The reconstitution of immunocompetence by GM-CSF or IFNg after pharmacological suppression

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Abbreviations

APC	Antigen presenting cells
CFU	Colony forming unit
CMV	Cytomegalus virus
ConA	Concanavalin A
CsA	Cyclosporine A
CSF	Colony-stimulating factor
CTL	Cytotoxic T-cell
Dex	Dexamethasone
EBV	Epstein Barr virus
FKBP	FK-binding protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSH	Glutathion
GVHD	Graft versus host disease
HLA	Human leukocyte antigen
i.p.	intraperitoneally
i.v.	intravenously
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
NO	Nitric oxide
NOS	Nitric oxide synthase
SD	Standard deviation
SEM	Standard error of the mean
TCR	T-cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
U	Unit
Vs.	versus

1. Introduction

1.1 Improvement and risk in transplantation medicine

In the second half of the 20th century, transplantation of organs and tissues to cure diseases has become a clinical reality. Success has been achieved as a direct result of the progress in understanding the cellular and molecular biology of the immune system, responsible for graft rejection. This understanding has led to the development of several immunosuppressive pharmaceuticals of which the first powerful immunosuppressant, cyclosporine A, has become available in the early eighties. Immunosuppressive agents are now part of nearly every transplantation procedure to assure the continued function of the transplanted organ. However, many of these drugs have deleterious long-term effects either on the function of grafts or, more importantly, on the function of other vital organs such as liver and kidney. New immunosuppressive drugs are constantly under development, but organ transplantation remains a therapy that requires patients to choose between the risk of their primary illness and its treatment on one hand, and the risks of life-long systemic immunosuppression on the other. Furthermore, chronic immunosuppression, which is mandatory after most transplantations, predisposes patients to cancer and to infections of all kinds [1-4]. Therefore, some investigators suggested to evaluate each patient individually before transplantation in the context of previous and recent infections, condition regimen, transplant complication, the degree of neutropenia, and immunodeficiency [5] to reduce the risk of infection. Others suggested to enhance immune recovery by infusions of virus-specific donor lymphocytes, inducing efficient prophylaxis and treatment of at least Epstein-Barr virus (EBV) and cytomegalus virus (CMV) infections [3]. The goal to reach in general is to find a novel and potentially powerful approach for the treatment of post-transplant infections, which frequently may result in the death of the patient.

Besides, promising alternatives to established immunosuppressive treatment in transplantation therapy include (1) the modulation of donor grafts to reduce their immunogenicity; (2) the removal of passenger leukocytes; (3) transplantation into immunologically privileged sites, e.g. testis or thymus; (4) the encapsulation of foreign tissue, and, finally, (5) the induction of a state of immunologic tolerance.

It is the last of these alternatives that has perhaps the most promising and most generic applicability as a future therapy. Recent reports documenting long-term graft survival in the absence of immuno-

suppression suggest that tolerance-based therapies may soon become clinical reality. Of particular interest in this respect are transplantation strategies focusing on the induction of donor-specific T-cell non-responsiveness.

1.2 Transplant rejection; The immune response to foreign tissue

The most important process that participates in the response to foreign tissue in transplantation is the recognition of non-self antigens. Basic elements of this response are schematically summarized in fig. 2 [6].

The response to non-self antigens involves both cellular and humoral immunity with the goal to reject the antigen. Rejection is graded in hyperacute, acute and chronic, depending on the immune response and the time transplants remain in the recipient. In the case of hyperacute rejection, which classically occurs in inter-species organ transplantation (xenotransplantation), transplant recipients are already sensitized prior to transplantation, e.g. by bacterial infections, induction of cross-reacting antibodies, pregnancy, or blood transfusion. In humans, the situation is as follows: since these patients have preformed antibodies against human leukocyte antigens (HLA), rejection occurs within a short time after transplantation. In the absence of a hyperacute rejection response, transplanted tissues often engender an acute rejection response, resulting in rejection within days, weeks or months. On the contrary, chronic rejection often occurs months to years after transplantation. The pathological hallmark for chronic rejection is fibrosis, leading to the distortion of normal organ architecture and consequently the loss of function. Chronic rejection is one of the most serious problems in the presence of continued immunosuppression. Beyond this, immune system components of the graft are able to mount an immune response towards the host. Graft versus host disease (GVHD) in the case of solid organ transplantation, however, is not a typical complication. Nevertheless, donor immune cells may persist at low levels in the recipient, leading to a state of "microchimerism" [7,8]. Microchimerism, defined as the coexistence of cells of different genetic origin within one individual may be essential for the sustained survival of allografts [9-12], i.e. transplantation within the same species, and efforts are made to co-transplant bone marrow together with an organ to induce tolerance by this mechanism [13,14].

The major histocompatibility complex (MHC) plays the defining role in the acceptance or rejection of a graft. The higher the homology between the donor graft and the host in respect to the MHC, the greater is the likelihood of graft acceptance. MHC surface proteins are divided into MHC class I and MHC class II antigens and play a pivotal role in the determination of self versus non-self by the

immune system. Foreign MHC class I or II molecules are recognized by the host's T-cells, which then generate an immune response. In general, MHC class I molecules present self-antigens derived from intracellular degradation of proteins. These processed antigens are presented in the extracellular domain of the MHC molecule and are recognized by the T-cell receptor (TCR) of $CD8^+$ T-cells. On the contrary, foreign antigens, endocytosed and processed by the host antigen presenting cells (APC) are presented to the host immune system ($CD4^+$ T-cells) on their MHC class II molecules [15]. APC, e.g. macrophages or dendritic cells, not only process and present antigens to the immune system, but also provide accessory cytokines and co-stimulatory molecules, needed for the initiation of a maximal T- and B-lymphocyte immune reaction. Since T- and B-cells are the predominant cells responsible for graft rejection, the blockage or removal of APC inhibits the sensitization, activation and proliferation of T-cells, and consequently prolongs the survival of transplanted grafts [16]. In addition, CD95 ligand (CD95L), derived from dendritic cells as the resident APC in skin, induces killing of CD95-expressing CD 4^+ T-cells [17], thus further extending graft survival. Hence dendritic cells can both activate and suppress the host immune system as a function of co-stimulatory molecules or CD95L, respectively [18]. The presence or absence of dendritic cells in a graft may therefore be a primary determinant of its acceptance. Another possibility to enhance graft survival is to pretreat transplants, e.g. islets of Langerhans, with anti-MHC antibodies [19]. However, the extent to which enhancing antibodies of host origin may contribute to graft survival is not known. Without pharmacological intervention, grafts are destroyed either directly by cytotoxic T-cells or indirectly by molecules such as cytokines, responsible for the accumulation of T- or B-lymphocytes [20]. A detailed overview of immunobiology and immunopharmacology of allograft rejection is given in a review from Sutanthiran and Strom [6].

1.2.1 The role of T-cells in graft rejection

$CD4^+$ T-cells play a pivotal role in the initiation of graft rejection, since $CD4^+$ knockout mice fail to reject grafts while $CD8^+$ do not [21]. Although this effect may be strain-dependent [22], it can be concluded that $CD4^+$ T-cells can both initiate and mediate allograft rejection, whereas $CD8^+$ T-cells, especially cytotoxic T-lymphocytes (CTL), are primarily mediators of graft destruction. CTL can destroy graft tissue either by a direct cell-cell interaction *via* CD95/CD95L or the delivery of cytotoxic molecules like granzymes. Apoptosis *via* CD95/CD95-L has been implicated in clonal selection and control of lymphocyte activation [23-26] as well as in killing mediated by cytotoxic T-cells [27]. Furthermore, Afford *et al*. recently showed that CD95-dependent apoptosis in chronic liver rejection might be increased by the CD40 molecule on leukocytes, endothelium or epithelial cells [28]. Another cytotoxic molecule released by CTL is perforin. Perforin may act directly by forming holes in the target cells, or also indirectly by increasing the porosity of the target cell membrane, thereby enhancing the entry of cytotoxic granzymes. The importance of perforin in transplantation has been shown in perforin knock out (k.o.)-mice, since these animals are deficient in the ability to lyse allospecific targets *in vitro* [29]. In addition to CTL, i.e. $CD8^+$ T-cells, cytotoxic $CD4^+$ T-cells can also mediate cytotoxicity *via* the perforin pathway [30].

Controversial discussed are the roles of specific T helper-cells of the Th1- and Th2-type in graft rejection [31]. In general, the Th1-type immune responses are proinflammatory and promote CTL development and delayed hypersensitivity responses. For example, Th1-type T-cells are producers of the lymphotoxins interleukin-2 (IL-2), interferon-gamma (IFNγ) and transforming growth factor-beta (TGF-β), which activate both T-cells and macrophages and by this means induce rejection. In contrast, Th2-type cells produce IL-4, IL-5 and IL-10 engendering immunosuppressive or down-regulatory effects on the Th1-like immune system. However, it has been suggested that in the absence of $CD8^+$ T-cells, $CD4^+$ T-cell production of Th2-type cytokines can also mediate graft rejection [32]. While T-cells exposed to antigen, e.g. grafts in the presence of IL-4 are driven towards a Th2-type immune response, the presence of IFNγ directs T-cells towards a Th1-type response. A more potent determinant of Th1- versus Th2-type polarization of the immune response to foreign tissue is IL-12 [33]. Thus and most important, Th1- and Th2-type cytokines can, depending on the sequence and intensity of their production, determine the nature of an immune response.

While the role of the lymphocytes discussed above in graft rejection is defined, this is not true for natural killer (NK) cells. Although NK cells have been shown to be implicated in xenograft rejection [34], NK cells by themselves usually do not directly contribute to graft rejection. However they are part of the graft-infiltrating cell population and therefore may share graft damage.

A further problem in transplantation derives from a possible pre-sensitization of patients, usually resulting from blood transfusions that patients receive in the course of treatment for their primary disease, from pregnancy, prior transplantation or disease. Pre-sensitization results in the formation of antibodies to multiple MHC antigens and patients are at the risk of hyperacute rejection. Therefore, they have to be overcome either by selecting the donor graft on the basis of non-reactivity to recipient preformed antibodies or by immunoabsorption of the host's preformed antibodies before transplantation. Furthermore, pre-sensitization has been shown to be controlled by transfusion with donor cells in the presence of CTLA4-Ig [35]. Current clinical protocols propose the removal of white blood cells before blood transfusion or concurrent treatment with immunosuppressive agents to prevent sensitization.

1.2.2 The role of macrophages in graft rejection

As described in the section above, T-cells are the key mediators in graft rejection. Macrophages may also be involved in the initiation and propagation of the immune response, mediating graft rejection [34]. The role of macrophages is emphasized by the finding that rejected xenografts are predominantly infiltrated by these cells. Furthermore, cytokines, released by either macrophages or T-cells, can play both destructive and immunomodulatory roles in graft rejection [36]. As most prominent cytokines, tumor-necrosis-factor (TNF), IFNγ or IL-1 contribute to graft destruction either directly or by activating the effector cells. In contrast, IL-4, IL-10 and TGF-β are thought to be capable of impairing graft rejection [37,38].

