

Macrophage Priming and Lipopolysaccharide-triggered Release of Tumor Necrosis Factor α during Graft-Versus-Host Disease

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Summary

In this report we have investigated macrophage (M ϕ) activity and tumor necrosis factor α (TNF- α) production during graft-vs.-host disease (GVHD). TNF- α production by M ϕ requires two signals: priming of M ϕ by interferon followed by triggering of TNF- α production and release by lipopolysaccharide (LPS). The state of M ϕ activation was examined in nonirradiated B6AF1 recipient mice injected with either 60×10^6 (acute GVHD) or 30×10^6 (nonlethal GVHD) parental B6 lymphoid cells. During the early phase of acute GVHD, administration of normally sublethal amounts of LPS triggered release of significant amounts of TNF- α into the serum resulting in death of the animals within 36 h. Normal animals treated with the same dose of LPS neither died nor produced detectable amounts of serum TNF- α . In vitro studies demonstrated that M ϕ were primed during GVHD. The level of M ϕ priming was greater during acute GVHD than nonlethal GVHD since 100-fold less LPS was required to trigger killing of a TNF- α -sensitive cell line by M ϕ from acute GVHD animals. The amount of TNF- α released into the serum after LPS injection increased during the course of the GVHD and was significantly greater in acute GVH-reactive mice. Endogenous LPS was detected in the serum of acute GVH-reactive animals coincident with the onset of mortality. The data provide evidence that during GVHD M ϕ are primed as a result of the allogeneic reaction and that endogenous LPS therefore triggers M ϕ production of TNF- α resulting in the symptoms characteristic of acute GVHD.

GVHD remains a major risk factor in allogeneic bone marrow transplantation. The characteristic features of acute GVHD include severe immunosuppression, diarrhea, weight loss, and the development of pathologic lesions in lymphoid and nonlymphoid tissues (1-7). During the course of experimental GVHD, T and B lymphocyte functions are suppressed (2, 3, 7), however, macrophages (M ϕ)¹ are activated as demonstrated by an increase in their phagocytic and bactericidal activity (8, 9).

Two factors, IFN- γ and LPS, mediate the activation of normal M ϕ in a synergistic manner (10-12). In human bone marrow transplant recipients, an increase in the serum concentration of IFN- γ occurs before clinical manifestation of GVHD (13). Indeed a large proportion of the T cell clones that can be isolated from such patients produce IFN- γ (14).

During murine GVHD, IFN- γ production has been demonstrated by in situ labeling of spleen cells (15) and a decrease in the severity of GVHD-associated intestinal lesions after treatment of animals with anti-IFN- γ (16). LPS, the second or triggering signal involved in M ϕ activation, is an integral part of the cell wall of enteric Gram-negative bacteria. In experimental GVHD, the administration of normally nonlethal amounts of LPS rapidly results in the onset of shock leading to death within 48 h (17, 18). The symptoms of GVHD can be reduced or prevented by germ-free conditions or through antibiotic therapy, strongly suggesting that bacterial products are involved in the reaction (19-21).

Normal M ϕ exposed to LPS produce and release TNF- α , a protein that has been shown to mediate weight loss or cachexia, some inflammatory processes, septic shock, and damage to normal and tumor cells (22-27). Quantities of LPS that are insufficient to induce the production and release of TNF- α from normal, unprimed M ϕ , can trigger IFN- γ -primed M ϕ to produce significant quantities of TNF- α (12, 28). In murine GVHD, it has been demonstrated that

¹ Abbreviations used in this paper: M ϕ , macrophage(s); MCS, mouse Con A supernatant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRS, normal rabbit serum; PFC, plaque-forming cell.

the administration of anti-TNF- α reduces cutaneous and intestinal lesions and mortality (29). More recently, increased levels of TNF- α have been found in the serum of allogeneic bone marrow transplant recipients (30) and have been correlated with the severity of GVHD (31, 32).

To determine whether M ϕ are the effector cells responsible for TNF- α production during GVHD, we have examined the state of M ϕ activation, response to LPS, and serum levels of LPS in nonirradiated F₁ hybrid mice injected with parental strain lymphoid cells. The results demonstrate that during GVHD M ϕ are primed and can release TNF- α when triggered by LPS; that the level of M ϕ priming and in vivo production of serum TNF- α in response to LPS are related to the number of cells transplanted and the time posttransplant; and that endogenous LPS can be detected in the serum during acute GVHD.

