson Immunocytometric Systems, Mountain View, CA) using the Lysis II software.

Cytolytic Assays. Alloreactive cytotoxic T lymphocytes were generated in a 5-d mixed lymphocyte culture (16). Responder spleen cells ($2.5 \times 10^6/ml^{-1}$) were from adult 6-wk-old mice deficient for perforin, functional FasL (*gld/gld*), or both. All mice were of H-2^b haplotype. Stimulators were irradiated spleen cells ($2.5 \times 10^6/ml^{-1}$, 3,000 rad) from either DBA/2 mice (H-2^d) or C3H (H-2^k) mice. Target cells were activated splenocytes from DBA/2 mice stimulated with ConA (4 $\mu g/ml^{-1}$) for 3 d (2), A20.2J B cell lymphomas (H-2^d), WEHI 164 fibrosarcomas (H-2^d), and L929 (H-2^k) fibrosarcomas, all obtained from American Type Culture Collection (Rockville, MD). Mouse embryonic fibroblasts (MEF) were isolated from 12 to 14-d-old C3H embryos as described (2). Cytotoxicity on targets of hematopoietic origin was assayed after 4 h for ^{51}Cr release and DNA degradation (16). Adherent target cells were labeled with ^{51}Cr for 12 h as described elsewhere (16), and ^{51}Cr release was determined after a 20-h incubation. Recombinant mouse TNF-α was obtained from Dr. J. Browning, Biogen (Boston, MA).

Induction of GVHD. 6-wk-old female recipients were given a sublethal dose of Co irradiation (5 Gy; 1.2 Gy/min). Within 1 h, 40×10^6 of spleen cells isolated from female allogeneic donors were injected through the tail vein. Animals used in the study were BALB/c, C57BL/6, 129, (C57BL/6 × 129)F1, (C57BL/6 × 129)$P^{-/-}$, C57BL/6 *gld/gld*, and (C57BL/6 × 129)$P^{-/-}$*gld/gld* mice. Rate of survival was recorded daily. Mice with the *gld/gld* mutation were used at 5–6 wk of age. A minimum of four recipient animals were used in each experimental group.

Results and Discussion

Mutant mice deficient for both perforin and functional FasL were generated by intercrossing $P^{-/-}$ mice (16) with *gld/gld* mice (19) lacking functional FasL (17, 18). Animals homozygous for both nonfunctional genes were viable. Flow immunocytometric analysis of the spleen cell population of young animals (6 wk old) showed a phenotype similar to that observed in normal mice (Fig. 1). With progressive age (from 8 wk old onward), however, these mice developed a more severe lymphadenopathy than the one previously described in *gld/gld* mice (19). Lymphoid organs of $P^{-/-}$*gld/gld* mice were massively enlarged. Spleens derived from 16-wk-old $P^{-/-}$*gld/gld* mice were on average seven times heavier than those from age-matched $P^{-/-}$ mice, or 3.5 times heavier than the corresponding organs from *gld/gld* mice (Lowin, B., and J. Tschopp, unpublished results). A predominant $CD3^+B220^+CD4^-CD8^-$ population accumulated (Fig. 1) that resembled the T cell population found in *gld/gld* mice (19). This phenotype represented ~50% of all splenocytes in 16-wk-old double-mutant mice (Fig. 1). A high proportion of T cells in $P^{-/-}$*gld/gld* mice expressed activation markers such as CD69 (data not shown), pointing to a chronic activation of the immune system. Most mice died at an age of 4–5 mo.