1.2.3 The induction of immunologic tolerance as a mechanism to prevent allograft rejection

Transplantation tolerance can be defined as the inability of the graft recipient to express a graft destructive immune response due to clonal deletion, clonal anergy or suppression [39-42]. True tolerance is induced as a consequence of exposure to the specific antigen prior to transplantation, and is independent of the administration of immunosuppressants. In 1953 Billigham *et al*. already showed that tolerance can be induced by chimerism, which is produced by a donor leukocyte infusion prior to transplantation. Provided that these leukocytes are not recognized as foreign, e.g. in fetal or newborn animals [43], chimerism allows the permanent engraftment of skin from the leukocyte donor without graft rejection. An example of total donor leukocyte chimerism is the complete replacement of the immune system in bone marrow transplantation. The functional relevance of microchimerism has been debated extensively by Billingham and Starzl [9,43]. Recent results in a rat cardiac transplantation model show that early elimination of chimerism leads to chronic rejection, whereas a late elimination of chimerism had no effect on graft survival [44], suggesting an important role for the induction, but not for the maintenance of microchimerism. However, attempts to use a donor splenic cell infusion in order to prevent liver graft rejection and to augment chimerism have failed [45]. Achieving tolerance by chimerism is probably more difficult in humans than in animals, because of unacceptable complications in humans, resulting from whole-body irradiation. The clinical application of chimerism in case of transplantation is therefore not yet established [46]. Besides, and with regard to the complications in chimerism, the induction of T-cell anergy may be the predominant mechanism for tolerance [47,48].

Nevertheless, donor-specific tolerance continues to be the elusive “holy grail” and the cherished goal for the transplant clinician [49,50]. Clinical examples of tolerance, albeit infrequent, of grafts functioning without any exogenous immunosuppressive drug, do suggest that some transplant recipients of allografts develop tolerance to the transplanted organ.

1.3 Immunosuppressive therapy in the transplantation process

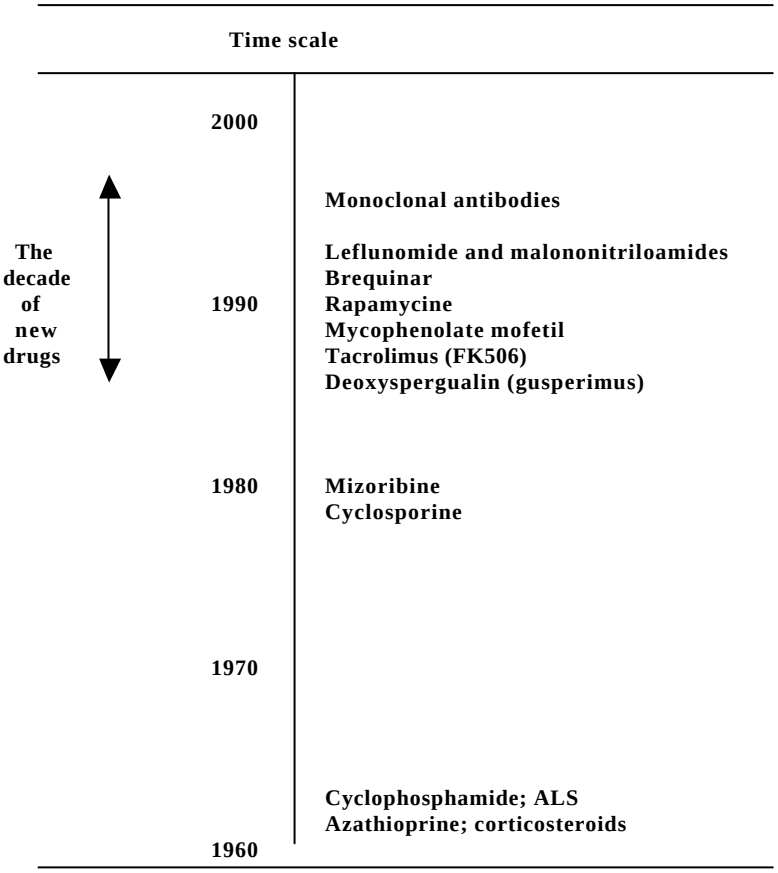
To avoid transplant rejection, an immune response to the graft, has to be prevented. As outlined before, T-cells play the dominant role in the rejection process, and therefore are the favourite target in the development of anti-rejection strategies in transplantation. Macrophages which participate in the initiation and propagation of rejection are not in the main focus. Strategies against the activation or proliferation of T-cells in transplantation include the induction of tolerance, T-cell anergy and finally immunosuppressive therapy by immunosuppressive drugs, the latter which is the topic of this section.

1.3.1 Development in transplantation surgery

There is evidence that successful isotransplantation surgery, i.e. intra-personal transplantation, was actually performed by ancient Hindu perhaps 2000 years ago. They constructed a nose using pedicle flap grafts from the patient's own forehead [51]. In 1597 a reconstructive rhinoplasty using skin flaps from the patient's arm was performed [52]. No further progress occured until the beginning of the 20th century, when animal and some human transplantation experiments were carried out. Scientists in these days recognized that the serious barriers to successful transplantation would not be technical, but biological. The "Laws of transplantation" were summarized in 1912 from G. Schoene. He reported that (1) Transplantation into a foreign species (xenotransplantation) invariably fails, (2) Transplantation into unrelated members of the same species (allotransplantation) usually fails, (3) Autografts (isotransplantation) almost invariably succeed, (4) There is a primary take and then delayed rejection of the first graft into an unrelated member of the same species, (5) There is an accelerated rejection of the second graft in a recipient that had previously rejected a graft from the same donor, or of a first graft that had been pre-immunized with material from the same donor, and (6) The closer the "blood relationship" between donor and recipient, the more likely is graft success [52]. In World War II, Sir Peter Medawar observed on burn victims receiving skin grafts that autografts, i.e. transplants from the recipient itself, succeeded. Furthermore he showed that allografts failed after an initial take, and that a second allograft from the same donor underwent hyperacute rejection (section 1.2). In 1944, Medawar further broadened our knowledge about the immunologic basis of allograft rejection by a series of experiments on rabbits [53]. Continuous analysis finally led him to the recognition that immunosuppression might overcome the laws of transplantation.

The capability of the immune system to recognize foreign proteins, e. g. in allotransplantation among non-identical individuals, leading to its destruction and rejection, led to the discovery and improvement of strategies for immunosuppression (fig. 1) [54].

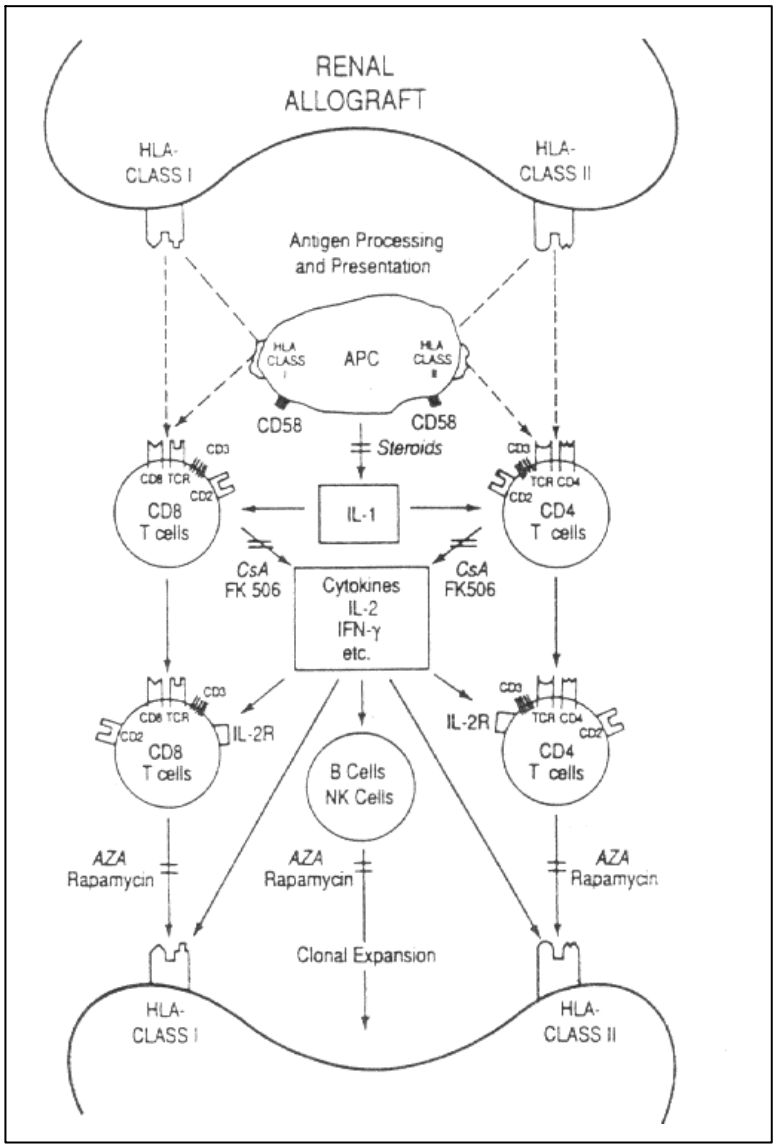
Fig. 1: Immunosuppressive drug development



After first efforts with radiation, therapy was supplanted by immunosuppressive drugs including glucocorticoids, azathioprine, and anti-lymphocyte serum [55,56]. The development of immunosuppressive agents like nitrogen mustard and corticosteroids and their evaluation in animal models soon led to the practical application of transplantation as a medical therapy [52]. The first successful human vascular organ graft, a kidney transplant, was performed in 1954, between monozygotic twins without the need for immunosuppression [57,58]. Moreover, in 1959 kidney transplantation was realized between unrelated individuals using immunosuppressive drugs [59]. Newer immunosuppressive drugs with better potency and wider margins of safety improved the outcome of renal allografts and generated the initial consistent success with cardiac, liver, lung, and pancreas grafts [60]. The first of these second-generation drugs was cyclosporine A. Newer agents include tacrolimus (FK-506), mono-

clonal antibodies like anti-CD3, and many other biological [61] and non-biological agents [62]. Multiple steps, such as cytotoxic T-cell activation and allo-antibody formation [63,64] are involved in the recognition of an allograft and the development of effector mechanisms, which result in allograft rejection. Each of these steps therefore represents a potential site that can be targeted in an anti-rejection strategy. The most important effector mechanisms and beneficial drugs against are given in figure 2 and below:

Figure 2: The anti-allograft response and potential sites for the its regulation [6]



APC = Antigen presenting cell

CsA = Cyclosporine A

AZA = Azathioprine

IL-2 = Interleukin-2

IFNg = Interferon-gamma

NK cells = Natural killer cells

1. anti-inflammatory and immunosuppressive: Corticosteroids, e.g. Dexamethasone
2. nonspecific inhibition of cell division : Cyclophosphamide, Azathioprine
3. selective inhibition of the *de novo* purine synthesis in lymphocytes: Mycophenolate mofetil

(MMF), Mizoribine

4. inhibition of the pyrimidine synthesis : Brequinar
5. inhibition of the interleukin (IL-)2 gene transcription: Cylosporine A and Tacrolimus [65]
6. inhibition of the IL-2 action on effector cells : Sirolimus (rapamycine)
7. monoclonal antibodies (Mab) with specific sites of action: (a) Anti-cd3Mab, (b) Anti-IL-2 receptor Mab [66], (C) Anti-T cell receptor Mab.
8. polyclonal antybodies against human thymocytes: Antithymocyte globulin (ATGAM)
9. costimulatory pathway blockade: Cytotoxic T lymphocyte antibody (CTLA4Ig), anti-CD40 Mab [67]
10. adhesion molecule blockade: Leukocyte function-associated antigen (LFA)-1, intercellular adhesion molecule 1 (ICAM-1),VCAM.

