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Proliferative and Interferon Responses by Peripheral Blood Mononuclear Cells After Bone Marrow Transplantation in Humans

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The capacity of peripheral blood mononuclear cells from bone marrow transplant recipients to proliferate and produce interferon in response to mitogens and specific antigens was tested. Proliferation in response to phytohemagglutinin or pokeweed mitogen occurred in cells from more than 90% of the recipients, and interferon was present in 60 to 70% of the supernatants from these cultures, even when tested as soon as 8 weeks after transplantation. Proliferation in response to bacterial antigens was infrequent, and interferon release was not detected. In the early post-transplantation period (less than 13 weeks), cells from only two of four cytomegalovirus (CMV) antibody-positive patients proliferated normally in response to CMV antigen and interferon release was detected only once. In the late post-transplantation period (more than 13 weeks), in only two of five instances did cells proliferating in response to CMV antigen release interferon. The response to CMV antigen of mononuclear cells from many transplant recipients differs from that of cells from normal controls.

Bone marrow transplant (BMT) recipients have a gradual and sequential recovery of normal peripheral blood elements and normal immune function (8, 15). Before complete hematological and immunological reconstitution, they have a high risk of developing severe infections with a broad range of bacterial and nonbacterial pathogens (6, 7, 33, 35). Bacterial infections usually occur during the period of granulocytopenia and deficient immunoglobulin synthesis, which occurs in the early post-transplant period and which also follows bone marrow graft rejection. Viral and other nonbacterial infections can also occur in the early postgrafting period, but more commonly occur 2 to 12 months after transplantation when normal granulocyte function has been restored. The human herpesviruses are the major cause of the severe virus infections which complicate BMT (26, 27, 29; Winston et al., Medicine, in press). Herpes simplex infections occur most commonly during the first post-transplantation month, and cytomegalovirus (CMV) infection, a major cause of morbidity and mortality after transplantation, tends to occur during the second month (27). Cell-mediated immunity appears to be the dominant factor in host resistance to these viruses (1, 10, 11, 24, 25), and thus prolonged inability of the mononuclear cells from BMT recipients to respond adequately to specific antigens may be the basis of their increased susceptibility to herpesvirus infections.

In normal individuals, interferon is produced by peripheral mononuclear cells in response to nonspecific mitogens and specific antigens, including viral antigens (12). Furthermore, recent evidence suggests that the capacity of circulating mononuclear cells to produce interferon in response to a herpesvirus antigen correlates with the host's resistance to infection by that virus (28, 30, 31). Thus, it is possible that the prolonged susceptibility of BMT recipients to herpesvirus infections might reflect the inability of these patients' mononuclear cells to produce interferon. We report here measurements of the capacity of mononuclear cells from BMT patients to undergo blastogenic transformation and to produce interferon after exposure to nonspecific mitogens and specific microbial antigens, including antigen prepared from human cells infected with CMV.

MATERIALS AND METHODS

Patient population and transplantation procedure. Patients with severe aplastic anemia or acute myelogenous leukemia were accepted for BMT. They

were placed in isolation and underwent transplantation with minor modifications of the protocol utilized by Thomas et al. (4). They received 1×10^8 to 3×10^8 nucleated bone marrow cells per kg, intravenously, from HL-A, -B, and -D identical sibling donors. All blood products administered post-transplantation were irradiated (5,000 rads). Methotrexate was administered to reduce the possibility of graft-versus-host disease (GVHD): 10 mg/kg on days 1, 3, 6, and 11 and then weekly for 13 weeks. Clinically significant GVHD was treated with rabbit anti-human thymocyte serum or steroids.

Reagents. The source of mitogen and its final concentration in the standard transformation assay were: phytohemagglutinin (PHA-P, Difco Laboratories) reconstituted with 5 ml of medium, 1:500; pokeweed mitogen (PWM, Difco), 1:50; concanavalin A (Sigma Chemical Co.), 2 μg/ml; monilia antigen (Hollister-Steer) 1:200; streptococcal antigen (Varidase, American Cyanamid Co., Lederle Laboratories Div.) 50 U/ml; and diphtheria and tetanus toxoids (Biological Laboratories, Commonwealth of Massachusetts), 100 and 20 μg/ml, respectively. Partially purified Sendai virus-induced human leukocyte interferon was the generous gift of K. Cantell (State Serum Institute, Helsinki). This preparation, assayed in human cells, contained 2×10^6 IU/mg of protein (assayed in parallel with the British standard of human interferon, code 69/19, Division of Biological Standard).