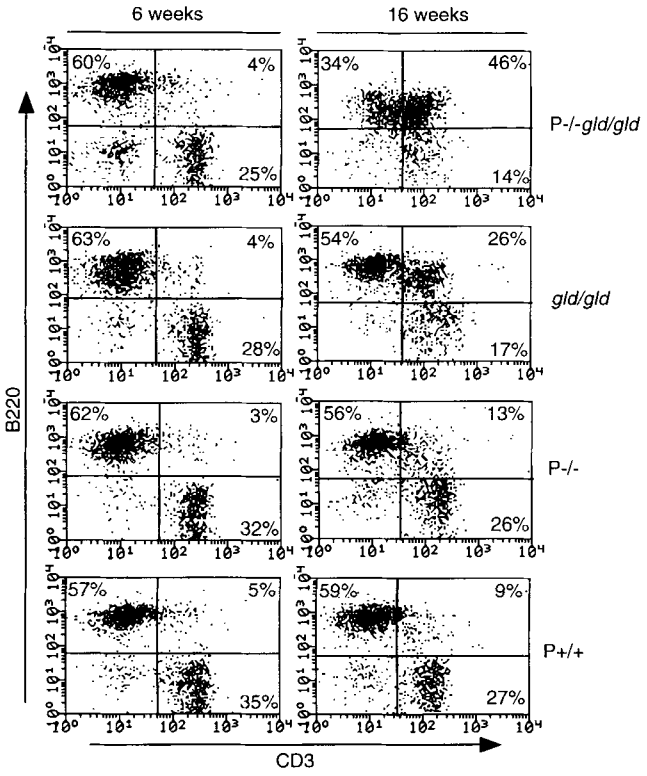

Figure 1. Lymphocyte populations in the spleen of $P^{-/-}$, *gld/gld*, and $P^{-/-}$*gld/gld* mice. Spleen cells were analyzed for the expression of CD3 and B220 by flow cytometry. Normal development of splenocytes derived from 6-wk-old $P^{-/-}$*gld/gld* mice and accumulation of $CD3^+B220^+$ cells in splenocytes from 16-wk-old $P^{-/-}$*gld/gld* and *gld/gld* mice. The $CD3^+B220^+$ population was $CD4^-CD8^-$ (data not shown).

T cells from double mutant animals exhibited no lytic activity on several target cells tested. Whereas alloreactive T cells (H-2^b) derived from 6-wk-old $P^{-/-}$ and *gld/gld* mice were able to kill ConA-stimulated allogeneic lymphoid cells (H-2^d) as well as the B cell lymphoma A20, splenocytes from $P^{-/-}$*gld/gld* mice were completely inactive (Fig. 2). The differences in cytotoxicity are unlikely to be caused by large differences in the allogeneic reaction, since IFN-γ concentration in the supernatants of the respective mixed leukocyte reactions was similar (3,000 ± 500 U/ml supernatant). These data thus confirm that perforin- and Fas-dependent pathways are responsible for all detectable lytic activity against lymphoid targets in short term cytotoxicity assays (2–4, 20). Cytolytic activity was also tested in long-term (20 h) cytotoxicity assays against nonhematopoietic target cells, that is, the fibrosarcoma WEHI-164 (H-2^d), L929 (H-2^k), and MEF (H-2^d). As shown in Fig. 3 *a*, MEF cells were not lysed in the absence

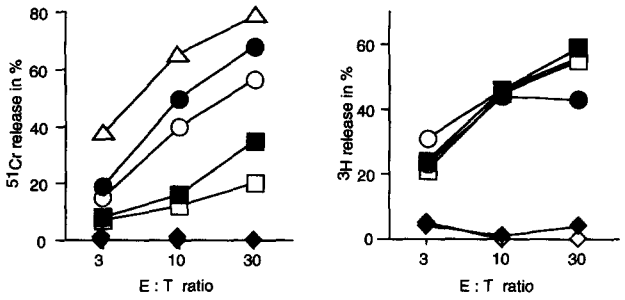

Figure 2. Killing of hematopoietic target cells by $P^{-/-}$*gld/gld* CTL. CTL activity of MHC alloantigen-specific T cells (H-2^b anti-H-2^d) from normal mice (*triangles*), $P^{-/-}$ mice (*squares*), *gld/gld* mice (*circles*) and $P^{-/-}$*gld/gld* mice (*diamonds*) was tested on ConA-activated spleen cells (*filled symbols*) and on the B cell lymphoma A20 (*open symbols*). The lytic activity was determined in both standard ^{51}Cr release (*left*) and DNA degradation (*right*) assays.

of both perforin and functional FasL, but surprisingly, killing of WEHI-164 and L929 was not significantly affected. The sensitivity pattern of target cells correlated with their respective susceptibility to TNF-α (Fig. 3 *b*), a cytokine mainly secreted by activated macrophages, NK cells, and some subpopulations of T cells (9). TNF-α has been shown in vitro to exhibit slow cytolytic activity (21), and in vivo, the production of TNF-α has been correlated with tissue injuries occurring in many immunopathological situations

