Selective Reconstitution by GM-CSF of the Immune Response in Human Immunosuppressed Cells

DISSERTATION
der Universität Konstanz

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Konstanz, July 2002

Tag der mündlichen Prüfung: 21. 10. 2002
1. Referent: Prof. A. Wendel
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Acknowledgement

This thesis was prepared at the Faculty of Biochemical Pharmacology in the University of Konstanz, Germany, under the auspices of Prof. Dr. Albrecht Wendel.

I am grateful to my supervisor Prof. Wendel for his long standing support, both spiritually and materially, for my study in Germany. I am indebted to him for his trust and understanding, his generosity and kindness, his open-mind-advice and encouragement. I have been greatly motivated by his initiative suggestions and stimulating ideas along with all the stages of the study project.

Equally important, the spirit and know-how of running a successful research group, a practice undergoing here introduced by my supervisor, on which all my study has been largely benefited, namely the trust, mutual benefit and social interactions, will definitely be integrated into the philosophy of my future career.

I am also indebted to Prof. Albrecht Wendel, Prof. Volker Ullrich and Dr. Thomas Hartung for their assessment or critical reading of this thesis.

Special thanks go to Dr. Rudolf Lucas for his friendship and for his active involvement in this project. His contribution to the coordination is also much appreciated.

I would like to express my thankfulness to Mrs. Gudrun Kugler for her kindness and being always ready-to-help.

Furthermore, I would like to thank Dr. Thomas Meergans for methodological help and advice; Dr. Margarete Odenthal from the University of Cologne, for the introduction of gene expression array technique; Prof. Ansgar W. Lohse and Dr. Marcus Schuchmann from University of Mainz, for the organization of the clinical blood samples and their constructive suggestion for the study; Dr. Thomas Hartung for his critical and stimulating ideas. The critical reading and correction from Drs. Juerg Hamacher, Jutta Schlepper-Schaefer and Gerald Kuenstle on the manuscripts of publications are also greatly appreciated. Thanks also go to Dr. Jutta Schlepper-Schaefer and Matthias Kresse for the correction of the German abstract.

Last, but not least, I would like to thank all members of the "Arbeitsgruppe Wendel" for their kindness and helps during all the stages of my study. The research work in this dissertation has been greatly benefited by stimulating discussion with them and by the friendly working atmosphere created by them.

This work is dedicated to my wife Ningli and my son Wenjia, for their love, their patience, their companion and their non-stop support, the invisible but indispensable part of this thesis. This work is also devoted to my parents, who built up the basis for everything.

Jian Xu

Konstanz, July 2002

Contributions (1999 - 2002)

Jian Xu, Rudolf Lucas, Marcus Schuchmann, Simone Kühnle, Thomas Meergans, Ana P. Barreiros, Ansgar W. Lohse, Gerd Otto, Albrecht Wendel. **Restoration of innate, but not of specific immune response in glucocorticoid-immunosuppressed human blood by GM-CSF.** (submitted to Nature Medicine)

Jian Xu, Rudolf Lucas, Marcus Schuchmann, Ana P. Barreiros, Ansgar W. Lohse, Gerd Otto, Albrecht Wendel. **Restoration of TNF production, but not lymphocyte proliferation, in steroid-treated liver transplant patients by GM-CSF.** (As invited speaker at the conference of Transplantation at the Universe: Infection in Organ Transplantation. 2001 Bremen, Germany)

Jian Xu, Rudolf Lucas, Marcus Schuchmann, Ana P. Barreiros, Ansgar W. Lohse, Gerd Otto, Albrecht Wendel. **GM-CSF restores TNF production, but not lymphocyte proliferation, in blood from immunosuppressed liver transplant patients.** (Peer-reviewed poster presentation in The Spring Congress of German Association of Experimental and Clinic Pharmacology and Toxicology, Frühjahrstagung der Deutschen Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie 2002, Mainz, Germany)

Abbreviations

AML	acute myelogenous leukaemia
APC	antigen presenting cell
BMSC	bone marrow stem cells
BMT	bone marrow transplantation
CD	Cluster of differentiation
Cdk	Cyclin-dependent kinase
CMV	Cytomegalovirus
Con A	Concanavalin A
Dex	Dexamethasone
Egr	early growth response
ELISA	enzyme linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony stimulating factor
GPDH	glyceraldehyde 3-phosphate dehydrogenase
GVHD	graft-versus host-disease
h	Hour
HSP	heat shock protein
Jab1	c-Jun activation-domain binding protein 1
ICAM	intercellular adhesion molecule
ICE	IL-1β-converting enzyme
IL	Interleukin
JAK	Janus kinase
LPS	Lipopolysaccharide
LAT	linker for activation of T-cell
MAP	Mitogen activated protein kinases
MHC	major histocompatibility complex
NF-κB	Nuclear factor-kappa B
NO	nitric oxide
NS	not significant
PAF	platelet activating factor
PBMC	periphreal blood mononuclear cell
PBSC	peripheral blood stem cells
RPA	ribonuclease protection assay
STAT	signal transducer and activator of transcription
TACE	TNF-α converting enzyme
TCR	T cell receptor
TNF	tumor necrosis factor

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1. Introduction

1.1 Transplantation immunology

Solid organ transplantation is a therapeutic option for many human diseases. Liver, kidney, heart, and lung transplantation have become standard therapy for selected end-stage diseases. Moreover, pancreas (including islet cell) and small bowel transplantation are also being developed in this regard.[1] The number of solid-organ transplant recipients is steadily increasing. Indeed, since the first renal transplantation in 1954, more than 600,000 solid-organ transplantations have been performed worldwide.[2] The quality of life and survival rates following organ transplantation have greatly improved due to advances in understanding of the human immune system in recognition of foreign organs, the application of immunosuppressant agents, and the advances in surgical techniques.

Immunological basis for alloimmune responses

Many processes participate in the response to foreign grafts. These include the local inflammatory response to surgery, the processes that initiate wound repair and vascular endothelialization, and the immune response to the recognition of none-self antigen.[3] The basic elements of this response are schematized in Figure 1.

The response to non-self antigen involves both cellular and humoral immunity. The goal of such a response is to reject that antigen, and its nature and intensity are determined by two factors. The first factor is the biology of the foreign tissue, whereas the second factor is the host response upon encountering that specific foreign tissue. These responses fall into three categories: hyperacute, acute and chronic rejection.

The host immune response to foreign organs is mediated by mononuclear cells, composed of CD4+ and CD8+ T cells, macrophages or other APC, natural killer cells, and B cells. There are two known pathways involved, namely the direct and indirect antigen presentation pathways. Several mechanisms mediating rejection of a foreign tissue or graft destruction are available to the immune system of a mammalian host.

T cells have a critical role in allograft rejection across histocompatibility differences within the same species. T cells express clonally distributed antigen receptors (TCR) that recognise processed antigen fragments as peptides presented by antigen-presenting cells in the groove of MHC molecules. In general, CD4+ T cells recognise antigenic peptides presented

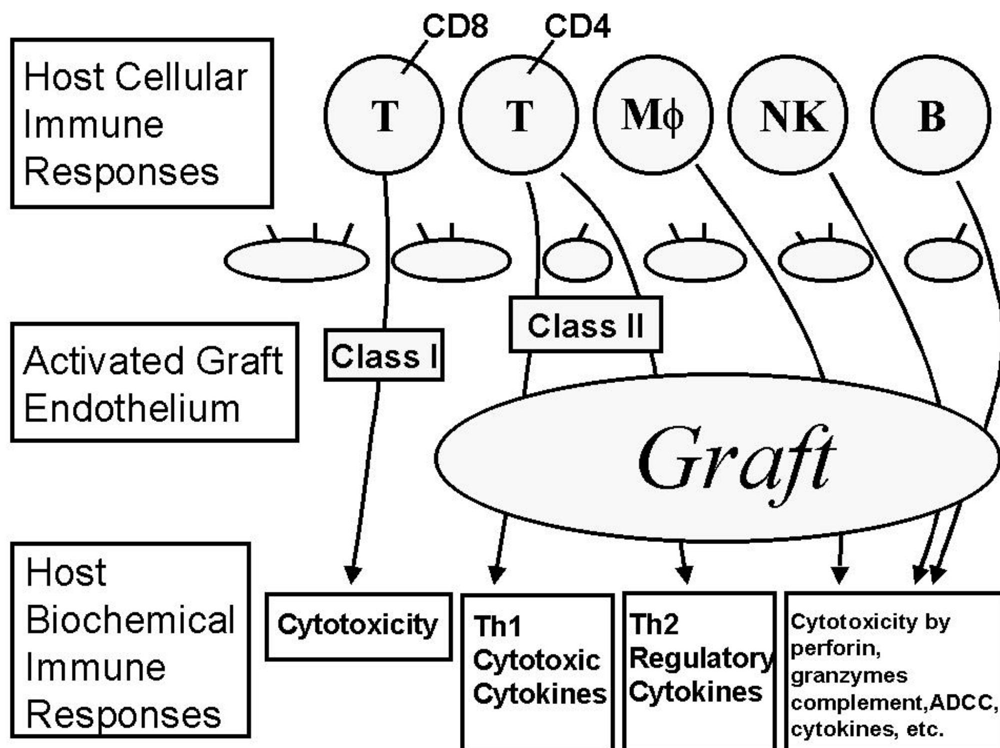

Figure 1. Overview of host immune response to transplanted tissue.[3]

Host immune response is mediated by mononuclear cells composed of CD4+ and CD8+ T cells (T), macrophages (Mϕ), natural killer cells (NK), and B cells (B). CD8/ T cells interact with major histocompatibility complex (MHC) class I plus peptide present on graft, including its endothelial cells; CD4+ T cells interact with MHC class II plus peptide present on both graft and endothelial cells. Endothelial cells are depicted as activated in response to inflammatory cytokines, expressing surface molecules that include both MHC molecules and adhesion molecules. Bottom depicts mechanisms of graft destruction associated with various responding host immune cells. Finally, also depicted is possibility of deviation of immune response by Th2-type cytokines produced by CD4+ T cells. ADCC, antibody-dependent cell-mediated cytotoxicity.

by class II MHC, and CD8+ T cells recognise antigen peptides presented by class I MHC. CD4+ and CD8+ T cells collaborate in the rejection of vascular grafts. Natural killer cells mediate rejection of MHC incompatible haemopoietic stem cell grafts in rodents and, presumably, also in human beings, but they are not involved in rejection of organ grafts. The role of T cells in allograft rejection has been shown by experiments where *in vivo* T cell depletion has allowed engraftment of genetically disparate haemopoietic stem cells and skin grafts from the same donor. Furthermore, depletion of T cells from the haemopoietic stem cell grafts prevents graft-versus-host disease. Antigen-presenting cells have an obligatory role in presenting antigenic peptides from the graft and eliciting rejection. Culturing thyroid tissue for several days before transplantation was sufficient to prevent rejection of the thyroid tissue graft. The culture was associated with loss of passenger leucocytes, which would have

provided antigen presenting function *in vivo* and allowed direct recognition of donor alloantigens by recipient T cells. In other circumstances, however, recipient antigen-presenting cells can take up, process, and present donor alloantigens to recipient T cells, a process called indirect recognition. Graft-versus-host disease was also prevented by eliminating competent antigen-presenting cells from bodies of recipient animals before transplantation of T cell-repleted marrow grafts from MHC incompatible donors. Thus, graft rejection and graft-versus-host disease stem from cognate interaction of T cells with antigen presenting cells, which allows recognition of alloantigen and delivery of accessory activation signals. Presentation of alloantigens by incompetent antigen-presenting cells facilitates tolerance.[4]

1.2 Immunosuppressive strategies in organ transplantation

In the absence of immunosuppression, transplanted organs invariably undergo progressive immune-mediated injury. Over the past 50 years, immunosuppressive drug regimens have evolved greatly and transformed solid-organ transplantation into a routine clinical procedure with impressive short-term results obtained in solid organ transplantation. Improved short-term survival is a consequence mainly of better prevention and treatment of acute rejection. In contrast, long-term graft survival remains a major problem, mainly due to chronic rejection, or due to the side effects or the lack of specificity of the immunosuppressive agents.[5] Hence, the ideal immunosuppressive agent should be able to (1) selectively inhibit alloantigen immune responses (2) prevent chronic allograft rejection and (3) be free of major side-effects.

The currently established immunosuppressive agents in transplantation

All current immunosuppressive drugs target T cell activation and cytokine production, clonal expansion, or both. In the clinic, the standard transplantation immunosuppressive protocols consist of *initial* and *maintenanc*e therapies to prevent allograft rejection and short courses of more aggressive immunosuppressive therapy to treat episodes of acute rejection.

Immunosuppression is initiated at high levels in the immediate post-transplant period when the risk of graft rejection is maximal. In most patients, initial immunosuppression consists simply of higher doses of the agents used in maintenance therapy. Induction of immunosuppression, however, involves the additional administration of potent anti-T-cell antibody preparations or IL-2 response blocking agents. These substances are successful in reducing the incidence and severity of early acute rejection. The major questions remaining

unanswered are whether or not these agents still show a benefit when combined with more potent immunosuppressive agents. Maintenance immunosuppression is best achieved with combinations of immunosuppressive agents, aiming to minimise the side-effects of any single drug, while maintaining adequate overall immunosuppression by targeting multiple steps in T cell activation. This is usually achieved by combining corticosteroids with a calcineurin inhibitor (cyclosporine or tacrolimus) and an anti-proliferative agent such as azathioprine or mycophenolate mofetil (Table 1). Among them, the corticosteroids are non-specific anti-inflammatory agents, which inhibit cytokine production by T cells and macrophages, thereby disrupting T cell activation and macrophage-mediated tissue injury. The action mode for corticosteroids is multifaceted, affecting transcriptional and post-transcriptional events.[6-8] A well-known mechanism is mediated through inhibition of nuclear factor κ B (NFκB) activation, by binding to glucocorticoid response elements in the promoter regions of cytokine genes.[9-11]

New immunosuppressive strategies in transplantation

Given the central role of the CD4+ T cell in allograft rejection, it is self-evident that most new immunosuppressive strategies have sought to inhibit the activation of this cell type. The more sophisticated ultimate aim, however, would be to inhibit only those T cells that respond to donor antigen, thus achieving immunological non-responsiveness to the transplant with maintainment of a fully functional residual immune system. Several ligand/receptor interactions occur between the T cell and the antigen-presenting cell during antigen presentation. While some simply mediate cell-cell adhesion, others transduce activation signals to either the T cell or the antigen-presenting cell.[12] Agents have been developed to block these interactions. For example, the blockade of T cell costimulation results in T cell anergy and thus may render the recipient's CD4+ T cells unresponsive to donor antigen, while the blockade of T cell adhesion molecules may inhibit the activation and recruitment of immune cells into the allograft, thus extending allograft survival. T-cell activation may also be inhibited when T cell accessory molecules are blocked. Recently, it has been found that peptides derived from class I and class II MHC molecules have an immunomodulatory effect on T cell activation. Thus these molecules are expected to give rise to a new class of immunosuppressive drugs (Table 2).

Table 1. Immunosuppressive drugs used in organ transplantation [9-11]

Immunosuppressive drugs		Mechanism of action	
		Molecular target	Molecular effect
INDUCTION	ATG/ALG	Binds multiple antigens on lymphoid cells	Complement-mediated lysis Opsonisation and clearance Modification of cell surface receptor
	OKT3	Binds T cell CD3	Complement-mediated lysis Opsonisation and clearance Modification of CD3 receptor
	Daclizumab	Binds α-subunit of IL-2 receptor	Down-regulation of receptor CD4 T cell depletion
	Basiliximab	Binds α-subunit of IL-2	Down-regulation of receptor CD4 T cell depletion
MAINTENANCE	Corticosteroids	Cytosolic receptors; Heat shock proteins	Blocks transcription of cytokine genes
	Cyclosporine	Binds cyclophylin; Inhibits calcineurin	Inhibits IL-2 production; Stimulates TGF-β production
	Tacrolimus (FK506)	Binds FKBP-12 Inhibits calcineurin	Inhibits IL-2 production; Antagonises TGF-β
	Azathioprine	Metabolites bind DNA	Inhibits purine synthesis; Blocks DNA and RNA synthesis
	Mycophenolate mofetil	Inhibits inosine monophosphate phosphate dehydrogenase	Blocks de-novo pathway of purine synthesis (selective for lymphocytes); Blocks glycosylation
	Sirolimus	Binds FKBP12; Blocks p70 S6 kinase	Blocks IL-2-induced cell cycle progression

Table 2. Novel immunosuppressive drugs

Interruption Category	Site of action	Agent used
TCR / MHC binding	CD4/MHC class II	Anti-CD4 mAbs
T cell costimulation	CD28/B7 CD40/CD154	CTLA4-Ig Anti-CD154 mAbs
Cell adhesion	LFA-1/ICAM-1	Anti-LFA1 mAbs Anti-ICAM-1 mAbs
Accessory molecule interactions	CD2/LFA3 CD45	Anti-LFA3 mAbs Anti-CD2 mAbs Anti-CD45

Gene therapy

Major advances have been made in techniques to deliver genetic material into cells. Experimentally, these techniques have been useful in the dissection of the immunobiology of transplant rejection. In clinical transplantation, gene therapy may enable immunomodulatory agents to be expressed specifically within the graft, thereby overcoming the difficulties of systemic immunosuppression. However, for approaching existing problems associated with transplantation, gene therapy is most likely to be used only as a complementary approach, for example, by means of introducing genes blocking T-cell activation in the graft in order to reduce immunogenicity, or by means of introducing genes for donor-specific MHC antigen into the recipient before transplantation to induce transplantation tolerance.[13]

Tolerance induction strategies

The ultimate goal in transplantation is the development of donor-specific tolerance, a goal which has been sought by transplant biologists for almost half a century.[14, 15] Immunological tolerance can be defined as a state in which (1) the immune system does not mount a pathologic response against a specific antigen (or antigens), (2) there is no

requirement for ongoing exogenous immunosuppression, and (3) response to other antigens is maintained.[15]

Although the precise mechanisms are not understood, most strategies developed to induce tolerance involve a combination of pretreating the recipient with donor antigen and short courses of immunosuppressive or immunomodulatory therapy.[12] Tolerance induction requires that alloreactive T cell clones are rendered non-reactive, which may be achieved through clonal anergy, activation-induced apoptosis, or induction of regulatory/suppressor cell function. Anergy and deletion mechanisms may occur within the thymus (central tolerance) or in the peripheral immune system (peripheral tolerance), whereas regulatory cells typically work in the periphery. Research into the mechanisms of activation-induced apoptosis has highlighted the importance of T cell IL-2 production.[16] Thus, IL-2 has a dual function: it is essential for T cell proliferation but is also required for cell death. These findings may explain why calcineurin inhibitors, in particular cyclosporine, prevent the development of tolerance in some experimental models.[17]

Immunosuppressive drugs developed in the past two decades have improved the short-term survival of organ allografts, but tolerance has not been achieved and almost all transplant recipients continue to require drugs throughout life.[4, 18] Moreover, challenges to achieve clinical transplantation tolerance still sustain.