Materials and Methods

Mice. Male C57BL/6 (B6) and C57BL/6 \times AF1 (B6AF₁) mice were bred and maintained in our laboratory and used at 12–18 wk of age.

Reagents and Media. Recombinant murine TNF- α (4×10^4 U/ μ g) was purchased from Genzyme Corp. (Boston, MA). LPS (*Escherichia coli* 0111:B4, lot 437627; Calbiochem Corp., La Jolla, CA) was dissolved in PBS and sterilized by irradiation (15,000 rad). RPMI 1640, HBSS, PBS (Gibco Laboratories, Grand Island, NY), and FCS (Sterile Systems, Logan, UT) contained <0.25 ng/ml endotoxin as quantified by the Limulus amoebocyte lysate assay (M. A. Bioproducts, Walkersville, MD). Con A was purchased from Pharmacia (Uppsala, Sweden). All glassware used to prepare reagents or carry out assays was heated at 180°C for 4 h.

Induction of GVHD. Single cell suspensions of donor spleen and lymph nodes were prepared in HBSS. Recipient B6AF₁ animals were injected intravenously with 30 or 60×10^6 B6 or 60×10^6 B6AF₁ lymphoid cells.

Direct Plaque-forming Cell (PFC) Assay. Spleen cells were assayed for the total number of PFCs to SRBC using the technique of Cunningham and Szenberg (33) as modified in this laboratory (34).

Cell Lines. The cell lines used included L5178Y, a DBA/2-derived lymphoma; L929, a C3H/HeN-derived fibrosarcoma; 3T6, a Swiss mouse embryo-derived fibroblast line; YAC-1, an A/Sn-derived lymphoma; and MDW4, a DBA/2-derived tumor cell line. Cultures were maintained in RPMI 1640 plus 5–10% FCS at 37°C in a 5% CO₂/air atmosphere. All cell lines were free of mycoplasma infection as determined by periodic monitoring using inoculated 3T6 cultures and 6-mercaptopurine deoxyribose (Bethesda Research Laboratories, Gaithersburg, MD) (35).

Interferon Supernatant. Mouse Con A supernatant (MCS) was prepared as a source of IFN- γ by culturing 3×10^6 B6AF₁ spleen cells/ml for 48 h (37°C/5% CO₂/air) in RPMI 1640 supplemented with 10% FCS, 5×10^{-5} 2-ME, and 5 μ g/ml Con A. The supernatants were collected, filter sterilized, and frozen (–70°C) until used.

M ϕ Cytotoxicity Assay. M ϕ cytotoxic activity was determined as previously described (36). Peritoneal cells were collected from mice injected 3 d previously with aged Brewer's thioglycollate medium (10% wt/vol) (Difco Laboratories, Inc., Detroit, MI). The cells were washed twice and adjusted to 2×10^6 cells/ml in ice-cold HBSS and 100- μ l aliquots were plated into 96-well flat-bottomed microtiter plates (Costar 3596; Rochester Scientific Co.,

Rochester, NY). After a 1.5-h incubation (37°C, 5% CO₂/air) the cultures were washed vigorously four times with warm HBSS to remove nonadherent cells, the wash medium was removed, and 100 μ l of assay medium (RPMI 1640 plus 10% FCS plus 10 U penicillin/ml plus 100 μ g streptomycin/ml) either alone or containing additional reagents was added to the adherent monolayers. The monolayers consisted of >95% M ϕ as determined by morphology, Diffquick staining and phagocytosis of latex beads (Sigma Chemical Co., St. Louis, MO). After a 4-h incubation (37°C, 5% CO₂/air) 10^4 ¹¹¹In-labeled L5178Y cells in 100 μ l assay medium were added to the cultures. Cells were labeled by adding 10 μ Ci [¹¹¹In]-Oxine (1 mCi/ml, sp act 10 mCi/ μ g In) (Amersham, Oakville, Ontario, Canada) to 7.5×10^6 cells in 0.5 ml RPMI 1640 plus 10% FCS at 22°C for 10 min, before washing and resuspending the cells in assay medium. After a 48-h incubation (37°C, 5% CO₂), the plates were centrifuged (5 min at 500 g) and 100- μ l aliquots of supernatant were removed and counted in a gamma counter (LKB, Turku, Finland). The percent specific lysis was calculated from six replicates as follows: $100 \times [(test\ cpm - spontaneous\ cpm)/(total\ cpm - spontaneous\ cpm)]$. Spontaneous release cultures contained normal M ϕ monolayers and target cells in assay medium only. Total cpm was determined from cultures of 10^4 labeled target cells in 100 μ l assay medium resuspended with 100 μ l of 4% NP-40 (BDH Chemicals, Montreal, Quebec, Canada).