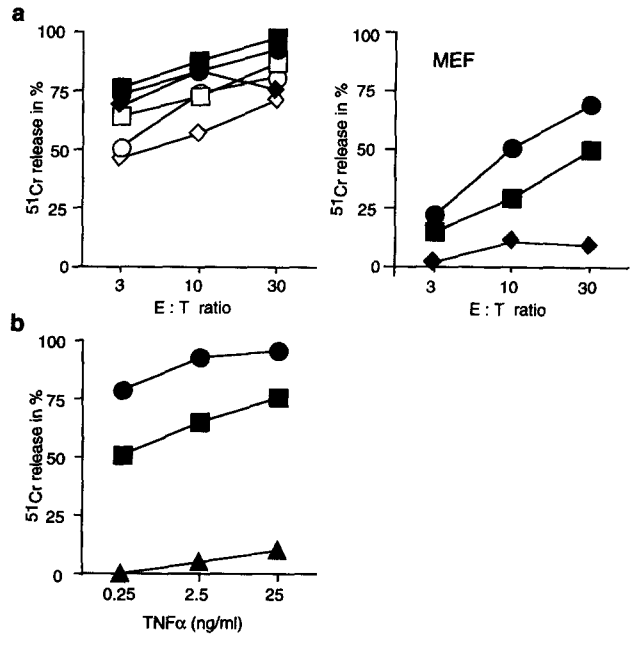

Figure 3. Killing of nonhematopoietic target cells by $P^{-/-}$*gld/gld* CTL. (*a*) As in Fig. 2, alloantigen-specific T cells (H-2^b anti–H-2^d and H-2^b anti–H-2^k) were generated in a 5-d mixed lymphocyte culture ($P^{-/-}$ [*squares*], *gld/gld* [*circles*], $P^{-/-}$*gld/gld* [*diamonds*]), and their lytic activity was tested on the fibrosarcomas WEHI-164 (H-2^d, *open symbols*) and L929 (H-2^k, *filled symbols*, *left*), and on primary MEF (H-2^k, *right*). (*b*) TNF-α sensitivity of the same target cells as in (*a*) was determined in a 20-h cytotoxicity test in the presence of increasing amounts of recombinant mouse TNF-α (WEHI-164 [*squares*], L929 [*circles*], MEF [*triangles*]).

(22–24). This suggests that TNF-α–mediated cytotoxicity is at least in part responsible for the lytic activity remaining in $P^{-/-}$ and FasL-deficient animals. Other cytolytic molecules, such as lymphotoxins (9) or NK lysins (25), may also contribute to the residual cytotoxicity, since neutralizing anti–TNF-α antibodies only partially inhibited this activity (10–30% specific reduction).

GVHD remains the main complication after allogeneic BMT. Since the effector molecules leading to lesion formation are only poorly understood, we investigated the role of perforin- and Fas-mediated cytolytic pathways in a murine model of acute lethal GVHD whereby donor splenocytes from C57BL/6 or 129 (H-2^b) mice are injected into sublethally irradiated allogeneic BALB/c (H-2^d) mice (26). Fig. 4 *a* shows that all BALB/c recipients injected with C57BL/6 or 129 spleen cells (40×10^6) died within 7 d, whereas injection of spleen cells from $P^{-/-}$ mice or functional FasL–deficient mice delayed the onset of the disease (survival mean 17 and 24 d, respectively). In contrast, mice given cells from $P^{-/-}$*gld/gld* animals were unaffected and survived (survival mean >50 d).