Challenges in clinical transplantation tolerance

Creating continuing tolerance would avoid the current requirements for life-long immunosuppression and many of the associated complications, however, there remain three key issues that need to be resolved and which are unknown so far. The first is the effect of tolerance on the development of chronic rejection: one major point that confronts any transplant program is the prevention of chronic rejection and yet the pathophysiology of chronic rejection is poorly understood. The second is the relationship between tolerance and specific infections. It remains to be proven whether tolerated grafts will continue to survive if the host is forced to mount an immune response to infection or trauma. The third is the risk of malignancies potentially associated with tolerance strategies.[19] On top of this, potential pitfalls exist, such as (1) the specificity of induced tolerance; (2) durability: it is not known what will be required to maintain graft tolerance for a lifetime; (3) recurrence of disease: if the organ replacement is due to autoimmune disease, such as lupus nephritis and autoimmune diabetes, then such diseases may affect a healthy graft. How tolerance-based transplantation will function for patients with underlying autoimmune diseases is not known.[3]

Last but not least, another major factor limiting the optimal use of organ transplantation and hence limiting the number of tests on alternative drugs or treatments inducing transplantation tolerance, is the shortage of donated human organs.[20] Cadaveric donor organs are sufficient for only 10% of total need at the time. The gap between requests for donor organs / tissues and their availability continues to grow.[3] One possible source that remains underutilized is human fetal tissues, but ethical and legal barriers to their use may be insurmountable.[3] A second approach is the development of bio-engineered tissues which could take many forms: for example the organs from transgenic and knockout animals. These strategies are still premature in their development and clinically unproven. In addition, the potential use of xenografts raises special ethical and psychological issues for physicians and recipients. More importantly, it even introduces novel infectious pathogens into the human population.[21-25] Therefore, from an infectious disease standpoint, this issue remains exceedingly complex.[3, 20] Consequently, there is still a long way to go before an ideal immune tolerance can be achieved in organ transplantation.[26]

Withdrawal of steroid

Because of troublesome side effects associated with steroid use, such as osteoporosis, diabetes and hypertension, many transplant centres have tried to withdraw steroids from stable, solid organ transplant recipients.[27-31] The ability to wean liver transplant recipients of steroids depends on both their primary immunosuppressive regimen and their primary disease state.[32] Slow steroid withdrawal in transplant patients, using conventional immunosuppression, reduces side effects, but bears a high risk of late rejection.[33, 34] In addition, the management of a recurrent autoimmune process, and associated other medical conditions will continue to cause problems for the successful complete and sustained long-term freedom from steroids.[35] Furthermore, there are still certain percentages of organ transplant recipients requiring even life-long steroid or reintroduce steroid, both in children [36] and adults,[35] even after many years post successful organ transplantation.

Immunosuppressive therapy is undergoing an exciting period of change, as increasing numbers of drugs make the transition from the laboratory bench to the clinical arena.[12] Nevertheless, several important issues have yet to be addressed, such as (1) specificity and the potency of the drug; (2) the drug combination for best efficiency; (3) the reduction of chronic

rejection and (4) the issue of cost-effectiveness, all of which require considerable extended follow-up studies.[12]

1.3 Infections in organ transplant recipients

Advances in immunosuppressive therapies as well as the improvement in surgical techniques have made organ transplantation a routine hospital procedure. Nevertheless, immunosuppression-associated impairment of the inflammatory response often leads to an inadequate defense against infections, which remains the most common life-threatening complication of long-term immunosuppressive therapy.[21, 37, 38] The risk of infection in the solid organ transplant recipient is determined primarily by two factors: the intensity of exposure to potential pathogens (epidemiologic exposure) and the combined effect of all of the factors that contribute to a patient's susceptibility to infection, namely, the net state of immunosuppression, which is the result of a complex interaction among multiple factors, for example the nature of the immunosuppressive therapy, such as dose, duration, and temporal sequence of individual agents.[23, 24]

The immunosuppressive programs used in all forms of solid-organ transplantation are quite similar, with either cyclosporine or tacrolimus being the cornerstone of maintenance antirejection therapy plus steroids. As a result, similar patterns of infection occur in all forms of organ transplantation giving rise to a consistent timetable when different infections can take place after transplantation.[21-24]

As represented in Figure 2, three pivotal segments are to be included: the first month, one to six months, and more than six months after transplantation. Clinically, this timetable may serve as a tool for developing a differential diagnosis in transplant recipients who present with infectious diseases, a tool for detecting excessive environmental exposure to pathogens that cause deviations from the timetable, and as a guide to the design of cost-effective, targeted preventive strategies.[24]

In the first month post transplantation, immunosuppression has just started and the cumulative exposure to immunosuppressive drugs as well as exposure to environmental sources of infection is small. In addition, rejection and infection with immunomodulating viruses are usually not yet problematic. The risk of infection is therefore related largely to nosocomial bacterial pathogens that typically take advantage of the post-operative hospitalized patient. The only likely fungal pathogens commonly encountered during this time period are *Candida* spp., and the only common virus is herpes simplex virus (HSV). Common

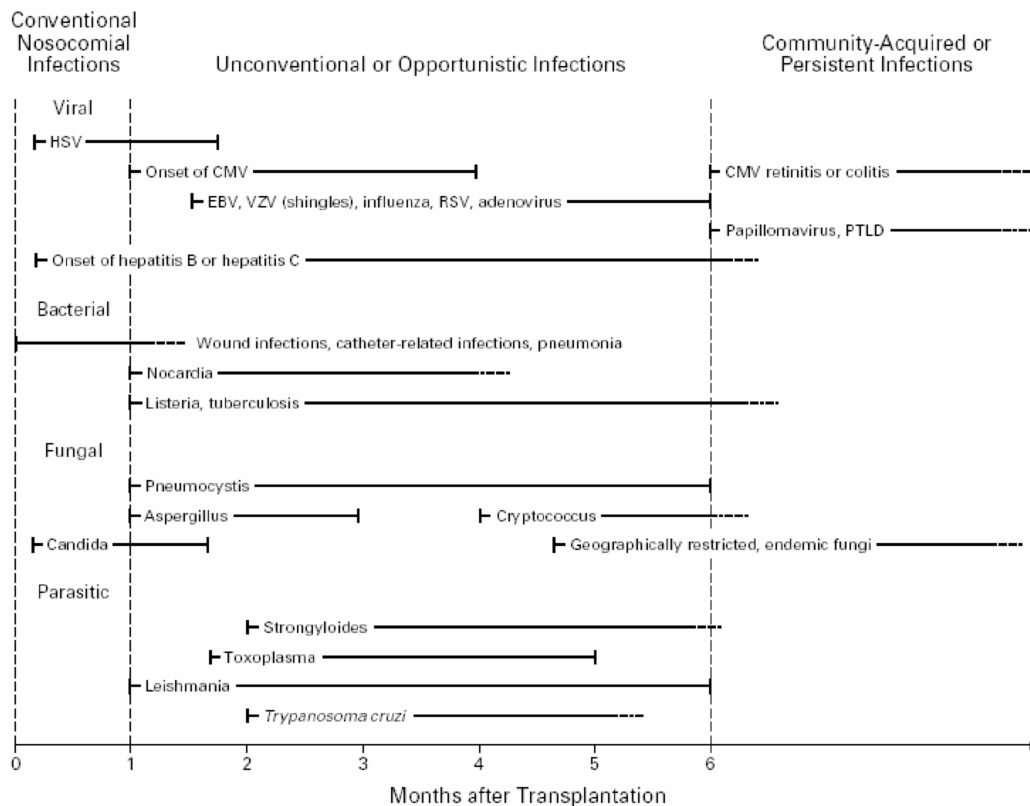

Figure 2. Usual Sequence of Infections after Organ Transplantation.[3]

Exceptions to the usual sequence of infections after transplantation suggest the presence of unusual epidemiologic exposure or excessive immunosuppression. HSV denotes herpes simplex virus, CMV cytomegalovirus, EBV Epstein–Barr virus, VZV varicella–zoster virus, RSV respiratory syncytial virus, and PTLD post-transplantation lymphoproliferative disease. Zero indicates the time of transplantation. Solid lines indicate the most common period for the onset of infection; dotted lines and arrows indicate periods of continued risk at reduced levels.

types of infection include pneumonia, urinary tract infection, intravenous catheter-related infection, and wound infection. Rarely, the allograft harbours pathogens and is itself a source of infection. The emergence of a previously unrecognized latent pathogen that was present in the recipient prior to transplantation is also relatively rare.

Infection between the first and sixth months following transplantation is dominated by viral and fungal pathogens. Iatrogenic immunosuppression remains fairly intense during this time, and problems with graft rejection often arise, adding to the degree of immunosuppression. Opportunists thrive in this setting, and suspicion of infection with such organisms must be high.

In the late post-transplantation period, i.e. more than 6 months following transplantation, if problems with rejection or chronic viral infection persist, the risk remains essentially the same as in the second period; thus, these patients remain at a very high risk of opportunistic

infections. If, however, the patient is clinically improving and is on a stable immunosuppressive regimen with good allograft function, the risk of infection resembles that of any minimally immunosuppressed individual in the community. Urinary tract infections in transplant recipients more commonly are complicated by pyelonephritis, bacteremia, and relapse than in other hosts.

However, the epidemiology of infections in the population of transplant recipients is changing because of the use of prophylactic regimens, vaccination, new immunosuppressive regimens, and careful control of infectious exposures.[24, 25]

1.4 Control of infections in organ transplantation

Antimicrobial therapy and its problems

The optimal approach to handling infection in the solid organ transplant recipient is prevention. There are three ways to use antimicrobial therapy in transplant recipients.[21] (1) Therapeutic use is the treatment of established clinical infection. (2) Prophylactic use is the administration of antimicrobial agents to an entire population of patients in order to prevent a form of infection that is important enough to justify such an intervention. (3) Pre-emptive use is the administration of therapy to a subgroup of patients defined by clinical or epidemiologic characteristics or by the results of a laboratory test that predicts a high rate of clinically significant disease. Because of the emphasis on the prevention of infection, particular attention is paid to prophylactic and pre-emptive strategies. In any case, exogenous immune suppression in all patients must be reduced as much as possible, in order to optimize both the prevention and the treatment of infection, an existing risk of graft rejection.[24]

Control of viral infection in organ transplant recipients

Control of viral infection in organ transplant recipients requires attention to the following interventions: i) prevention, whenever possible of viral acquisition; ii) the proper deployment of active and passive immunisation, with hyperimmune globulin preparations directed against cytomegalovirus, hepatitis B, varicella, and, perhaps, respiratory syncytial virus; and iii) the prescription of antiviral agents at critical points in the post-transplant course. Two important principles should be kept in mind when approaching this problem: prevention is the goal, as treatment of established infection is extremely difficult; and effective preventive strategies must be linked to the intensity of the immunosuppressive program employed. To achieve these goals, the addition of pre-emptive therapy to standard prophylactic regimens represents a significant advance.[39]

Cytomegalovirus (CMV) continues to be a cause of substantial morbidity and death after solid-organ transplantation. There are 3 major consequences of CMV infection: CMV disease, including a wide range of clinical illnesses; superinfection with opportunistic pathogens; and injury to the transplanted organ, possibly enhancing chronic rejection.[40]

Immunisation regimens in solid-organ transplant recipients

Solid-organ transplant recipients are at increased risk of various infectious diseases, some of which are vaccine preventable. Indeed, only in these cases, immunisations are among the most efficient interventions available. Solid-organ transplant recipients would greatly benefit from effective immunisations, provided the recommendations are based on a careful risk-benefit analysis, in which the effectiveness of the vaccine is weighed against possible adverse reactions, including graft rejection.[2]

1.5 Biochemical pharmacology of GM-CSF

Granulocyte-macrophage colony stimulating factor (GM-CSF) was first identified based on its ability to stimulate the clonal proliferation of myeloid precursors *in vitro*.[41-43] Endogenous GM-CSF, a heavily glycosylated polypeptide, was the first human myeloid haematopoietic growth factor to be molecularly cloned, after the gene sequence of endogenous human GM-CSF was first identified in 1985.[42] Within a few years, three different synthetic human GM-CSFs were produced, using recombinant DNA technology in bacterial (for Molgramostim), mammalian (for Regramostim) and yeast (for Sargramostim) expression systems.[41, 43]

The biologic effects of GM-CSF are mediated via binding to receptors expressed on the surface of target cells, which include granulocyte, erythrocyte, megakaryocyte, and macrophage progenitor cells, as well as mature neutrophils, monocytes, macrophages, dendritic cells, plasma cells, certain T lymphocytes, vascular endothelial cells, uterine cells, and myeloid leukemia cells. Molecular cloning studies have shown that the GM-CSF receptor is composed of two distinct subunits: the α subunit, which is unique to the GM-CSF receptor, and the common β (βc) subunit, which is shared with the receptors for IL-3 and IL-5.[44] The signal transduction pathways that occur after GM-CSF binds to the GM-CSF receptor are under evaluation. There appear to be at least two distinct signalling pathways, each involving a distinct region of βc. The first, which leads to induction of c-*myc* and activation of DNA replication, involves activation of a Janus kinase (JAK2), that is physically associated with βc.

Regulation of gene expression by JAK2 appears to be mediated by production of a DNA-binding complex containing the signal transducer and activator of transcription (STAT) proteins. The second pathway involves activation of *ras* and mitogen-activated protein kinases, with consequent induction of c-*fos* and c-*jun*, which are genes involved in regulation of hematopoietic differentiation.[41, 45, 46]

Although results from GM-CSF (-/-) (knock-out or gene targeted) mice (Table 3) demonstrate that endogenous GM-CSF is not an essential growth factor for basal hematopoiesis, but is rather critical in pulmonary homeostasis,[47-49] exogenous application of this drug has revealed diverse biologic effects playing a vital role in various functions of the immune system, including responses to inflammation and infection, as well as in hematopoiesis.[41-43] As a growth factor used for enhancing immune responses, GM-CSF is also known to recruit and activate antigen-presenting cells (APCs). Very recently, GM-CSF overexpression experiments within pancreatic beta-cells indicated that it would recruit, expand, and activate APCs, such as macrophages. Moreover, infiltration of such cells does not overtly harm, but may even protect pancreatic function, as seen with the delay in chemically induced diabetes.[50]

Table 3. Features of GM-CSF (-/-) mice

Features	References
1. Normal development	[47, 48]
2. No major abnormalities in hematopoiesis up to 12 weeks of age	
3. Fertile	
4. Development of abnormal lungs	
5. Extensive lymphoid hyperplasia in airways	
6. Granular eosinophilic material and lamellar bodies in alveoli	
7. Numerous large intraalveolar phagocytic macrophages	
8. Subclinical lung infections involving bacterial or fungal organisms	
9. Critical in regulation of surfactant homeostasis and alveolar macrophage innate immune functions in the lung	[49]

Clinical indications for the use of recombinant human GM-CSF have expanded considerably since the drug first became available in the early 1990s for acceleration of myeloid engraftment in neutropenic patients. Moreover, a variety of potential clinical uses for

GM-CSF are under investigation, such as prophylaxis or adjunctive treatment of infection in high-risk settings or immunosuppressed patient populations, the use as a vaccine adjuvant, and the use as immunotherapy for malignancies. Interestingly, through post-transplant immunization with GM-CSF producing tumor vaccines, the graft-versus-tumor effect can be sustained.[51] The selected putative applications of GM-CSF both in preclinical and clinical settings are summarized in Table 4.

GM-CSF not only plays a vital role in hematopoiesis by inducing the growth of several different cell lineages, it also enhances numerous functional activities of mature effector cells involved in antigen presentation and cell-mediated immunity, including neutrophils, monocytes, macrophages, and dendritic cells. More than a decade of *in vitro* and *in vivo* research using GM-CSF has shown that the name of this CSF is restrictive, because it describes only one aspect of the numerous biologic effects that have now been attributed to GM-CSF.[41] Based on the increasing variety of biologic effects being attributed to endogenous GM-CSF, additional clinical uses are under investigation. It is likely that the future will see applications of GM-CSF in a variety of settings beyond those classically associated with myelosuppression.