TNF Assay. Blood samples were collected by the cardiac puncture of mice under ether anesthesia. The blood was allowed to clot at 4°C overnight and the serum was separated and frozen at –70°C until assayed. Serum TNF activity was determined using an L-929 cytotoxicity assay (37). Subconfluent monolayers of L-929 cells were trypsinized, washed, and 6×10^4 cells/well in RPMI 1640 plus 10% FCS were plated into 96-well microtiter plates (Costar Data Packaging, Cambridge, MA). The cells were allowed to adhere for 1 h (37°C/5% CO₂), the supernatant was removed, and 50 μ l of RPMI 1640 supplemented with 10% FCS and 2 μ g/ml actinomycin D (Sigma Chemical Co.) were added. Twofold serial dilutions of serum samples in 50 μ l of RPMI 1640 + 10% FCS were added to the cultures and after an 18-h incubation (37°C/5% CO₂), the cell viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) (38). MTT product that was reduced during a 4-h incubation was solubilized by the addition of 100 μ l 0.04 N hydrochloric acid in isopropanol followed by 100 μ l of distilled water. Reduced MTT was measured on an EAR 400 AT ELISA plate reader (SLT Lab Instruments, Salzburg, Austria) at 570 nm (690 nm reference wavelength). The concentration of TNF in samples was determined from a standard curve of recombinant murine TNF- α (Genzyme Corp.).

Rabbit Anti-murine TNF- α . Rabbit anti-murine TNF- α was generously provided by Dr. Anthony Cerami, Dr. Kirk Manogue, and Dr. Barbara Sherry (Laboratory of Medical Biochemistry, The Rockefeller University, New York) (25). The ability of rabbit anti-murine TNF- α to inhibit serum TNF activity was determined by the addition of 5 μ l of antiserum to L929 cultures before the addition of serum samples. Inhibition of M ϕ -mediated cytotoxicity was determined by the addition of 5 μ l of antiserum to M ϕ monolayers 1 h before the addition of L5178Y target cells. Cytotoxic function of IFN- γ plus LPS-activated M ϕ was inhibited 98% by the antiserum.

LPS Determination. The chromogenic limulus amoebocyte lysate assay, (Whittaker Bioproducts, Inc., Walkersville, MD) was used to assay for serum LPS. Sterile serum samples diluted 1:200 with pyrogen-free water (Whittaker Bioproducts, Inc.) and further twofold serial dilutions prepared with pyrogen-free water con-

taining 0.5% normal B6AF1 mouse serum were assayed. Colored reaction product was quantified at 405 nm on an EAR 400 AT ELISA plate reader (SLT Labinstruments). LPS concentrations in samples were interpolated from a LPS standard curve prepared from *E. coli* 0111:B4 (Calbiochem Corp.) diluted in pyrogen-free water supplemented with 0.5% normal B6AF1 mouse serum.

Results

GVHD-induced Immunosuppression and Mortality. Non-irradiated B6AF1 mice transplanted with either 30 or 60 × 10⁶ B6 lymphoid cells developed GVHD as characterized by the onset of severe immunosuppression. Spleen cells from either transplanted group failed to produce any significant antibody response after injection on day 10 with a T cell-dependent antigen, SRBC (Table 1).

GVHD-induced mortality was, however, dependent upon the number of B6 cells injected. Mortality was first observed on day 17 posttransplant. By day 35 the mortality within the group receiving 60 × 10⁶ cells (acute GVH) was 71%, whereas only 4% of animals transplanted with 30 × 10⁶ cells (nonlethal GVH) had died.

