The Role of Lipopolysaccharides during Acute Graft-Versus-Host Disease

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ABSTRACT

In this study we have shown that translocation of intestinal LPS into the portal and systemic circulatory systems during graft-versus-host disease (GVHD) is predictive of morbidity and mortality. Randomized C57BL/6 x AF1 ($B6AF_1$) mice were injected with C57BL/6 (B6) lymphoid cells and sacrificed at predetermined times after transplantation for bacterial lipopolysaccharide (LPS) tissue analysis. The liver, the spleen and the sera from some acute GVH reactive mice first tested positive for LPS from day 2, 4 and 16 post transplant, respectively. Total hepatic and splenic LPS in acute GVH reactive mice peaked, and LPS was first detected in the sera of these same animals, at a time coincident with the onset of mortality. These results show that LPS is present to initiate tumour necrosis factor alpha (TNF-α) release from interferon gamma (IFN-γ) primed macrophages, resulting in the manifestations of acute GVHD.

RESUME

Cette étude démontre que l'entrée du lipopolysaccharide (LPS) intestinal dans [illegible] culations hepatique et systémique au cours de la maladie de [illegible] "hôte (GVHD) prédit la mortalité. Des souris C57BL/6 x AF1 [illegible] au hasard ont été injectées de cellules lymphatiques [illegible] ur ensuite être sacrifiées à des temps prédéterminés pour l'analyse de la présence du LPS dans leurs tissus. Le foie, la rate et le sérum des animaux atteints de la maladie GVHD ont démontré des taux croissants de LPS à partir de 2, 4 et 16 jours, respectivement. Les taux de LPS dans les organes ont plafonné et LPS est apparu pour la première fois dans le sérum à un temps coincident avec le début de la mortalité. Ces résultats démontrent que LPS entre dans la circulation hépatique et ensuite la circulation systémique au cours de la maladie GVHD. Ceci appuit notre hypothèse, qui stipule que LPS stimule la production de TNF-α par les macrophages exposés à l'interféron, ainsi produisant les symptômes de la maladie GVHD aigué.

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LIST OF ABBREVIATIONS

Apo/B:	Apo receptor B
$B6AF_1$.	$C57BL/6xAF_1$
B6:	C57BL/6
BPI:	bactericidal permeability increasing protein
CD14:	cluster differentiation factor 14
CR3:	complement receptor 3
GVHD:	graft-versus-host disease
HBSS:	hank's balanced salt solution
HDL:	high density lipoprotein
IL-1:	interleukin 1
IL-6:	interleukin 6
IFN-γ:	interferon gamma
LAL:	limulus amoebocyte lysate
LBP:	LPS binding protein
LDL:	low density lipoprotein
LFA-1:	leukocyte function associated 1
LPS:	lipopolysaccharide

m-RNA	messenger ribonucleic acid
NK:	natural killer
p95	glycoprotein 95
p150	glycoprotein 150
PFC:	plaque forming cell
PGE.	prostaglandin E
SRBC:	sheep red blood cell
TNF-α:	tumour necrosis factor alpha

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INTRODUCTION

GVHD

This review although not extensive will address graft-versus-host disease (GVHD) by looking at the cells and factors involved in the mechanism of disease, the triggering antigens and the effectors of pathology. More extensive book reviews of GVHD are available.[1,2]

GVHD History

GVH reactions were first observed at the turn of the century by Murphy[3], and then by Danchakoff[4] in chorioallantoic membrane grafting studies. Rat tumour cells grafted onto chick embryos along with adult avian spleen or bone marrow, caused enlargement and nodulation of host spleens. However, it was not until the 1950s that Simonsen[5] and Dempster[6], independently put forth the idea of graft cells mounting an immune response against host tissues. Each with their canine kidney allograft experiments, incorrectly hypothesized that lymphocytic and reticulo-

endothelial cell infiltrates in the renal cortex were of local graft origin. However, continued work by Simonsen[7] and murine tolerance induction experiments by Billingham[8,9] and Brent[8], finally affirmed the occurrence of the GVH reaction and established the requirements needed for the development of GVHD Lymphoid tissue hypoplasia, alopecia, diarrhoea and wasting were observed in newborn mice or chickens grafted with immunocompetent adult spleen cells. In 1966 Billingham[9] stated the three requirements for the development of the GVH reaction:

1) The cells of the graft must be immunologically competent.
2) The host must be incapable of mounting an immune response against the graft.
3) The host must differ genetically from the donor so that the graft may recognize and react against the foreign host.

During the last twenty years research on the development of the GVH reaction has shown that in addition to class II major histocompatibility (MHC) donor/host genetic incompatibility, class I MHC or minor histocompatibility antigen (MiHA) differences alone will induce GVH reactions.[10,11] Using different strain combinations it was found that GVH reactions of greater intensity are elicited when donor and host differ at the class II MHC region, or at both the MHC and MiHA.[12,13]

GVHD Assays

The classical assays used to measure the intensity of the GVH reaction include the splenomegaly assay, the weight loss and mortality assay, the immune suppression or plaque forming cell (PFC) assay to sheep red blood cells (SRBC), and the histopathological analysis of lymphoid and non-lymphoid tissues.

1) Splenomegaly

The consistent occurrence of splenomegaly early after GVH reaction induction led to the development of this assay.[7] Splenomegaly peaks 6 to 10 days after transplantation and is measured by calculating the splenic index Based on the fact that the ratio of spleen weight to the body weight is a constant, the splenic index is determined by the division of the spleen to body weight ratio of the experimental animals, by the spleen to body weight ratio of the control animals. Through convention, a spleen index greater than 1.3 is indicative of a significant degree of GVH reactivity.

2) Weight loss and Mortality

The degree of weight loss, the time of onset of death, and the total percentage of animals which succumb to the GVH reaction, are indicative of disease intensity and severity. The weight loss and mortality assays have shown that the donor/host strain combination employed, and the donor cell dose injected for the transplant, influence the severity of the resulting GVH reaction.[14]

3) Immune Suppression

The GVH reaction consistently induces suppression of both humoral and cell mediated immune responses. The PFC response to the T cell dependent antigen SRBC, is suppressed early after GVH reaction induction. The severity and time of peak suppression, are both strain and dose dependent. In the experiments presented in this thesis, the GVH reactions were induced by injecting 60×10^6 parental B6 strain lymphoid cells into $B6AF_1$ mice In this parent F_1 combination the PFC response to SRBC is maximally suppressed by day 10 post-transplant.[14]

Acute GVHD

GVHD can be acute or chronic in nature, depending on onset, severity, progression and target Chronic GVH reactive mice may exhibit slight weight loss, and survive longer than 50 days post transplant, whereas acute GVH reactive mice show severe weight loss, and die by day 35 post transplant.[12] Acute GVHD, which was the focus of our study, has an early onset, a rapid severe progression and targets lymphoid and epithelial tissues such as skin, liver and gut Mice develop a hunched posture, become cachectic, and have diarrhoea. Unfortunately, the etiology of acute GVHD is still controversial. The experimental model used to study GVHD is the parent -> F1 transplant scheme, in which the parental lymphoid cells can react against the antigens on the hybrid cells of the other parent, but the F1 hybrid does not recognize the transplanted parental cells as foreign. Work to date, suggest that effectors of immune suppression, and tissue damage during GVHD, include T, NK, NK-like cells and macrophages. Early suppression, appears to be monokine mediated, as an influx of macrophages to the spleen, lymph nodes and thymus produce large quantities of prostaglandin E. Later, thymic atrophy, and pathological changes to the adrenal glands, epithelial and lymphoid tissues, establish persistent immunosuppression [14]

GVHD Pathological injury

GVH-induced pathological lesions are observed in both lymphoid, and non-lymphoid tissues. The thymus, the spleen, the lymph nodes, the liver and the intestine of GVH reactive animals, show altered structure and impaired function In 1979, Seemayer[15] observed an initial enlargement of spleen and lymph nodes, followed by a progressive loss of structure, necrosis and tissue wasting The thymus suffered stress induced cortical involution, with loss of cortical-medullary demarcation and Hassal's corpuscles, medullary macrophage accumulation with lymphocytic invasion, and epithelial cell damage. In addition, Seemayer[15] demonstrated a lymphocytic infiltration of the liver's portal tract, bile duct and lobules, resulting in vacuolization and necrosis. Intestinal lymphoid infiltration was also seen, along with crypt epithelial cell damage. Mowat's[16] studies on intestinal GVHD, have shown an early increase in crypt cell mitotic activity, crypt and villus lengthening, and an increase in intraepithelial lymphocytes. Late during the GVH reaction, he observed villus atrophy and crypt cell necrosis, with loss of mucosal lymphoid cells.