Preparation of CMV antigen. Human embryonic lung fibroblasts were infected with the AD 169 strain of CMV at a multiplicity of 0.1 plaque-forming units per cell. When 90 to 100% of the cells showed cytopathic effect, they were extracted with glycine-buffered saline (0.043 M glycine and 0.15 M NaCl, pH 9.0) as previously described (36). Antigen was titered in a complement fixation test (22; see below) with 1.5 U of complement. The antigen used in the blast transformation assay had a complement fixation titer of 1:64 with known positive serum. Control antigen prepared from uninfected human embryonic lung fibroblasts cells did not react with the positive serum.

Isolation of lymphocytes. Mononuclear cells were isolated in Ficoll-Hypaque gradients from heparinized blood (100 U/ml; Abbott Laboratories) (2). The interface cells from a normal individual consisted of greater than 85% mononuclear cells, of which 98% were viable as judged by trypan blue exclusion. The yield of cells represented 50% of the total mononuclear cells present in the initial blood sample. Cells that formed rosettes with sheep erythrocytes were enumerated (16).

Method of culture. Gradient purified cells (usually 10^6) were incubated at 37°C in tissue culture tubes (16 by 125 mm; Falcon, 3033), with or without plant mitogens or antigens, in 1.0 ml of RPMI 1640 containing 10% human AB+ serum (CMV complement fixation antibody titer of <2) for the specified time interval (usually 3 or 6 days) in a 10% CO_2 atmosphere. Six hours before harvest, 1.0 μCi of [^{3}H]thymidine (2 Ci/mM) was added in 50 μl of medium.

Harvest of cultures. Tubes were shaken by a Vortex mixer, and 50-μl portions were spotted on Whatman filter paper (no. 3 filters; Blaston, Ltd.). The remaining cells were centrifuged (200 × *g*), and the supernatants were stored at −20°C for interferon assay. Filters were dried and soaked overnight in 10% trichloroacetic acid at 4°C, washed twice in 5% trichloroacetic acid, washed twice in ethanol-acetone (1:1) for 30 min at 37°C, and rinsed with acetone. Dried filters were counted in a Beckman 3320 spectrometer in Liquifluor (New England Nuclear Corp.) for a sufficient time to achieve a counting accuracy of ±5%. Duplicate samples from duplicate cultures were averaged to determine the mean counts per minute at each experimental point.

Interferon Assay. Interferon in cell culture supernatants was assayed by a modification of the technique of Havell and Vilček (18), in which flat-bottom microtiter plates were seeded with 10^4 human foreskin fibroblasts (cell strain 350 Q) in 50 μl of Dulbecco medium with 10% fetal calf serum and incubated at 37°C for 5 days in a 10% CO_2 atmosphere. Serial twofold dilutions of cell supernatant (or interferon standard) were then prepared in medium with 2% serum at 4°C, and 50 μl of each dilution was inoculated into four replicate wells. After 18 h, the wells were drained, washed twice, and infected with 50 μl of medium containing 10^3 plaque-forming units of vesicular stomatitis virus (Indiana strain; purified "B" particles) (19). Twenty-eight hours postinfection, when the cytopathic effect in the virus control wells involved 90 to 100% of the cells, the plates were fixed, and stained with crystal violet. The plates were examined with an inverted microscope, and wells in which fewer than 25% of the cells exhibited cytopathic effect were scored as "protected." The interferon titer of each sample was defined as the reciprocal of the highest dilution at which at least three of the four replicate wells were "protected." Titers were corrected to international units on the basis of the titer of a human leukocyte interferon standard which was assayed in each test. This interferon standard on repeat testing had an average deviation from the mean of less than one (twofold) dilution. The titer deviated from the mean by more than one dilution in only 11% of the tests. Furthermore, each unknown sample was tested in quadruplicate, and rarely was one of the four replicates nonidentical. The average deviation of a replicate from its mean was less than 1/16 of a dilution.