Mφ Priming during GVHD: LPS-triggered Expression of Mφ-mediated Cytotoxic Activity. The state of Mφ activation was examined to determine whether an alteration in Mφ function was related to the symptoms characteristic of acute GVHD. Activation of Mφ cytotoxic activity occurs via a two-stage process. After exposure to IFN-γ, Mφ are primed and are then responsive to a second triggering signal provided by LPS. It is only after LPS triggering that the primed cells become cytotoxic (11). On day 14 after transplantation, Mφ isolated from animals undergoing an acute, lethal GVHD expressed cytotoxic activity when incubated with 2.5 ng/ml LPS, thus indicating that the cells had been primed (Table 2). Cytotoxic activity against L5178Y target cells appeared to be dependent upon the amount of LPS that the effector cells were exposed to. As expected, normal Mφ were non-cytolytic unless incubated with MCS, as a source of IFN-γ, and LPS. The absence of cytotoxic activity against the NK cell-sensitive/macrophage-resistant tumor cell lines YAC-1

and MDW4 (36) demonstrated that lytic function was not mediated by NK cells (data not shown).

It has been reported previously that IFN-γ and LPS affect Mφ in a synergistic manner such that cells exposed to greater concentrations of IFN-γ require less LPS in order to be triggered (10). The LPS dose-responses of Mφ from animals undergoing acute and nonlethal GVHD were therefore compared. As can be seen in Fig. 1, on day 12 after transplantation, the concentration of LPS required for expression of cytotoxic activity by Mφ from B6AF1 mice receiving 60 × 10⁶ B6 cells was ~100 times lower than that required to trigger a similar response in Mφ from B6AF1 mice transplanted with a nonlethal dose of 30 × 10⁶ B6 cells. Mφ from F1 mice transplanted with 60 × 10⁶ B6 cells expressed maximal cytotoxic activity in the presence of 5 ng/ml LPS, whereas

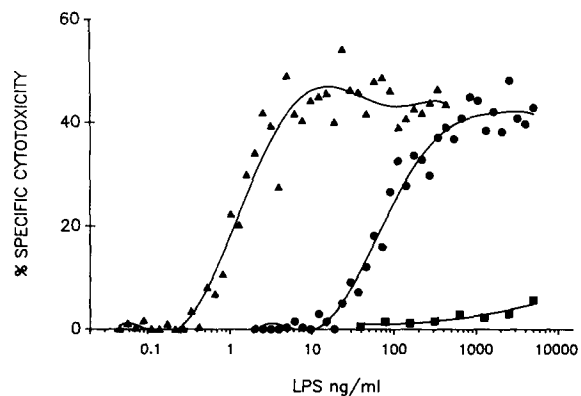


Figure 1. Mφ cytotoxic activity in response to LPS during acute and nonlethal GVHD. Mφ-mediated cytotoxic activity was determined against ¹¹¹In-labeled L5178Y target cells in a 48-h assay as described in Materials and Methods. LPS dose-response of Mφ from normal B6AF1 mice (■), and from B6AF1 mice 12 d after transplantation with 60 × 10⁶ B6 lymphoid cells (▲) or 30 × 10⁶ B6 lymphoid cells (●). Each value represents the mean of six replicates of pooled Mφ effector cells. Similar results were obtained in two separate experiments.

Table 1. Immunosuppression and Mortality during GVHD

Donor strain	Recipient strain	No. of cells transplanted	Total PFC to SRBC per spleen*	Mortality (dead/total)		
				7 d [†]	14 d	35 d
			× 10 ⁻³			
B6	B6AF ₁	60 × 10 ⁶	0.05 ± 0.05	0/60	0/60	43/60
B6	B6AF ₁	30 × 10 ⁶	0.1 ± 0.06	0/60	0/60	2/60
B6AF ₁	B6AF ₁	60 × 10 ⁶	126.8 ± 20.4	0/20	0/20	0/20
-	B6AF ₁	-	111.6 ± 25.6		ND	

* On day 10 after transplantation representative experimental mice from each group were injected with 5 × 10⁸ SRBC and 4 d later the number of PFC from individual spleens was determined. The values presented represent the mean ± SEM determined from six to nine mice.

[†] Days after transplantation.