1.3.2 Corticosteroids

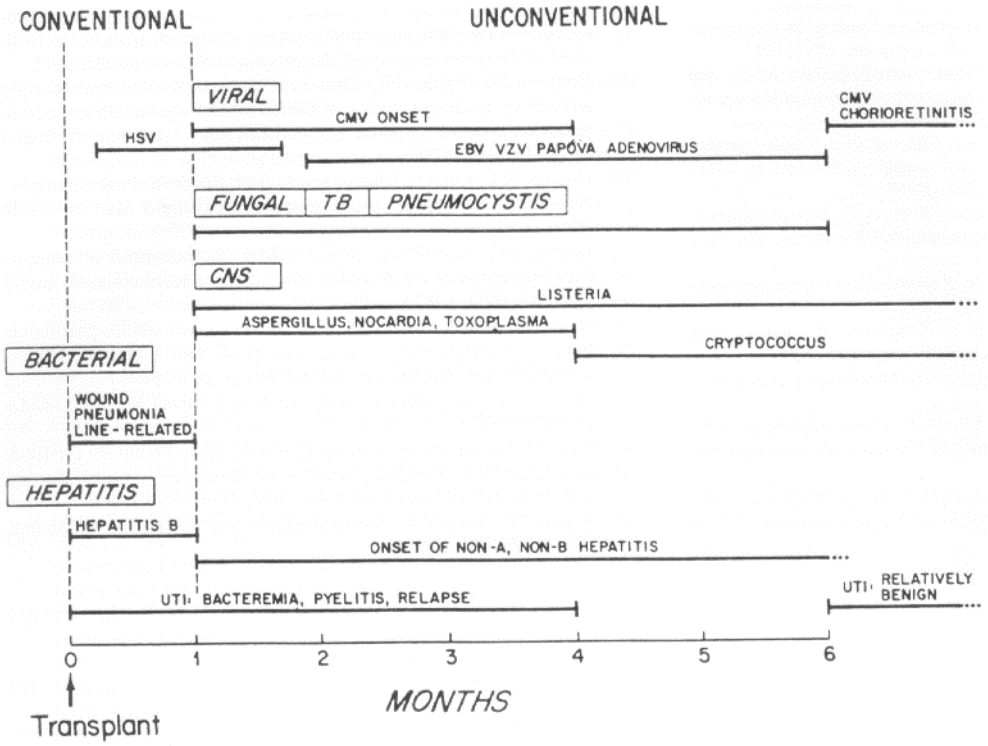
Glucocorticoids such as dexameathsone were developed the years from 1959 to 1962 and remained, together with azathioprine (Imurek®, Imuran®), the most important immunosupressive drugs for nearly 20 years. They are among the most widely used drugs for the treatment of inflammatory diseases. Furthermore in organ transplant regimens they are at high doses used for the induction of immunosuppression and are at lower doses part of the maintenance protocol in combination with other drugs. Popular indications for corticoid treatment are acute and chronic inflammatory conditions such as asthma, allergies, arthritis and connective tissue diseases [68]. After binding to their receptors which are found on most, perhaps all, cells in the body they cross cell membranes, combine with specific binding proteins and are transported across the nuclear membrane. Corticosteroids show direct effects on monocytes and macrophages by inhibiting the NF-κB pathway and decreasing the release of proinflammatory cytokines, e.g. TNF, IL-2 [69], IL-6 [70,71] or IL-1β [72-74]. In endothelial cells and fibroblasts, the release of the pro-inflammatory cytokines IL-8 and granulocyte macrophage colony-stimulating factor (GM-CSF) is blocked [73,75-77]. Moreover, glucocorticoids induce the expression of the anti-inflammatory scavenger interleukin-1 receptor (IL-1R) [78-81], thus balancing the pro- and anti-inflammatory site of the immune system in parallel [71]. Dexamethasone treatment also reduces the numbers of GM-CSF dependent eosinophils [82,83] and neutrophils [84], and enhances the macrophage colony-stimulating factor (M-CSF) and GM-CSF stimulated proliferation of bone marrow-derived macrophages [85]. Other data indicate a downregulation of GSH

levels [86] and an IL-2 reversible state of T-cells anergy after dexamethasone treatment [87].

One major complication of corticosteroids is the rampant spread of infection, especially when given in combination with other immunosuppressive agents. In certain animal models, as well as in clinical situations, steroid treatment worsened the outcome of infectious disease [88-90]. Numerous human studies with dexamethasone failed due to an increased infection rate, predominantly caused by cytomegalovirus (CMV) [91, 92]. In animals dexamethasone likewise augmented the risk for leishmaniosis [93], latent tuberculosis [94] and biliary tract infection [95]. On the contrary, steroid therapy demonstrated a benefit in several pediatric [96] and adult [97,98] septic patients, in newborn rats with septic shock [99] or in hepatic insufficiency [100]. When administered before antibiotics, dexamethasone improved the neurologic outcome and subsequent meningeal inflammation [101].

Taken together, glucocorticoids due to their immunosuppressive capacity greatly improved transplant surgery. However, numerous clinical studies revealed an increase of infectious disease in immunosuppressed patients. Thus immunosuppressive therapy is a double edged sword that on the one hand reduced graft rejection but on the other hand increased the risk of infection. Figure 3 illustrates the occurence of opportunstic infections after organ transplantation under immunosuppression.

Figure 3: Framework for the occurrence of opportunistic infections after organ transplantation



1.3.3 Immunophilin-binding drugs

One of the most important groups of immunosuppressive agents is the one of the immunophilin-binding drugs, including cyclosporine A (CsA, Sandimmun®), tacrolimus (FK-506, Prograf ®) and sirolimus (rapamycine). Immunophilins are ubiquitous, abundant and highly conserved proteins that are active as protein-folding enzymes. Binding of the hydrophobic immunosuppressive drugs to the immunophilin results in an active immunophilin-drug complex. While CsA binds to the immunophilin cyclophilin, tacrolimus and sirolimus bind to the FK-506 binding protein 12 (FKBP-12). However, the CsA-cyclophilin complex and the tacrolimus-FKBP-12 complex inhibit the enzyme calcineurin, whereas the sirolimus-FKBP-12 complex binds to the ”target of rapamycin” (TOR) protein. The molecular mechanisms of CsA, tacrolimus and sirolimus are discussed in detail in the following sections [102].

1.3.3.1 The calcineurin inhibitors: Cyclosporine A and tacrolimus

The development of CsA was a breakthrough for the outcome in organ transplantation. Since the mid-1980s the standard immunosuppressive regimen has been based on cyclosporine, azathioprine, and corticosteroids. However, despite fundamental advances were achieved by these drugs, several problems, especially by drug toxicity, resistant acute rejection, chronic rejection, and secondary infections remained. Therefore, new, selective, and powerful drugs, like sirolimus, mycophenolate mofetil (MMF), sodium brequinar, mizoribine, leflunomide and 15-deoxyspergualine have been discovered [103-105].

Although CsA and tacrolimus are chemically unrelated, they have similar modes of action in the prevention of allograft rejection. However, CsA and tacrolimus form different intracellular drug-immunophilin complexes with cyclophilin or the FK-binding protein (FKBP-12), respectively. Both complexes lead to a specific and competitive binding to calcineurin, thereby inhibiting its phosphatase activity [106-109]. The target of both drugs, calcineurin, is a critical link in the sequence of steps starting with the initial engagement of foreign antigen by the T-cell receptor and ending with cytokine transcription and T-cell activation. Inhibition of calcineurin diminishes the dephosphorylation and consequently activation of the nuclear factor of activated T-cells (NFAT), responsible for the transcription of cytokines like interleukin-2 (IL-2). Thus, the inhibition of NFAT prevents the proliferative response of T-lymphocytes [110-112]. Furthermore, CsA and, to a weaker extent, tacrolimus inhibit the induction of nitric oxide synthase (NOS) through different intracellular mechanisms [113]. As nitrite and nitrate levels rise in the acute phase of rejection [114,115], this may have an important effect on the outcome of transplantation. Both drugs are metabolized by the cytochrome P450 system [116,117], which plays an important role in detoxification processes. Thus, these immunosuppressives increase toxification processes of other xenobiotics. Nowadays almost all current immunosuppressive protocols are based on either CsA or tacrolimus; however, both drugs have a neurotoxic, nephrotoxic, and diabetogenic potential and thus research for new immunosuppressive agents continues.

1.3.3.1.1 Cyclosporine A

Cyclosporine A (CsA; Sandimmun® and Sandimmun Neoral®), a neutral lipophilic cyclic polypeptide consisting of 11 amino acids with a molecular weight of 1202, is produced by the fungus species *Tolypocladium inflatum*. It was introduced in clinical trials 1979 by Calne *et al.* [118,119] and further refined in a combination with corticosteroids by Starzl *et al.* [120]. As a potent inhibitor of almost all known lymphokines, including IFNγ, IL-2 and IL-7, it affects both the T-cell and B-cell proliferation [121,122]. Furthermore, CsA can mediate tolerance by the induction of apoptosis in T-lymphocytes [123,124]. Detailed information about the molecular mechanisms of CsA on T-cells are given in different reviews [122,125]. CsA, mainly active against T helper cells [126], revealed significant improvement in 1 year patient and graft survival in renal transplantation [127,128], and sustained long-term benefits within 3 and 5 year trials [129-132]. Promising results were obtained by applying CsA together with steroids, resulting in a 1-year survival rate of almost 70 % after liver [120], heart [133-136] and lung transplantation [137,138]. CsA has a narrow therapeutic window, with high doses associated to toxicity and low levels associated to rejection [139-141]. Because it binds to red blood cells, therapy with CsA is usually monitored by whole blood or plasma through concentrations [126]. Halloran *et al.* recently showed that CsA can only inhibit about 50 % of calcineurin activity [142,143], explaining a sufficient activity to mount immune responses for host defense and the inadequate immunosuppression of CsA as monotherapy on the other hand in their experiments. A low bioavailability of CsA (5 % to 10 %) is frequently observed prior to renal transplantation, which increases up to 20 % or 40 % after transplantation, a phenomenon attributed to defective transport mechanisms for the drug in uremic patients [144]. Furthermore, liver diseases and numerous other drugs affect CsA levels by induction or suppression of the cytochrome P450 enzyme system in the liver [144]. To improve the limited and sometimes unpredictable absorption of CsA from the gut, a new formulation, named Neoral®, with a better bioavailability and predictability of pharmacokinetic properties was performed [145].

However, CsA uptake is linked to numerous severe side effects. Hepatotoxicity, tremors, hirsutism, gingival hypertrophy, anorexia, gout, paresthesia or hyperesthesia and hypomagnesemia occur in up to 20 % of patients [125]. A further problem which predominantly concerns women is hypertrichosis,

i.e. an extreme increase in hair growth. Moreover, CsA is unlikely to be genotoxic [105], whereas it might increase carcinogenicity [146-148].