In summary, GVH-associated tissue damage, may be due to direct targeting of lymphoid and epithelial cells, or due to an innocent bystander effect.[16] The antigen targeted by the GVH effector cell is unknown, however widespread increases in epidermal and epithelial Ia expression, may promote the GVH response Lymphoid tissues such as the spleen, the

thymus and the lymph nodes, are primary targets of the GVH reaction. Epithelial cells in the liver, the lungs, the gut, the tongue, the pancreas and the salivary glands, also appear to be major targets of GVH-induced injury [15] In particular, intestinal pathology involving diarrhoea, malabsorption and enhanced epithelial permeability to luminal contents including intestinal bacteria, is thought to be a central component of the disease process.[17-19] In support of a role for endotoxin, animals kept under germ-free conditions, or treated with antibiotics, or antibodies to LPS develop less severe GVHD [20-23]

GVHD Mechanism

Today, GVHD remains a major complication of allogeneic bone marrow transplantation, and is a distinctive syndrome characterized by immunosuppression, hepatosplenomegaly, wasting and mortality. Despite GVHD, bone marrow transplantation is the treatment of choice for disorders such as aplastic anaemia, severe combined immunodeficiency and leukaemia.[12,16] Unfortunately, the mechanisms behind the development of GVHD morbidity and mortality, following allogeneic bone marrow transplantation, have not been firmly established.

Studies attempting to identify the GVHD effector cells have implicated many cells including T, NK, NK-like cells and macrophages.[14] In 1957, Simonsen[7] induced GVH reactions using only white blood cells, and in 1962

Gowans[24] induced lethal GVH reactions in F_1 rats with small lymphocytes from the thoracic duct. Subsequent studies, showed that only lymphocytes from peripheral blood, thoracic duct, lymph nodes or spleen would induce GVH reactions. During the last ten years, using monoclonal antibodies and T cell clones, the potential of different T cell subtypes to induce GVH reactions has been studied. In particular, depletion studies by Korngold & Sprent[25], have shown that both helper and cytotoxic cells, are involved in the induction of GVHD across the entire H-2, or class I MHC differences, while GVHD induced across class II MHC differences, implicated helper cells only, and in contrast, GVHD induced across MiHA differences implicated mainly cytotoxic cells.

In addition to cells of the T lineage, NK cells have been implicated in GVHD pathogenesis. In 1981, Dokhelar et al.[26] noted an increase in NK cell activity during the onset of the GVH reaction. During the mid-eighties, several studies by Ghayur et al.[27,28] employing NK deficient beige (bg/bg) mutant mice, or anti-asialo GM-1 antibody, demonstrated a donor derived allo-induced ASGM-1+ population essential in the induction of GVH tissue damage.

During acute GVHD, macrophage function is activated, while T and B cell function is suppressed.[27] Macrophages were shown to be strongly activated, to produce tumour necrosis factor alpha (TNF-α), by interferon gamma (IFN-γ, presence demonstrated in both clinical and experimental GVHD) and bacterial LPS.[29-33] These two biological response modifiers, act

synergistically on macrophages; IFN-γ upregulates TNF-α m-RNA, thus priming the cell, for LPS triggered translation and release of TNF-α.[30-33]

Parent->F_1 model

The experimental system used to study acute GVHD, involves a single intravenous injection of parental strain pooled spleen and lymph node cells, into F_1 hybrids. The parent->F_1 hybrid model meets the requirements for the development of GVHD, while enabling the study of the GVH reaction without the use of irradiation or drugs, which may contribute to and confound the underlying mechanisms of the disease. The development of GVHD, requires that the donor cells be immunocompetent, and allogeneic to the recipient, and that the recipient be immunocompromised, and therefore unable to reject the cells received. When the above requirements are met, the grafted donor cells mount an immune response against the host, causing inflammation with excessive cytokine production, and injury to both lymphoid and non-lymphoid tissues. The strain combination used in this study, differed at the entire *H-2* complex (B6 -> B6AF_1). The parental B6 cells, will be allostimulated to mount a response against the A strain antigens expressed on the F_1 tissues. Previous work, has shown that the nature and severity of the resulting GVH reaction, is both strain and dose dependent. In this combination, 60 x 10^6 parental cells will cause an acute GVH reaction.

Parameters

The hallmarks of acute GVHD include, severe immunosuppression, hepatosplenomegaly, cachexia and high mortality. These parameters of GVHD were measured as described in the materials and methods. The presence of endotoxin or LPS, a structural component of the cell wall of gram negative bacteria was assessed, in the tissues of GVH reactive mice, using the Limulus Amoebocyte lysate system (LAL).

The LAL assay

In 1885, Howell[59] observed that the blood of the horseshoe crab, or Limulus polyphemus clots in the presence of endotoxin. In the 1950s, Bang's[60] discovery of the agent responsible for the clotting of Limulus blood, led to the development of Limulus amoebocyte lysate reagent. The LAL reaction is a cascade of enzymatic reactions, which culminate in the formation of a gel clot, as coagulogen substrate is cleaved. Comparative testing of endotoxin using the chromogenic and gel clot methods (described below), has shown that the results of the two procedures are very similar and within the accepted error of both tests.[61]

Macrophages

General properties

Macrophages are mobile phagocytic cells, that play effector roles in host defence, against microbes and tumours, inflammation, healing and cell mediated immunity. In addition to the classical properties of endocytosis, monocytes and macrophages have a large secretory repertoire which includes proteinases, hydrolases, enzyme inhibitors, arachidonic metabolites, lipoproteins, clotting factors, complement components, growth regulating factors, oxygen intermediates and endogenous pyrogens. The function of a given macrophage, will depend on its age, state of activation and location. In fact, macrophages make up a heterogeneous population, and within different organs the phenotype of the resident macrophage population will change. As discussed above, macrophages are activated during acute GVHD, and have been shown to produce TNF-α, resulting in symptoms characteristic of this disease.[34]

Tumour necrosis factor alpha(TNF-α)

Macrophages are the principal source of TNF-α *in vivo*. TNF-α is a protein that mediates many activities including cytotoxicity, cytostasis, shock and cachexia.[35,38] Reflecting its important role in numerous physiological processes, TNF-α and cachectin, once thought to be two separate molecules, were isolated independently. TNF-α was discovered at the turn of the century by Coley[39] by injecting *Streptococcus* and *Serratia* into

human tumours, and observing haemorrhagic necrosis and decreased tumour mass. Cachectin was discovered during the 1970s by Cerami[36], using trypanosomiasis as a model system for wasting or cachexia in chronic disease. Cerami[36,37] and his co-workers, showed that a transferable serum factor produced by macrophages, would suppress *de novo* triglyceride synthesis. Finally, independent research on TNF-α and cachectin culminated in 1985, when purification, sequencing and bioactivity studies, showed that these were identical molecules.[36]