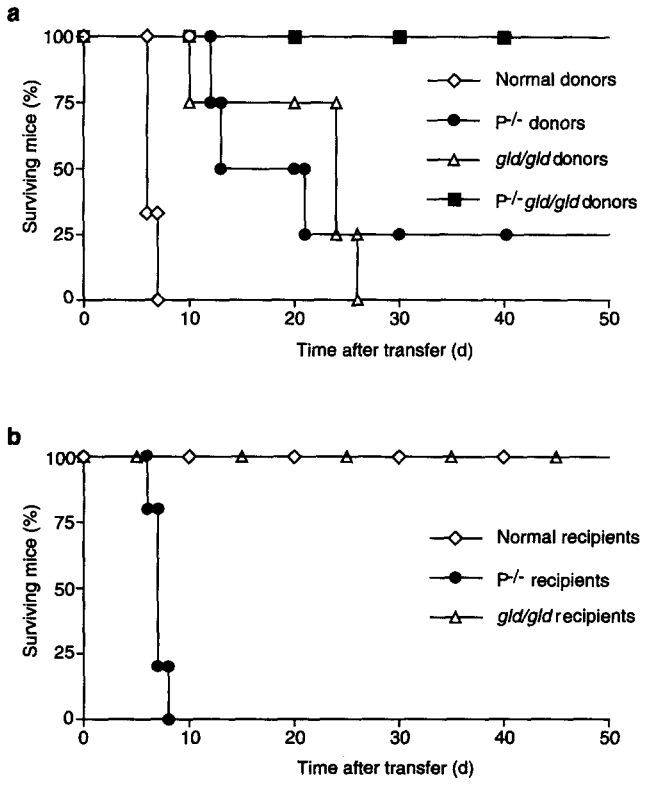

Figure 4. (*a*) Both perforin- and Fas-mediated cytotoxic pathways participate in lethal acute GVHD. Spleen cells from normal C57BL/6, 129, (C57BL/6 × 129)F1 donors, $P^{-/-}$ and functional FasL–deficient mice (*gld/gld*) were transferred into sublethally irradiated BALB/c recipients, and the rate of mortality of the recipients was recorded. Animals given only sublethal irradiation survived indefinitely (survival mean >50 d). Data are representative of three experiments with at least four mice per group (including control donors) per experiment. (*b*) Resistance to lethal acute GVHD is perforin dependent. Normal BALB/c spleen cells were injected into sublethally irradiated normal [C57BL/6, 129, (C57BL/6 × 129)F1], $P^{-/-}$, or *gld/gld* recipients.

Whereas sublethally irradiated BALB/c mice die within 7 d when inoculated with normal C57BL/6 spleen cells (Fig. 4 *a*), irradiated C57BL/6 (or 129) recipients injected with the same number of BALB/c spleen cells survive and show no signs of illness (Fig. 4 *b*) (26, 27). Whether this difference of sensitivity reflects the ability of the C57BL/6 mice to eliminate potentially alloreactive BALB/c CTL from the graft or is caused by a low frequency of BALB/c anti-C57BL/6 CTL precursors in BALB/c splenocytes is not known. We therefore transferred normal BALB/c spleen cells into sublethally irradiated C57BL/6 recipients deficient for either perforin or functional FasL. BALB/c splenocytes failed to induce any symptoms of lethal GVHD in normal and *gld/gld* recipients (Fig. 4 *b*). In contrast, C57BL/6 recipients lacking functional perforin became susceptible to BALB/c splenocytes and died within 7–8 d. Thus, normal sublethally irradiated C57BL/6 recipients contain radioresistant cells that are able to suppress the effector function of BALB/c splenocytes solely through a perforin-dependent pathway. The cell population responsible for this inhibition might include host antidonor radioresistant CTL, NK cells, or natural suppressor cells appearing after total body irradiation (27, 28).

In the murine model of lethal GVHD studied here, both perforin and FasL appear to play a crucial role in mortality. Other lytic effector molecules still detected in perforin–FasL–deficient lymphocytes contribute little, although our study cannot exclude the involvement of TNF-α or other cytokines in milder forms of GVHD, as suggested by others (15, 23). In vivo, perforin's implication in the clearance of lymphocytic choriomeningitis virus infection and fibrosarcoma tumor cells is well established (4, 29), whereas the Fas system was shown to be involved in the elimination of activated lymphocytes in the periphery (7, 30). This study now demonstrates that FasL also participates in pathological tissue damage during GVHD, in agreement with the observation that injection of anti-Fas antibodies into adult mice can cause rapid death due to liver failure (31). In view of the massive upregulation of FasL in functional Fas receptor–lacking *lpr* (lymphoproliferation) mice (32, 33), the severe wasting syndrome (GVHD) observed when *lpr* bone marrow–derived cells are adoptively transferred into a syngeneic wild-type recipient (34) may also be explained. Tissues attacked in FasL-mediated GVHD probably include those that abundantly express Fas, such as liver, lung, or heart, whereas perforin, with no requirement for a specific receptor (35), may eventually destroy any organ.

Although both perforin- and Fas-dependent pathways are responsible for the mortality caused by acute GVHD in BALB/c mice, perforin-mediated cytotoxicity appears to be the only pathway required for the rejection of allogeneic spleen cells. $P^{-/-}$ mice still reject heart allografts (36), and thus effector mechanisms used to reject tissues and organs of different origin seem to differ.

GVHD remains a major barrier to a wider application of allogeneic BMT for a variety of diseases. Provided that the active role of FasL and perforin in the progression of acute GVHD can be illustrated in humans, the development of therapeutic strategies aimed at controlling the two cytolytic pathways during BMT may be an approach for decreasing the risk of GVHD.

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