The incidence of post-transplant lymphoma in the kidney transplant population has been reported to be between 2 and 4 % using maintenance CsA therapy [149]. The most common complications combined with the intake of CsA are hyperkalemia in 95 %, hypertension in up to 95 %, hyperuricemia in 80 %, or nephrotoxicity and a variety of infections in about 30 % of patients. It should be remarked that in many patients CsA-induced nephrotoxicity is functional rather than anatomic and is caused primarily by preferential constriction of the afferent renal arteriole [150]. The inherent nephrotoxicity of CsA was the impetus for the use of lower doses.

1.3.3.1.2 Tacrolimus

Tacrolimus (FK-506, Prograf®), a lipophilic macrolide lactone with a molecular weight of 822, is derived from the soil fungus *Streptomyces tsukubaensis*. It was discovered in 1984, and its immunological efficiency demonstrated in 1987 [151,152]. In 1989 tacrolimus was introduced as a potent alternative to CsA by Starzl *et al*., and it was first used for liver allografts failing from either acute or chronic rejection under CsA [153-155]. In contrast to CsA, tacrolimus blocks only the T-cell dependent immune responses, while T-cell independent B-cell responses are preserved. T-cell stimulation through alternative pathways is therefore not affected [105]. Tacrolimus specifically inhibits the synthesis of IL-2, IL-3, IL-4, colony stimulating factors (CSF) and IFNγ [104,107] and thereby inhibits the proliferation of cytotoxic T-lymphocytes in a fashion similar to CsA [156,157]. Kino *et al*. [158] in 1987 already reported that tacrolimus inhibits the activation of lymphocytes *in vitro* 10 to 100 times more potently than CsA. Tacrolimus mainly improved the outcome in liver transplantation when compared to CsA. An improvement in the quality of life of liver transplant patients on tacrolimus in contrast to CsA has been reported by Felser *et al*. [159] and Kino *et al*. [107]. In several studies, a significantly lower incidence of liver rejection under tacrolimus than under CsA was revealed [160-163] and thus, a large percentage of CsA-treated patients with refractory rejection was successfully switched to tacrolimus [164,165]. Detailed information from several studies comparing CsA and tacrolimus can be taken from Jain *et al*. [166] and Gummert *et al*. [167].

Like CsA, lipophilic tacrolimus is highly bound to plasma proteins, red blood cells and lymphocytes [168] and has a half-life of 8 - 24 h [169]. Metabolism in the intestinal cell wall or in the liver by cyto-

chrome P450 [170,171] leads to at least 15 metabolites, of which some show pharmacological activity. Analogous to CsA, tacrolimus has severe adverse effects [172], including nephrotoxicity, neurotoxicity, and new-onset diabetes. Hypertrichosis as a severe side effect in CsA treated women was not found with tacrolimus. However, when used as primary drug, tacrolimus was found to be more significantly nephrotoxic and neurotoxic and was associated with anemia.

1.3.3.2 TOR inhibitors: Sirolimus

Sirolimus (rapamycine), discovered in the mid-1970s [173], is a natural fermentation product of *Streptomyces hygroscopicus* with a molecular weight of 914. Sirolimus which is not yet clinically used is available only in an intravenous form. Intravenous administration in rabbits results in a half-life of 13 hours, with over 97 % partition into red and white cells [53]. As a lipophilic macrolide it is virtually insoluble in water, but readily soluble in organic solvents. Similar to CsA and tacrolimus, sirolimus is a prodrug that first must complex with its immunophilin before it can block immune cell activation [105]. Although sirolimus has structural similarities to tacrolimus and binds to the same immunophilin, FKBP-12, the sirolimus-FKBP-12 complex does not block calcineurin activity. Thus sirolimus does not inhibit early T-cell activation [111], nor reduce the synthesis and the release of IL-2 [174,175]. However, sirolimus inhibits the T- and B-lymphocyte proliferative responses to a number of stimuli [174-176] by blocking kinases involved in the progression of the cell from the G1- to the S-phase [177]. Furthermore, lymphocyte responses to IL-2, IL-4 and IL-6 are blocked [178]. Information about the immunopharmacology, which is not yet cleared in detail, can be taken from the review of Abraham *et al.* [179]. When administered together with CsA, sirolimus shows synergistic effects both *in vitro* and in small animal models [180] since it inhibits several CsA-resistant pathways in both T- and B-cell stimulation [181]. In rodents, sirolimus was shown to be a potent inhibitor of the rejection of both xeno- [182] and allogenic [183] skin grafts and in highly histoincompatible heart grafts [184,185]. Furthermore, sirolimus prolonged allograft survival in different animal models and was at least as effective as CsA-based immunosuppression [186,187].

The introduction of sirolimus brought a clear progress in human studies of renal [188] and hepatic allograft survival [189]. Furthermore, the nephrotoxicity associated with CsA and tacrolimus was avoided by the use of sirolimus in several animal studies, possibly due to the lack of calcineurin inhibition [190,191]. However, in dogs [184] and also baboons [186], sirolimus was reported to be extraor-

dinarily toxic, thus disturbing gastrointestinal functions and producing diarrhea, vomiting, severe ulceration and vasculitis from the mouth to the colon. While dogs were unusually sensitive to the toxicity of sirolimus, other animals were characterized by only weight loss, testicular atrophy and lethargy.

1.3.3.3 Inhibitors of *de novo* purine synthesis: inosine 5´-monophosphate dehydrogenase (IMPDH) inhibitors: Mycophenolat Mofetile

Mycophenolat mofetile (MMF) is a semisynthetic ester pro-drug of the active agent mycophenolic acid (MPA), isolated from the fungus *Penicillium glaucum*. MMF *in vivo* is phosphorylated to MPA, which non-competitively and irreversibly inhibits the enzyme inosine monophospate dehydrogenase (IMPDH). IMPDH inhibition blocks the *de novo* synthesis of the purine guanosine, thus selectively depleting guanine nucleotide pools, i.e. GTP. Consequences of a depletion of GTP are an inhibition of DNA synthesis and replication in T- and B-lymphocytes [192] and finally a prevention of lymphocyte proliferation [104]. Late downstream effects of a reduced number of T-cells are a decrease in IL-2 and IL-4, regulating the IFNγ production and consequently the synthesis of the major histocompatibility (MHC) antigens. The potential immunosuppressive properties of MPA were first demonstrated by Mitsui and Suzuki in 1969 [193]. But the failure to prolong mouse skin graft survival, due to very rapid metabolism of MPA in mice in contrast to other species, substantially delayed its further studies. Finally, the production of the ester prodrug MMF with a higher bioavailability showed promising results in early clinical studies of cadaveric kidney [194] and liver transplantation [195]. MMF has recently been shown to reduce refractory rejection in kidney transplantation [196-198] when administered together with CsA or steroids. A slight benefit in the rate of rejection was also observed, when MMF plus tacrolimus were given, but in these groups the rate of postoperative infection was more than 50 % [166]. MMF has to be administered orally with a high bioavailability, and it is eliminated in the bile [104]. However, MMF has a low potency as it reduces the activity of its target by only 50 % [143] and is effectively inactivated. Therefore, high doses are required to maintain and assure immunosuppressive levels of the drug in the blood. Since MMF is well tolerated, and only some cases of gastrointestinal toxicity were reported, it is recently used to reduce the doses of CsA or tacrolimus and thereby chronic nephrotoxicity of these substances [199].

1.3.4 Immunosuppressive therapy: a double-edged sword

Successful allotransplantation requires the use of immunosuppressive agents [60]. Most of them, e.g. CsA, tacrolimus or sirolimus, were discovered by screening microbial products for immunosuppres-

sive activity or by simulating genetic defects known to produce severe immune impairments [200]. New immunosuppressive agents that are more potent, selective, or reliable are at hand or in development. While the calcineurin inhibitors CsA and tacrolimus predominantly affect the T-cell function, the inhibitors of purine synthesis, MMF, and TOR, sirolimus, possess additional mechanisms directly affecting B-cell activity, antibody formation or antigen-presenting cell functions. All such drugs are known to predispose to neoplasia, but also to infection, the latter which is the topic of this thesis. Of chronically immunosuppressed patients 10 to 45 % develop neoplasia within 10 years after transplantation, and within 20 years 40 to 75 % do so [201,202]. In addition, many immunosuppressive drugs have deleterious long-term side effects, such as cyclosporine- or tacrolimus-induced nephrotoxicity [203,204]. However, the development of other immunosuppressive drugs, such as MMF, has reflected a clear benefit in patient and graft survival, as they reduce the incidence and severity of rejection. A detailed overview of pharmacokinetics and -dynamics, clinical trials or molecular mechanisms of clinically used immunosuppressants are given in the reviews of Gummert *et al.* [167], Halloran [205] or Kundu *et al.* [206], respectively. Nowadays the combination of different immunosuppressive drugs are clinically used, thus decreasing the doses of nephrotoxic drugs and consequently severe side effects. Both, double [207] and triple drug protocols [208] have already been evaluated in organ allografting. First results show that the triple drug regimen is more effective than the double drug protocols as it allows a more flexible immunosuppression. An overview, summarizing therapeutical strategies concerning transplantation is given in the review of Welsh [209].

Last but not least infections due to immunosuppression remain a clinical problem since the most frequent cause of mortality and severe morbidity in transplant recipients in the first few months after surgery is not graft rejection but viral, bacterial or fungal infection [1,2,210]. In the first three months after surgery patients receive high dosages of immunosuppressive drugs, since immunogenicity of the graft is rather high. In this period bacterial infections, which can be controlled in some cases by antibiotics, commonly appear.

Thereafter immunosuppression is reduced, because the allograft is more and more accepted by the recipients immune system. Consequently the risk of infection is diminished. However, viral infections, e.g. CMV with a lethality up to 90 %, can be observed in this phase [210]. Therefore regular serological analysis from blood and urine specimen is performed in clinical routine to control the bacterial, viral and fungal status of patients. Methods for a successful reconstitution of the suppressed immune

functions without increasing the risk of graft rejection have not yet been investigated. Therefore, this subject was elected in the present thesis.

1.4 Strategies to reconstitute the suppressed immune response

As outlined before post-transplant therapy has improved patient and graft survival. Nevertheless, a high percentage of patients still suffer from infectious diseases days, weeks or months after surgery due to the use of immunosuppressive drugs. Since a high rate of patients die from these infections, it was investigated whether immunocompetence after pharmacological immunosuppression in case of infection can be systematically reconstituted without risking graft rejection. Previously, Bundschuh *et al*. showed that the two pro-inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-gamma (IFNγ) can reactivate the murine immune system after immunoparalysis. Therefore it was examined whether these two cytokines have got the potential to reactivate the immune system after pharmacological suppression induced by several clinically used drugs in case of infection. Furthermore, it was studied if such cytokine intervention badly influences graft acceptance. In the following chapters, the characteristics of the immuno-stimulators GM-CSF as well as IFNγ are described.

1.4.1 GM-CSF

1.4.1.1 The mode of action of GM-CSF

In the mid 60s, a new group of hematopoietic growth factors, the colony-stimulating factors (CSF), were discovered by Metcalf, Bradley, Pluznick and Sachs [211-216]. In humans, four different CSF were characterized: granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF or CSF-1) and interleukin-3 / multi colony-stimulating factor (IL-3). Together with erythropoietin and interleukins, CSF have the capacity to stimulate the proliferation and differentiation of stem cells to myeloid blood cells, i.e. monocytes, granulocytes, mega-caryocytes, mast cells and erythrocytes in hematopoiesis. Besides their role in hematopoiesis importance should be attached to their immunomodulatory capacities. For detailed information about the biological effects, molecular structures, receptors, and signalling pathways of CSF the reader is referred on different reviews [217-227].