1.6 Reconstitution potential of GM-CSF for the immune response

GM-CSF, a drug already approved for hematological indications in humans,[42] has been indicated *in vitro* and *in vivo* to enhance the synthesis and release of pro-inflammatory cytokines.[52-56] Systemic injection of GM-CSF increases survival in a murine model of acute leukemia.[57] Our laboratory has previously shown that GM-CSF potentiates the immune response to endotoxin [58] and can restore the impaired immune response in lipopolysaccharide (LPS)-desensitized mice,[59] as well as in refractory human monocytes.[60] Others found that anergic monocytes from sepsis patients were reactivated [61, 62] and hyporesponsiveness of whole blood, induced by trauma, sepsis, or cardiac surgery could be overridden *in vitro* [63] by GM-CSF. Although GM-CSF is certainly efficient in facilitating the reappearance of the neutrophils, platelets and the erythroid lineage, the potency of this cytokine in terms of immune reconstitution is still uncertain.[64]

Table 4. Brief summary of the putative applications of GM-CSF [41]

Therapeutic Use	Preclinical actions or clinical results (selected)
Fungal infections	Increases receptor expression on macrophages. Decreases incidence of fungal infections versus placebo in autologous bone marrow transplantation (AuBMT) patients.
HIV infection and its complications	Suppresses HIV expression. Increases CD4 count. Enhances antiretroviral activity of zidovudine and stavudine. Decreases viral load.
Vaccine adjuvant	Increases class II MHC expression and stimulates T-cell immune responses. Enhances antibody response to hepatitis B vaccine.
Antitumor therapy	Enhances monocyte cytotoxicity against human tumor cells. Prolongs disease-free survival and overall survival compared with historical controls in patients with advanced melanoma.
Immunotherapy for acute myelogenous leukemia (AML)	Enhances activated killer cell function. Decreases risk of relapse compared with controls.
Mucositis, stomatitis, diarrhea	Stimulates the migration and proliferation of endothelial cells and promotes keratinocyte growth. Reduces incidence or severity of mucositis, stomatitis, diarrhea under immunosuppression.
Wound healing	Decreases time to wound healing. Intradermal injections of rHuGM-CSF results in enlarged keratinocytes, keratinocyte proliferation, thickening of the epidermis, and enhances healing.

1.7 The use of GM-CSF in transplantation

Administration of GM-CSF after bone marrow transplantation (BMT) enhances myeloid and platelet recovery, significantly reduces the duration to re-engraftment, the number of antibiotic treatment days, and the period of hospitalisation.[65] Moreover, GM-CSF is cost effective in the treatment of patients with relapsed Hodgkin's disease, who received intensive chemotherapy and autologous bone marrow transplantation,[66, 67] suggesting the use of GM-CSF as an adjunct to autologous/syngeneic bone marrow transplantation.[68]

Nevertheless, the potential of GM-CSF to accelerate the recovery of neutrophils, monocytes and lymphocytes, indicates a need for caution in the use of GM-CSF after allogeneic marrow transplantation.[69] Fortunately, administration of GM-CSF after allogeneic BMT does not appear to be associated with an increased incidence of chronic graft-versus host-disease (GVHD) or relapse, or of other adverse effects, such as the development of myelodysplasia.[70] Instead, GM-CSF has found an application in harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors for allogeneic marrow transplantation.[71] Additionally, as a growth factor, GM-CSF may increase numbers of circulating peripheral progenitor cells to serve as the source for marrow transplantation,[72] leading to less serious infections and a decreased hospital stay for the GM-CSF treated patients. Future trials with combinations of sequentially used cytokines may lead to a more rapid recovery of red blood cells and platelets, in addition to granulocytes.[73]

GM-CSF starts to find its applications or *ex vivo* clinical researches in organ transplantation.[5, 74-76] A detailed description of the clinical studies of GM-CSF in transplantation is listed in Table 5.

Table 5. The clinical studies of GM-CSF in transplantation

Category	Indications	Type of study	References
Bone marrow transplantation	To shorten the period of neutropenia	*In vivo* study in a primate model	[77]
	To accelerate neutrophil recovery	phase I/II study and clinical study	[78-80]
	To reconstitute granulo-monocytopoiesis faster	*Ex vivo* clinical study	[81]
	To sustain neutrophil recovery	Clinical study	[82]
	To restore neutrophil competence	Clinical study	[83]
	To enhance the graft-versus-leukemia reactivity	Clinical study	[84]
	To maintain haemopoiesis	Clinical study	[85]
	To accelerate hemopoietic recovery	Clinical study	[86]
	To accelerate granulocytic recovery	Clinical study	[87]
	To enhance peripheral progenitor cell yield with marrow reconstitution	*In vivo* mouse study and Clinical study	[88-90]
	To boost faster leukocyte recovery	Clinical study in patients with lymphoid malignancies.	[91]
Stem cell transplantation	To accelerate myeloid recovery, with a decreased incidence of bacterial infections	Clinical study in patients with lymphoma	[92]
	To facilitate a rapid myeloid engraftment	Clinical study in acute leukemia patient with myelofibrosis	[93]
	To facilitate myeloid recovery and the regeneration of immune system	Clinical study together with G-CSF	[94]
	To prime autologous peripheral blood stem cells (PBSC) or bone marrow stem cells (BMSC)	Cell collection for organ transplantation	[95, 96]
Solid organ transplantation	To restore neutrophil count in renal transplantation patients with leukopenia	Clinical study in renal transplantation	[74]
	To increase the absolute neutrophil count and CD34+cell numbers	Clinical study in heart-lung transplantation	[5]
	To increase neutrophil count in the treatment of neutropenia	Clinical study in pediatric orthotopic liver transplantation	[75]
	To increase the respiratory burst of human neutrophils	*ex vivo* study in liver transplantation	[76]

1.8 Aims of the study

Routine immunosuppression therapy in organ transplantation impairs the host immune defense against infections, which remain the major cause of morbidity and mortality following solid organ transplantation.[23, 24] Therefore, it is important to create a status in immunosuppressed organ transplant recipients with a reactivated immune resistance to infections, without causing graft rejection. Such a status requires a preferential reactivation of the effectors of the innate immune response, *i.e.* macrophages and/or neutrophils, by pharmacological intervention, without restoring the suppressed specific immune response, characterized e.g. by IL-2 production by T-cells, implicated in graft rejection.[97-99]

GM-CSF is a promising candidate for such an intervention, based on what has been discussed above. As a drug already approved for the increase of leukocyte counts in humans,[42] GM-CSF has been additionally found to potentiate the immune response to endotoxin [58] and to restore the impaired immune response in LPS-desensitized mice, as well as in refractory human monocytes.[59] It has also been found that anergic monocytes from sepsis patients were reactivated by GM-CSF.[60] Moreover, hyporesponsiveness of whole blood, due to trauma, sepsis, or cardiac surgery could be overridden *in vitro* by GM-CSF.[63] Further, our lab recently demonstrated in a murine model that GM-CSF reconstituted the immunosuppressed macrophage response *ex vivo* and *in vivo*, while the IL-2 and IFNγ response of T-cells remained silent. Most importantly, the immunosuppressed mice survived an otherwise lethal bacterial infection when pre-treated with GM-CSF, without inducing graft rejection after skin allotransplantation (Kühnle and Wendel, submitted). Consequently, aiming at extending the previous finding to humans, the aims of the study are:

(1) To investigate the reconstitution potential of GM-CSF in immunosuppressed human blood, with particular attention to factors relevant in innate immunity, using the gene-array technology.

(2) To check the lack of reactivation of immunosuppressed lymphocytes by GM-CSF.

(3) To explore the possible mechanism supporting such potential.

2. Materials and methods

2.1 Chemicals and reagents

Human recombinant GM-CSF (LEUCOMAX® 400: Molgramostim) was purchased from ESSEX Pharma GmbH (Munich,Germany), while human recombinant IL-1β and TNF (for bioassay) were a gift from Dr. Stephen Poole, from NIBSC (National Institute for Biological Standards and Control, South Mimms, Hert, United Kindom). Dexamethasone (Dexa-Allvoran®) was purchased from TAD Pharmaceuticals (Cuxhaven, Germany), and LPS (from *Salmonella abortus equi*) was obtained from Sigma (Deisenhofen, Germany), while Concanavalin A (Con A) from Sigma (Deisenhofen, Germany), α^{32}P-ATP from ICN Biomedicals Inc. (Costa Mesa, California, USA), RPMI 1640 medium with and without phenol red from Invitrogen GmbH (Karlsruhe,Germany) and Biochrom (Berlin, Germany) respectively, were obtained. The RNeasy preparation kit was purchased from QIAGEN GmbH (Hilden, Germany) and the cDNA expression array (Atlas Human Arrays 1.2) from Clontech Laboratories, Inc. (Palo Alto, California, USA). The Live/Dead viability/cytotoxicity kits were purchased from Molecular Probes (Leiden, The Netherlands). BCA protein assay reagents and ECL® western blot kit were obtained from PIERCE (Rockford, Illinois,USA) and from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA), respectively. The ribonuclease protection assay (RPA) kit RPA II™, the *in vitro* transcription kit MAXIscript™, the nonisotopic labeling kit BrightStar™ psoralen-biotin and the nonisotopic detection kit BrightStar™ BioDetect™ were all obtained from Ambion Ltd. (Huntingdon, Cambridgeshire, United Kingdom). The cytokine multi-probe template sets hCK-2 and hCK-3, including templates for TNF, IL-1, IL-1β, IL-1ra, IL-6 and LT-β were purchased from PharMingen (Hamburg, Germany). The neutralizing monoclonal anti-human IL-2 antibody was from R&D systems GmbH (Wiesbaden-Nordenstadt, Germany).

2.2 Human blood sampling

Blood from 10 healthy donors was used in order to select the optimal working concentration of the immunosuppressive drug dexamethasone. In order to test the potential clinical relevance of our findings, this study included 10 patients with an average age of 58.6 years (from 39 to 69), who all underwent orthotopic liver transplantation at the University Hospital of Mainz. Decompensation of liver function was due to chronic liver diseases, such as primary biliary cirrhosis, chronic viral hepatitis B or C, autoimmune hepatitis or acute liver

failure. All these patients were treated with methyl prednisolon (12 mg for 9 patients and 36 mg for 1 patient) combined with tacrolimus. From these 10 patients, blood was taken at the lowest level of immunosuppression by tacrolimus (Cmin), within 1 month after transplantation. For comparison, also included were additional 10 liver transplant patients whose blood was drawn later than one month post transplantation. All patients gave written informed consent to transplantation and follow-up examinations.

2.3 Preparation of human PBMC and lymphocytes

Peripheral blood mononuclear cells (PBMC) were prepared from healthy donors or liver transplant patients in cell preparation tubes (Vacutainer CPT, Becton Dickinson Company, Franklin Lakes, New Jersey, USA) according to the manufacturer's instructions. After centrifugation (20 min, 1650 $\times$ g), the white layer above the gel containing the PBMC was removed and the cells were washed 3 times with RPMI 1640. The purified PBMC, adjusted to 5×10^6 cells/ml with 200 µl of RPMI 1640 medium supplemented with 2.5 IU/ml heparin (Liquemin, Hoffmann La Roche, Grenzach-Whylen, Germany) and 100 IU/ml penicillin/streptomycin (Biochrom, Germany) were added to endotoxin-free sterilized 1.5 ml tubes, followed by incubation at 37°C and 5% CO_2 subsequently with dexamethasone (1 µM) for 1 h, GM-CSF (50 ng/ml) for 1 h, and stimulated with LPS (100 ng/ml) for 1 and 16 h, or with ConA (5µg/ml) for up to 72 h. PBMC were collected by centrifugation (300 $\times$ g, 10 minutes), pooled and subjected to total RNA isolation prepared for cDNA expression array analysis (1-h-LPS-stimulation), or collected for viability/proliferation assay and for western blot (6 to 72-h-LPS or Con A-stimulation). Cell-free supernatants were kept at –80°C until ELISA measurement. Lymphocytes were prepared as the non-adherent fraction from PBMC upon growth adherence for 2 h (non-adherent fraction of PBMC).

2.4 ELISA

Cytokines in cell-free supernatants were quantified by sandwich enzyme-linked immunosorbent assay (ELISA). Antibody pairs for TNF-α, IL-1β, IL-1ra, IL-2 and IL-6 were purchased from Endogen (Munich, Germany). Human recombinant TNF (Bender, Vienna, Austria), IL-1β, IL-1ra and IL-6 (Endogen) were used as standards. ELISA plates were coated overnight and were processed as previously described. [100] The colorimetric measurement at 450 nm with reference wavelength of 690 nm was performed using an ELISA Reader (SLT, Crailsheim,Germany) with the built-in software for quantification.

2.5 Human TNF bioassay

The bioactivity of TNF, assessed as cytotoxicity in WEHI 164 subclone 13 fibrosarcoma cells, was evaluated using the ethidium homodimer-1 incorporation assay (Molecular Probes), as described previously.[101] WEHI cells were grown in RPMI 1640 medium, supplemented with 10% FCS and antibiotics. Cells were plated at 3×10^4 cells/well in 100 µl in flat-bottomed 96-well plates. Transcriptional inhibition was obtained upon addition of actinomycin D (1 µg/ml), followed by loading of 100 µl of samples or standards. Each sample was tested in duplicates of eight serial dilutions. Human recombinant TNF (NIBSC) was used as a standard. After 18 h of incubation at 37°C and 5% CO_2, cells were centrifuged for 5 min at $300 \times g$ and washed with phenol-red-free RPMI 1640 medium. Upon incubation of the positive control wells with 33% ethanol for 1 h, cells were stained with ethidium homodimer-1 for 1 h before fluorescence measurement. Plates were read in the multilabel plate reader Victor II, using an excitation wavelength of 530 nm and an emission wavelength of 620 nm. The bioactive human TNF was quantified using the standard curve, plotted as the percentage of cell lysis versus quantity of TNF standards.

2.6 Viability / Proliferation assay

Live cells in 96-well-plates were washed 3 times with Dulbecco's PBS and resuspended in serum-free-medium or D-PBS containing 1 µM of Calcein AM (Molecular Probes) and were then kept at 37°C for 1 h, followed by fluorescence measurement in the multilabel plate reader Victor II (Wallac), set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The negative control were prepared by treating live cells with 33% ethanol for 60 minutes before staining with Calcein AM. Viability/proliferation was presented as percentage (%) of the control live cells based on the fluorescence reading defined.

2.7 cDNA expression array

After PBMC treatment, cells were pooled and subjected to total RNA isolation (Qiagen, Hilden, Germany). mRNA expression was analyzed using the Atlas Human Arrays 1.2 (Clontech, Palo Alto, California, USA). The assay followed the manufacturer's manual. Briefly, cDNA synthesis was performed in the presence of reverse transcriptase and α^{32}P-dATP (ICN Biomedicals, Costa Mesa, California, USA). The purified probes with a total

radioactivity of 5×10^6 cpm were hybridized to identical human cDNA array membranes, which contained 1176 previously characterized human genes. The signal intensity was measured using a PhosphoImager system after 1-2 days exposure time and was quantitated with the ImageMaster VDS software package (Pharmacia Biotech, San Francisco, California, USA). The levels of expression were normalized using several highly expressed housekeeping genes whose respective expression levels were the same in cells of all settings. The cDNA expression array was performed 2 times for all the settings and a 1.5-fold or more up-/down-regulation is considered to be significant.

2.8 Ribonuclease Protection Assay (RPA)

The ribonuclease protection assay was performed according to the manufacturer's manual. Briefly, for *in vitro* transcription by MAXIscript™, cytokine multi-probe templates (hCK-2 and hCK-3) were used. The resulting antisense transcript was then labelled with nonisotopic labeling kit BrightStar™ (psoralen-biotin) under long wavelength (365nm) UV light after purification. Sample total RNA, isolated from treated PBMC with RNeasy preparation kit, was hybridized with the labelled RNA probe at 42°C overnight and treated with RNase for 30 minutes at 37°C using ribonuclease protection assay kit RPA II™. The remaining "RNase-protected" probes were purified and resolved on 5% polyacrylamide/TBE gel with 8 M urea. The gel was then transferred to positively-charged nylon membrane Hybond™-N (Amersham Pharmacia Biotech) by electroblotting, followed by crosslinking nucleic acids at 80°C for 1 h and by detection using BrightStar™ BioDetect™. The housekeeping genes (L32 and GAPDH) included in the multiprobe sets allowed assessments of total RNA levels for normalizing sampling.

2.9 Western blotting

Cell extracts of 20 µg total protein, determined with the bicinchoninic acid assay (Pierce, Rockford, IL, USA) to confirm equal loading, were separated on a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Germany). The membrane was probed with a mouse anti-$p27^{kip1}$ antibody (Pharmingen, San Diego, California, USA), rabbit anti-Cdk2 antibody and anti-Jab1 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), and mouse anti-cyclin D2 antibody (Pharmingen, San Diego, California, USA), respectively. Immunoprecipitates were detected by a horseradish-

peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham, Piscataway, New Jersey, USA).

2.10 Statistical analysis

Data were presented as mean ± SEM and data sets were subjected to one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests (GraphPad Prism, GraphPad Software Inc., San Diego, USA). $p < 0.05$ (indicated as *) was considered significant, while NS stands for not significant.

3. Results

3.1 Concentration-dependent inhibition by dexamethasone of LPS-induced TNF-α production in relation to viability of PBMC

To explore the reconstitution capacity of GM-CSF in immunosuppressed PBMC, this study used a cellular model, in which purified PBMC from 10 healthy donors were exposed to dexamethasone and then stimulated with LPS in the absence or presence of GM-CSF. The optimal working concentrations of GM-CSF (50 ng/ml) and LPS (100 ng/ml) for the priming or stimulation of TNF production were based on what was previously reported.[60] Since dexamethasone has been reported to cause cell death or apoptosis of monocytes [102, 103] or lymphocytes,[104] this study first examined the viability of the dexamethasone-treated PBMC. As shown in Figure 3 A, in the range from 0.1 to 10 µM dexamethasone, cells maintained their viability within 16 h. At 72 h after treatment, a viability loss occurred at dexamethasone concentrations higher than 1 µM. Since 1 µM dexamethasone was sufficient for almost complete inhibition of LPS-induced TNF-α production (Figure 3 B), without inducing significant cytotoxicity, this concentration was chosen for all further experiments.