TNF/cachectin is a 17-kDa protein, encoded by genes within the major histocompatibility complex on chromosome 6 in humans and chromosome 17 in mice.[40] Synthesized as a propeptide, TNF-α is cleaved and secreted as a 157 residue protein, which aggregates into a trimeric active form. Many cells including T lymphocytes, NK cells and smooth muscle cells have been reported to produce TNF-α; however, the main source of TNF-α *in vivo*, is the macrophage.[41] Macrophages in the lung, the liver, the peritoneum and the bone marrow, have been shown to produce TNF-α.[37] TNF-α gene transcription, is upregulated by IFN-γ and LPS; however, biosynthesis is regulated posttranscriptionaly, possibly through effects on mRNA stability.[40] Glucocorticoids may also play an influential role as potent inhibitors of TNF-α biosynthesis.[40,41] Once secreted, TNF-α has a very short half-life. The molecule is rapidly sequestered by TNF-α receptors, which are found on all somatic tissues except red blood cells. In addition to metabolic effects resulting in fat

resorption, TNF-α alters immune function.[40] The effects of TNF-α at the cellular and tissue levels are widespread. The multitude of metabolic effects of TNF-α include. decreased adipocyte expression of lipoprotein lipase, inhibition of fat cell differentiation, transcriptional suppression of various adipose tissue-specific proteins, decrease in muscle cell transmembrane potential, increased in muscle cell glucose transport, and neutrophil activation. Neutrophils exposed to TNF-α become more phagocytic, endothelial adherent and cytotoxic. TNF-α is directly cytotoxic to endothelial cells. TNF-α stimulates endothelial production of IL-1 and procoagulant factor, and decreases the production of thrombomodulin, possibly leading to disseminated intravascular coagulation, hemorrhagic necrosis, and thrombosis. TNF-α also suppresses erythropoiesis, has a direct and indirect pyrogenic effect on the neurons of the hypothalamus, stimulates bone resorption, causes cartilage degradation, induces the production and release of PGE-2, collagenase, acute-phase reactants, and suppresses albumin and transferrin synthesis.

TNF-α/cachectin has been implicated in the pathogenesis of many diseases, including malaria and GVHD.[36] In fact, antibodies to IFN-γ, LPS and TNF-α have been shown to decrease the severity of GVHD.[38,40-43]Using the parent into F_1 model, we have recently shown that GVHD morbidity and mortality is associated with increased TNF-α serum levels, and that GVHD macrophages are primed and produce lethal amounts of TNF-α when triggered intravenously with LPS.[34]

LPS

In 1936, Shear and Andervont[44] discovered that bacterial polysaccharide was the bacterial component causing haemorrhagic necrosis of murine transplantable tumours. Today, bacterial lipopolysaccharide (LPS), a primary component of the outer membrane of gram-negative bacteria, is known to mediate shock and wasting through stimulation of TNF-α in many disease states.[36,40,41] This amphiphilic compound, composed of a conserved lipid A molecule and highly variable polysaccharide core and species specific side chain regions, is prevalent in the mammalian gastrointestinal tract. The bioactivities of bacterial LPS, which include lethality, local Shwartzman reaction and pyrogenicity, are carried out by its lipid A portion. The lipid A acylation and phosphorylation pattern, and the polysaccharide chain length determine the toxicity of each LPS strain.[45] The supramolecular structure of LPS, is also believed to influence its pathophysiological effects. X-ray diffraction and fluorescence polarization studies, have shown that LPS plays a role in the outer membrane, architecture and function of gram-negative bacteria. The well ordered, dense and rigid arrangement of lipid A and associated proteins confer great impermeability to the bacterial outer membrane.[46] Shed LPS forms aggregates, which are believed to take on lamellar and micellar states. Clearance of endotoxin is believed to involve disaggregation, and uptake by lipoprotein and protein LPS ligands.[45,46]

LPS Clearance

In vivo, LPS clearance is managed by low (LDL) and high density lipoproteins (HDL) and a family of LPS binding proteins which include: macrophage scavenger receptors (apo/B, CR3, LFA-1, p150 and p95), neutrophil bactericidal / permeability increasing protein (BPI) and hepatocyte LPS binding protein (LBP).[47-58] The removal of LPS from the blood is biphasic in nature. The initial phase of clearance, due to phagocytic uptake of LPS by liver Kupffer cells, splenic and alveolar macrophages and leukocytes has a $T_{1/2}$ of 15 minutes. The second phase of clearance, due to removal by the adrenals and liver by HDL and LDL respectively has a $T_{1/2}$ of 15 hours.[47] Studies have shown that these lipoproteins prolong the half life of LPS in the serum, reduce its toxicity by inhibiting LPS stimulated TNF-α, IL-1 and IL-6 monokine secretion and direct its uptake to tissues that metabolize cholesterol (HDL) and contain macrophages (LDL/LPS complex uptake via macrophage scavenger receptors).[47]

Acute phase proteins interfere with LPS lipoprotein complex formation, more specifically LBP complexes reversibly with LPS, delaying the ultimate formation of LPS/lipoprotein complexes.[48,50] LBP/LPS complexes bind the CD14 macrophage receptor, stimulating TNF-α production through a yet to be determined signalling mechanism.[52,53]LPS mediates shock and multi-organ failure through its stimulation of TNF-α production.[37]

The liver plays a crucial role in the slow elimination of endotoxin from the system. Labelling studies have shown that LPS is directed towards the Kupffer cells of the liver, from which it passes sequentially to the hepatocytes, the gall bladder and finally, is found in the faeces virtually undegraded (macrophages and neutrophils deacylate and dephosphorylate the lipid A portion of LPS).[47,56,57]

The Purpose of This Study

In a recent paper, we proposed a mechanism for acute GVHD, in which alloantigen induced cytokine production activates NK-like GVH effector cells, and primes macrophages. Subsequently, epithelial injury mediated by GVH effector cells, results in gram-negative bacterial translocation from the gut, through the portal system to the liver, and later to the spleen and the systemic circulation. Finally, LPS release due to the killing of bacteria, by primed hepatic and splenic macrophages, results in the triggering of excessive TNF-α release, causing additional tissue injury, and resulting in mortality.[34]

In order to substantiate the model described above, we set out to determine if LPS appeared in mice during the natural course of the GVH reaction. In this thesis, the appearance of LPS in the tissues of GVH reactive animals is described. The results demonstrate, that early during the course of acute GVHD, and prior to weight loss, LPS appears, first in the liver and then in the spleen of GVH reactive mice; and that late in the course of acute GVHD, and concomitant with the onset of mortality, LPS levels rise in the organs, and LPS is detected in the serum.

METHODS

Experimental Design

Purpose

In order to determine if bacterial LPS is present during the course of acute GVHD randomized GVH reactive mice were sacrificed at predetermined times post transplant for tissue endotoxin analysis. Animal weight, immune function, spleen index, liver index and survival were recorded as parameters of GVHD severity.

Transplant

Mice

Male C57BL/6 (B6), C57BL/6 x AF1 (B6AF1) and CBA mice were bred and maintained in our laboratory and used at 12-18 wk of age.

Experimental Design

To determine if bacterial LPS was present during the course of acute GVHD 142 randomized recipients of 60 x 10^6 spleen and lymph node (lymphoid) cells were sacrificed at predetermined times post transplant for

tissue endotoxin analysis. Early during the course of the GVH reaction 8 animals were sacrificed per time point (days 2,4,8,10,12 and 14), following the onset of mortality 12-15 GVH reactive animals were sacrificed per time point (days 16,17,18 and 19). Animal immune function, spleen index, weight and survival were recorded as parameters of acute GVHD severity. To determine the effect of the transplant procedure and host vs graft reactivity on LPS translocation 39 syngeneic ($B6AF_1$ lymphoid cells) and 54 allogeneic (irradiated (irr.) CBA lymphoid cells) transplants were performed and assayed as for GVH animals with 3 and 5 animals sacrificed per time point, respectively. The allogeneic cells were irradiated so that they would not introduce a GVH component to the host vs graft reaction.

GVHD induction

Single cell suspensions of B6 donor spleen and lymph nodes were prepared in Hank's Balanced Salt Solution (Gibco Laboratories, Grand Island, NY) in which LPS was undetectable, by gently pressing the tissue through a fine mesh screen. Recipient B6AF1 animals were injected intravenously with $60x10^6$ B6 lymphoid cells. Control animals were injected with either 60×10^6 B6AF1 (syngeneic) or irradiated (irr. 2000 rad) CBA (allogeneic) lymphoid cells.