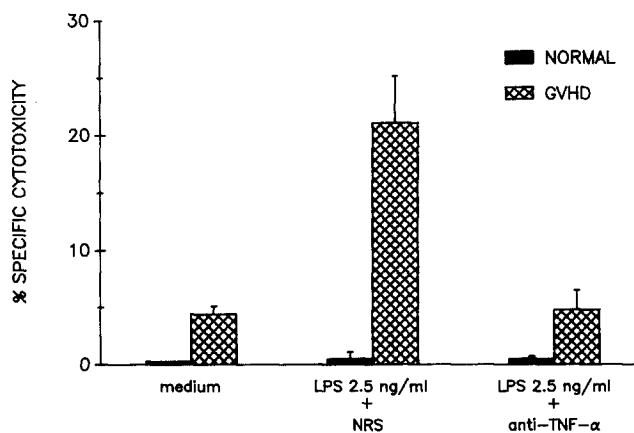


Figure 2. Inhibition of GVH-primed and LPS-triggered M ϕ cytotoxic activity by anti-murine TNF- α . M ϕ -mediated cytotoxicity against ^{111}In -labeled L5178Y target cells was determined in a 48-h assay as described in Materials and Methods. M ϕ were isolated from normal B6AF $_1$ mice or B6AF $_1$ mice 14 d after transplantation with 60×10^6 B6 lymphoid cells. 5 μl of normal rabbit serum (NRS) or anti-murine TNF- α was added 1 h before addition of target cells. Results represent the mean percentage specific cytotoxicity \pm SEM from three experiments.

500 ng/ml LPS was required to trigger a similar response in M ϕ from F $_1$ mice transplanted with 30×10^6 B6 cells. The results clearly indicate that M ϕ priming occurred to a greater extent during acute GVHD. M ϕ from normal F $_1$ animals expressed only minimal cytotoxic function even in the presence of 5,000 ng/ml LPS.

M ϕ -mediated killing of L5178Y target cells has been shown

Table 2. Activation of M ϕ Cytotoxic Activity by LPS during Acute GVHD

Treatment in vitro	M ϕ -mediated specific cytotoxicity against L5178Y*	
	Normal B6AF $_1$	Acute GVHD B6AF $_1$ †
	%	
Medium	0.5 \pm 0.9	3.7 \pm 1.3
LPS (2.5 ng/ml)	-1.7 \pm 0.4	30.9 \pm 3.8
LPS (50 ng/ml)	-2.0 \pm 3.6	55.5 \pm 4.0
IFN §	1.6 \pm 1.4	6.7 \pm 0.7
IFN § + LPS (2.5 ng/ml)	53.0 \pm 3.3	54.3 \pm 4.2

* $\bar{X} \pm$ SEM of three experiments. M ϕ -mediated cytotoxicity was determined against ^{111}In -labeled L5178Y target cells in a 48-h assay as described in Materials and Methods.

† B6AF $_1$ animals were transplanted with 60×10^6 B6 cells and their peritoneal M ϕ were collected and used on day 14 posttransplant.

§ IFN from mouse Con A supernatant was added at a concentration of 200 $\mu\text{l}/\text{ml}$. Activation of normal M ϕ -mediated cytotoxic function by MCS, in combination with LPS, was completely inhibited by the addition of R46A2 mAb against murine IFN- γ .

Table 3. Lethal Effect of LPS during GVHD

Donor strain	Recipient strain	No. of cells transplanted	LPS*	Mortality within 36 h of injection (dead/total)	
				7 d†	14 d
B6	B6AF $_1$	60×10^6	μg		
			10	24/24	24/24
			2	1/12	13/22
B6	B6AF $_1$	30×10^6	0	0/12	0/12
			10	6/24	7/24
			2	ND	1/24
B6AF $_1$	B6AF $_1$	60×10^6	0	0/12	0/12
			10	0/12	0/12
			100	0/6	0/6
-	B6AF $_1$	-	10	0/12	0/12

* Animals were injected intravenously with LPS in 0.2 ml PBS or PBS alone.

† Days after transplantation.

to be mediated by TNF- α , a protein produced and released by M ϕ after activation with IFN- γ and LPS (12). As is demonstrated in Fig. 2, addition of anti-murine TNF- α to the M ϕ cytotoxicity assay completely inhibited the LPS-triggered cytolytic activity of M ϕ primed during the acute GVHD, confirming that it was mediated by TNF- α .

Sensitivity to LPS is Related to the Severity of the GVHD. Since we demonstrated in vitro that the degree of M ϕ priming was related to the severity of the GVHD, we next investigated the in vivo effect of LPS. The lethal effects of LPS were related to the length of time after transplantation, the number of lymphoid cells transplanted, and the dose of LPS. In acute GVH-reactive mice, intravenous injection of 10 μ g of LPS, either 7 or 14 d after 60×10^6 B6 cells were transplanted, resulted in the death of 100% of the animals within 36 h (Table 3). The animals characteristically developed a hunched posture, piloerection, and severe diarrhea within 2–6 h after LPS injection. A dose of 2 μ g of LPS on day 7 resulted in 1 of 12 animals dying, whereas greater than 50% died, given the same dose on day 14. LPS also had a significant effect on animals transplanted with 30×10^6 B6 cells. Although the injection of 2 μ g on day 14 resulted in the death of only 1 of 24 animals, \sim 25% died after injection with 10 μ g of LPS either 7 or 14 d posttransplant. Mortality was not observed in normal B6AF₁ mice injected with either 10 or 100 μ g of LPS (Table 3).