Some interferon samples were tested after being heated to 56°C for 1 h, after dialysis overnight against pH 2.0 glycine buffer (0.005 M), or after exposure to rabbit anti-type 1 human leukocyte interferon (34) obtained from the Development and Applications Branch, National Institute of Allergy and Infectious Diseases.

Complement fixation test. A microtiter method was used as described by Lennette (22). Four units of CMV antigen and 1.5 U of guinea pig complement (Flow Laboratories) were used. A unit of complement was the amount of complement which caused complete lysis of sensitized sheep erythrocytes incubated with 4 U of standard CMV antigen. The end point was the highest dilution of serum giving greater than 50% fixation as approximated by the erythrocyte button. Sera exhibiting fixation at dilutions of ≥1:2 were considered reactive.

RESULTS

The peripheral blood mononuclear cells of normal adults regularly proliferate and produce interferon in response to mitogens (Table 1).

Maximal levels of interferon were produced after day 3 of incubation with PHA and PWM. Thus, a 3-day incubation period was used to determine the concentration dependence of the interferon response of mononuclear cells from normals and BMT recipients to plant mitogens. On the basis of experiments with PHA (Fig. 1) and analogous experiments with PWM and concanavalin A (data not shown), a single final concentration of each mitogen was chosen (PHA, 1/500 of standard solution; PWM, 1/50 of standard solution; concanavalin A, 2.0 μg/ml) to be used in testing the capacity of cells from BMT recipients to produce interferon in vitro. The concentrations chosen stimulate maximal interferon levels in normal individuals.

Control experiments demonstrated that neither the nonspecific mitogens nor the CMV antigen had any direct antiviral activity or any depressant effect on the titer of the interferon standard. Supernatants from unstimulated mononuclear cells were also found to be free of antiviral activity. It was noted that some stimulated culture supernatants (but not purified interferon) had antiviral activity only when very dilute (>1:32), whereas when undiluted they had no antiviral activity. This prozone effect, which appears to be due to the simultaneous production of interferon and an antagonist (5), which has been reported previously (12), made it impossible to screen samples at a low dilution.

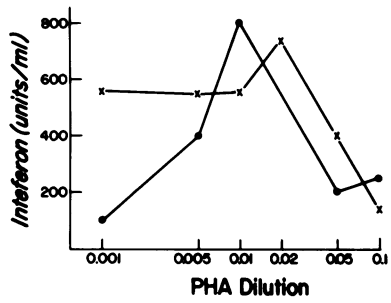

FIG. 1. *Interferon production by mononuclear cells cultured with various concentrations of PHA. Ficoll-Hypaque gradient peripheral blood leukocytes prepared from normal individuals* (×) *or BMT recipients* (●) *were cultured for 3 days with various concentrations of PHA. The concentration of interferon per milliliter of supernatant fluid from each of these cultures was determined in quadruplicate.*

Mixing experiments demonstrated that these undiluted cell supernatants could abolish the protective effect of added standard human leukocyte interferon.

The mononuclear cells from the majority of BMT recipients produced interferon after stimulation by nonspecific mitogens (Table 1). When adequate numbers of mononuclear cells were available, a blastogenic response to PHA or PWM was demonstrable in more than 90% of the recipients. Interferon was present in 60 to

TABLE 1. *Blastogenic transformation and interferon production by mononuclear cells from normal patients and BMT recipients*

Group studied	Mitogen or antigen[a]	Total tests[b]	Blastogenic response[c] (% positive)	Interferon response total group[d] (% positive)	Interferon response recent transplantation[e] (% positive)
Normal patients	PHA	10 (8)	100	100	
	PWM	8 (6)	100	100	
Transplant recipients	PHA	21(19)	90	62	85
	PWM	17 (14)	95	70	71
	ConA[f]	13 (10)	64	15	14
	Allogenic lymphocytes	5 (5)	80	20	
	Monilia	12 (9)	42	8	
	Streptococcal	11 (8)	18	0	
	Tetanus	12 (10)	16	0	
	Diphtheria	5 (5)	20	0	

[a] Ficoll-Hypaque purified mononuclear cells were incubated for 3 days with plant mitogens or 6 days with antigens.

[b] Numbers in parentheses indicate the total number of patients tested.

[c] Incorporation of [^{3}H]thymidine into cell DNA was assayed. A positive response was defined as incorporation which was twofold greater in the presence of mitogen or antigen than in the control cultures. The percent of total tests which were positive is tabulated.