Assessment of GVHD

GVHD was assessed by immunosuppression, weight loss and spleen and liver index. Immunosuppression was assayed by determination of the number of splenic plaque-forming cells (PFC) to SRBC using the technique of Cunningham and Szenberg[62] as modified in this laboratory.[63] Briefly, ten days after transplantation B6AF1 recipients received an i.v. injection of $5x10^8$ SRBCs, four days later individual spleen cell suspensions were incubated at 37°c with SRBC and complement for 60 minutes in glass slide chambers. The resulting PFCs were counted.

Animal weight was recorded daily. Spleen and liver indices were determined by the method of Simonsen[7] which measures the degree of hepatosplenomegaly.

LPS Determination

The Chromogenic Limulus Amoebocyte Lysate (LAL) 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 containing 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, Salzburg, Austria). LPS concentrations in samples, were interpolated from an LPS standard curve prepared from E. coli 0111:B4 (Whittaker Bioproducts, Inc.) diluted in pyrogen-free water supplemented with 0.5% normal B6AF1 mouse serum.

The Pyrotel LAL Assay (Associates of Cape Cod, Inc., Woods Hole, MA) was used to assay for hepatic and splenic LPS. Tenfold serial dilutions of sterile organ homogenates were prepared in pyrogen-free water. LPS standards were prepared from E. coli 0113 (Associates of Cape Cod, Inc.) in sterile organ homogenates of normal B6AF1 liver or spleen and diluted with pyrogen-free water. The total amount of LPS per organ was determined from the highest dilution at which gel clot formation occurred.

RESULTS

Acute GVHD induces immunosuppression and hepatosplenomegaly The nature of GVHD induced immunosuppression which can develop as early as day 2 post transplant and reach maximal intensity by day 7, depends on the strain and dose of parental cells injected. Nonirradiated $B6AF_1$ mice transplanted with 60 x 10^6 B6 lymphoid cells developed acute GVHD as characterized by severe immunosuppression and splenomegaly. The PFC assay, showed that acute GVH reactive mice were immunosuppressed whereas, untreated, syngeneic and allogeneic controls expressed immune function. Spleen cells from $B6AF_1$ mice transplanted with B6 cells, failed to produce an antibody response following injection on day 10 with the T cell-dependent antigen, SRBC. In contrast, spleen cells from untreated $B6AF_1$ mice, or those transplanted with either 60 x 10^6 $B6AF_1$ or 60 x 10^6 irr. CBA lymphoid cells produced a normal antibody response to SRBC (Table 1). Calculations of the spleen and liver indices, showed that acute GVH reactive mice developed hepatosplenomegaly whereas, untreated, syngeneic and allogeneic controls did not.

$B6AF_1$ mice transplanted with B6 cells, developed hepatosplenomegaly, a donor cell-induced recruitment of host myeloid and erythroid

Table 1. PFC response to SRBC of $B6AF_1$ recipients during acute GVHD.

Donor Strain	Total PFC to SRBC* per spleen (x 10^{-3})
——	320 ± 83
B6AF1	266 ± 18
irr. CBA	179 ± 54
B6	0

* $B6AF_1$ mice were untreated or transplanted with either 60 x 10^6 B6, $B6AF_1$ or irr. CBA lymphoid cells. On day 10 following transplantation, representative experimental mice from each group were injected with 5 x 10^8 SRBC and 4 days later the number of PFC from individual spleens was determined. The values presented represent the mean ± SEM determined from 4 to 10 mice.

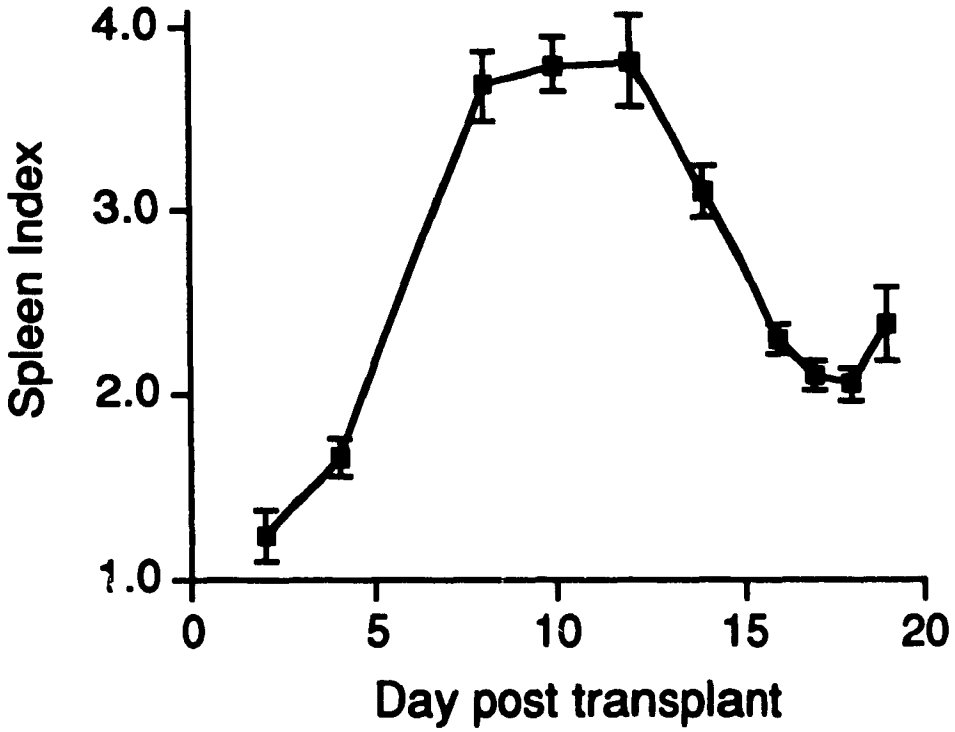

Fig 1. Spleen index & SEM of $B6AF_1$ recipients during acute GVHD. $B6AF_1$ mice were transplanted with either 60 x 10^6 B6, $B6AF_1$, or irr. CBA lymphoid cells. From days 2 to 14 inclusive 8 $B6AF_1$ mice transplanted with B6 lymphoid cells (GVH) were killed for each time point . From days 16 to 19 inclusive the number of mice killed for each time point was increased to 12-15. Three $B6AF_1$ mice transplanted with $B6AF_1$ lymphoid cells (syngeneic) and 5 $B6AF_1$ mice transplanted with irr. CBA lymphoid cells (allogeneic) were killed was calculated as the spleen to body weight ratio of the experimental group divided by the spleen to body weight ratio of the untreated $B6AF_1$ group. The spleen index of $B6AF_1$ mice treated with $B6AF_1$ or irr. CBA cells was always within a range of 0.8 to 1.1.