TNF- α Production during GVHD: Triggered Release by LPS. To determine whether TNF was produced in response to LPS and also to induce maximal TNF release, animals were in-

Table 5. Inhibition of LPS-induced Serum TNF from Acute GVHD Mice by Anti-murine TNF- α

Day injected with LPS [†]	No. of mice	Serum TNF activity*	
		Anti-TNF- α [§]	Control
		<i>U/ml</i> $\times 10^{-3}$	
7	5	<0.20	18.4 \pm 8.3
14	5	<0.20	63.3 \pm 9.7

* $\bar{X} \pm$ SEM from individually determined samples. TNF activity was determined using the MTT dye reduction assay on actinomycin D-treated L929 cells as described in Materials and Methods.

[†] B6AF₁ animals transplanted with 60×10^6 B6 cells were injected intravenously with 25 μ g LPS in 0.2 ml PBS on the day posttransplant indicated. 2 h later blood samples were collected as described in Materials and Methods.

[§] 5 μ l of rabbit anti-murine TNF- α was added to TNF assay cultures immediately before addition of serum samples.

^{||} 5 μ l of normal rabbit serum (NRS) was added to the TNF assay cultures. NRS did not itself have any cytotoxic activity against L929 cells.

jected intravenously with 25 μ g of LPS and bled 2 h later (Table 4). In acute GVH-reactive mice, injection of LPS 7 d after transplantation induced serum TNF levels of 14,759 U/ml. On day 14, LPS-triggered serum TNF levels were \sim 3.5-fold greater. Mice receiving a nonlethal transplant of 30×10^6 B6 cells produced significantly lower concentra-

Table 4. LPS-triggered Production of Serum TNF during GVHD

Donor strain	Recipient strain	No. of cells	LPS [†]	7 d*		14 d	
				No. of mice	TNF [§]	No. of mice	TNF [§]
					<i>U/ml</i> $\times 10^{-3}$		<i>U/ml</i> $\times 10^{-3}$
B6	B6AF ₁	60×10^6	+	5	14.8 \pm 7.4	9	52.9 \pm 8.8 ^{††}
			-	7	<0.02	10	<0.02
B6	B6AF ₁	30×10^6	+	6	1.3 \pm 0.5	10	12.0 \pm 5.9
			-	4	<0.02	5	<0.02
B6AF ₁	B6AF ₁	60×10^6	+	ND		6	0.08 \pm 0.08
			-	ND		9	<0.02
-	B6AF ₁	-	+	10	0.5 \pm 0.3	10	<0.02
			-	5	<0.02	10	<0.02

* Days after transplantation.

[†] Animals were injected intravenously with 25 μ g LPS in 0.2 ml PBS or PBS alone. 2 h later blood samples were collected as described in Materials and Methods.

[§] $\bar{X} \pm$ SEM of individually determined samples. TNF activity was determined using the MTT dye reduction assay on actinomycin D-treated L929 cells as described in Materials and Methods.

^{||} $p < 0.005$ on day 7 as compared with day 14 after transplantation with 60×10^6 B6 cells.

^{††} $p < 0.001$ on day 14 after receiving 60×10^6 B6 cells as compared with day 14 after receiving 30×10^6 B6 cells.

Table 6. Presence of Endogenous LPS in the Serum of Acute GVHD Reactive Mice

Days posttransplant*	Mortality	Positive for LPS [†]	No. of animals tested	LPS
	%	%		pg/ml [§] × 10 ⁻³
10	0	0	8	<0.12
12	0	0	8	<0.12
14	0	0	8	<0.12
16	14.9	40	15	2.2 ± 1.5

* 142 B6AF₁ mice were transplanted with 60 × 10⁶ B6 lymphoid cells. Animals were randomly selected for testing on the day posttransplant indicated.

[†] Serum samples were collected aseptically and tested for LPS as described in Materials and Methods.