3.2 Optimization of the gene array working conditions

The arrays chosen for this study contained 1176 human cDNAs encoding proteins with a wide range of functions partially characterized for PBMC. Similar numbers of PBMC obtained from healthy donors were pooled prior to total RNA isolation and cDNA expression array analysis. Figure 4 shows overview images of 4 identical arrays hybridized with [^{32}P]-labelled cDNA probes prepared from human PBMC that were either left untreated or stimulated with LPS in the presence or absence of dexamethasone and/or GM-CSF. To compare transcript levels in cells of all settings, appropriate internal reference genes were used for normalization. This study measured 6 housekeeping genes as internal standards, which encode for ubiquitin, G3PDH, HLA class I histocompatibility antigen C-4 alpha subunit (HLAC), β-actin, 60S ribosomal protein L13A and 40S ribosomal protein S9. As indicated in Figure 4 (6 spots in the bottom single row), the signal intensities of these 6 internal standard genes were similar among different settings, allowing a comparison between multiple array experiments by

normalizing the data sets to the average expression level of these housekeeping genes. By comparing the signal intensities obtained from untreated cells (Figure 4A) with cells treated

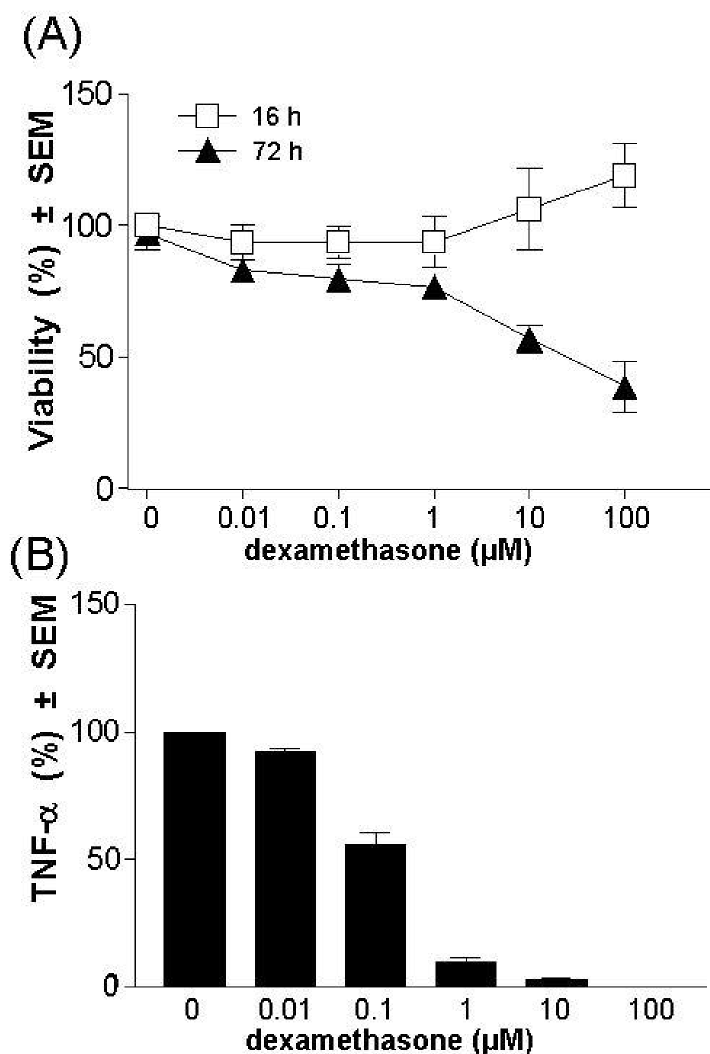

Figure 3. Concentration-dependent effect of dexamethasone on viability of human PBMC and on the suppression of LPS-stimulated TNF-α release

(A). PBMC (5×10^6 cells/ml) were preincubated for 1 h with different concentrations of dexamethasone and then incubated with LPS (100 ng/ml) for 16 h or 72 h at 37°C and 5% CO_2. Viability was measured in serum-free-medium containing 1 µM of Calcein-AM after 1 h of staining by fluorescence measurement on Victor II at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A negative control was prepared by treating live cells with 33% ethanol for 60 minutes before staining with Calcein-AM. Data represent means ± SEM of the healthy donor group (n =10).
(B) TNF-α was assessed by ELISA in supernatants of cells preincubated for 1 h with different concentrations of dexamethasone and then incubated for 16 h with LPS (100ng/ml).

with LPS only (Figure 4B), a differential LPS-inducible gene expression pattern was obtained. In this matrix, the extent of either suppression by dexamethasone or the influence of GM-CSF was quantified by comparing Figure 4C or Figure 4D with Figure 4B, respectively.

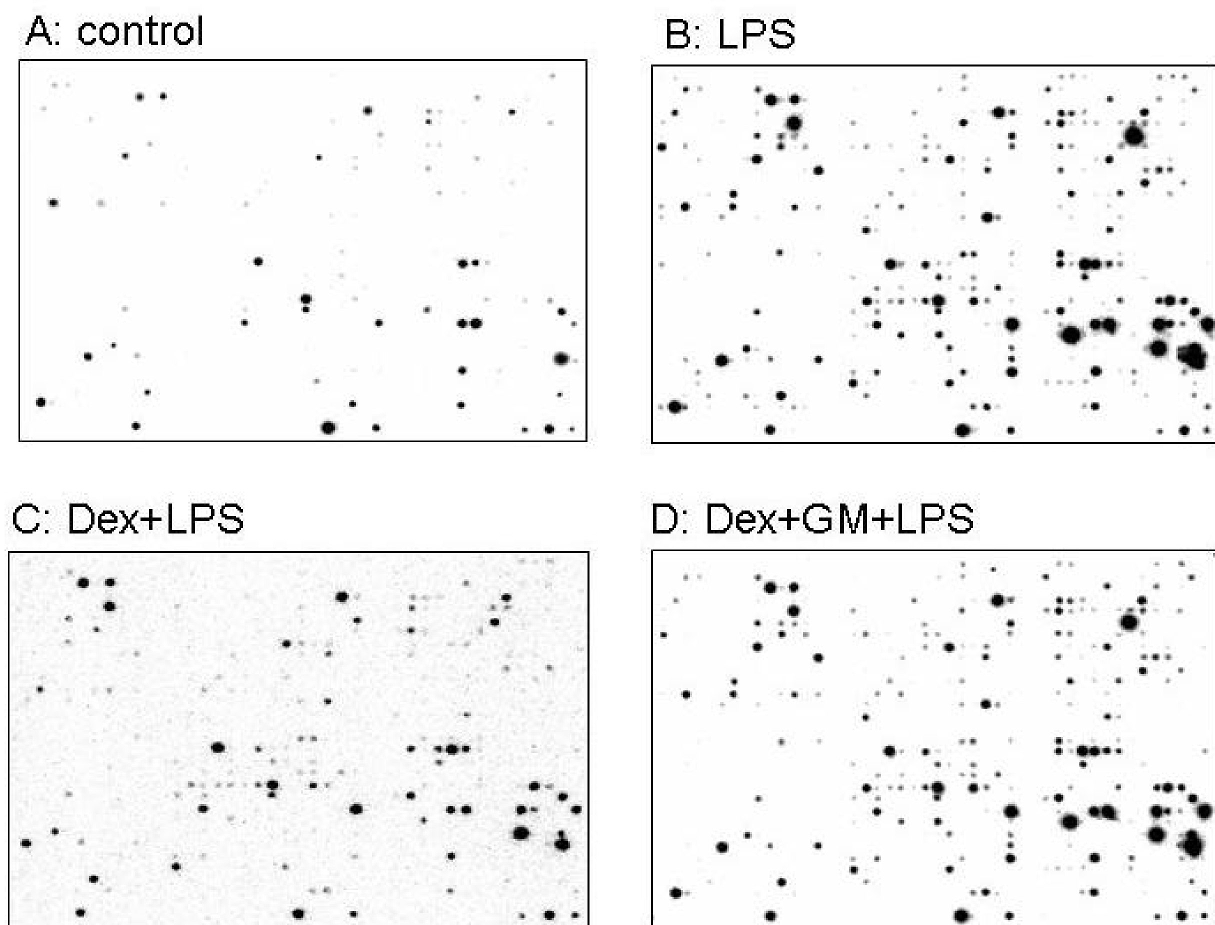

Figure 4. GM-CSF inducible changes in gene expression profile on cDNA array membranes of human PBMC exposed to various stimuli.

PBMC (5×10^6 cells/ml) were subsequently treated with dexamethasone (1 µM) for 1 h, with GM-CSF (50 ng/ml) for another 1 h and with LPS (100 ng/ml) for an additional h at 37°C and 5% CO_2. Total RNA isolated from untreated or treated PBMC in 4 settings pooled from healthy donors was used as a template to synthesize α^{32} P-radiolabelled cDNA probes of equal specific activity (5×10^6 cpm). The probes were hybridized to 4 identical cDNA array membranes. Following washing steps, the membranes were imaged by PhosphorImager. The membranes contain 1176 genes, divided into various functional groups. The housekeeping genes coding for ubiquitin, G3PDH (glyceraldehyde 3-phosphate dehydrogenase), HLAC (HLA class I histocompatibility antigen C-4 alpha subunit), β-actin, L13A (60S ribosomal protein L13A) and S9 (40S ribosomal protein S9), used as internal references, were located in the bottom single row on each membrane.

3.3 GM-CSF restored the release of TNF-α, but not of IL-1β, in immunosuppressed blood

In order to investigate the potential of GM-CSF to restore TNF-α production upon LPS stimulation, this study first used an experimental dexamethasone-suppression model, in which

whole blood or PBMC from 10 healthy donors were exposed to dexamethasone and then stimulated with LPS. The results in Figure 5A show that in the absence of dexamethasone, GM-CSF itself did not induce TNF-α protein release, as assessed by ELISA, but rather primed the cells for the LPS-inducible TNF-α production. In dexamethasone-exposed whole blood, the LPS-induced TNF-α release was significantly reduced, but in the presence of GM-CSF, LPS-inducible TNF-α production was significantly increased (Figure 5A), although not to the level of GM-CSF-primed naive cells from healthy donors. Interestingly, another macrophage-mediated response, i.e. IL-1β release, was not restored by GM-CSF, although its production was significantly primed by GM-CSF in the non-suppressed setting (Figure 5B).

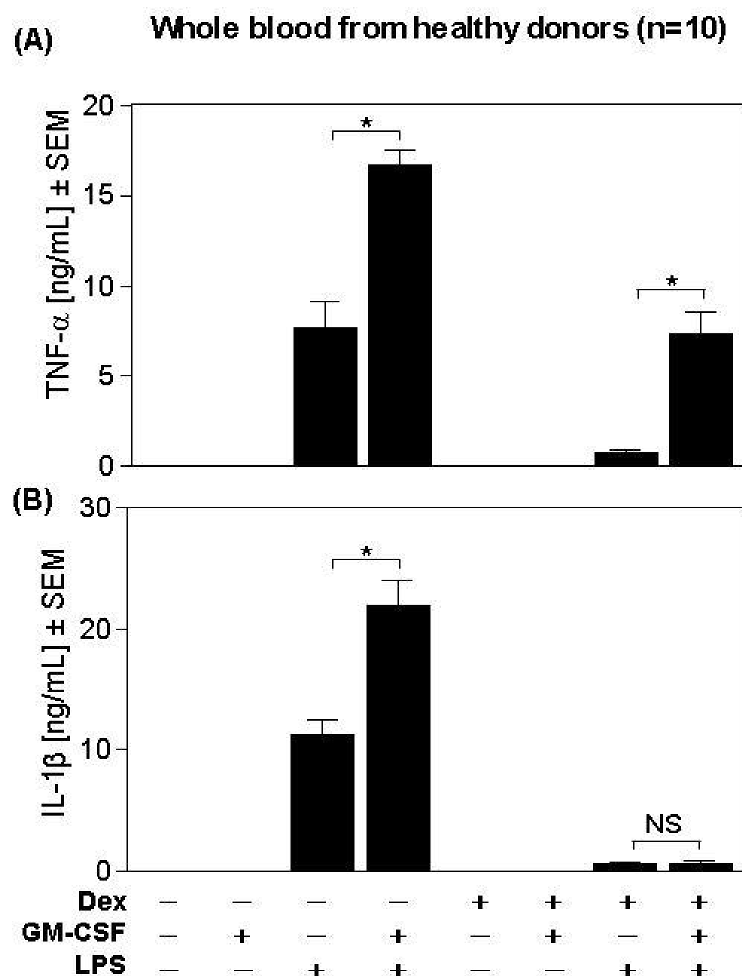

Figure 5. Reconstitution of TNF-α but not IL-1β production by GM-CSF in dexamethasone suppressed blood from healthy donors.

Whole blood was incubated with or without dexamethasone (1 μM) for 1 h, with GM-CSF (50 ng/ml) for another 1 h and stimulated with LPS (100 ng/ml) for 16 h. Cell-free supernatants were then subjected to ELISA for cytokine release measurement. The amount of TNF-α (A) and IL-1β (B) released was expressed per milliliter whole blood. Data represent mean ± SEM of the results from 10 healthy donors.

This study next examined the potential of GM-CSF to restore TNF-α production at the mRNA level, using a cDNA expression array. For these array experiments, the LPS stimulation model was used, i.e. PBMC's purified from 10 healthy donors were sequentially treated with dexamethasone (1 μM) for 1 h, with GM-CSF (50 ng/ml) for 1 h and then with LPS (100 ng/ml) for 1 h. This study examined 6 housekeeping genes as internal standards, encoding for ubiquitin, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), human lymphocyte antigen C-4 alpha subunit (HLAC), β-actin, 60S ribosomal protein L13A and 40S ribosomal protein S9. As shown in Figure 6A, in the original grid on the array membrane, the signal intensities of these 6 internal standard genes were equal among different settings, indicating a similar level of housekeeping gene expression in cells of all settings. Based on these control data, the expression levels of the other genes on the array were examined, with special emphasis here on TNF-α and IL-1β. As is visually apparent in the selected grid of the array, TNF-α mRNA expression was suppressed by dexamethasone, which could be significantly restored by GM-CSF, with an upregulated expression of up to 4-fold, almost reaching the level of the LPS-treated setting (Figure 6B), while no significant influence on IL-1β mRNA levels by GM-CSF was detected (Figure 6C).

Subsequently, this study investigated whether these observations also hold true in clinical samples. As shown in Figure 7A, exogenous GM-CSF restored the LPS-induced TNF-α release in blood taken from 10 immunosuppressed liver transplant patients *in vitro*, as measured by ELISA. Since the bioactivity of TNF-α can be neutralized by its soluble receptors sTNF-R1 and sTNF-R2, the latter of which is cleaved by the same convertase that mediates the shedding of TNF-α from its membrane-bound pro-form, [105] this study also performed a TNF-α cytotoxicity assay in WEHI 164 cells. The study found that the TNF-α restored by GM-CSF in the blood from immunosuppressed liver transplant patients was bioactive (Table 6) and that the TNF-α concentrations estimated in the bioassay were comparable to those measured by ELISA. In accordance with the previous results, also in this *ex vivo* immunosuppressed blood setting, GM-CSF did not restore the IL-1β response (Figure 7B).

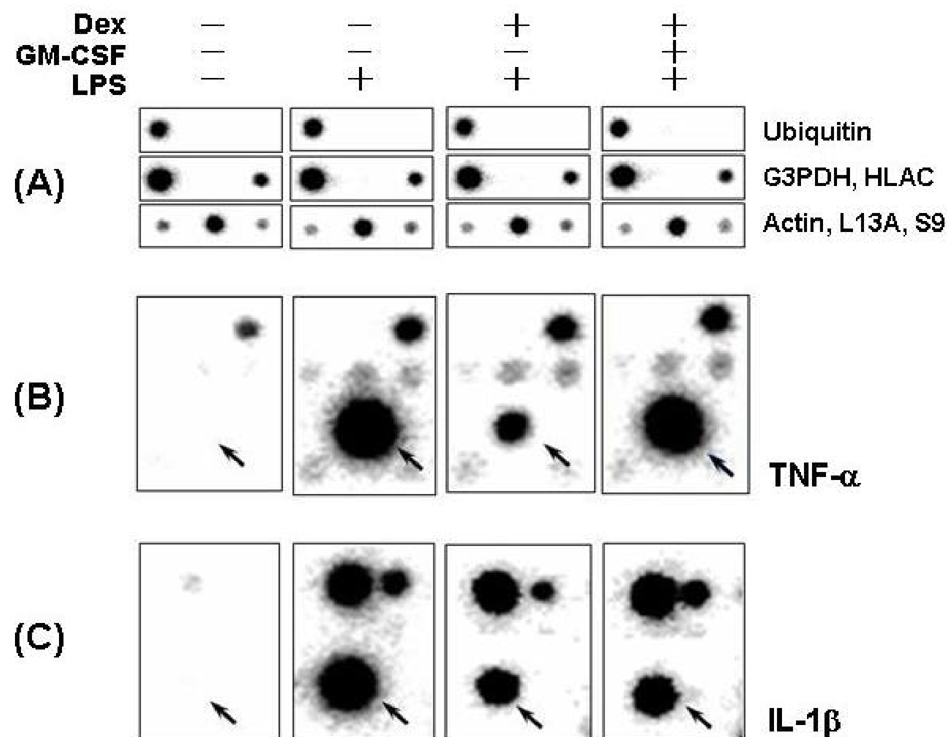

Figure 6. Differential reconstitution by GM-CSF of macrophage response: upregulated mRNA expression of TNF-a but not of IL-1b in human immunosuppressed PBMC.