[d] Supernatants from the cultures described in footnote *a* were assayed in quadruplicate for interferon. The percent of supernatants tested which contain interferon is tabulated.

[e] Seven patients were tested in the period before 8 weeks post-transplantation.

[f] ConA, Concanavalin A.

70% of the supernatants of these cultures, although not always in the same cultures in which blastogenesis occurred. Cells from seven BMT recipients were tested within 8 weeks after transplantation. Supernatants from six of the seven cultures contained interferon after stimulation by PHA; five contained interferon after stimulation by PWM; in only one was interferon release stimulated by concanavalin A. Thus, one characteristic of engrafted mononuclear cells early after BMT is their ability to release interferon in response to plant mitogens.

Interferon release and blastogenic response were not invariably coupled (Table 1). The divergence between these two responses by mononuclear cells of selected patients is shown in Table 2. Cells from patient A initially showed a relatively low stimulation index in response to PHA, but produced large amounts of interferon; subsequently, the blastogenic response increased and interferon production fell. Cells from patient B in the early post-transplant period produced interferon in response to PHA, but did not proliferate. Patient C had cells which did not produce interferon when stimulated by PHA, but responded to PWM with blastogenesis and interferon production. The cells from patient D responded in a fashion opposite to that described for patient C. Thus, in a given patient, the character of the response to one plant mitogen did not necessarily predict the response to a second. This observed dissociation between proliferation and interferon release was not seen with mononuclear cells from the normal population studied.

TABLE 2. *Response of mononuclear cells from BMT recipients to nonspecific mitogens*[a]

Patient	Post-transplant (days)	Mitogen	Stimulation[b] index	Interferon[c] (U/ml)
A	39	PHA	24	1,600
	55	PHA	158	800
	103	PHA	119	800
	412	PHA	119	44
B	38	PHA	0.5	400
	52	PHA	7	570
C	614	PHA	90	0
		PWM	14	200
D	68	PHA	88	140
		PWM	7	0

[a] Patients were selected solely to demonstrate unique combinations of blastogenic and interferon responses.

[b] Stimulation index represents incorporation into cells exposed to mitogen divided by incorporation into unexposed cells and is an average of two samples removed from each of two duplicate cultures.

[c] Interferon concentration was measured from the same mononuclear cell cultures used to determine the blastogenic response and is an average of four determinations of supernatants pooled from the duplicate cultures described in footnote *b*.

In most BMT recipients, however, interferon release and blastogenic response to PHA paralleled one another (Fig. 2). Patient 1 experienced a depression of both responses midway during an episode of progressive GVHD, and patient 2 experienced an increase in these responses after successful therapy of GVHD. In patient 2, both responses were again depressed concurrently with an episode of interstitial pneumonia. Interferon production did not correlate with the percent of T lymphocytes (E-rosetting cells).

Concanavalin A, allogenic lymphocytes, and monilia less frequently stimulated blastogenesis and were relatively poor inducers of interferon (Table 1). Bacterial antigens rarely stimulated either blastogenesis or interferon.

Although it was of interest to determine whether peripheral mononuclear cells from BMT recipients could produce interferon in response to nonspecific mitogens and to nonviral specific antigens, it was especially pertinent to determine whether they would respond to herpesvirus antigens. For this reason, the capacity of mononuclear cells from these patients to re-

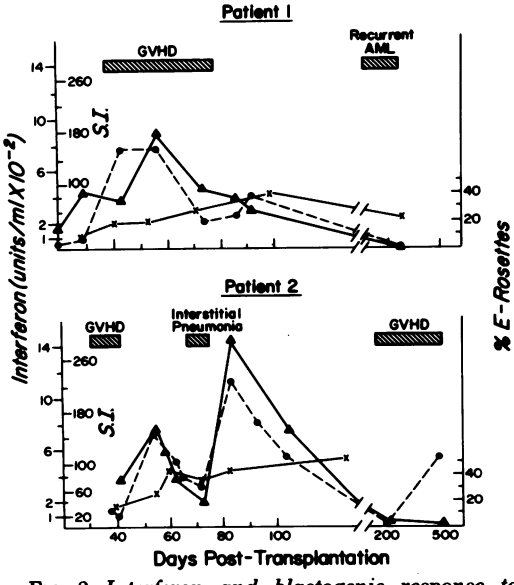

FIG. 2. *Interferon and blastogenic response to PHA in two patients after BMT. PHA-stimulated, Ficoll-Hypaque-separated mononuclear cells from BMT recipients were assayed for E-rosetting cells* (×) *and, in duplicate, for blastogenesis* (●). *Supernatants from these cultures were assayed for interferon* (▲) *in quadruplicate.*