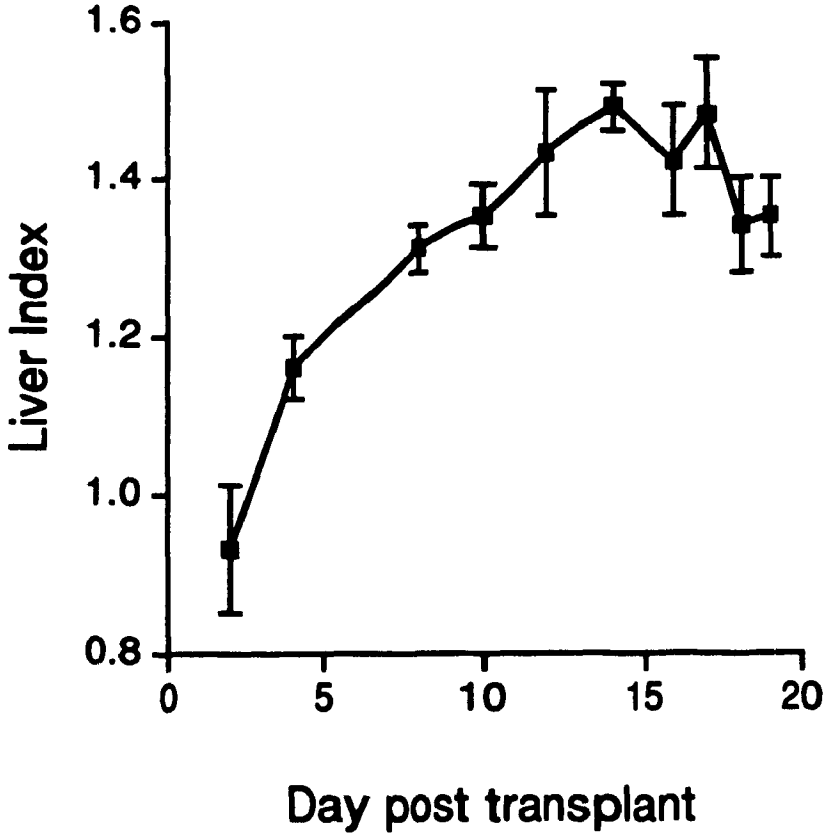

Fig 2. Liver index & SEM of $B6AF_1$ recipients during acute GVHD. $B6AF_1$ mice were transplanted with either 60 x 10^6 B6, $B6AF_1$, or irr. CBA lymphoid cells. From days 2 to 14 inclusive 8 $B6AF_1$ mice transplanted with B6 lymphoid cells (GVH) were sacrificed for each time point . From days 16 to 19 inclusive the number of mice sacrificed for each time point was increased to 12-15. Three $B6AF_1$ mice transplanted with $B6AF_1$ lymphoid cells (syngeneic) and 5 $B6AF_1$ mice transplanted with irr. CBA lymphoid cells (allogeneic) were sacrificed for each time point from days 2 to 19. The liver index was calculated as the liver to body weight ratio of the experimental group divided by the liver to body weight ratio of the untreated $B6AF_1$ group. The liver index of $B6AF_1$ mice treated with $B6AF_1$ or irr. CBA cells was always within a range of 0.7 to 1.1.

cells characteristic of GVHD. As predicted by the work of Simonsen, the spleen index peaked 10 to 12 days after transplantation with an index of 3.8. a value which is well above the spleen index of 1.3 considered predictive of GVHD (Fig 1). In contrast, the spleen indices of untreated $B6AF_1$ mice, or of those transplanted with either $B6AF_1$ or irr. CBA lymphoid cells were uncharacteristic of GVHD. The liver index peaked 14 days after transplantation with an index of 1.5, a value which is well above the liver indices of untreated $B6AF_1$ mice or of those transplanted with either $B6AF_1$ or irr. CBA lymphoid cells (Fig 2). These results, suggest that the $B6AF_1$ mice transplanted with B6 lymphoid cells developed severe acute GVHD.

Acute GVHD induces weight loss and mortality Nonirradiated $B6AF_1$ mice transplanted with 60×10^6 B6 lymphoid cells developed acute GVHD, as characterized by severe weight loss and mortality. Daily assessments of body weight and population survival analysis, showed that acute GVH reactive mice developed severe weight loss and the population as a whole suffered a high rate of mortality whereas, untreated, syngeneic and allogeneic controls maintained their pretransplant weights and failed to show mortality. Significant weight loss was first observed in $B6AF_1$ recipients of B6 cells on day 10 post transplant and reached 22% of the mean pretransplant body weight by day 19. Weight loss was not observed in untreated $B6AF_1$ mice or in those transplanted with 60×10^6 $B6AF_1$ or

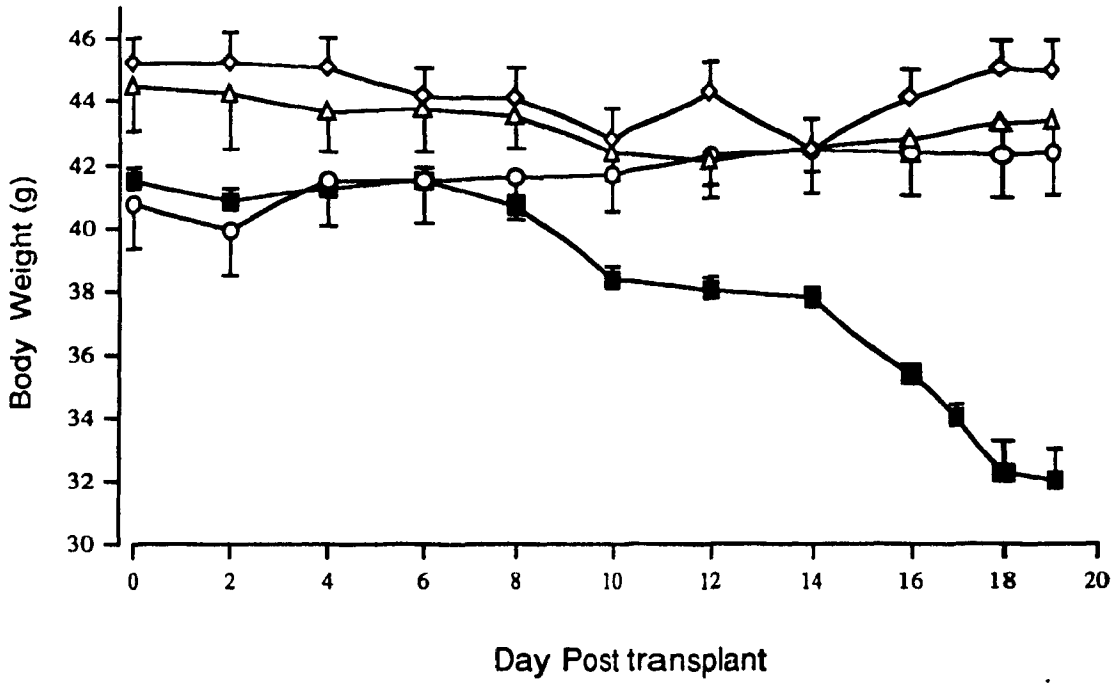

Fig 3. Body weight of B6AF$_1$ recipients during acute GVHD. B6AF$_1$ mice were untreated (O) or transplanted with either 60 x 10^6 B6 (■), B6AF$_1$ (◇), or irr. CBA lymphoid cells (△). The values plotted represent the mean ± SEM determined from 142 recipients of B6 cells, 39 recipients of B6AF$_1$ cells, 54 recipients of irr. CBA cells or 24 untreated mice.

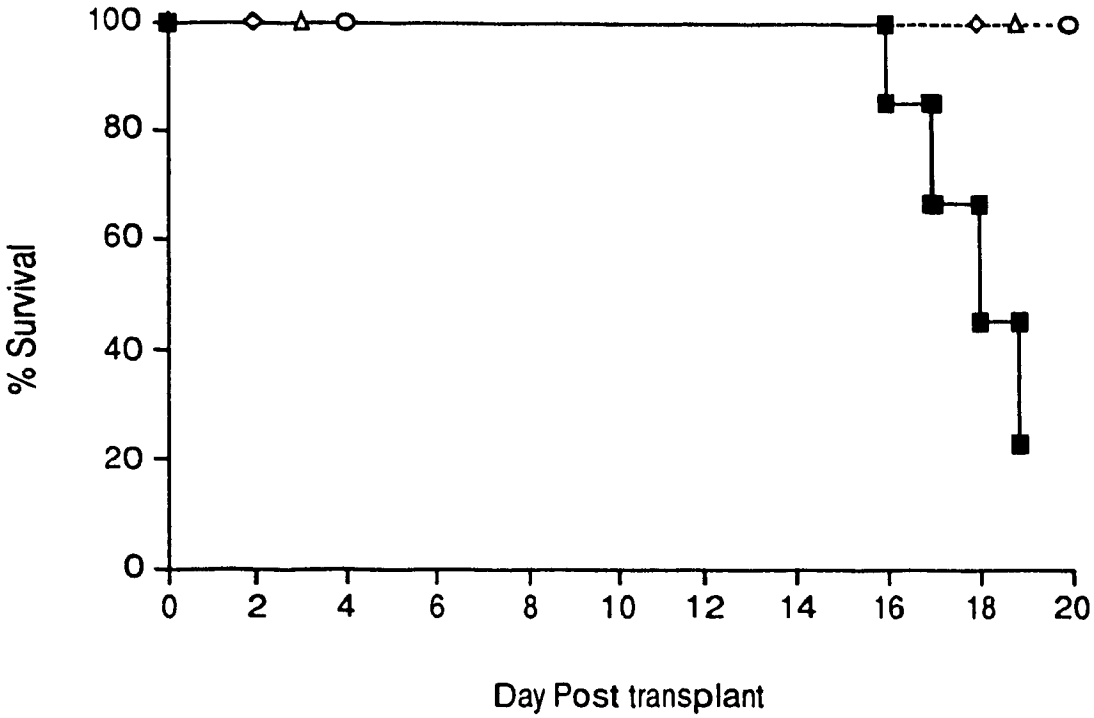

Fig 4. Survival of $B6AF_1$ recipients during acute GVHD. $B6AF_1$ mice were untreated (o) or transplanted with either 60 x 10^6 B6 (■), $B6AF_1$ (◇), or irr. CBA lymphoid cells (△). The values plotted represent the percent of live mice from a pool of 142 recipients of B6 cells, 39 recipients of $B6AF_1$ cells, 54 recipients of irr. CBA cells or 24 untreated mice.