PBMC (5×10^6 cells/ml) were subsequently treated with dexamethasone (1 µM) for 1 h, with GM-CSF (50 ng/ml) for another 1 h and with LPS (100 ng/ml) for an additional h at 37°C and 5% CO_2. α^{32} P-radiolabelled (5×10^6 cpm) cDNA probes was generated from total RNA (from the pooled PBMC after treatment, n = 3) and hybridized to 4 identical cDNA array membranes containing 1176 genes each. The membranes were imaged by PhosphorImager. The location of the detected housekeeping genes (A) were indicated in original grid on the membrane of the array. The arrows in the selected part of the array membrane indicated the detected signals for mRNAs of TNF-α (B) and IL-1β (C), with the normalized expression rate of (Dex+GM-CSF+LPS) over (Dex+LPS), or restoration rate: 4.2 for TNF-α and 1.0 for IL-1β respectively. Dex stands for dexamethasone.

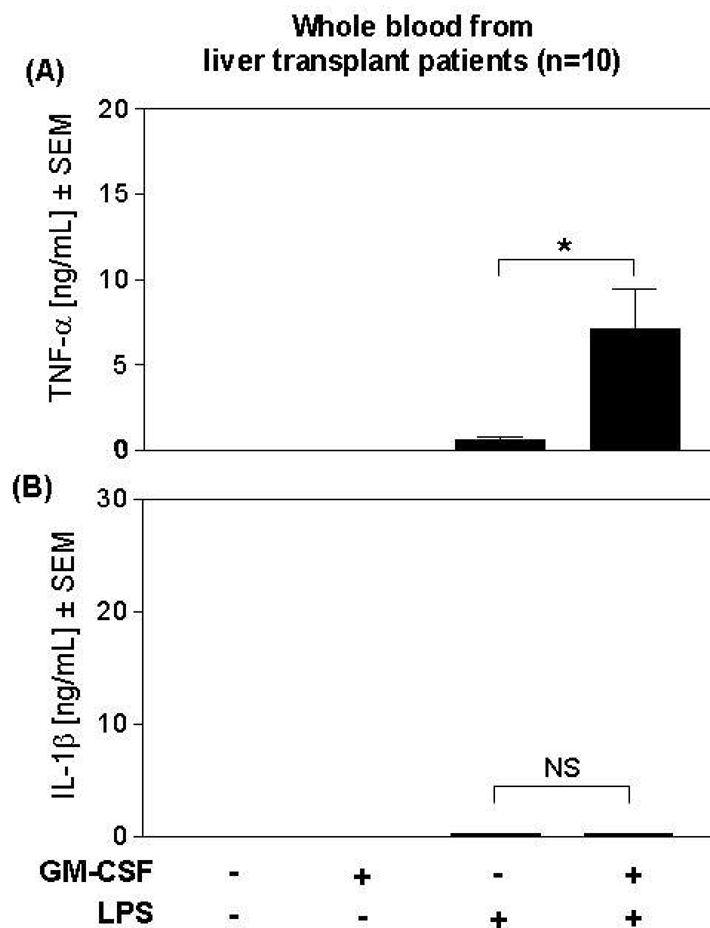

Figure 7. Reconstitution by GM-CSF of the macrophage-mediated TNF-a but not IL-1b release in immunosuppressed blood from liver transplant patients.

Whole blood taken from liver transplant patients was treated exactly as in Figure 5, without further dexamethasone treatment. After 16 h of incubation at 37°C with 5% CO_2, released cytokines were measured in cell-free supernatants by ELISA. The amount of TNF-α (A) and IL-1β (B) released was expressed per milliliter whole blood. Data represent mean ± SEM of the results from 10 liver transplant patients.

Table 6. Bioactivity of LPS-elicited TNF-α release potentiated by GM-CSF in immunosuppressed blood from liver transplant patients.

Treatment	TNF-α (pg/ml) ± SEM	
	Bioassay	ELISA
Untreated	0 ± 0 #	0 ± 0
GM-CSF	0 ± 0 #	0± 0
LPS	530 ± 50 #	640 ± 170
GM-CSF + LPS	6870 ± 1440 #	7080 ± 2350

The bioactivity of TNF-α, assessed as cytotoxicity in WEHI 164 subclone 13 fibrosarcoma cells, was evaluated using the ethidium homodimer-1 incorporation assay.[101] The bioactive human TNF-α was quantified using the standard curve, plotted as the percentage of cell lysis versus the quantity of TNF-α standards. For comparison, results obtained in ELISA from the same samples were also given. # represented no significant difference (n=10, p> 0.05) compared to the results measured by ELISA.

3.4 GM-CSF did not restore T-cell response and T-cell proliferation

Since the rationale of our pharmacological approach is the presumption to preferentially restore the innate immune defense without reactivating the adaptive immune system, it was necessary to examine whether GM-CSF activated T-cell responses in immunosuppressed blood. This study chose the Con A-stimulated IL-2 production of whole blood and Con A-induced proliferation of PBMC (T-cell) as markers for a typical T-cell response. As shown in Figure 8A, GM-CSF enhanced the Con A-stimulated IL-2 production in blood from healthy donors, in agreement with what was published,[106] and the Con A-induced proliferation of PBMC as well (Figure 8B), but failed to induce IL-2 release in dexamethasone-suppressed blood from healthy donors (Figure 8A) and in blood from liver transplant patients (Figure 8C). Consistently, this study also found that GM-CSF did not restore the Con A-stimulated proliferation of immunosuppressed PBMC (Figure 8B, 8D).

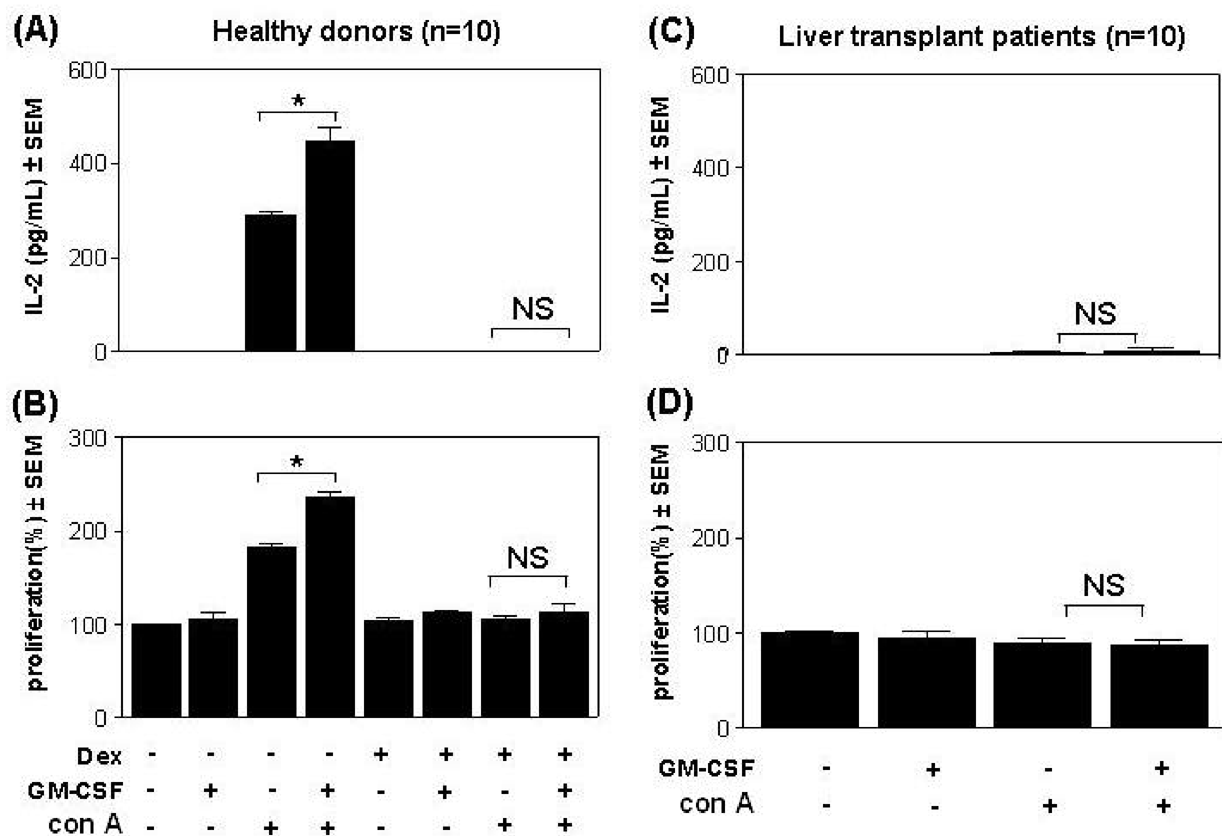

Figure 8. No augmentation by GM-CSF of T-cell IL-2 response and T-cell proliferation in immunosuppressed human blood in contrast to non-suppressed blood.

(1) IL-2 response: Both the dexamethasone-treated blood from healthy donors (as described in Figure 5) and the blood from liver transplant recipients were treated with or without GM-CSF (50 ng/ml) for 1 h followed by stimulation with 5 µg/ml Con A. After 72 h of incubation at 37°C with 5% CO_2, the released IL-2, expressed per milliliter whole blood, was measured in cell-free supernatants by ELISA.
(2) Proliferation assay: PBMC were treated the same as above for whole blood. The proliferation assay was performed by fluorescence measurement using Calcein-AM staining (Molecular Probes, Leiden, the Netherlands). The negative control was prepared by treating the cells with 33% ethanol for 60 minutes. Proliferation of PBMC was presented as percentage (%) of viable cells of the control. Data represent the mean ± SEM of the healthy donor group (n=10) (A and B) and of the transplant recipient group (n=10) (C and D).

3.5 IL-1β restored Con A-induced proliferation of lymphocytes in immunosuppressed PBMC, independently of IL-2

As indicated above, exogenous GM-CSF could not induce Con A-stimulated proliferation of lymphocytes in immunosuppressed PBMC. Moreover, in the same setting, GM-CSF did not upregulate IL-1β (data not shown), but rather increased the expression of IL-1ra (Table 7), which is known to neutralize IL-1β bioactivity.[107] Therefore, this study next investigated whether IL-1β could restore the Con A-stimulated proliferation during immunosuppression. The results of the proliferation test indicated that exogenous IL-1β induced a Con A-stimulated proliferation of lymphocytes in dexamethasone-suppressed PBMC (Figure 9A) and PBMC from liver transplant patients (Figure 9B). In addition, such restored proliferation could not be blocked by a neutralizing anti-human IL-2 monoclonal antibody, which could completely inhibit the Con A-induced proliferation of lymphocyte (data not shown), indicating an IL-2-independent mechanism involved (Figure 9B).

Table 7. Enhancement by GM-CSF of IL-1ra release in immunosuppressed blood from liver transplant patients (n=10).

	Treatment			
	Untreated	GM-CSF	Con A	GM-CSF/Con A
IL-1ra(pg/ml)	950	6880	9890	22570
± SEM	± 350	± 2000	± 1720	± 3620 §

Whole blood was treated and the release of IL-1ra was measured as described in Figure 8.
§ significant difference (n=10, $p < 0.01$) as compared to the purely Con A treated setting.

3.6 The IL-1β - restored Con A-induced proliferation was associated with down-regulation of $p27^{kip1}$ and up-regulation of Cdk2

Recently, it was demonstrated that $p27^{kip1}$, a member of the family of cyclin-dependent-kinase (Cdk) inhibitors (CKIs),[108, 109] plays an important role in the regulation of T-cell proliferation.[110] Indeed, down-regulation of the expression of $p27^{kip1}$,[111, 112] which functions as a binding inhibitor of the Cdk2/Cyclin E,[113, 114] as well as expression and activation of Cdk2,[115] are required for the proliferation of T cells. Therefore, this study examined whether these cell cycle proteins were implicated in the restoration of the Con A-

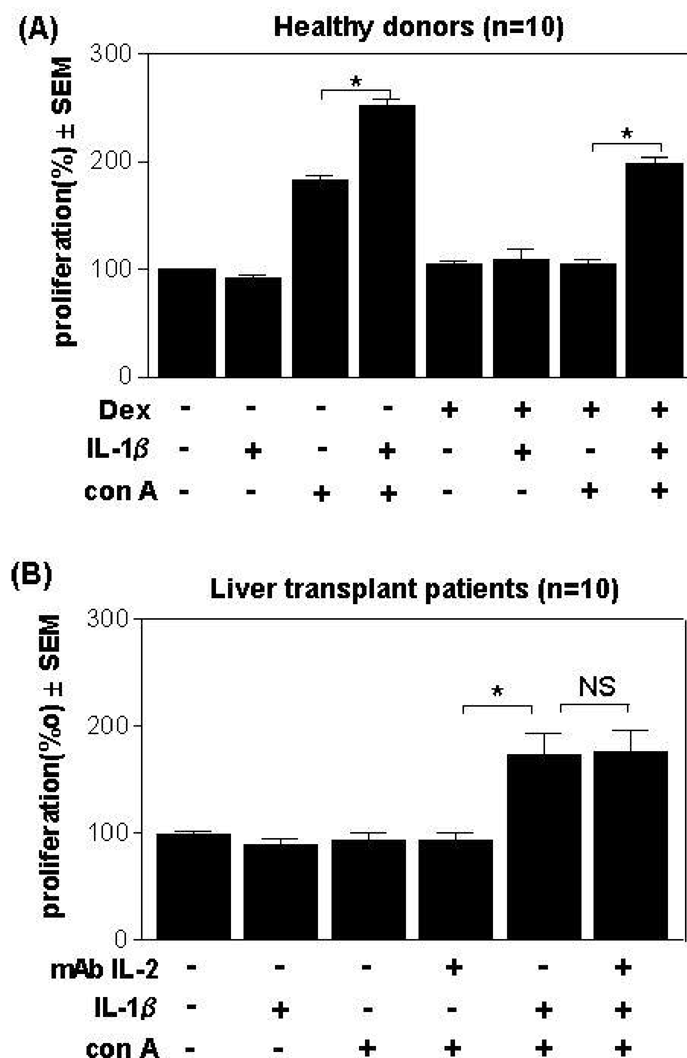

Figure 9. Induction by exogenous IL-1b of Con A-stimulated IL-2-independent proliferation of lymphocytes from immunosuppressed blood.

Lymphocytes obtained by growth adherence of PBMC from healthy donors and immunosuppressed liver transplant patients were treated as described in Figure 8 (proliferation assay), except that IL-1β (100 ng/ml) rather than GM-CSF was applied. Proliferation was presented as percentage (%) of live cells of the control. Data represent mean ± SEM of (A) the healthy donor group (n=10) and of (B) the transplant patient group (n=10). The neutralizing monoclonal anti-human IL-2 antibody (mAb IL-2) (R&D systems, Wiesbaden-Nordenstadt, Germany) was applied at a final concentration of 0.5µg/ml.

stimulated proliferation by IL-1β in the immunosuppressed PBMC. As indicated, a down-regulation of $p27^{kip1}$ (Figure 10A) and an up-regulation of Cdk2 (Figure 10B) protein expression in both Con A-treated non-immunosuppression settings was observed in cells from healthy donors, while in the Con A-treated dexamethasone suppression settings, the $p27^{kip1}$ down-regulation and Cdk2 up-regulation only occurred in the presence of IL-1β (Figure 10C,

D). Further experiments with immunosuppressed PBMC from liver transplant patients confirmed these observations (Figure 10E, F). The correlation of regulation of p27^{kip1} and Cdk2 with proliferation data (Figure 9 and Figure 10) suggested the involvement of these proteins in the IL-1β-restored Con A-induced proliferation of immunosuppressed lymphocytes.

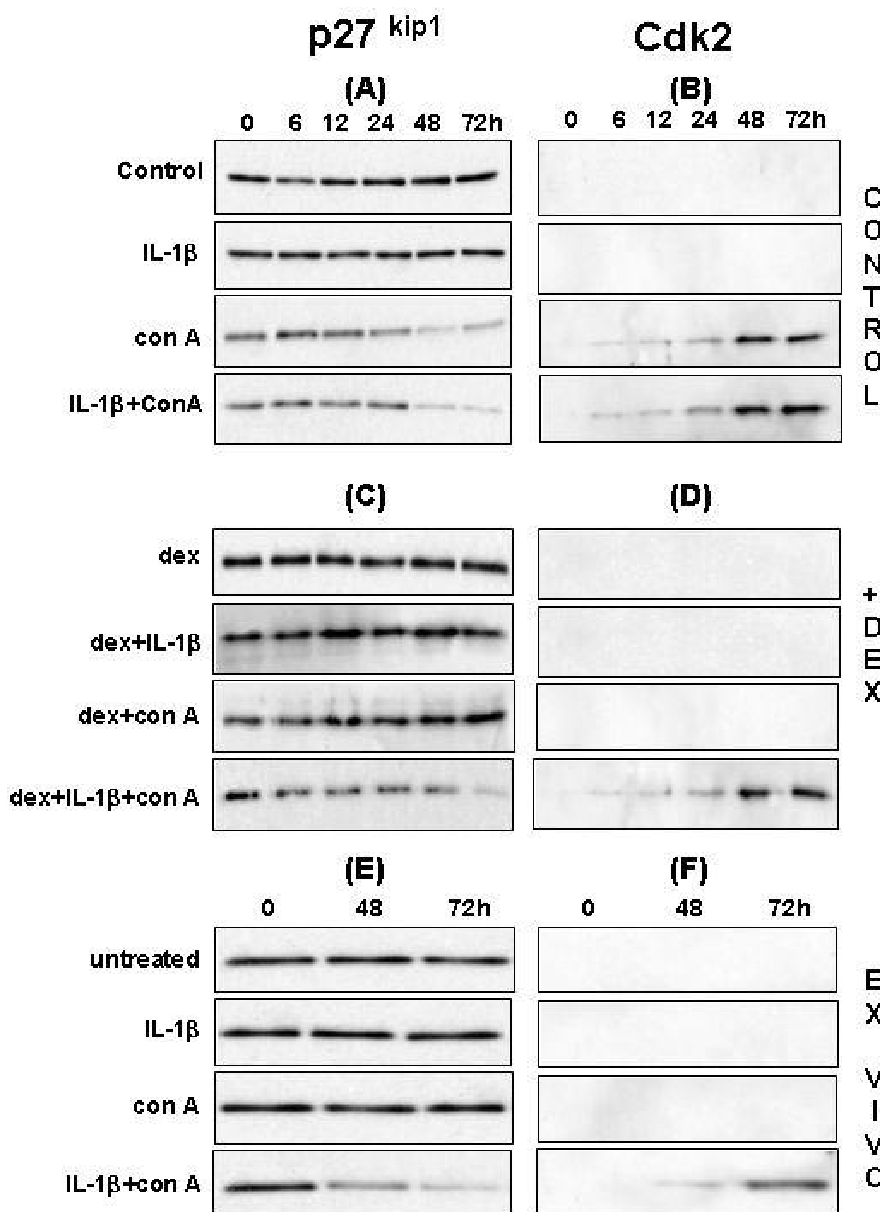

Figure 10. Down-regulation of p27^{kip1} and up-regulation of Cdk2 during IL-1β restored Con A-induced proliferation of immunosuppressed lymphocytes.