Murine GM-CSF, cloned in 1984 [219] and human GM-CSF, cloned in 1985 [228,229] share a protein sequence homology of only 60 % [229]. Receptors for GM-CSF are found on leukemic cell lines and on mature neutrophils, monocytes and eosinophils [230]. GM-CSF activity is species-restricted, and as a multilineage hematopoietin [231] GM-CSF stimulates the proliferation of eosinophilic and neutrophilic granulocytes as well as macrophages [216,231,232]. Consequently, GM-CSF plays an important role in the inflammatory immune response [216,233,234]. However, deficiency of GM-CSF in mice did not affect hematopoiesis [235,236], but the animals developed abnormal lungs with extensive infiltration of lymphocytes and some had subclinical bacterial or fungal infections [235]. In excess, GM-CSF was shown to induce blindness and muscle wasting [237] in mice, but had no effect on leukemic transformation [238].

1.4.1.2 The endogenous production of GM-CSF

GM-CSF, an approximately 14 kDa protein, can be expressed by several cell types, i.e. T-lymphocytes [239,240], B-lymphocytes [241], macrophages [242], fibroblasts [243], mast cells, endothelial cells and NK cells in reponse to cytokines [239,244], antigens [245] or inflammatory agents. Thus, human [246,247] and murine [248] monocytes or macrophages activated by LPS rapidly release *de novo* synthesized GM-CSF. Also, on maturation of monocytes to macrophages, the secretion of GM-CSF was greatly increased *in vitro* [249,250].

1.4.1.3 GM-CSF in inflammation

In mice, GM-CSF was reported to augment a systemic cytokine release in response to an inflammatory stimulus in normal as well as in experimentally immunocompromised mice [251,252], thus increasing LPS-induced murine lethality [253,254]. Administration of GM-CSF to cancer patients resulted in an enhanced release of LPS-induced TNF and IL-1 from monocytes *ex vivo* [255-257]. Hence, stimulated macrophages can mediate an amplification of the local inflammatory response by activating mature white cells that migrate to the inflammatory site, ensuring their retention in the region of inflammation and by enhancing proliferation and differentiation of progenitor cells [216,231,232]. GM-CSF was revealed to increase the number of circulating leukocyte [237,258,259], neutrophil, monocyte, eosinophil [223,260], and megakaryocyte counts [261]. Moreover, an anti-apoptotic effect could be demonstrated [262], since blocking autocrine GM-CSF induced lymphoid cell death. However, GM-CSF was shown to exert predominantly pro-inflammatory rather than myeloproliferative effects [237,258]. Interestingly, GM-CSF levels in humans in case of infection were not elevated [263-265], whereas an increase in plasma GM-CSF in conjunction with infections in immunosuppressed renal transplant patients has been described [266]. In contrast, pharmacological intervention by cyclosporine A or dexamethasone was shown to downregulate the GM-CSF secretion in endothelial cells [267] or fibroblasts [268]. In healthy individuals circulation, GM-CSF has rarely been found at detectable levels, but is extractable from all major organs at higher concentrations [269], where it acts locally in a paracrine manner [270].

Several reports revealed increased phagocytosis of *Candica albicans* [271,272] and *Leishmania tropica* [273], or intracellular killing of *Leishmania donovani* by GM-CSF primed macrophages. Moreover, GM-CSF restored the *Stapylococcus aureus* killing capacity of neutrophils, suppressed by dexamethasone [274], and, when given to monkeys, enhanced killing of an *E. coli* strain by granulocytes *ex vivo* [275]. Such a priming effect was also found in monocytes of patients who had undergone high dose chemotherapy, even weeks after the cessation of the GM-CSF therapy [276]. Parasite reduction could be significantly increased, when a combination of GM-CSF and IFNγ were used [277]. Nevertheless, the molecular basis of those clearing effects is yet unknown. Data, indicating that the effect lies in an induction of oxidative burst by GM-CSF are discussed controversially. Nathan *et al*. reported that while GM-CSF does not affect the respiratory burst of adherent monocytes, it can affect that of monocytes in suspension [278].

Taken together, GM-CSF exerts its anti-infectious potential in various animal models where macrophages are important for the hosts defense system.

1.4.1.4 Clinical significance

Hematopoietic growth factors generally have made a significant impact in the treatment of cancer and AIDS, primarily in the prevention of infections associated with HIV disease or chemotherapy-induced neutropenia, in bone marrow transplantation, in chemotherapy-induced thrombocytopenia and in chemotherapy-induced anemia [279,280]. Both, the hematopoietic and the pro-inflammatory effect of GM-CSF, which was introduced into clinical practice in 1991 and is now available as Molgramostim (Leukomax®) or Sargramostim (Leukine®) [281], are important for its usefulness. Most studies mainly tested GM-CSF in bone marrow transplant patients [223,282-284] and in the treatment and prevention of infections [281,285]. Short pretreatment with GM-CSF before chemotherapy reduced the hematopoietic toxicity of cytostatics and thereby also enabled the dose intensity of protocols to be increased [286]. Other studies suggested that GM-CSF ameliorates the outcome for patients with graft failure without exacerbating graft-versus-host disease [238]. Improved host defense on the other side, might have a bearing for the treatment of infectious diseases. Thus, therapy with GM-CSF could significantly reduce the incidence of infections and diminished the demand for antibiotics. In humans, GM-CSF administration was shown to be protective in viral [287] or parasite infections [288], and in resistance to *Salmonella typhimurium* [265] and *Leishmania* [289].

In cancer treatment, GM-CSF secreting tumor vaccine, i.e. the insertion of the GM-CSF gene into cancer cells that are used to immunize patients, is discussed [290-292]. Such vaccine already was effective in curing cancer in mice, in patients with renal cell carcinoma [293] and in malignant melanoma patients [294], but failed in other studies of large tumors burdens that secrete immunosuppressive factors like IL-10 [292], in non small lung cancers [295], in metastatic renal cell carcinoma [296] and in prostate cancer [297]. Promising results were also found in the successful combat of chronic hepatitis C by GM-CSF [298]. Otherwise, an excess activation of neutrophils by GM-CSF can induce significant tissue damage of inflamed sites [299] and might increase [300] or decrease [301] the risk for the acute respiratory distress syndrome (ARDS). A variety of pronounced side effects was associated with high doses of GM-CSF, but at lower pharmacologically active doses, GM-CSF was generally well tolerated in both, monkeys and humans [302]. In summary, potential adverse side effects of GM-CSF application have to be carefully considered [283,303] and weighed up against the benefit of immunostimulation. The appropriate use of CSF in clinical practice is further discussed in several reports [304-307].

1.4.2 Interferon-gamma (IFNg)

1.4.2.1 The mode of action of IFNg

IFNγ, first described in 1965 as a component in supernatant derived from T-lymphocytes, was shown to augment various biological activities of macrophages [308], including intracellular killing of parasites and increased oxidative metabolism [309], enhanced expression of MHC class II antigens [310], or increased tumor cell killing [311]. Since then, the 34 kDa protein IFNγ attracted much interest from clinical investigators, as it is essential for natural as well as acquired resistance to infection and cancer. Because of its immune-regulatory consequences, IFNγ was also termed “immune-interferon” [312].

1.4.2.2 The endogenous production of IFNg

IFNγ is produced exclusively by NK cells and some sub-populations of T-lymphocytes [313,314]. Production of IFNγ by mononuclear cells was only described by the group of Fultz *et al.* [315]. To be activated, T-lymphocytes require a combination of three signals which are (1) a specific ligand binding to the T-cell receptor, (2) a balanced assembly of cytokines, e.g. IL-1, IL-6, TNF or IL-12, and (3) contact with accessory cells through cell adhesion molecules.

1.4.2.3 IFNg in inflammation

IFNγ release can be induced by preparations of Gram-positive bacterial compounds like *Staphylococcus aureus* [316] and *Listeria* [317], as well as from Gram-negative endotoxins. While the secretion of IFNγ in case of stimulation by Gram-positive bacteria is restricted to NK cells, Gram-negative bacteria induce NK cells, as well as the T-lymphocytes, i.e. $CD4^+$ and $CD8^+$ cells to release the cytokine [318]. Nevertheless, in the lethal *Shwartzman* reaction, caused by two consecutive injections of endotoxin, the elimination of NK cells, but not of $CD4^+$ or $CD8^+$ T-cells, is enough to prevent the toxic manifestations of the reaction [319]. While several cytokines, like TNF [320], IL-12 [318], IL-2 [321] and IL-1 [322] share synergistic effects with IFNγ, others, like IL-4 [323-325], IL-10 [326], TGF-β [327, 328], IFN-α and IFN-β [329], but also TNF [330] were described as antagonists.

IFNγ is generally assumed to play a primordial role in the defense against intracellular bacteria and parasites [331,332]. In fact, most of the pathogens are found in mononuclear cells. This IFNγ dependent pathway is complemented by a cytotoxic T-cell pathway, which kills phagocytes or other cells that harbour microbial pathogens [333]. Furthermore, exogenously administered IFNγ has been found to act prophylactically against a variety of experimental virus infections, such as CMV infection in mice [334] or rats [335]. However, in the case of HIV, the activation of monocytoid cells by IFNγ was found to stimulate rather than inhibit virus replication [336,337]. Furthermore, IFNγ is well known to potentiate the respiratory burst responsiveness of macrophages to stimulants, resulting in an increased production of highly reactive oxidants, such as H_2O_2 [338] and the superoxide anion [309], as well as nitric oxide (NO) [339]. The production of NO again is associated with an augmented defense against bacterial infection [150,340], enhanced anti-viral effects [341] and the killing of tumor cells. Nevertheless, macrophages activated by IFNγ have been found to have a reduced ability to ingest a variety of obligate intracellular microorganisms, e.g. *Rickettsiae, Trypanosoma cruzi* and *Leishmania amastigotes* [342]. Furthermore, significant side effects of NO may also cause undesirable cell and tissue damage.

Another well documented action of IFNγ is the induction of MHC I and II on antigen presenting cells (APC), responsible for the recognition of viral, bacterial, tumor, transplant or auto-antigens on foreign cells, which are the preferred target for cytotoxic T-cells. Thus it was suggested that IFNγ is

crucial to allograft rejection [343]. And indeed, treatment of skin allograft recipients with anti-IFNγ has been found to delay rejection of the graft [344].

1.4.2.4 Clinical significance

The application of IFNγ is controversially discussed. IFNγ was most often safe and well-tolerated, but sometimes induced severe toxicity. Furthermore, it has been reported to augment the anti-tumor effects of TNF in animal tumor models by initializing complete necrosis of tumor tissue [345]. Therapy of autologous bone marrow transplantation [346], human pleural adenocarcinoma [347], ovarian cancer [348,349], colon carcinoma [350], human myelogenous leukemia [351], multiple myeloma [352], but also atopic dermatitis [353-355], furunculosis in HIV [356], visceral leishmaniosis [357,358] and *Borrelia burgdorferi* infection [359] with IFNγ has been shown to be safe and effective. First clinical studies were performed by Boehringer Ingelheim with *rhu*IFNγ (Imukin®). In case of granulomatosis, Imukin was reported to significantly reduce the risk for severe infections from 70 % (placebo group) to 23 % (Imukin group) in a dose range of 1,5 to 50 µg/kg. No toxicity, teratogenicity or side effects were found in such clinical studies [360]. Furthermore, like GM-CSF, IFNγ is tested in current clinical approaches of gene therapy studies to design more selective and effective anti-cancer drugs by introducing cytokine genes into tumor cells [361,362]. However, IFNγ was reported to play an enhancing role in ischemia-reperfusion [363] and to hasten the progress of HIV infection [364].