[§] $\bar{X} \pm$ SEM of LPS-positive samples.

tions of serum TNF. As is shown in Table 4, neither normal nor syngeneic transplanted B6AF₁ animals produced significant amounts of TNF after LPS injection. Spontaneously released TNF could not be detected in the serum of any of the GVH-reactive animals injected with PBS. The TNF in the sera of acute GVH-reactive mice injected with LPS was determined to be entirely TNF- α , as its activity was completely inhibited by the addition of anti-murine TNF- α (Table 5).

Detection of Endogenous Serum LPS during Acute GVHD To determine whether endogenous LPS was present in B6AF₁ mice during acute GVHD, serum samples were assayed for LPS at various times after transplantation with 60 × 10⁶ B6 lymphoid cells (Table 6). In a large group of transplanted animals, the first death occurred on day 16 posttransplant. LPS was detected in the serum of 40% of randomly selected animals on day 16, but not at any of the prior time points. By day 18 posttransplant, 2 d after LPS could be detected in the serum, mortality increased to 49%. LPS bound to lipoproteins (39) or to LPS-binding protein (40) in the serum may be much less active than unbound LPS in the limulus assay, therefore, the results presented are perhaps a conservative estimate of the actual levels of serum LPS.

Discussion

Our results demonstrate that M ϕ are primed during the course of the GVHD and that the level of M ϕ priming is greater in mice undergoing acute lethal as opposed to non-lethal GVHD. As a consequence of M ϕ priming, normally insignificant amounts of LPS trigger TNF- α release into the serum leading to death of acute GVH-reactive animals and in vitro result in the expression of M ϕ -mediated tumor cytotoxicity. Coincident with the time of onset of mortality, endogenous LPS can be detected in the serum of acute GVH-

reactive animals. The results demonstrate that M ϕ can act as a source of TNF- α during GVHD.

IFN- α , IFN- β , and IFN- γ can activate or prime M ϕ and there is evidence of production of all three types of IFN during GVHD (14, 15, 41). The greatest priming effect on M ϕ is known to be mediated by IFN- γ (42). M ϕ were found to be primed to a much greater extent during the acute as compared with the nonlethal GVHD (Table 2 and Fig. 1). The importance of IFN- γ within the network of cellular and cytokine interactions that occurs during GVHD is demonstrated by the reduction in the severity of GVHD-induced intestinal lesions after anti-IFN- γ treatment (16).

Priming of normal M ϕ results in a large reduction in the amount of LPS needed to trigger both TNF- α release and M ϕ -mediated cytotoxic function (10–12, 28). F₁ mice injected with 60 × 10⁶ parental strain cells were especially sensitive to LPS since all of the animals died within 36 h of injection of 10 μ g LPS whereas normal F₁ animals were resistant to 100 μ g LPS (Table 3). The terminal symptoms characteristic of acute GVHD occurred soon after intravenous injection of 10 μ g of LPS into animals transplanted 7 or 14 d previously with 60 × 10⁶ B6 cells. The symptoms, including a hunched posture, piloerection, and diarrhea, are also characteristic of animals injected with TNF- α (43).

Significant amounts of TNF- α were found in the serum of animals undergoing GVHD 2 h after an intravenous injection of LPS (Table 4). The amount of serum TNF- α was related to the number of donor cells transplanted and time posttransplant. Without the administration of LPS, TNF- α could not be detected in the serum of any of the GVH-reactive animals examined. However, a reduction in mortality and epithelial lesions does occur in GVH-reactive mice treated with anti-TNF- α , despite the absence of detectable serum TNF (29). The absence of circulating TNF- α may be due to rapid binding of TNF- α by liver, kidney, skin, and gastrointestinal tissues resulting in a short serum half-life (44, 45). In human allogeneic bone marrow recipients, TNF- α can be detected in the serum (30) and does appear to be associated with GVHD (31, 32).