Whole cell lysates of lymphocytes from healthy PBMC untreated (A) (B) or treated with dexamethasone (C) (D) and from liver transplant recipients (E) (F) were prepared at the indicated times after Con A (5 µg/ml) stimulation. The lysates (20 µg) were tested for p27^{kip1} protein expression by immunoblotting with anti-p27^{kip1}Ab (A) (C) (E). The same membrane was sequentially stripped and probed with anti-Cdk2 antibody for Cdk2 protein detection (B) (D) (F). Results are representative of three different experiments.

3.7 The IL-1β - restored Con A-induced proliferation was correlated with the upregulation of Jab1 expression

The proliferation of mammalian cells is under strict control, and the cyclin-dependent-kinase inhibitory protein $p27^{kip1}$ is an essential participant in this regulation both *in vitro* and *in vivo*.[116] Several studies indicated that $p27^{kip1}$ is a substrate of the ubiquitin/proteasome system, and that its breakdown depends on its phosphorylation status.[117-119] But this is only half of the story: recently it was found that the c-Jun activation domain-binding protein 1 (Jab1) controls the activity of $p27^{kip1}$ by facilitating its degradation.[120] Therefore, this study further investigated the involvement of Jab1 in the IL-1β-restored Con A-induced proliferation from immunosuppressed PBMC. As indicated, Jab1 up-regulation only occurs in the settings of cell proliferation in healthy PBMC treated with/without dexamethasone (Figure 11 A, B) and in the PBMC from liver transplant patients (Figure 11 C). The up-regulation of Jab1 is positively correlated with Cdk2 and reversely correlated with $p27^{kip1}$ expression in the same settings (compared to Figure 10).

3.8 GM-CSF differentially up-regulated LPS-induced gene expression in dexamethasone suppressed human PBMC

Representative subsets of genes from Figure 4 which were induced by LPS, but suppressed by dexamethasone, are presented as a close-up in Figure 12. The arrows indicate those genes encoding for proteins with various functions which were restored or increased in the presence of GM-CSF. These proteins include: (1) several transcription factors including the NF-κB56 subunit precursor and Egr-1, (2) cytokines or factors, mainly produced by monocytes or macrophages in PBMC under LPS stimulation, namely TNF-α, IL-6, IL-8 and platelet-activating factor receptor (PAF-R), (3) other components of innate immunity, such as matrix metalloproteinase 14. Notably, in contrast to TNF-α, the gene expression of IL-1β was only partly suppressed by dexamethasone and was not up-regulated by GM-CSF under the conditions of our model (data not shown).

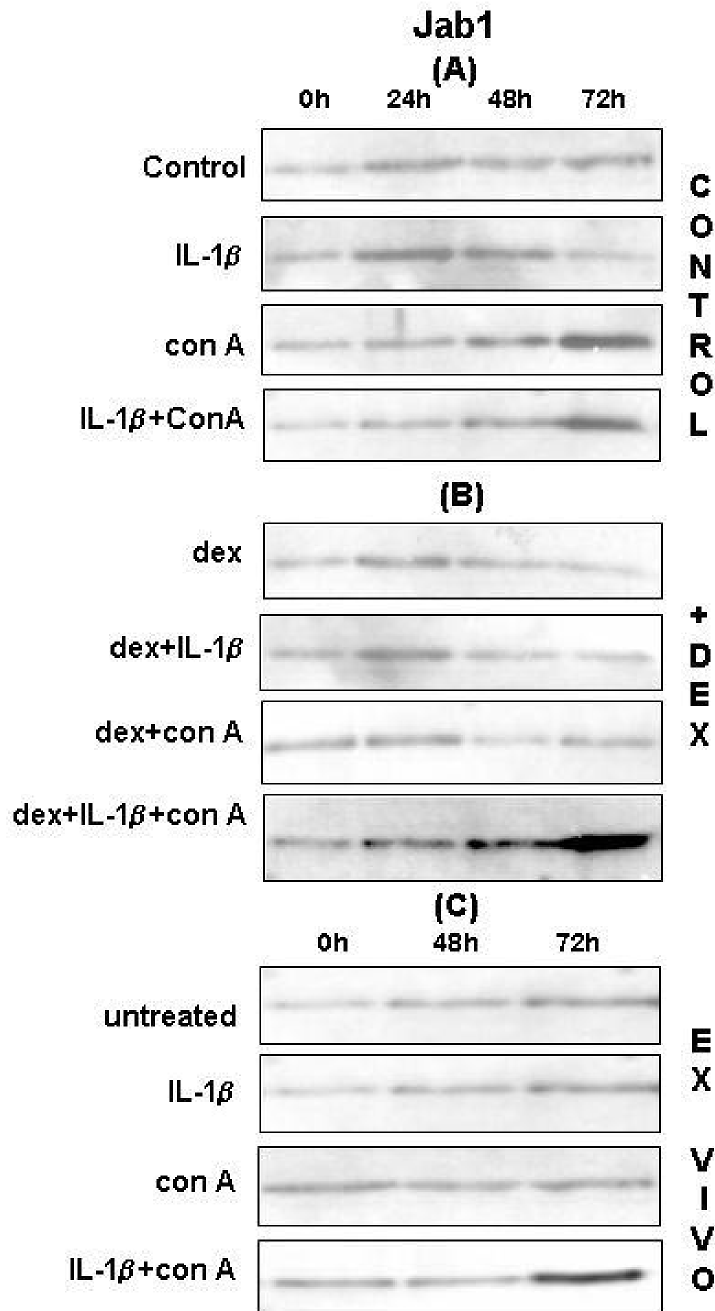

Figure 11. Up-regulation of Jab1 in IL-1b - restored, Con A-induced proliferation of immunosuppressed lymphocytes.

Whole cell lysates of lymphocytes from healthy PBMC untreated (A) or treated with dexamethasone (B) and from liver transplant recipients (C) were prepared at the indicated times after Con A (5 µg/ml) stimulation. The lysates (20 µg) were tested for Jab1 protein expression by immunoblotting with anti-Jab1Ab. Results are representative of three different experiments.

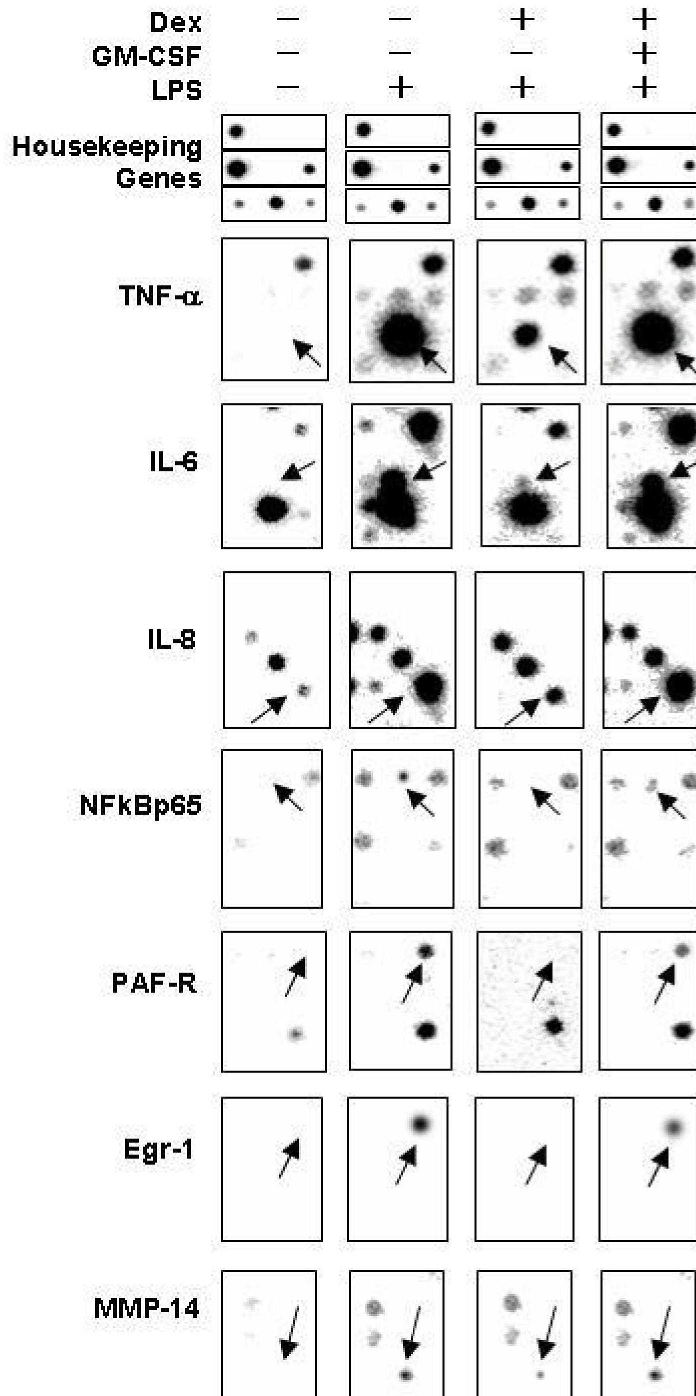

Figure 12. GM-CSF susceptible reconstitution of gene expression in dexamethasone- suppressed human PBMC.

Presented here are the selected grids from Figure 4 as a close-up check. Signal intensity was quantified using ImageMaster software and the expression rate was obtained based on this quantification. The housekeeping genes included genes coding for ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, HLA class I histocompatibility antigen C-4 alpha subunit, β-actin, 60S ribosomal protein L13A and 40S ribosomal protein S9. The arrows indicated the location of other genes coding for TNF-α, IL-6, IL-8, NF-κBp65, PAF-R, Egr-1 and MMP-14.

Table 8. Genes restored by GM-CSF in immunosuppressed, LPS-stimulated human PBMC (restoration rate [(a)] > 90%)

ID [(b)]	Gene Code	Protein/Gene	Fold [(c)]
L25080	A01g	transforming protein rhoA H12	3.3
M90813	A04h	Cyclin D2	4.0
U09579	A07k	Cyclin-dependent kinase inhibitor 1	2.2
L36645	B10e	Ephrin A receptor 4 precursor	3.2
M33336	B14g	cAMP-dependent protein kinase I alpha regulatory subunit	3.2
L35253	B07h	Mitogen-activated protein kinase p38 (MAP kinase p38)	3.0
L31951	B05j	c-jun N-terminal kinase 2	2.0
X08004	B02n	ras-related protein RAP-1B	1.8
L19067	C12c	NF-kappaB transcription factor p65 subunit	2.0 [(d)]
X01394	C08f	tumor necrosis factor precursor	4.7
Y09392	C02g	WSL protein	1.9
U28014	C06h	Caspase-4 precursor (CASP4)	1.9
X04106	C02i	Calcium-dependent protease small (regulatory) subunit; calpain;	1.7
M74524	D05a	Ubiquitin-conjugating enzyme E2 17-kDa (UBE2A)	2.6
M60974	D01b	growth arrest & DNA-damage-inducible protein (GADD45)	3.3
U12979	D14j	Activated RNA polymerase II transcriptional coactivator p15	2.1
X78710	D04k	metal-regulatory transcription factor	3.4
X61498	D05m	nuclear factor NF-kappa-B p100 subunit	2.0 [(d)]
M68891	D08m	Endothelial transcription factor GATA2	8.0
X52541	E10a	Early growth response protein 1 (hEGR1)	2.7
M62831	E11a	transcription factor ETR101	2.0
U30504	E03d	transcription initiation factor TFIID 31-kDa subunit	2.1
M59079	E13d	CCAAT-binding transcription factor subunit B (CBF-B)	2.1
L11672	E07e	zinc finger protein 91 (ZNF92)	2.0
M33374	E05h	cell adhesion protein SQM1	2.3
X07979	E09i	Fibronectin receptor beta subunit (FNRB); CD29 antigen	2.5
D10202	E09j	Platelet-activating factor receptor (RAF-R)	3,5
X72304	E10k	corticotropin releasing factor receptor 1 precursor (CRF-R)	3.5
M29696	E14k	Interleukin-7 receptor alpha subunit precursor (IL-7R-alpha)	2.1
Y00371	F02a	70-kDa heat shock protein	2.1
X07270	F04b	heat shock 90-kDa protein A	1.9
M23452	F11e	Macrophage inflammatory protein 1 alpha precursor (MIP1-α)	1.8
X53799	F10g	Macrophage inflammatory protein 2 alpha (MIP2-α)	1.8
X78686	F12g	Granulocyte chemotactic protein 2	1.9
Y00787	F14g	interleukin-8 precursor (IL-8)	3.7
J04130	F03h	Macrophage inflammatory protein 1 beta precursor (MIP1-β)	3.9
X04602	F13i	interleukin-6 precursor	2.6
U29607	F12k	methionine aminopeptidase 2 (METAP2)	2.3
D00759	F01l	Proteasome component C2	1.8
D00760	F02l	Proteasome component C3	1.9
D00761	F03l	Proteasome component C5	1.8
D00762	F04l	Proteasome component C8	2.1
D26512	F09m	matrix metalloproteinase 14 precursor	2.4

(a) Restoration rate was defined as the ratio of the results obatined from the settings (Dex+GM-CSF+LPS) and (Dex+LPS). (b) GenBank accession number. (c) Fold increase is defined as the factor that is added by GM-CSF on top of the increase found by LPS alone of the dexamethasone suppressed response. (d) restoration rate was less than 50 %.

Table 8 presents a quantification of expression of those genes that were sensitive to a restoration by GM-CSF to at least 90% of the LPS-inducible signal. The data in the table demonstrate that GM-CSF restored the expression of various genes, including proteasome subunits C2, C3, C5 and C8, 70 kDa heat shock protein, as well as metal-regulatory transcription factor and transcription factor GATA2. Other genes coding for proteins with functions not restricted to the immune system were also restored by GM-CSF: (1) cyclin D2, a cell-cycle regulator found to be induced by LPS in macrophages,[121] (2) the cell adhesion protein SQM1, (3) the fibronectin receptor beta subunit (FNRB) (cell-cell interactions) and (4) WSL protein and caspase-4 precursor (apoptosis).

In contrast, the data in Table 9 also demonstrate that the LPS-inducible lymphocyte response, like the expression of CD27 and T-cell specific RANTES, was not up-regulated by GM-CSF in dexamethasone-suppressed PBMC. Similar results were obtained for the genes of LAT (linker for activation of T-cells) and trans-acting T-cell specific transcription factor GATA3.

Table 9. Genes neither restored nor up-regulated by GM-CSF in immunosuppressed human PBMC

ID (a)	Gene Code	Protein/Gene	Fold (b)
AF036906	C03e	linker for activation of T-cells (LAT)	1.1
M63928	C03g	CD27L antigen receptor precursor; T-cell activation CD27 antigen	1.1
M15796	C06l	Proliferating cyclic nuclear antigen (PCNA)	1.0
X55122	E08c	trans-acting T-cell specific transcription factor GATA3	1.0
J03132	E14g	intercellular adhesion molecule-1 precursor (ICAM1);	1.2
M37435	F03e	Macrophage-specific colony-stimulating factor (CSF-1; MCSF)	1.2
M21121	F10e	T-cell-specific rantes protein precursor;	1.1
K02770	F10i	Interleukin-1 beta precursor (IL-1β)	1.0

(a) GenBank accession number. (b) The fold was defined as the upregulation ratio by GM-CSF as in Table 8.

3.9 Verification of array data by Ribonuclase Protection Assay

Despite its reproducibility, gene array analysis is only semi-quantitative.[122, 123] Therefore, the ribonuclease protection assay technique was used to cross-check for a selection of some genes of major interest susceptible to alterations in expression by GM-CSF in dexamethasone-suppressed human PBMC. As demonstrated in Figure 13, by using this

alternative method, the LPS-induced, dexamethasone suppressed expression of TNF-α, IL-1ra and IL-6 was restored in the presence of GM-CSF, while the expression of IL-1β remained suppressed, which is fully consistent with the results obtained from the cDNA array.