In sum, cytokines such as GM-CSF and IFNγ are demonstrated to modulate the function of monocytes and have been used to experimentally probe the immunotherapeutic potential of monocytes against microorganisms and malignancy. However, monocytes rarely act alone but communicate with other leukocytes involved in cell-mediated immunity. In particular, monocytes cooperate with T-helper (Th1 and Th2) sub-populations of peripheral lymphocytes. Preclinical studies in humans suggest that GM-CSF and IFNγ are the most promising biological response modifiers for augmenting monocyte-mediated immunity [307].

1.5 Experimental animal models of macrophage- and T-cell dependent inflammation

This section was initiated with the aim to describe the different animal models applied in the present work. Experimental animal models are the necessary basis for such preclinical research programs,

although extrapolation of animal studies to the clinical situation is difficult. Due to the easy handling and low costs of purchase and keeping, rodents were selected for larger study scales. In the following section, the experimental murine models, by which the effects of stimulative cytokines on different cells of the immune system were studied are described.

1.5.1 Endotoxic shock as a model for "sterile" infection

The most common animal model used to study the mechanisms following an overactivation of the immune system is the endotoxic shock model. However, the significance of endotoxin (lipopolysaccharide; LPS) in the induction of sepsis is not yet clearly defined [365]. One mechanism how LPS could be part of the induction of sepsis might be the leakage of bacteria from the gut under shock conditions [366]. In experimental animal models, sepsis can be reproduced by an injection of LPS, a component of the outer cell membrane of Gram-negative bacteria. Mice, challenged with a lethal dose of endotoxin, die within 72 hours due to cytokine-induced multi-organ failure. The major mechanism by which the immune system reacts towards a direct stimulation by LPS, is the release of endogenously produced pro-inflammatory cytokines, like TNF, IL-1, IL-6, IL-8 and others by macrophages [367-370] or of IFNγ by activated T-cells [371]. There is evidence, that those pro-inflammatory cytokines play the central role in the pathogenesis of shock. Mice, deficient in IL-1 [372] or TNF [373,374] are resistant against endotoxic shock. In contrast, IFNγ contributes to the aggravation of a septic insult as it increases the sensitivity of macrophages stimulated with LPS [375,376]. Nevertheless, animals deficient in the IFNγ receptor are more susceptible towards infection with *Listeria monocytogenes* [377] or *Mycobacterium bovis* [332], indicating an important role of IFNγ in host defense.

The experimental LPS shock, induced by a single bolus injection of purified LPS in naive mice, closely resembles the clinical appearance of human endotoxic shock as it can appear after transplantation under pharmacological immunosuppression. Since an inflammatory response of especially macrophages is evoked in this particular model it was selected to examine the possibilities of GM-CSF and IFNγ to reactivate the pharmacologically suppressed macrophage activity in transplanted and non-transplanted animals.

1.5.2 T-cell activation by Concanavalin A (ConA)

Concanavalin A, a protein of the jack bean *Canavalis ensiformis*, was first purified in 1919. Since the 1960s, this plant lectin and mitogen became a very important tool in immunology, but its toxicol-

ogy, cytopathology and signal transduction pathway have not been characterized properly yet. ConA induces the secretion of several pro-inflammatory cytokines, e.g. TNF [378], IL-2 [379,380], and IFN γ [309]. Like in the LPS shock model described above, pro-inflammatory cytokines play the central role in the pathogenesis of disease. While the injection of LPS preferentially stimulates macrophages, the overactivation of T-cells occurs here. Secreted pro-inflammatory cytokines induce a hepatitis-like syndrome, and ConA treated mice die within 8 hours from fulminant liver failure, combined with severe hematological changes [381]. By interfering in the signalling cascade, immunosuppressive agents protected mice from cytokine-related lethality [381]. Like for macrophages, it was tested whether or not a pharmacologically suppressed T-cell response in transplanted and non-transplanted animals can be reconstituted by the pro-inflammatory cytokines GM-CSF or IFNγ in the ConA model.

1.5.3 The infection with *Salmonella typhimurium*

The use of immunosuppressive drugs during transplantation has markedly increased the acceptance of different grafts and the survival rates of patients. However, opportunistic infections with various microorganisms are an immense problem [382-384]. In this context, bacterial and fungal infections are a major threat to immunocomprised patients after transplantation. Because infection with strains of *Salmonella* is a problem in immunocompromised patients [385] the effects of immunosuppressive treatment were tested in an infection model with *Salmonella typhimurium*. The authors of this study showed an aggravation of murine salmonellosis in immunosuppressed mice, most likely by an inhibition of the T-cell function [386]. After parenteral infection, most of the salmonellae were phagocytosed and killed [387]. The remaining bacteria multiplied mostly in the liver and spleen, resulting in an ongoing infection due to an increasing bacterial load in these organs. Replication took place extracellularly and, probably, also within professional phagocytes [387,388]. Several investigators reported that the pro-inflammatory cytokines GM-CSF [265,389] and IFNγ [390,391] played a key role in the resolution of *Salmonella* infection. However, Leshem *et al.* have not found such beneficial effects of GM-CSF [392] in bone marrow transplanted mice, but suggested to treat immunocompromised patients with IL-1. Others reported a synergistic effect of IL-1 and GM-CSF in the treatment of *Salmonella typhimurium* infection, resulting in a significant augmentation of survival [393] and new reports give rise to a beneficial treatment of *Salmonella* infection with IL-18 [394]. An infection model with *Sal-*

monella typhimurium was selected for the present work to determine the effects of the cytokines GM-CSF and IFNγ on the pharmacologically suppressed immune system in transplanted and non-transplanted animals. These experiments were expected to yield proof-of-principle whether our hypothesis was correct or not.

1.6 Aims of this study

The use of immunosuppressive drugs in transplantation remains a double-edged sword, because, even though these drugs effectively reduce the incidence of graft rejection, inactivation of the immune system also renders patients prone to infectious disease. Hence, viral, bacterial and fungal infections are the prime cause of death in the first few months after surgery. Consequently, the therapeutic goal to be reached for treatment of transplant patients in case of infection is defined as a short-term reactivation of the immune system by pharmacological intervention, thus fortifying the defense against infectious disease without hazard to the graft. Two recombinant drugs, GM-CSF and IFNγ, are at hand with a promising pharmacological impact.

Thus the present study was initiated with the aim to investigate the capability of GM-CSF and IFNγ to modulate the immune response under immunosuppression. In detail, the aims of this study were the following ones:

1. To investigate the immunomodulatory potency of GM-CSF and IFNγ on naive and immunosuppressed monocytes/macrophages in the LPS shock model *in vivo* and *ex vivo*.
2. To examine the immunomodulatory effect of GM-CSF and IFNγ on naive and immunosuppressed T-cells in the ConA model *in vivo* and *ex vivo*.
3. To determine whether GM-CSF and IFNγ enable successful combat of *Salmonella typhimurium* in immunosuppressed CBA/Ca mice.
4. To clarify whether GM-CSF and IFNγ negatively affect the outcome of graft acceptance.
5. To determine whether GM-CSF and IFNγ allow successful handling of bacterial infection by *Salmonella typhimurium* in immunosuppressed and transplanted CBA/Ca mice.
6. To study mechanisms responsible for the reconstitution of the immune system.
7. To give prospects for the applicability of GM-CSF and IFNγ in clinical use in humans.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals applied parentally to animals

Yeast-derived *rmu* GM-CSF (1 x 10^7 U/ml) was kindly provided by Dr. F. R. Seiler (Behring-Werke, Marburg, Germany) and *rmu* IFNγ was a generous gift from Dr. G. R. Adolf (Bender & Co, Vienna, Austria). The endotoxin content of both, GM-CSF and IFNγ was less than 1 Endotoxin Units / mg protein according to the supplier's information. *Salmonella abortus equi* endotoxin was purchased from Metalon (Königswusterhausen, Germany). Concanavalin A (ConA) was obtained from Sigma (Deisenhofen, Germany). *Salmonella typhimurium* strain nr. 15277 was from ATCC (Manassas, VA, USA). Dexamethasone (Dexa-Allvoran®) was provided by TAD Pharmazeutisches Werk (Cuxhaven, Germany), Cyclosporine A (Sandimmune®) by Novartis Pharma GmbH (Nürnberg, Germany), tacrolimus (Prograf®) by Fujisawa München (Germany), and mycophenolate mofetil (CellCept®) by Roche Reinach (Switzerland). Sirolimus (SDZ-RAD) was a generous gift from Dr. W. Schuler (Novartis Pharma, Basel, Switzerland). All reagents were diluted in pyrogen-free saline (Braun, Melsungen, Germany). The anaesthetics Chloralhydrat and Enfluoran were supplied by Sigma (Deisenhofen, Germany) and Abbott Chemicals München (Germany), respectively. Pentobarbital (Nembutal® or Narcoren®) was purchased from Sanofi Withrop (München, Germany).

2.1.2 Cell culture material

Cell culture plates (96-well) and petri-dishes were purchased from Greiner (Nürtingen, Germany). Cell culture medium RPMI 1640 with 2 mM L-glutamine, FCS and penicillin/streptomycin were from Biochrom (Berlin, Germany).

2.1.3 Other reagents

Tetramethylbenzidine (TMB), NADH, NADPH and other enzymes were obtained from Boehringer Mannheim (Mannheim, Germany). Ovine anti-mouse TNF antisera was prepared in our laboratory as described [395]. Unless further specified, all other reagents were purchased from Sigma (Deisenhofen, Germany).

2.2 Animals

Eight to twelve week old male Balb/c mice were obtained from the animal house of the University of Konstanz (Konstanz, Germany). For the bacterial infection experiments, specific *Salmonella*-resistant CBA/Ca mice were purchased from Harlan (Austerlitz, Netherlands) and then bred from the animal house of the University of Konstanz. Animals were housed at a constant day/night cycle of 12 h at 22°C and 55% relative humidity in a 12 h day/night rhythm with free access to food (Altromin 1313, Altromin Co., Lage, Germany) and water. All animals received humane care according to the EU (European council directive 86/609/EEC) and the national German regulations, and the directions of the University of Konstanz ethical committee were followed.

2.3 Animal experiments

2.3.1 Skin Transplantation

2.3.1.1 Donor operation

After breaking the donors' neck, the animal was attached on a plate using an adhesive tape. The dorsal skin of the tail was cut at its base and at its middle part, respectively, and afterwards incised lengthwise deep to cartilage. Tail skin then could be removed from the cartilage using a pair of forceps. The graft, stored in PBS (4°C) was immediately excised and cleaned of remaining fat and tissue under the microscope. Skin pieces for transplantation had a size of approximately 1 x 1 cm.