60 x 10^6 irr. CBA lymphoid cells (Fig 3). In mice undergoing acute GVHD, mortality was first observed on day 16 post transplant and by day 19, the cumulative survival was 24% of recipients. Survival was constant at 100% throughout the experiment for untreated B6AF1 mice or those transplanted with 60 x 10^6 $B6AF_1$ or 60 x 10^6 irr. CBA lymphoid cells (Fig 4).

LPS is present in the Liver, the Spleen and the Serum during Acute GVHD

To determine whether detectable levels of LPS were present in the liver, the spleen and the serum of GVH reactive mice, tissues from randomly selected mice were assayed for LPS at predetermined times post transplant. LPS levels of >1-10^2ng/organ were first detected in the liver of $B6AF_1$ recipients of B6 cells on day 2 post transplant. Between 4 and 14 days post transplant, an increasing percentage of mice tested had LPS present in their liver. An increase in the total amount of LPS levels to >10^2-10^4 ng/organ, was found in the livers of some of the acute GVH reactive mice, between days 16 and 19 post transplant (Fig 5).

LPS levels of >1-10^2ng/organ were first detected in the spleen of $B6AF_1$ recipients of B6 cells on day 4 post transplant. As was observed in the liver of these same animals, elevated levels of total LPS >10^2-10^4 ng/organ were observed in the spleen from day 16 onwards (Fig 6).

LPS was first detected in the serum of GVH reactive mice on day 16 post transplant. The animals in which serum LPS could be detected also

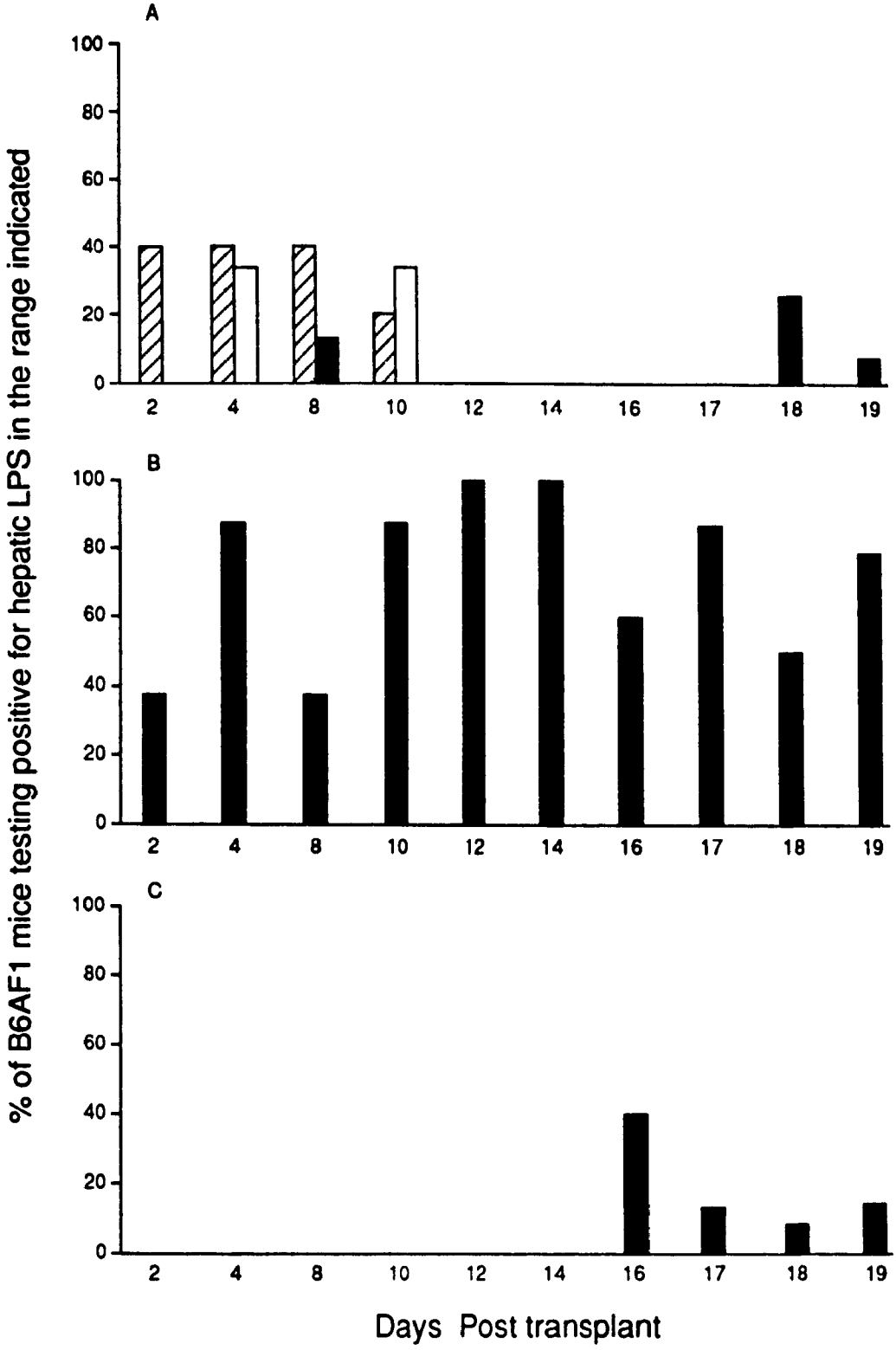

Fig5. The presence of LPS in the liver of $B6AF_1$ recipients during acute GVHD. $B6AF_1$ mice were transplanted with either 60 x 10^6 B6 (■), $B6AF_1$ (▨), or irr. CBA lymphoid cells (□). Livers from randomly selected mice were homogenized and tested for LPS using the Pyrotel LAL assay as described in the materials and methods. From days 2 to 14 inclusive 8 $B6AF_1$ mice transplanted with B6 lymphoid cells (GVH) were sacrificed at each time point. From days 16 to 19 inclusive the number of mice sacrificed for each time point was increased to 12-15. Three $B6AF_1$ mice transplanted with $B6AF_1$ lymphoid cells (syngeneic) and 5 $B6AF_1$ mice transplanted with irr. CBA lymphoid cells (allogeneic) were sacrificed for each time point from days 2 to 19. The data are presented as the percentage of organs testing positive within the LPS range indicated: (A)0.5-1ng/organ, (B)>1-10^2 ng/organ and (C)>10^2-10^4ng/organ. LPS was undetectable in the syngeneic and allogeneic groups after day 10 and LPS was never detected in the untreated $B6AF_1$ mice.