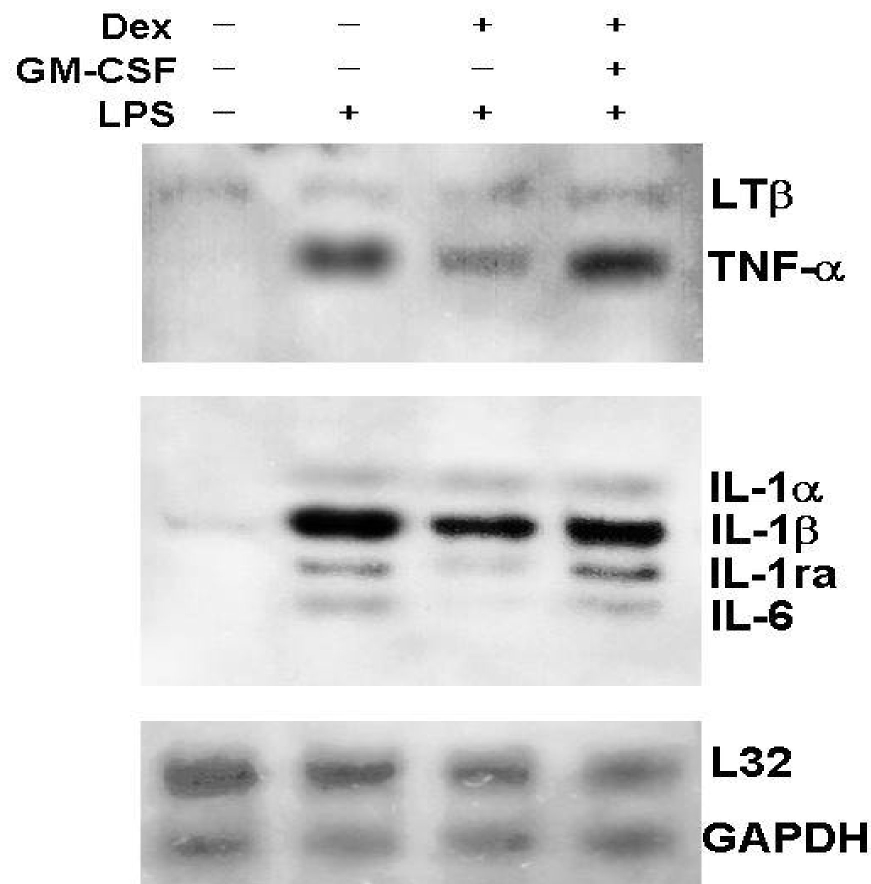

Figure 13. Verification of selected array data by ribonuclease protection assay (RPA).

Total RNA (20 µg) collected from treated human PBMC was hybridized at 42°C overnight to the psoralen-biotin labelled RNA probes, which were transcribed *in vitro* from DNA templates encoding TNF-α, IL-1α, IL-1β, IL-1ra, IL-6 and two housekeeping genes L32 and GAPDH, then treated with RNase for 30 minutes at 37°C. The remaining RNase-protected probes were purified and resolved on denaturing (8M urea) 5% polyacrylamide gel, and transferred to positively-charged nylon membrane by electroblotting, followed by RNA crosslinking and detection with BrightStar™ BioDetect™ kit (Ambion) exposed to X-ray film to visualize protected bands. Results are representative of 3 different experiments.

3.10 Evaluation of array data by ELISA and Western blot analysis

To check whether the changes in mRNA levels detected by the array hybridizations translate into similar changes in protein abundance for a representative subset of genes, ELISA assays for TNF-α, IL-1β and IL-6 were carried out using culture media collected from the experiments used for RNA isolation. The results (Figure 14) demonstrate that in supernatants from dexamethasone-treated cells stimulated with LPS, TNF-α release was inhibited and was restored to control release levels in the presence of GM-CSF. A comparable result was obtained for IL-6, while similar to the finding in the array experiments described above, the release of IL-1β remained suppressed. The results demonstrate that the restored TNF-α and IL-6 release was due to an increased protein production following a reconstitution of mRNA by GM-CSF.

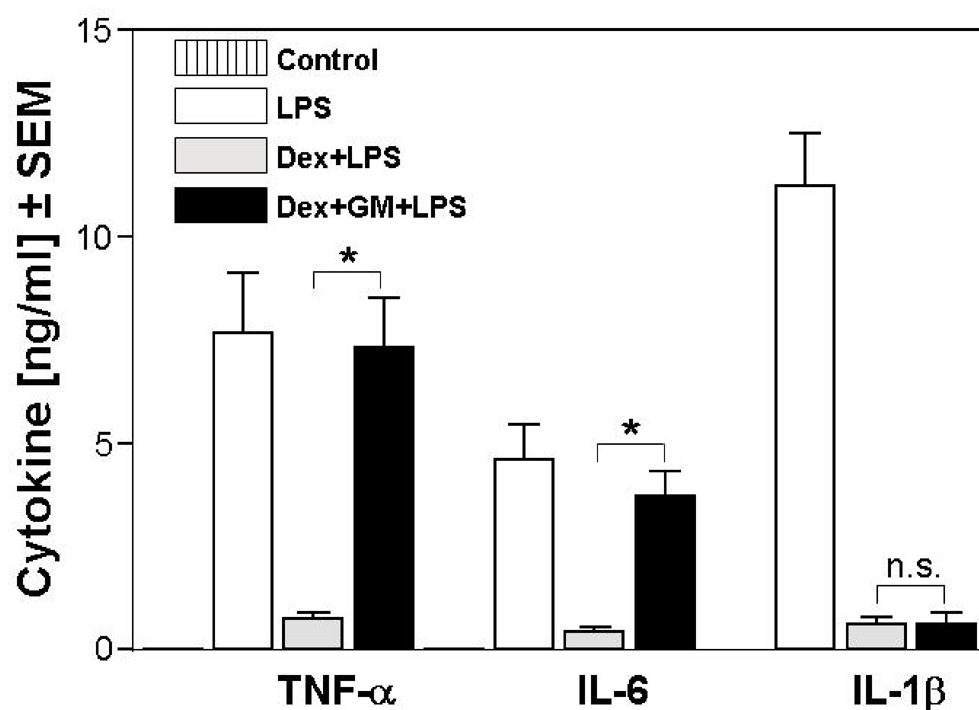

Figure 14. Comfirmation of array data with ELISA

PBMC (5×10^6 cells/ml, 200µl) was subsequently treated with dexamethasone (1 µM) for 1 h, with GM-CSF (50 ng/ml) for another 1 h and with LPS (100 ng/ml) for 16 h at 37°C and 5% CO_2. Cell-free supernatants were then obtained by centrifugation (300 × g, 10 min) and subjected to ELISA for TNF-α, IL-6 and IL-1β measurement. Data represent mean ± SEM of the healthy donor group (n=10) and $p < 0.05$ (indicated as *) was considered significant.

Finally, a further independent confirmation of these results was obtained in pre-exposed PBMC supernatants analyzed by Western blotting. The data in Figure 15 demonstrate that GM-CSF upregulated expression of cyclin D2 in PBMC suppressed with dexamethasone, matching with the result from the cDNA expression array (compared to cyclin D2 result in Table 8).

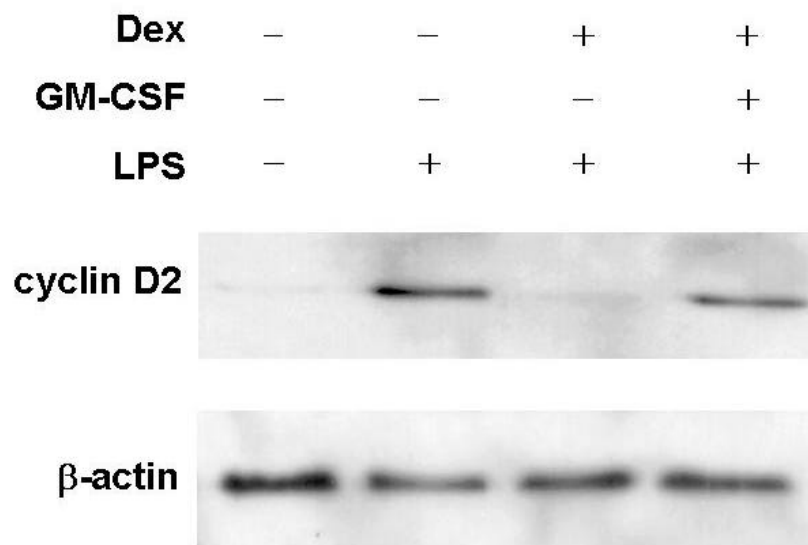

Figure 15. Comfirmation of array data with Western blot

Whole cell lysates (20 μg) of treated PBMC were tested for cyclin D2 protein expression by immunoblotting with anti-cyclin D2. The same membrane was sequentially stripped and probed with anti-β-actin antibody for actin protein detection to confirm equal sample loading. Results are representative of 3 different experiments.

4. Discussion

During the past two decades, the development of new immunosuppressive drugs with improved efficacy and decreased toxicity has led to a substantial improvement of the survival of organ transplant patients and of short-term graft survival for all organs.[124] However, immunosuppression itself impairs the inflammatory response, which attenuates the signs and symptoms of invasive infection, the most common life-threatening complication of long-term immunosuppressive therapy. The prevention or effective treatment of infection is therefore still a primary goal in organ transplantation.[21] Indeed, a broad range of potential sources of infection, ranging from latent viruses to pathogens of both community and hospital origin, as well as the adverse effects of antimicrobial drugs used for prophylaxis and therapy, resulting both from the duration of the therapy required and from interactions with the immunosuppressive drugs cyclosporine and tacrolimus, represent important challenges to overcome.[21] It is therefore desirable, in immunosuppressed organ transplant recipients, to create a status with a preferential reconstitution of the innate immune response which will contribute to recognition and control of the infectious agents,[125, 126] while keeping the adaptive immune response silent. Such a hypothesis has been tested in our laboratory *in vivo* where immunosuppressed mice survived a lethal *Salmonella typhimurium* infection when pre-treated with GM-CSF, without inducing graft rejection after skin allotransplantation, as a consequence of the reconstitution of the immunosuppressed pro-inflammatory TNF-α response, while the response of T-cells remained silent (Kühnle and Wendel, submitted). The study reported here translates these findings to an *in vitro* property of GM-CSF in an *ex vivo* setting using immunosuppressed blood from liver transplant patients, as compared to blood from healthy donors.

4.1 The experimental systems

To investigate the differential reconstitution potential of GM-CSF, whole blood incubation was performed, firstly in dexamethasone-suppressed blood from healthy donors, in order to normalize all conditions required for the study, and secondly *ex vivo* in the blood from immunosuppressed liver transplant recipients, in order to confirm and emphasize the potential clinical implications. The LPS-response-model was used to check for the reconstitution potential of GM-CSF, especially on TNF-α response, while the Con A-

response-model was applied to investigate the non-reactivation of immunosuppressed lymphocyte by GM-CSF, in terms of IL-2 release and lymphocyte proliferation, the typical T-cell responses.

For a closer look of the effect of GM-CSF on the immune response under immunosuppression, a population of PBMC was chosen as the target of investigation, instead of a purified single cell type or cell line. The rationale of this selection is to be able to pick up any possible action of GM-CSF in the presence of all major immune cells and their cross-talk, in order to approach reality as closely as possible *in vitro*.

In order to find out to which genes, besides the gene for TNF-α, the differential reconstitution potential of GM-CSF extends in humans, this study has used the gene array technology. Such experimental approach offers the possibility to study simultaneously the expression of almost any given gene without biasing conclusions drawn from a subset of genes presumed to be involved in a particular process.[123, 127-131] In fact, by using gene array or similar techniques, such as serial analysis of gene expression (SAGE), a comprehensive gene expression profile of resting or stimulated human monocytes/macrophage or lymphocyte has been studied.[127, 129, 132-138] Recently, a stereotyped and specific gene expression program, with a remarkable degree of heterogeneity and also a degree of order, has been reported in the human innate immune responses to bacteria.[139, 140] The study reported here presents the gene profile of primary human immune cells which is complex due to two variants studied, i.e., the absence or presence of GM-CSF under immunosuppression.

4.2 The reconstitution potential of GM-CSF on TNF-α release

Based on the previous finding on the capacity of GM-CSF to potentiate or reactivate the impaired immune response in immunocompromised mice or human cells,[58, 60, 63] the study reported here translates these findings consistently to an *in vitro* property of GM-CSF in an *ex vivo* setting using immunosuppressed blood from liver transplant patients, as compared to the blood from healthy donors. Also in this experimental approach, a recovery of the proinflammatory TNF-α response was observed without reactivation of the T-cell response, in terms of IL-2 production and proliferation. As a mechanistic rationale, the results from the cDNA expression array suggest that the restoration of TNF-α production by GM-CSF may result from the restored mRNA expression of the cytokine. Furthermore, the results obtained

from the TNF-α bioassay excluded the possibility that the restored TNF-α protein was neutralized by binding to its soluble receptors.[141, 142]

4.3 TNF-α and infection in organ transplantation

Concerning the possible role of TNF-α in graft rejection in organ transplant patients, only a few reports are published. In fact, neither in renal nor in liver transplant patients an association between TNF-α producer genotype and rejection was found at the mRNA level.[143-145] In addition, GM-CSF, the TNF-reconstituting agent in our model, has been safely applied following liver transplantation in the treatment of neutropenia.[75]

Bacterial infection occurs in up to 68% of liver transplant recipients, commonly with infection of the liver, biliary tract, peritoneal cavity, blood stream and surgical wound. Most such infections occur in the first 2 months following transplantation, although Listerial infection may occur at any time following transplantation.[24] Although this study presented no direct evidence in humans that the increased TNF-α levels will protect transplant patients from bacterial infection post-transplantation, many reports demonstrate that this cytokine is crucial in the host defense in various infection models.[52-56] Indeed, the neutralization of TNF-α bioactivity, as recently approved for the treatment of Crohn's disease or rheumatoid arthritis, was shown to be associated with the development of active tuberculosis in some patients.[146, 147] Moreover, at the protein level, excessive inhibition of TNF-α production due to routine immunosuppression was suggested to lead to an insufficient response to infectious stimuli in kidney [148] as well as in liver transplant [149] recipients. The results from additional experiments using later-stage-post-transplantation (but within 12 months) blood demonstrated a similar reconstitution of TNF-α release by GM-CSF to early-stage-post-transplantation blood (data not shown). Therefore, the GM-CSF-restored TNF-α response could be of benefit for the liver transplant recipient in overcoming bacterial infection both in early and late stage post transplantation.

In addition to bacterial infection, viral infection remains the major complication in later stage of post-transplantation.[21, 24] There are reports demonstrating that TNF-α protects against virus infection *in vitro* and *in vivo* by means of improving viral clearance [150] or by means of inhibiting viral replication [151, 152] including cytomegalovirus (CMV), the very fatal agent for immunocompromised individuals such as organ transplant patients.[153-155] It was also found that TNF-α may inhibit early transgenic expression by CMV promoters *in*

vivo, a mechanism which is independent of adaptive immunity and is likely secondary to innate immune responses to virus infection.[156] Recently, TNF-α has been shown in a TNF-knock-out model to be necessary for adhesion molecule expression and recruitment of leukocytes to inflammatory sites, and thus, elimination of infectious viral agents.[157]

4.4 The differential regulation of TNF-α and IL-1β by GM-CSF

Notably, in the immunosuppression model used here, GM-CSF preferentially restored production of TNF-α, rather than that of IL-1β, both at the mRNA and protein level, which has been confirmed by RPA and ELISA, indicating that different mechanisms are involved in the regulation of these predominantly macrophage-mediated responses by GM-CSF. Recently published results indicate that monocytes/macrophages can indeed distinctly modulate TNF-α and IL-1β production,[158, 159] however, without giving clues to any particular mechanism(s). Indeed, it has been shown that nitric oxide (NO) can activate TACE (TNF-α converting enzyme), an enzyme contributing to the shedding of membrane-bound TNF-α, and thus increase TNF-α release.[160] In contrast, NO was reported to inhibit ICE (IL-1β-converting enzyme) by S-nitrosylation of the enzyme, resulting in a decrease of IL-1β release,[161, 162] which might represent a possible mechanism of the differential restoration capacity of GM-CSF for TNF-α rather than IL-1β production. However, this study did not observe an increased NO production in the presence of GM-CSF under immunosuppression (data not shown), indicating that alternative mechanisms might be involved in such a differential modulation.

4.5 The role of IL-1β in T-cell proliferation and its implication for the study

It is striking that exogenous IL-1β restored the Con A-induced T-cell proliferation in immunosuppressed blood, independently of IL-2. This indicates that IL-1β may have an important function that has not been recognized in case of immunosuppression.

It is known that the commitment of a cell to proliferate is a multistage process characterized by inducible expression of cyclins and cyclin-dependent kinases (Cdk) and a concomitant decrease of Cdk inhibitors.[116, 163, 164] One such key player among the members of the CKI family is p27kip1 [108, 109] as a regulator of T-cell proliferation.[110, 165] The down-regulation of $p27^{kip1}$ expression is required for the development, proliferation and

immunoresponsiveness of T cells.[111, 112] In addition, $p27^{kip1}$ acts as a tight binding inhibitor of Cdk2/Cyclin E [113, 114, 166] the expression and activation of which is critical in cell proliferation.[115] Interestingly, this study found that downregulation of $p27^{kip1}$ and upregulation of Cdk2 protein expression only occurred in the setting of Con A-stimulated control and IL-1β-restored Con A-stimulated immunosuppressed lymphocytes.

Jab1 has been described as a coactivator of AP1 transcription factor, and is a subunit of a large protein complex (called the COP9 signalosome). A recent study has found that the Jab1 protein can cause breakdown of the $p27^{kip1}$ protein in mammalian cells.[120] In the study reported here, Jab1 expression is found inversely correlated with $p27^{kip1}$ expression levels. Therefore, Jab1, as a negative regulator of $p27^{kip1}$,[167-179] may be also associated with the Con A-induced proliferation restored by IL-1β.

Although other CKIs or cyclin/Cdks may also get involved in Con A-induced proliferation restored by IL-1β, this study did not observe a similar regulation in such factors as p21 and cyclin D/E in the presence of IL-1β under immunosuppression (data not shown). Hence, downregulation of $p27^{kip1}$ and upregulation of Cdk2 and Jab1 expression might represent a mechanistic explanation for the IL-1β-mediated restoration of proliferation of the immunosuppressed lymphocytes, which could represent an important function for IL-1β that deserves further investigation.