Although priming of M ϕ facilitates their ability to produce TNF- α , LPS is required to trigger TNF- α release from M ϕ (12). Endogenous LPS was found in the serum of animals undergoing acute GVHD and the time of detection was coincident with the onset of mortality (Table 6). Translocation of Gram-negative bacteria across the damaged gastrointestinal epithelium and into the portal circulation may be the prime source of LPS during GVHD. Increased bacterial translocation has been demonstrated in immunosuppressed animals (46). Thus the immunosuppression and lesions of the intestinal epithelium that are characteristic of GVHD would promote the translocation process. Augmentation of M ϕ -mediated bactericidal and phagocytic activity during GVHD (8, 9) prevents the occurrence of active infections during the period of severe immunosuppression associated with GVHD, yet at the same time, results in the presence of LPS in the serum. Under normal circumstances, LPS is rapidly taken up in the liver (47), however, the appearance of LPS in the serum on day 16 posttransplant suggests that the liver's ability to scav-

enge LPS has become saturated. During GVHD, interaction between LPS and primed M ϕ that accumulate in a number of organs, including the liver and spleen (4, 48), would provide a ready source of TNF- α . Although T cells, NK cells, and mast cells can produce TNF- α (49–51), M ϕ are a principal source of TNF- α and the only cell type known to release the cytokine in response to LPS.

The possibility that LPS may mediate some of the symptoms characteristic of GVHD has been suggested by a number of observations. The hepatic lesions that develop in mice during experimental endotoxemia are strikingly similar to those that occur during GVHD (52). A significant reduction in the incidence and severity of GVHD can be obtained through gastrointestinal decontamination, germ-free conditions, and protective laminar flow environments (19–21). In (C57BL \times CBA)F₁ mice transplanted with CBA lymphoid cells two mechanisms, one associated with LPS and the other LPS independent, have been demonstrated to be involved in GVHD-associated tissue injury (53). Furthermore, passive immunization of mice with an antiserum to that portion of the LPS molecule common to all forms of LPS improved survival after acute GVHD (54).

It appears that at least three phases are involved in the development of acute GVHD. The initial afferent phase that occurs during the lymphoproliferative response to alloantigen involves the excessive production of cytokines, including IFN- γ (13–15) produced by CD4⁺, CD8⁺, and/or NK cells (55). As a result of the interaction with released cytokines, NK or NK-like cells are activated and priming of M ϕ occurs. The second phase involves an initial injury to epithelial tissues, including the epithelium of the gastrointestinal tract, which appears to be mediated by activated NK or NK-like cells (56–59). In addition, primed M ϕ may produce TNF- α as a result of interaction with glycosylated proteins released from damaged tissue and thus also contribute to the initial tissue

destruction (60). In the third phase, as a result of injury to the gut epithelium, increasing amounts of Gram-negative bacteria enter the portal circulation and are rapidly taken up and killed by fixed M ϕ in the liver (i.e., Kupffer cells) and splenic M ϕ . Bacterial-derived LPS from the killed Gram-negative bacteria then triggers the release of nonphysiologic amounts of TNF- α from primed M ϕ resulting in cachexia, further tissue injury, shock, and death of the transplant recipient. The three phases therefore include an afferent phase that leads to the activation of two different effector cells, NK or NK-like cells and M ϕ , which in turn mediate the two efferent phases of tissue damage. The evidence supportive of the mechanisms described here includes the prevention of GVHD-related pathologic lesions and immunosuppression after treatment with anti-IFN- γ (16, 61), or after elimination of NK or NK-like cells (56–59), prevention of pathologic lesions by treatment with anti-TNF- α (29), and reduction of tissue injury through bacterial decontamination (19–21, 53).

The consequences of M ϕ priming can be beneficial or detrimental depending upon the degree to which the cells have been primed. In the case of nonlethal GVHD, relatively low levels of M ϕ priming and the ability to produce TNF- α were observed. TNF- α can augment host resistance to bacterial, viral, and fungal infections (62–64) and therefore a moderate degree of M ϕ priming is beneficial during GVHD. In the present study animals with nonlethal GVHD survived for >6 mo in a conventional housing facility despite severe prolonged immunosuppression. TNF- α production by primed M ϕ during nonlethal GVHD may also be the underlying mechanism of the graft-versus-leukemia effect which results in a decreased incidence of leukemic relapse in bone marrow transplant recipients with low-grade GVHD. Conversely, excessive priming of M ϕ resulting in overproduction of TNF- α can lead to progressive GVHD and death of the transplant recipient.

We thank Drs. Anthony Cerami, Kirk Manogue, and Barbara Sherry from the Laboratory of Medical Biochemistry, and The Rockefeller University for generously providing rabbit anti-murine TNF- α . We also thank Michel Emond, Ailsa Lee Loy, and Rosmarie Siegrist-Johnstone for their expert technical assistance, and Christine Pamplin for typing the manuscript.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. K. S. Price is the recipient of a studentship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Quebec.

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Received for publication 16 September 1991 and in revised form 16 October 1991.

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