2.3.1.2 Recipient operation

The recipient was anaesthetized by an injection of a 3,6 % chloralhydrate solution (Sigma, Deisenhofen, Germany) that achieved an aesthetic effect lasting for 30 to 60 min. After generously shaving the neck of the recipient the graft bed was disinfected with an iodide solution. The graft bed was prepared by carefully removing a piece of native skin that was slightly bigger than the graft without damaging the transparent panniculus carnosus containing vessels that reestablish blood supply to the graft. By suturing the graft with 8 – 12 single stitches of a 5-0 filament (Heiland, Hamburg, Germany) it was adjusted to the recipient skin and again disinfected with the iodide solution. A vaseline gauze was put on the graft, followed by a soft cotton gauze which was then covered with a Band-Aid. To allow unrestricted motion, wholes for both forepaws were cut in the cotton gauze. The bandage had to be attended tight enough to remain in place but free enough to allow the animal to eat and breathe. On the seventh postoperative day the bandage and the sutures were removed under ether-

anaesthesia. Feasible rejection of the graft was assessed from day 7 onwards by morphological changes. The time point of rejection was defined as complete necrosis of the graft.

Bacterial infection in transplanted CBA/Ca mice was induced on day 7 and survival was monitored over a period of up to 4 weeks. Grafts and surrounding native skin of some transplanted mice were photographed regularly, using the macro lens of a Casio QV8000SX camera, and several probes were withdrawn for histological examination.

2.3.2 Treatment schedules

Animal experiments with both, transplanted and non-transplanted mice were started between 6 and 9 a.m.. All substances and bacteria were given i.v. or i.p. in a total volume of 300 µl per 30 g mouse.

2.3.2.1 LPS shock in non-transplanted animals

LPS was injected i.p. in a dose of 5 mg/kg in male Balb/c mice. The immunosuppressive agents Dex (5 µg/kg; i.p.; -6h), CsA (5 µg/kg; i.v.; -4 h), tacrolimus (dose range from 0 to 50 mg/kg, i.v.; -1h), sirolimus (dose range from 0 to 500µg/kg; i.v.; -1h) and MMF (dose range from 0 to 10 mg/kg; i.v.;-1h) were given at the time points indicated. GM-CSF or IFNγ (both 50 µg/kg; i.v.) were given 45 min before the challenge and survival was monitored for 72 h after the LPS challenge. Blood for determination of plasma TNF was obtained 90 min after the LPS challenge from the tail vein, samples were immediately centrifuged for 10 min (4°C) at 370 x g and supernatants were stored at - 80°C until ELISA measurements.

2.3.2.2 ConA-induced liver injury in non-transplanted animals

Liver injury was induced by an i.v. injection of ConA (25 mg/kg) according to Tiegs *et al*. [381] in male Balb/c mice, that were fasted over night. In the ConA experiments, Dex (1 mg/kg; i.p.), CSA (1 mg/kg; i.v.), tacrolimus (1mg/kg; i.v.) and sirolimus (1 mg/kg; i.v.) were given 1 h before the challenge. GM-CSF or IFNγ (each 50 µg/kg; i.v.) were given 45 min before the challenge. All mice were sacrificed by lethal anesthesia 8 h after the inflammatory stimulus.

Blood for determination of plasma TNF and IL-2 was obtained 90 or 240 min after the LPS challenge from the tail vein, or after 8 h for determination of IFNγ, respectively. Samples were immediately centrifuged for 10 min (4°C) at 370 x g and supernatants were stored at - 80°C until ELISA measurements.

2.3.2.3 Bacterial infection in non-transplanted animals

In the bacterial infection model male and female *Salmonella*-resistant CBA/Ca mice were used. Immunosuppression here was induced by Dex (1 mg/kg; i.p.) or CsA (1 mg/kg; i.p.) 2 days before the injection of *Salmonella typhimurium* (5 x 10^5 /kg; i.p.). Both, GM-CSF and IFNγ (each 50 µg/kg; i.v.) were given once on day 2 after infection (Dex treatment) or daily from day 2 until day 4 (CsA treatment). To examine the spreading of bacteria, some mice were sacrificed by lethal anaesthesia at different time points, otherwise survival was monitored over a period of 3 weeks after the infection. For determination of colony forming units (CFU), blood samples were obtained from the tail vein on day 7 after infection, diluted and immediately spread on blood agar plates.

2.3.2.4 Bacterial infection in transplanted animals

For transplantation experiments male and female *Salmonella*-resistant CBA/Ca mice were used. Continuous immunosuppression was induced by a daily injection of CsA (30 mg/kg; i.p.) or tacrolimus (1 mg/kg; i.p.) plus Dex or MMF (each 10 mg/kg; i.p.). After assessment of successful skin graft acceptance on day 7, mice were injected with *Salmonella typhimurium* (5 x 10^5 /kg; i.p.). While immunosuppression was continued daily, infected mice were treated for 4 consecutive days with either GM-CSF or IFNγ (each 50 µg/kg; i.p.) from day 7 to day 10 to reconstitute immune functions. Survival of transplanted mice was monitored for 4 weeks. To examine the spreading of bacteria in infected mice blood was withdrawn from the tail vein processed and spread on agar plates as indicated in chapter 2.7.

2.3.2.5 Sampling

For determination of TNF and IL-2 plasma levels, blood, withdrawn from the tail vein 90 or 240 min after administration of LPS or ConA, was collected in heparinized Eppendorf-cups (Eppendorf, Hamburg, Germany), immediately centrifuged (5 min, 13.000 x g, 4°C) and stored at – 80°C. All other blood samples (IFNγ, transaminases) were obtained after lethal anaesthesia of mice with 100 µl pentobarbital (45 mg/ml in saline) containing 5 mg/ml heparin. After midline laparotomy and opening of the chest, blood was withdrawn by cardiac puncture and immediately centrifuged for 5 min at 13.000 x g at 4°C to obtain the plasma.

Parts of the graft and surrounding skin were gathered from transplanted mice after breaking the animals neck. Tissue samples were immediately immersed in a 4 % buffered formalin solution as a fixation for histological studies.

2.4 *Ex vivo* experiments

2.4.1 Stimulation with LPS

Immunosuppression was induced by injection of Dex (5 µg/kg) or CsA (5 µg/kg) 2 h before anaesthesia. Afterwards, various monocyte/macrophage containing cell populations from peritoneum, lung, blood, spleen and bone marrow were isolated from naive or immunosuppressed Balb/c mice according to Bundschuh *et al.* [251]. After washing, diluting in RPMI 1640 medium containing 10 % FCS and 5 % penicilline/streptomycin, plated cells or blood samples were pre-incubated with GM-CSF or IFNγ (10 ng/ml) for 2 h before stimulation with LPS (1 µg/ml). After incubation for 6 h at 37°C, 5 % CO_2 and 20 % O_2, cells and blood were centrifuged for 10 min at 370 x g and 4°C, and supernatants were stored at - 80°C for cytokine determination by ELISA.

2.4.2 Stimulation with ConA

In the ConA experiments, spleen cells were obtained by spreading freshly isolated tissue from anaesthetized, naive mice through a cell strainer (100 µm, Falcon, Becton Dickinson, Heidelberg, Germany) into 5 ml medium (4°C). Cells were centrifuged at 400 x g for 10 min at 4 °C, resuspended in ammonium chloride solution (0.17 M NH_4Cl, 200 mM Tris/HCl; pH 7.2) and incubated at 37°C, 5% CO_2 and 20 % O_2 for 12 min to lyse erythrocytes. After washing twice in PBS, cells were plated in microtiter plates and pre-incubated with Dex, CsA, tacrolimus or sirolimus (100 ng/ml) for 90 min, and GM-CSF or IFNγ (10 ng/ml) for 45 min. Cells were incubated with ConA (5 µg/ml) for 48 h, centrifuged for 10 min at 400 x g and supernatants were stored at - 80 °C until cytokine detection by ELISA.

2.5 Enzyme, cytokine and nitrite/nitrate determination

2.5.1 Determination of enzymes

The extent of liver injury, induced by ConA, was assessed by the determination of plasma enzyme activity of alanine aminotransferase (ALT) according to Bergmeyer [396], using an Eppendorf ACP 5040 enzyme analyzer. Data are expressed in U/l plasma.

2.5.2 Determination of cytokines by ELISA

Various murine cytokines in plasma or supernatants were determined by ELISA (enzyme-linked immunosorbent assay). Specific anti-mouse antibody-pairs (biotinylated detection antibodies) were

used. All ELISAs were performed in polystyrene microtiter plates (Greiner, Nürtingen, Germany). For detection of TNF, a protein G+ purified polyclonal ovine anti-mouse TNF capture antibody (protein content 20 mg/ml, in-house preparation) and a biotyinylated rat anti-mouse secondary antibody (Endogen, USA) were used. IL-2 (R&D, Wiesbaden, Germany) and IFNγ (Pharmingen, Hamburg, Germany) were determined by specific anti-mouse monoclonal antibody pairs (biotinylated detecting mAb). Streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA, USA) and the peroxidase chromogen tetramethylbenzidine (TMB, Boehringer Mannheim, Mannheim, Germany) were used to detect the immunocomplex with an ELISA reader (SLT, Crailsheim, Germany). The detection limits of the assays were 10 pg/ml for TNF, 10 pg/ml for IL-2 and 50 pg/ml for IFNγ, respectively.

2.5.3 Determination of nitrite/nitrate

For the determination of nitrite release, cells were incubated for 20 h in RPMI 1640 medium, containing 10 % FCS and 1 % penicilline/streptomycin. Culture supernatants were assayed for nitrite by the Griess reaction. Briefly, 200 µl culture supernatant were transformed to 96-well microtiter plates, 20 µl N-(1-naphthyl) ethylendiamine (0.1 % in H_20) were added and absorbance was read after 3 min incubation time at 560/690 nm on an ELISA reader. The nitrite content in culture increased linearly within the time span of 20 h investigated.

2.5.4 Determination of reactive oxygen species

To determine the amount of generated peroxynitrite, the oxidation of luminol (Sigma, Deisenhofen, Germany) in whole blood was measured. Unless otherwise indicated, blood, diluted 1:1.25 in RPMI 1640 medium containing 10 % FCS, was pretreated in 96-well plates over night with 10 ng/ml GM-CSF or IFNγ, respectively. Luminol was provided short time before the addition of heat-inactivated *Salmonella typhimurium*. Afterwards, the chemiluminiscence over a period of 30 min was measured in a chemiluminator (Wallac, Turku, Finland, Germany).

2.6 Histological examinations

To investigate the morphological changes, deriving from rejection, the graft and surrounding skin from recipient mice was gathered and stored in 4 % formalin solution. After embedding in paraplast, 2 µm slices were cut and stained with hematoxylin and eosin. As parameters for histopathological

evaluation, the amount of necrosis, anomalies in the lamination of skin strati and the extent of regenerated vessels were assessed.

2.7 Determination of aerobic colony froming units (CFU)

After weighing, organs were passed through 100 µm nylon cell strainers (Falcon, Becton Dickinson, Heidelberg, Germany) with PBS and 100µl of the organ suspension or whole blood were spread on Columbia blood agar plates (Heipha, Biotest, Heidelberg, Germany). Aerobic CFU were counted after overnight incubation at 37°C and CFU per g organ or per 100 µl blood were calculated.

2.8 Statistical analysis

Data in all tables and figures are given as mean values +/- SD or SEM. Survival curves were created by using the method of Kaplan and Meier (GraphPad Prism, GraphPad Software Inc., San Diego, USA). For statistical comparison survival curves were analyzed using the *Logrank* test. Other data were analysed by using the one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. $P < 0.05$ was considered significant.