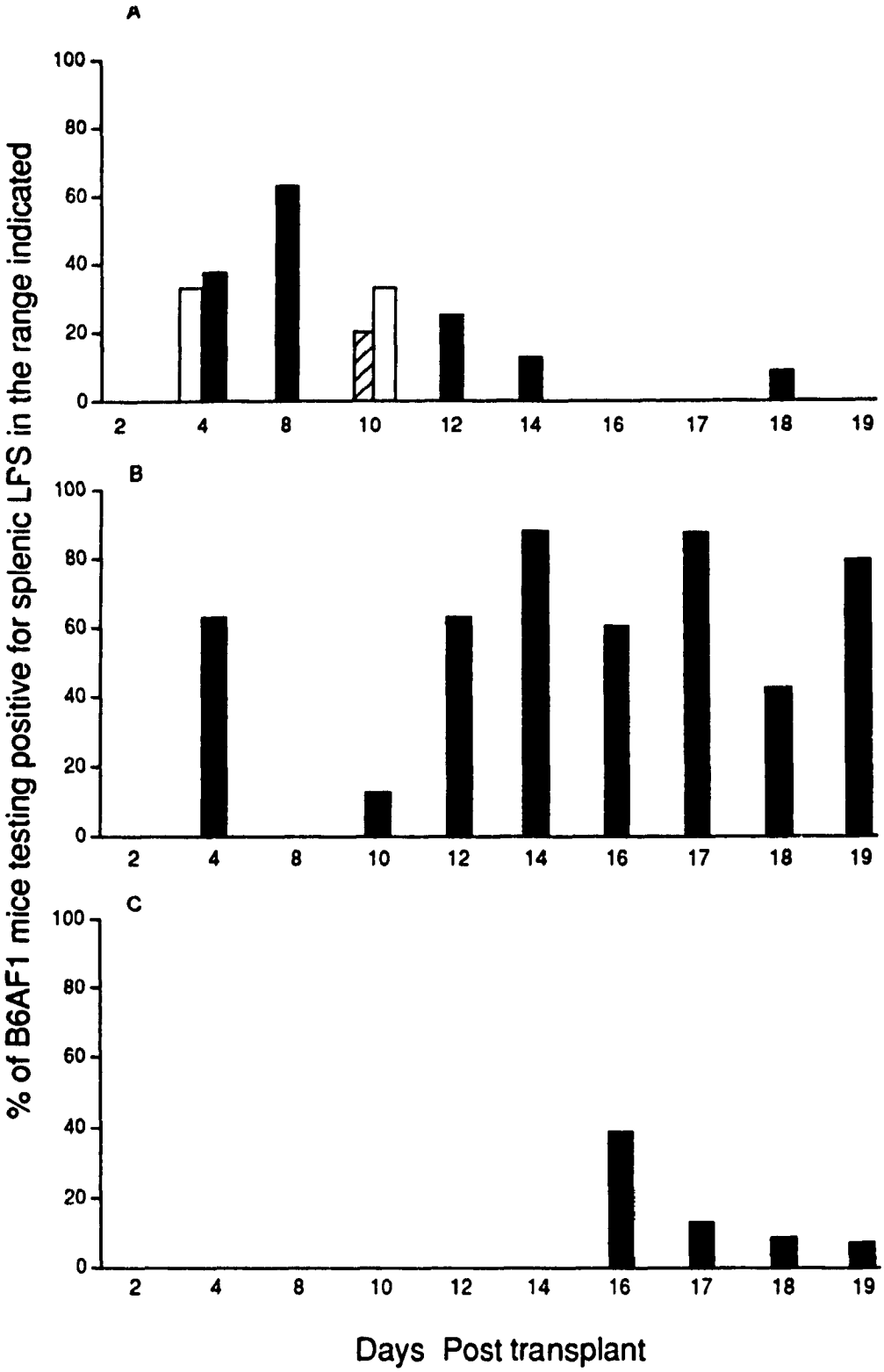

Fig 6. The presence of LPS in the spleen of $B6AF_1$ recipients during acute GVHD. $B6AF_1$ mice were transplanted with either 60 x 10^6 B6 (■), $B6AF_1$ (◪), or irr. CBA lymphoid cells (□).Spleens from randomly selected mice were homogenized and tested for LPS using the Pyrotel LAL assay as described in the materials and methods. From days 2 to 14 inclusive 8 $B6AF_1$ mice transplanted with B6 lymphoid cells (GVH) were sacrificed at each time point. From days 16 to 19 inclusive the numbe: of mice sacrificed for each time point was increased to 12-15. Three $B6AF_1$ mice transplanted with $B6AF_1$ lymphoid cells (syngeneic) and 5 $B6AF_1$ mice transplanted with irr. CBA lymphoid cells (allogeneic) were sacrificed for each time point from days 2 to 19. The data are presented as the percentage of organs testing positive within the LPS range indicated: (A)0.5-1ng/organ, (B)>1-10^2 ng/organ and (C)>10^2-10^4ng/organ. LPS was undetectable in the syngeneic and allogeneic groups after day 10 and LPS was never detected in the untreated $B6AF_1$ mice.

had greater levels of total LPS in their organs. Mortality was first observed on day 16 post transplant, coincident both with the appearance of LPS in the serum and with the increase in LPS levels in the liver and the spleen.

Detectable levels of LPS >0.5-1 ng/organ were measured in the liver and the spleen of some of the $B6AF_1$ mice transplanted with 60 x 10^6 $B6AF_1$ lymphoid cells, from day 2 up to day 10 post transplant. LPS was also detected in the liver and the spleen of some of the $B6AF_1$ mice transplanted with 60 x 10^6 irr. CBA lymphoid cells, on day 4 and day 10. LPS levels above 1 ng/organ were never detected in livers or the spleens from $B6AF_1$ mice transplanted with 60 x 10^6 $B6AF_1$ or 60 x 10^6 irr. CBA lymphoid cells. After day 10 post transplant, LPS was undetectable in the organs of both the syngeneic and the allogeneic controls (Figs 5 and 6). LPS was undetectable in the serum of the untreated, the syngeneic and the allogeneic controls (Fig 7).

LPS was undetectable in the tissues of all the untreated $B6AF_1$ animals throughout the experiment.