spond to CMV antigen was determined. Mononuclear cells from three BMT recipients who had no CMV complement fixation antibody before or during the observation period did not proliferate or release interferon in response to CMV antigen (Table 3). Nine additional patients were studied who had serological evidence of post-transplantation CMV infection (Table 4). Cells from five patients were not cultured until more than half a year after transplantation (patients 1 to 5). Cells from four of these patients proliferated in response to CMV antigen. Supernatants from cultures from patients 2 and 4 (two attempts, 6 weeks apart) contained interferon, whereas no interferon was released by cells from patient 5. In contrast, antibody-positive controls always proliferated in response to CMV antigen and interferon release always accompanied proliferation (Table 4). Patient 3 was studied when she had severe GVHD and was receiving immunosuppressive therapy.

Four additional antibody-positive patients were studied at 4 to 13 weeks after transplantation when the highest frequency of CMV infection occurs (27). Patients 7 and 9 had a positive blastogenic response, whereas the mononuclear cells from patients 6 and 8 had negative or borderline response during this period. Significant interferon (>10 U/ml) was transiently detected only in supernatants from patients 6 and 9.

In the subsequent post-transplantation period (more than 13 weeks), cells from patients 6 through 9 did not release interferon in response to CMV antigen, even when a proliferative response was readily detectable. This dissociation was not seen in the normal control group. In no instance was interferon detected in the absence of lymphocyte blastogenesis.

The interferon produced was trypsin sensitive (23), was completely destroyed by heating at 56°C for 1 h, was 75% destroyed by exposure to pH 2 for 16 h, and was partially inactivated by antibody to Sendai virus-induced human leukocyte interferon (34).

TABLE 3. *Response of mononuclear cells from CMV antibody-negative BMT recipients to CMV antigen*

Patient	Post-transplant (weeks)	CMV antibody[a]	Stimulation index	Interferon (U/ml)
1	2	<2	ND[b]	ND
	22	ND	1.0	0
	47	<2	0.8	0
2	8	<2	ND	ND
	30	<2	2.1	0
3	−1	<2	1.2	0
	7	<2	0.8	0
	11	<2	0.3	0

[a] Reciprocal of end point dilution in the complement fixation test.
[b] ND, Not done.

TABLE 4. *Response of mononuclear cells from CMV antibody-positive normal patients and BMT recipients to CMV antigen*

Patient	Post-transplant (weeks)	CMV antibody[a]	Stimulation index	Interferon (U/ml)
Normal controls[b]		16–128[c]	13.5 (±10.0)[d]	255 (±100)[d]
1	125	512	6.3	ND[e]
	133	ND	7.6	ND
2	42	128	45.6	ND
	56	ND	7.5	200
3	29	64	ND	ND
	36	ND	0.5	0
	44	ND	0.8	0
4	160	128	ND	ND
	169	ND	60	100
	175	ND	100	0
5	101	16	17.1	0
6	4	8	1.2	ND
	8	ND	3.0	50
	15	ND	0.7	0
	70	2	0.6	0
7	−1	256	ND	ND
	4	64	ND	ND
	13	ND	25.1	6
8	−1	256	39	100
	8	32	1.2	0
	11	32	1.4	0
	14	32	16	0
	19	32	4.6	0
9	5	8	ND	ND
	7	64	13.9	12.5
	10	64	20.5	0
	13	64	ND	25
	18	ND	24.6	0
	25	2,048	6.7	0
	36	ND	4.7	

[a] Reciprocal of end point dilution in the complement fixation test.
[b] Stimulation was determined with eight normal individuals, and the interferon in the supernatants of four such tests was determined. The stimulation index of five seronegative individuals never exceeded 2.3.
[c] Range.
[d] Mean and standard deviation.
[e] ND, Not done.