In line with the results from others [180, 181] and from our laboratory,[180, 181] this study found that GM-CSF enhanced the IL-1ra release not only after an LPS- (data not shown) but also after a Con A-stimulus in immunosuppressed human blood. Since GM-CSF did not restore the release of IL-1β, but rather led to its neutralization, these results might explain why GM-CSF did not restore the proliferation of immunosuppressed PBMC, in view of the observed effect of IL-1β on T-cell proliferation.

4.6 The possible mechanism underlying the reconstituional potential of GM-CSF

Based on the capacity of GM-CSF to potentiate or reactivate the impaired immune response in immunocompromised mouse or human cells,[58-60, 63] this study also disclosed GM-CSF-induced alterations in the expression of genes relevant to immune defense in immunosuppressed human PBMCs, making use of the gene array technology, confirmed in

detail by alternative techniques. The novelty of this part of the study consists in the finding that GM-CSF preferentially reconstituted the dex-suppressed gene expression, which favours the supposed hypothesis of selective restoration of innate immunity in organ transplantation recipients.

Receptor binding of GM-CSF initiates signal transduction ending up in induction of c-*myc* and activation of DNA replication, or activation of *ras* and mitogen-activated protein kinases (MAP), with consequent induction of c-*fos* and c-*ju*n,[41] which this study confirmed here (data not shown). In the context of our immunopharmacological question, the novel findings of this study are that GM-CSF restored the expression of these factors in dexamethasone-suppressed PBMC stimulated by LPS, in many cases close to the level reached by LPS alone (Table 8).

A further transcription factor detected here to be restored by GM-CSF was the p65 subunit precursor of NF-κB, proposed to be a critical regulator of many cytokine genes. Interestingly, NF-κB is also one of the most important targets of the suppressive action of corticosteroids, and is consequently held responsible for their anti-inflammatory effect.[9-11] Therefore, it seems likely that the partial reconstitution of NF-κB expression by GM-CSF may contribute to the restored expression of other cytokine genes as well. Given the fact that glucocorticoids inhibit LPS-induced TNF translation by inhibiting JNK/SAPK [182] and MAP38,[183, 184] a reconstitution of the transcription of these factors by GM-CSF might also be a possible way to restore TNF production in macrophages, although glucocorticoid induced the activation of JNK and MAP38 in human eosinophils.[185]

Notably, the induction of immediate early genes in cells of the immune system is a critical determinant of their defense response to pathogens, suggesting their transcription factor products dictate the patterns of expression of downstream, function-related genes. Compelling evidence from several other systems [186] indicates that the immediate early gene, Egr-1 (early growth response 1), another factor found in this study to be restored by GM-CSF, may be of particular importance to the immune system. Recently, the Egr-1 promoter has been shown to be responsive to the immune signals, i.e. that the LPS-induced Egr-1 is required for maximal induction of TNF-α gene expression.[187] The observation that various other transcription factors are upregulated by GM-CSF as listed in Table 8, points to a general mechanism by which GM-CSF restores the transcription process.

This study also demonstrates that GM-CSF restored gene expression of several LPS-induced cytokines which play a pivotal role in mediating inflammatory response. Among such cytokines: (i) TNF-α represents a key factor with a broad range of biological and

immunologic effects, including antibacterial and antiviral action, growth regulation, and immunomodulation [188, 189] and is crucial in the macrophage-mediated host defense in various infection models.[52-56] Indeed, the neutralization of TNF-α bioactivity as recently approved for treatment of Crohn's disease or rheumatoid arthritis was shown to be associated with the development of active tuberculosis in some patients.[146, 147] (ii) IL-8, a monocyte-derived cyto- and chemokine in LPS-stimulated PBMC, regulated primarily at the level of gene transcription [190-192] is implicated in a variety of inflammatory diseases,[193] and TNF-α has been shown to be one of the strongest activators of IL-8 expression.[191, 192] (iii) IL-6, also a key cytokine in inflammation and infection.[194] Indeed, results from a knock-out mice study demonstrated that the lack of IL-6 enhances the susceptibility to infection with herpes simplex virus type 1 [195] and to peroral infection with *Toxoplasma gondii*.[196] (iv) Monocyte-specific PAF-R, a G-protein-coupled receptor constitutively expressed on monocytes, mediates the pro-inflammatory action of platelet-activating factor (PAF) by ligand-receptor binding.[197] Establishment of PAFR(-/-) mice confirmed that the PAF receptor is responsible for pro-inflammatory responses, although its role in other settings remains to be clarified.[198] Importantly, the infection of peritoneal mouse macrophages by *Leishmania amazonensis* can be inhibited in the presence of PAF.[199] TNF-α and PAF interact in human monocytes during inflammatory processes through the TNF-α induced up-regulation of PAFR expression.[197] Therefore, this study conclude that the up to 3-fold enhancement of PAF-R mRNA by GM-CSF may contribute to the enhanced activity of monocytes or macrophages.

Importantly, other immunity components, induced by LPS, have been found to be upregulated by GM-CSF in dexamethasone-suppressed PBMC, including, for example, (1) proteasome components such as C2, C3, C5 and C8, multisubunits of protease, responsible for the generation of peptides loaded onto MHC class I molecules, (2) matrix metalloproteinase 14, expressed in macrophages,[200] a member of zinc-containing proteolytic enzymes that breakdown extracellular matrix proteins in normal physiological processes such as embryogenesis, tissue growth, and wound healing.[201] (3) METAP2, methionine aminopeptidase 2, which has a role in host cell immune response.[202, 203] (4) 70-kDa heat shock protein, which may improve the stability of mRNA for LPS-induced cytokines [204] and directly induces cytokines such as TNF-α.[204, 205] All these considerations suggest that an enhanced potential of the macrophage response in terms of gene expression from immunosuppressed PBMC can be elicited by GM-CSF.

In contrast, specific LPS-induced lymphocyte responses, such as the expression of CD27 and T-cell specific RANTES, were not up-regulated by GM-CSF. Furthermore, factors critical for T-cell activation such as LAT (linker for activation of T-cell) [206, 207] and trans-acting T-cell specific transcription factor GATA3 [208] could not be restored by GM-CSF, indicating a preferential restoration of the innate immune response under immunosuppression by the study drug.

Taken together, the data from this study using gene array technology, confirmed by RPA and ELISA, provide an extended overview and an improved insight into the complex network of genes affected by GM-CSF in human PBMC. The results argue for a differential restoration of or enhancing potential of GM-CSF on LPS-inducible gene expression in immunosuppressed human PBMC, encompassing on the one hand many genes derived from monocytes or macrophages, hence contributing to the non-specific immune response, as well as many genes of key transcription factors. On the other hand, expression of genes specific for LPS-induced lymphocyte responses remained unchanged. These results provide a mechanistic basis for the finding in this study proposing the potential therapeutic value of GM-CSF for the improvement of the resistance of immunosuppressed organ transplant patients against infections.

4.7 Prospective of GM-CSF in organ transplantation

To give a reasonable out-look of GM-CSF in organ transplantation, it is helpful to trace and review what has been published in the past in the field. As discussed in the Introduction, GM-CSF has found many applications in bone marrow transplantation and stem cell transplantation, and in some cases of solid organ transplantation (Table 5). From a PubMed-based search on several high-impact-factor journals, namely *Cell*, [44, 209-216] *Nature*,[217-225] *The New England Journal of Medicine*,[226-245] *Science*,[47, 246-253] *The Lancet*,[254-274] *Immunity*,[275, 276] *The Journal of Experimental Medicine*,[277-326] , a shift of the research focus can be observed, from purely basic research on GM-CSF, such as the purification,[253] gene cloning [249] and translation of mRNA for GM-CSF,[225] to pre-clinical studies, such as the gene knock-out study,[47] and further to the clinical investigations, such as the role of GM-CSF on accelerating recovery of neutrophils,[232] accompanied by the identification of newer features of GM-CSF.[275, 277] More than ten years of research on GM-CSF has shown that its name is restrictive, because it fails to describe the numerous biologic effects now

linked to GM-CSF.[41] The perspective for GM-CSF is likely that more applications of GM-CSF would be found with further characterization of new functions in a variety of settings.

In the study reported here, consistent with the clinical experience that steroid therapy of graft rejection fails when IL-1ra production is defective,[327] the inability of GM-CSF to restore the release of IL-1β, combined with the enhancement of the release of IL-1ra, is a favourable property for a drug supposed not to interfere with graft acceptance. Supportive arguments for such a use are all further known actions of GM-CSF, such as increased endocytosis and enhanced phagocytic and metabolic functions, and especially increased defense against intracellular fungi, bacteria, protozoa and viruses.[328] GM-CSF has also been shown *in vitro* to potentiate the immune response to endotoxin, [58] to restore the impaired immune response in refractory human monocytes,[59] to reactivate the anergic monocytes from sepsis patients,[60] to overcome the hyporesponsiveness of blood induced by sepsis and trauma [63] and to increase the respiratory burst of human neutrophils after liver transplantation.[76] In addition, GM-CSF has found clinical applications in the management of myelosuppression,[329] of microbial diseases to enhance macrophage functions [328] and in the treatment of neutropenia following pediatric orthotopic liver transplantation to increase neutrophil count,[75] and is still in emerging therapeutic use.[41] Together with our recent *in vivo* findings in mice that a lethal infection can be survived and skin transplants are still fully accepted after GM-CSF treatment, this study suggests that such a differential reconstitution of the immune competence extends also to humans. Therefore, the therapeutic use of GM-CSF in order to improve the resistance of immunosuppressed organ transplant patients against bacterial infections represents a promising subject for clinical studies in humans.

4.8 Brief summary

(1) Upon LPS or Con A stimulation, respectively, GM-CSF restored TNF-α production, both at the mRNA and the protein level, without inducing IL-2 production and T-cell proliferation, in dexamethasone-suppressed healthy blood and in blood from liver transplant patients.

(2) GM-CSF did not reconstitute the expression of IL-1β, neither at the mRNA nor at the protein level, but rather enhanced IL-1 receptor antagonist release, while exogenous IL-1β restored the Con A-stimulated proliferation of immunosuppressed lymphocytes,

independently of IL-2, which might explain the non-activation of immunosuppressed lymphocyte by GM-CSF.

(3) The results from the gene expression array suggest a preferential restoring potential of GM-CSF on dexamethasone-suppressed genes related to innate immunity, a possible mechanism underlying the study effect of GM-CSF.

(4) The IL-1β induced an IL-2-independent Con A-stimulated proliferation of immunosuppressed lymphocytes is characterized by down-regulation of $p27^{kip1}$ and up-regulation of Cdk2, the key regulators in lymphocyte development and proliferation, as well as by up-regulation of Jab1, the factor known to catabolize $p27^{kip1}$.

(5) In total, these findings support the potential clinical value of GM-CSF for the therapeutic improvement of resistance to infections in immunosuppressed organ transplant patients, thus proposing clinical phase II studies.

5. Abstract

Background & Aims: Infection remains the major complication of immunosuppressive therapy in organ transplantation. Therefore, reconstitution of the innate immunity against infections, without activation of the acquired immune response, in order to prevent graft rejection, is a clinically desirable status in transplant recipients. In extending previous findings on the pharmacology of Granulocyte-Macrophage Colony Stimulating factor (GM-CSF) from animal model to humans, this *ex vivo* study investigates reconstitution potential of GM-CSF in immunosuppressed blood from liver transplant patients.

Methods: In vitro and ex vivo immunosuppressed whole blood or PBMC from 10 healthy donors and from 10 liver transplant patients, whose blood was drawn within one month post liver transplantation, was stimulated with Lipopolysaccharide (LPS, 100 ng/ml) for 1 to 16 h or with Concanavalin A (Con A, 5 µg/ml) for 6 to 72 h after incubation with GM-CSF (50 ng/ml) for 1 h. ELISA, ribonuclease protection assay, Western-blot and gene array were used to compare *ex vivo* the cytokine release, mRNA, protein expression and the gene expression profile, altered by GM-CSF under immunosuppression in three groups: naïve blood, blood cells from 10 healthy donors (dexamethasone 1 µM, 1 h) or blood from 10 liver transplant patients (methyl prednisolon, average of 12 mg per day).

Results: GM-CSF restored Tumor Necrosis Factor (TNF)-α mRNA and protein expression to the stimulated non-suppression control levels, without inducing Interleukin (IL)-2 production and T-cell proliferation in immunosuppressed blood *in vitro* and *ex vivo*. In contrast, GM-CSF did not restore the expression of IL-1β mRNA and protein, but rather enhanced the release of IL-1β receptor antagonist up to more than 2 fold of the stimulated control levels. In contrast to GM-CSF, exogenous IL-1β (100 ng/ml) restored an IL-2-independent Con A-stimulated proliferation of immunosuppressed lymphocytes to the Con A stimulated control levels, characterized by down-regulation of $p27^{kip1}$ (from 48 h on) and up-regulation of Cdk2 and Jab1 (from 24 and 48 h on, respectively) within a 72 h investigation time after Con A challenge. This might explain why GM-CSF does not reactivate the lymphocyte response under immunosuppression. Furthermore, gene expression profiling indicated that many genes encoding for the innate immune response, which had been immunosuppressed in vitro, were essentially completely restored, while important markers for LPS-responses of lymphocytes, such as CD27 and T-cell specific RANTES, were not restored.

Conclusions: The selective restoration by GM-CSF of TNF-α and other factors important for the innate immune defense, without activating lymphocytes, suggests a therapeutic potential of GM-CSF in improving the resistance against infections upon organ transplantation.

6. Zusammenfassung

Hintergrund: Infektionen bleiben die Hauptkomplikation der immunsuppressiven Therapie bei Organtransplantationen. Aus klinischer Sicht ist es daher wünschenswert, unter Immunsuppression das angeborene Immunsystem für die Abwehr bakterieller Infektionen in den Transplantat-Empfängern wiederherzustellen, ohne jedoch die spezifische Immunantwort, welche zur Transplantat-Abstoßung führen kann, zu aktivieren. Ausgehend von vorangegangenen Ergebnissen zur Immunrekonstitution durch Granulocyten-Makrophagen-Kolonie-stimulierendem Faktor (GM-CSF) im Tiermodell, untersucht diese Arbeit ex vivo das Potential von GM-CSF zur Wiederherstellung der Immunantwort in immunsupprimiertem Blut von Lebertransplantationspatienten.

Methoden: *In vitro* durch Dexamethason (1 µM, 1 Std) immunsupprimiertes Vollblut oder periphere Blutmonozyten (PBMC) von 10 Gesunden oder *ex vivo* supprimiertes Blut oder PBMC von 10 Lebertransplantat-Patienten (Methylprednisolon, durchschnittlich 12 mg pro Tag) wurde als klinisches Ausgangsmaterial verwendet. Vollblut oder PBMCs wurden mit GM-CSF (50 ng/ml) inkubiert in Gegenwart von Lipopolysaccharid (LPS, 100 ng/ml) für 1 bis 16 Std oder Concanavalin A (Con A, 5 µg/ml) für 6 bis 72 Std. Anschließend wurde die Wirkungen von GM-CSF auf die Zytokin-Freisetzung (gemessen im ELISA), die mRNA (mit Ribonuclease Protection Assay) und das Genexpressionsprofil (mit Gene Array–Technik) sowie die Proteinexpression (mit Western Blotting) untersucht.

Ergebnisse: GM-CSF stellte nach Stimulation sowohl Tumor Nekrose Faktor (TNF)-α mRNA als auch TNF-Freisetzung auf die Werte stimulierter Kontrollen wieder her, ohne jedoch die Interleukin (IL)-2 Produktion und Lymphozyten-Proliferation in Immunsupprimiertem Blut *in vitro* und *ex vivo* zu induzieren. Demgegenüber beeinflusste GM-CSF weder die mRNA- noch die Protein-Expression für IL-1β. Die Freisetzung von IL-1β Rezeptor-Antagonist (IL-1ra) wurde jedoch gegenüber stimulierten Kontrollen mehr als verdoppelt. Im Gegensatz zu GM-CSF induzierte exogenes IL-1β (100 ng/ml) eine IL-2-unabhängige Proliferation immunsupprimierter Lymphozyten nach Con A-Stimulation, charakterisiert durch eine Herunterregulierung von $p27^{kip1}$ (nach 48 Std.) und eine Hochregulierung von cdk2 und von Jab1 (nach jeweils 24 oder 48 Std nach Con A). Die von GM-CSF induzierte Neutralisierung von IL-1 durch IL-1ra könnte somit die ausbleibende Lymphozytenantwort während der Immunsuppression in Gegenwart von GM-CSF erklären.

Zusätzlich zeigte das Genexpressions-Profil, dass viele weitere Gene durch GM-CSF *in vitro* hochreguliert wurden (auf bis zu 90% der LPS-stimulierten Kontrollen ohne Immunsupression), die für Funktionen des angeborenen Immunsystems kodieren. Im Gegensatz dazu wurden wichtige Marker einer LPS-Antwort von Lymphozyten, wie CD27 und T-Zell-spezifisches RANTES, nicht wiederhergestellt.

Schlussfolgerung: Die Studie zeigt, dass GM-CSF differenziell die TNF-α-Antwort, einen Schlüsselmediator gegen bakterielle Infektionen, im Blut immunsupprimierter Lebertransplantationspatienten wiederherstellen kann, ohne jedoch die T-Zell-Proliferation und IL-2 -Antwort zu aktivieren. Der zugrundeliegende Wirkungsmechanismus könnte eine differentielle Wiederherstellung von Genen durch GM-CSF sein, die funktionell hauptsächlich mit der angeborenen Immunabwehr azzoziiert sind. Dies ist von therapeutischer Bedeutung, um Patienten nach einer Lebertransplantation resistenter gegenüber Infektionen zu machen.

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8. Dedications

This work is dedicated to
my wife N ingli
and
my son Wenjia
for their love, companion and non-stop support,
the invisible but indispensable part of this thesis.

This work is also
devoted to my parents and parents-in-law
who built up the basis for everything.