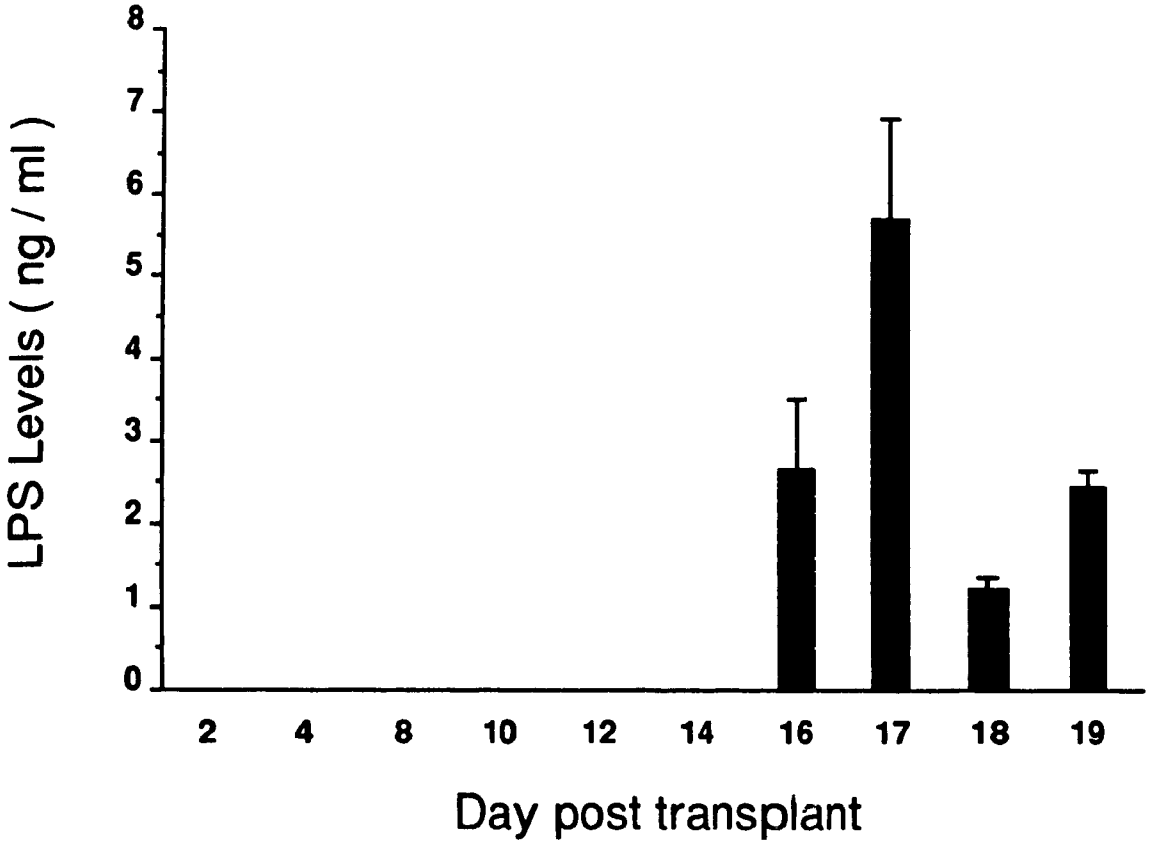

Fig 7. The presence of LPS in the serum of $B6AF_1$ recipients during acute GVHD. The bars indicate LPS levels in $B6AF_1$ mice transplanted with B6 lymphoid cells. LPS was undetectable in the serum of untreated $B6AF_1$ mice or those transplanted with $B6AF_1$ or irr. CBA lymphoid cells. The percent of serum samples testing positive for LPS using the chromogenic LAL assay, as described in the materials and methods, were 40%, 20%, 9% and 7%, on days 16, 17, 18 and 19 , respectively.

Discussion

Our results demonstrate the presence of LPS in the liver, the spleen and the serum of mice during acute GVHD. The percentage of GVH reactive mice positive for hepatic and splenic LPS increased with time post transplant Likewise, the total amount of LPS detected in each organ increased as the GVH reaction progressed reaching peak levels of 10^4ng/organ on day 16 post transplant. LPS appeared in the serum of the mice expressing peak hepatic and splenic LPS levels at a time coincident with the period of mortality: day 16 through day 19 post transplant. LPS appeared transiently at low levels of 0.5 to 1 ng/organ in the liver and the spleen of $B6AF_1$ mice transplanted with syngeneic (B6AF1) or irr. allogeneic (CBA) lymphoid cells, but was undetectable beyond day 10 post transplant. In addition, LPS was undetectable in the serum of both these groups and was never detected in the tissues of untreated $B6AF_1$ mice. The results demonstrate, that LPS is present to initiate TNF-α release from primed macrophages, at a time when animals display severe weight loss and mortality following acute GVHD.

We believe that the adverse symptoms of GVHD arise when intestinal endotoxin is not detoxified by the liver. A hundred years ago Pavlov[58]

proposed that the liver carried out a protective role against toxins. Since then a relationship between hepatotoxins, macrophages and intestinal endotoxins has been proposed. Work on liver cirrhosis has shown that LPS rather than intact bacteria is critical for liver damage to occur, and a synergism between LPS and hepatotoxins such as galactosamine and CCl_4 has been demonstrated in acute toxic liver injury. Finally, the clearance ability of the Kupffer macrophages has been associated with the sensitivity to LPS. It is hypothesized that hepatic damage resulting from a host of toxic or metabolic insults on sinusoidal, hepatocyte and Kupffer cells impairs the liver's ability to detoxify LPS and thus renders it susceptible to normally harmless levels of gut endotoxin ultimately leading to a spillover of toxic substances into the systemic circulation.[58]

Activation of TNF-α production by macrophages, requires priming by IFN-γ and triggering by LPS.[30-35]There is evidence of IFN-γ production during GVHD however, the presence of LPS had not been demonstrated.[64] We have previously shown *in vitro* and *in vivo* that macrophages from GVH reactive animals produced relatively large amounts of TNF-α following administration of LPS.[34] In the same study, we demonstrated macrophage priming during acute GVHD and showed that priming increases with the time post transplant. The priming sensitizes macrophages to LPS, so that normally insignificant quantities of LPS trigger TNF-α production. Further substantiating our hypothesis that LPS plays a crucial role in the disease process, animals dying of acute GVHD showed symptoms of septic shock

such as piloerection, hunching and diarrhoea. In contrast, animals kept under germ-free conditions or treated with anti-TNF-α do not develop severe GVHD.[20-23,42,43]Here we provide direct evidence that LPS, the trigger for TNF-α production by macrophages is present during acute GVHD. Early manifestations of LPS failed to cause weight loss or mortality, possibly due to insufficient macrophage priming and/or insufficient LPS/LBP complex formation, prior to day 10 post transplant. However, from day 10 onward weight loss increased dramatically, suggesting that hepatic and splenic scavenger functions were unable to efficiently abrogate LPS/LBP complex stimulation of TNF-α production. We believe that as the acute GVH reaction progressed increased gut injury, and subsequent increases in LPS and bacterial load saturated endotoxin scavenger mechanisms, resulting in elevated tissue LPS levels, causing massive TNF-α production leading to shock and death.

We propose that LPS may play a critical role in the pathogenesis of acute GVHD. Bacterial LPS will cross the gut during states of trauma which disrupt the mucosal barrier such as mechanical, thermal,irradiation or immunosuppressive insults.[18,19,35-71] E.Coli have been cultured from the liver and spleen of GVH reactive mice- K. Price unpublished observation). Conceivably, bacterial translocation into the portal circulation would be promoted in mice undergoing acute GVHD, by both the severe immunosuppression and the lesions to the gut mucosal barrier which develop.[14,17]Early translocation of LPS or gram-negative bacteria via the

hepatic portal system and possibly via the lymphatics, may be due to transient intestinal alterations resulting from the transplant or host vs graft reactivity rather than or in addition to acute GVHD injury. Moreover, vascular changes such as increased levels of vasoactive substances have been shown to increase intestinal permeability to LPS.[69] We believe that the later increase in LPS organ levels and its coincident appearance in the serum represents a saturation of hepatic and splenic clearance mechanisms resulting in an accumulation of LPS in the systemic circulation.

LPS is cleared from the blood biphasically through the action of numerous ligands. Macrophage scavenger receptors and low density lipoproteins promote the phagocytosis of LPS while, high density lipoproteins transport LPS to cholesterol metabolizing organs such as the adrenal glands.[47-58] It appears that the rapid but reversible formation of LPS/LPS binding protein (LBP) complexes delays the uptake of LPS by these lipoproteins and stimulates TNF-α production through ligation of the macrophage CD14 receptor.[47,55-57] Saturation of such clearance mechanisms occurring as a result of persistent immunosuppression and ongoing intestinal injury would cause LPS to accumulate in the serum of GVH reactive mice and presumably lead to lethal TNF-α and nitric oxide production.[36,37,55,70,71]

The present study has further substantiated the acute GVHD model which we proposed in a recent report. Briefly, acute GVHD can be divided into three phases; an initial afferent phase involving the activation of NK-like

GVH effector cells, and the priming of macrophages through alloantigen induced cytokine production; a second phase during which GVH effector cell mediated epithelial injury results in the translocation of gram-negative bacteria into the portal and systemic circulatory systems; a third phase during which hepatic and splenic primed macrophages kill gram-negative bacteria releasing LPS which triggers excessive production of TNF-α, a mediator of tissue injury, cachexia and death. This model for acute GVHD, permits several different approaches for the treatment and/or prevention of acute GVHD, since our model proposes that at least 3 different cells and their cytokines may be involved. Thus, the prevention of the effector function of any one of these cells could arrest the GVH reaction. In particular, our results suggest that the blocking of LPS stimulation of primed macrophages could prevent the pathological symptoms associated with acute GVHD.

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SUMMARY

In order to determine if bacterial LPS is present during the natural course of acute GVHD randomized GVH reactive mice were sacrificed at predetermined times post transplant for tissue endotoxin analysis.

· Liver, spleen and serum of acute GVH reactive mice display increasing LPS levels starting from day 2, 4 and 16 post-transplant respectively, suggesting that there is translocation of LPS from the gut.

· LPS levels in these organs peaked and LPS first appeared in the serum at a time coincident with the onset of mortality.

· LPS is present to initiate TNF-α release from primed macrophages and increasing LPS levels in liver, spleen and appearance in serum correlates with GVHD morbidity and mortality.