DISCUSSION

Mononuclear cells from the majority of BMT recipients proliferated and released interferon into the tissue culture medium after stimulation by plant mitogens, especially PHA and PWM

(Table 1). The kinetics of interferon production by BMT recipients was similar to that of normal individuals in timing of peak response and in the dose of PHA which produced the highest interferon levels (Fig. 1). In most cases, these responses to mitogens were detectable as soon as adequate cell numbers were available for testing.

In these patients, all lymphocytes were of donor origin and no residual recipient lymphocytes were detected after transplantation. Thus, the cell responsible for the production of interferon after stimulation by plant lectins appears early in the ontogeny of the immune system after BMT and may be analogous to fetal ontogeny, in which the capacity of mononuclear cells to produce interferon is a very early specific immune event (3, 32).

Blastogenic response of mononuclear cells from BMT recipients to bacterial antigens was infrequent, and interferon production was never stimulated by these antigens (Table 1). This lack of immune reactivity may represent the lack of bacterial antigens in the patients' relatively restricted environment. In this regard, it is noteworthy that antigen prepared from *Candida albicans*, an organism which frequently colonized patients early in the post-transplantation period, induced a blastogenic response in cells from 5 of 12 patients. In contrast to the lack of response to bacterial and fungal antigens, cells from seven of nine CMV antibody-positive patients proliferated in response to CMV antigen at some time during the study period (Table 4). Patient 6 had a borderline response, and the failure of patient 3 to respond can be explained by severe GVHD and iatrogenic immunosuppression. Specifically, during the first 13 weeks posttransplantation, cells from only two of four patients demonstrated a strong proliferative response to CMV antigen and only one released interferon. In the late post-transplantation period (more than 13 weeks), inteferon release was stimulated by CMV antigen in the cells from only two of five blastogenesis-positive patients studied. This contrasts sharply with the response of normal controls in whom interferon release parallels proliferation. The interferon released had some characteristics of immune (type 2) interferon (heat and acid lability), but is also partially neutralized by antibody to human leukocyte (type 1) interferon (34).

In animal models, allogenic GVHD produces a secondary immunoincompetence (9). Since clinical GVHD occurs in 75% of allogenic BMT recipients and since it is a significant cause of morbidity and mortality in 25% of BMT patients, we sought a correlation between GVHD and the capacity of mononuclear cells to produce interferon. In two patients who were sequentially studied (Fig. 2), progressive GVHD was associated with the inability of mononuclear cells to produce interferon, although the concomitant therapy for GVHD may have produced a secondary immunosuppression (Fig. 2, patient 1).

A decrease in blastogenesis and interferon production occurred concomitantly with interstitital pneumonia in one patient, although it is not possible to identify the primary event; i.e., whether inadequate cellular immunity resulted in a viral infection or whether such an infection could have reduced the capacity of the newly engrafted immune system to respond to other antigens (17, 20, 21).

A dissociation was sometimes present between blastogenesis and interferon production in response to plant lectins (Tables 1 and 2) and to CMV antigen (Table 4). These observations could indicate that the mononuclear cells producing interferon are not identical with those undergoing blastogenic transformation; that the two populations of cells mature at different times in the transplant recipient; or that they are differentially affected by clinical events. The latter phenomena is suggested by the loss of the mononuclear cell capacity to produce interferon in patients who had acquired this capacity and whose cells continue to be stimulated to divide by CMV antigen (Table 4, patient 9). A similar dissociation was observed by Epstein and Ammann in cells from patients with congenital immunoglobulin A deficiency (13) and in mononuclear cells of normal individuals who had received a booster dose of vaccinia virus (14).

In conclusion, within 8 weeks after transplantation, the mononuclear cells of BMT recipients are capable of proliferating and producing interferon in response to the plant lectins, PHA and PWM. However, the mononuclear cells from some CMV antibody-positive patients fail to proliferate normally and/or fail to produce interferon when exposed to CMV antigen. The failure to produce interferon continues for many patients for an extended period after transplantation. The clinical significance of these observations are unclear. However, in cardiac transplant recipients, poor proliferative and interferon responses to specific viral antigens were correlated with frequent infection with herpes simplex and varicella-zoster viruses (30). As was done with those patients, it will require a prospective clinical study in BMT recipients to further relate defects in immune function with clinical events such as CMV infection, GVHD, and immunosuppressive therapy.

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