3. Results

This work is the continuation of a thesis written in our group by Daniela Bundschuh [397], dealing with immunomodulation in models of the systemic inflammatory response syndrome in LPS tolerant mice. There, a special immune status, i.e. LPS-tolerance, was induced in mice by pretreating animals with low-dose LPS 24 h before a high-dose LPS challenge. While immune cells, and specifically macrophages, of naive mice respond on the injection of high-dose LPS with a marked release of cytokines, resulting in the death of the animals, immune cells of tolerant mice were shown to be immunoparalysed. That is, no increased cytokine levels and no elevated mortality was detected in tolerant mice. However, when pretreated with the pro-inflammatory cytokines GM-CSF or IFNγ, paralysed macrophages were reactivated and responded to stimulation with LPS with a release of several pro-inflammatory cytokines, resembling the pattern in naive mice [251]. Nevertheless, tolerance induction with this treatment regimen failed to influence the course of bacterial infection. Although GM-CSF and IFNγ increased TNF levels after fecal peritoneal infection in LPS tolerant mice, spreading of bacteria was unhindered and resulted in the death of the animals.

In the work presented here, the method of reactivating immunoparalysed macrophages was applied to investigate whether a pharmacologically suppressed immune system, as it occurs in transplantation, can be reactivated by the two cytokines GM-CSF and IFNγ. Macrophages and/or neutrophils, the effectors of the innate immune response, are the primary target population of such an immune defense fortification while suppression of T-cell functions, responsible for graft rejection, need to be maintained.

3.1 Determination of the lowest effective doses of immunosuppressants

The doses of immunosuppressants used in the clinic are very high to assure complete inactivation of the immune system. To investigate whether GM-CSF and IFNγ can in principle reactivate pharmacologically suppressed immune cells, the lowest effective doses of the different immunosuppressants used in this thesis were determined by their ability to prevent a lethal inflammatory response after stimulation with endotoxin (LPS) in a murine model.

In table 1, the mean survival times of animals given different doses of immunosuppressants before lethal LPS challenge are given as a readout for an effective immunosuppression over a period of 72 h.

Table 1: Dose-dependent immunosuppression of different immunosuppressive drugs *in vivo*

	Mean Survial [h] ± SD				
Drug ***Dosage [µg/kg]***	***Dex***	***CsA***	***tacrolimus***	***sirolimus***	***MMF***
1000	n.d.	n.d.	38 + 9 *	n.d.	26 + 5 $^{n.s.}$
500	72 ± 0 ***	72 ± 0 ***	n.d.	72 ± 0 ***	n.d.
100	72 ± 0 ***	72 ± 0 ***	47 ± 3 ***	n.d.	48 ± 3 ***
50	72 ± 0 ***	72 ± 0 ***	42 ± 7 ***	52 ± 5 ***	n.d.
10	n.d.	72 ± 0 ***	34 ± 10 $^{n.s.}$	n.d.	42 ± 5 ***
5	72 ± 0 ***	72 ± 0 ***	n.d.	39 ± 12 *	n.d.
0.5	56 ± 10 ***	62 ± 9 ***	n.d.	n.d.	n.d.
0	28 ± 5	30 ± 6	27 ± 3	22 ± 8	25 ± 10

Mice were pretreated with the indicated doses of immunosuppressive drugs 1 h before the LPS challenge (5 mg/kg). Survival over 72 h was monitored. Data are from 1 (tacrolimus, sirolimus, MMF) or 2 (Dex, CsA) independent experiments with n = 3 animals per group and given in means of survival [h] ± SD; n.d. not determinated. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ *vs*. LPS treated controls without immunosuppression. Data were analyzed with the *Logrank* test. $p < 0.05$ was considered significant.

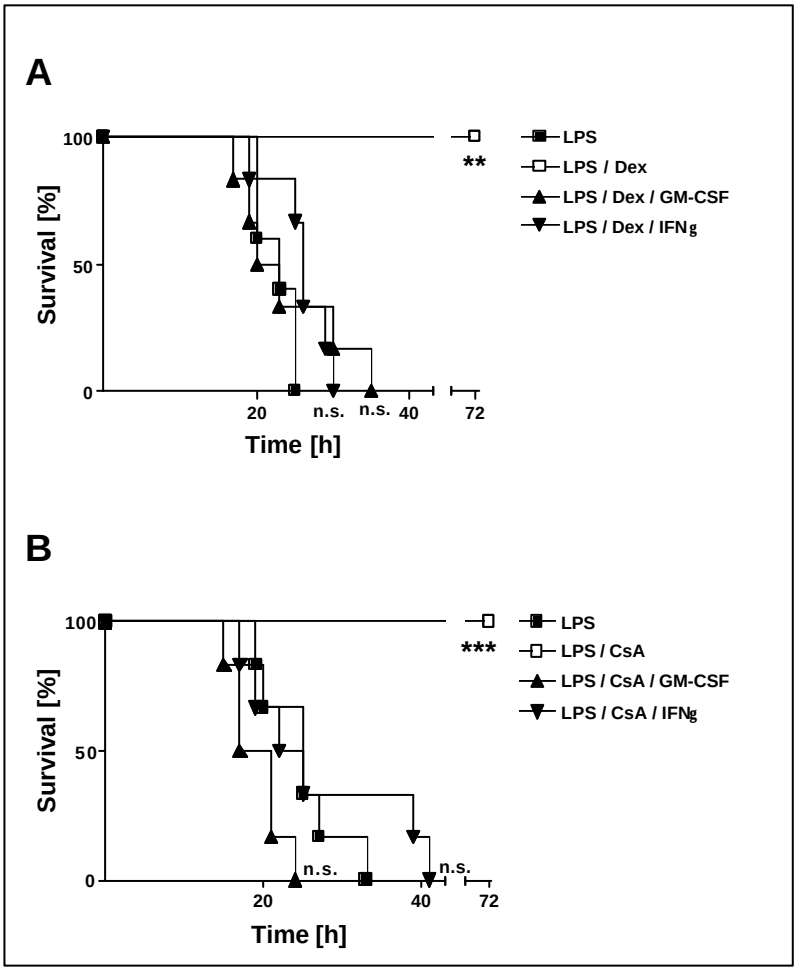
While Dex and CsA showed an effective immunosuppression already in the range of 5 µg/kg body weight, sirolimus was acting in dosages ≥ 500 µg/kg. Tacrolimus and MMF completely failed to protect against endotoxic shock. High doses of these substances had toxic effects, as animals receiving 1 mg/kg of either tacrolimus or MMF died sooner than did mice treated with lower doses. Furthermore, all animals with an elevated serum level of TNF died within the observation period of 72 h. In line with these findings, successfully immunosuppressed mice showed no increased serum TNF levels 90 min after the LPS challenge. Consequently, doses of 5 µg/kg body weight of Dex and CsA were used in further experimental settings in the endotoxic shock model.

3.2 Immune reconstitution of macrophages with GM-CSF or IFNg after pharmacological suppression

3.2.1 The influence of GM-CSF and IFNg pretreatment on LPS-induced plasma TNF-levels and mortality in immunosuppressed mice *in vivo*

Following the dose-finding experiments, it was examined, whether GM-CSF and IFNγ could reactivate the immune cell function after pharmacological suppression, as was the case for tolerant mice [251]. Mice immunosuppressed with 5 µg/kg Dex or CsA were treated with 50 µg/kg of either GM-CSF or IFNγ 45 min before injection of a lethal dose of LPS (5 mg/kg).

Figure 4: GM-CSF and IFNg increase mortality in Dex- and CsA treated mice

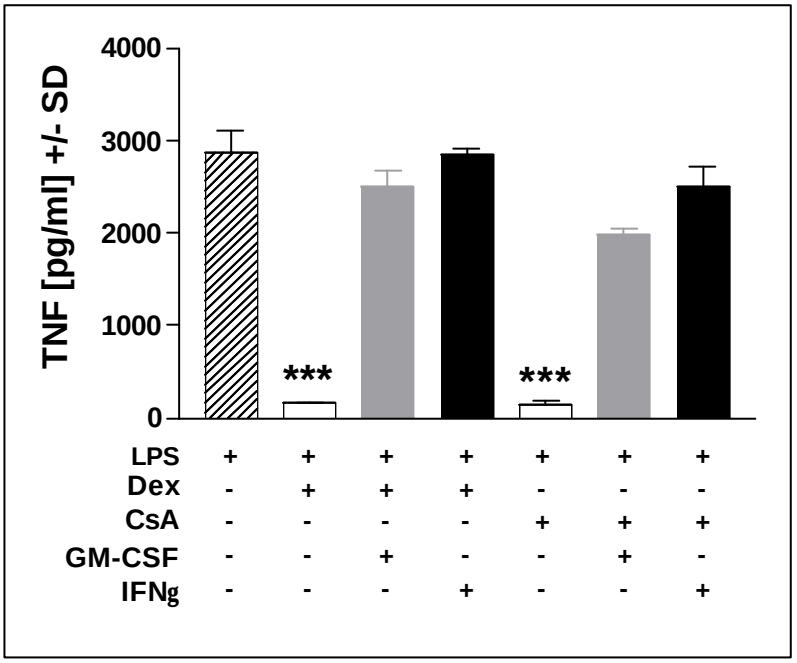


Six mice per group were immunosuppressed with **(A)** Dex (5 µg/kg; i.p.) 6 h or **(B)** CsA (5 µg/kg; i.v.) 4 h before LPS. Where indicated GM-CSF or IFNγ (both 50 µg/kg; i.v.) were given 45 min prior to the LPS challenge (5 mg/kg; i.p.). Survival over 72 h was compared between naive (■), immunosuppressed (□) and GM-CSF- (▲) or IFNγ- (▼) treated animals. Survival curves were analyzed using the *Logrank* test; *** $p<0.001$, ** $p<0.002$, n.s. not significant *vs*. LPS. $p<0.05$ was considered significant.

As indicated in fig. 4, mice with a pharmacologically suppressed immune system were not susceptible towards endotoxic shock. All animals in the Dex- and CsA-treated groups survived the period of 72 h without any symptoms of sepsis or septic shock.

Naive mice were not protected from endotoxic shock, i.e. all animals died within 30 h after the LPS challenge. Pretreatment with both GM-CSF and IFNγ restored susceptibility towards endotoxic shock in immunosuppressed animals. Such pretreatment led to the death of all the animals in both groups. Interestingly, death in the groups pretreated with GM-CSF and IFNγ occured nearly simultaneously. Mice pretreated with GM-CSF died after 24 ± 7 h (Dex) or 19 ± 3 h (CsA), whereas IFNγ pretreatment induced death after 26 ± 4 h (Dex) and 27 ± 10 h (CsA). Life and death in the different groups correlated with low and high levels of TNF in the animals' plasma (fig. 5):

Figure 5: GM-CSF and IFNg restore the pharmacologically suppressed TNF-release



Six mice per group were immunosuppressed with Dex (5 µg/kg; i.p.) 6 h, or CsA (5 µg/kg; i.v.) 4 h before LPS. GM-CSF or IFNγ (both 50 µg/kg; i.v.) were given 45 min prior to the LPS challenge (5 mg/kg; i.p.). 90 min after the LPS injection, blood samples for TNF determination by ELISA were obtained from the tail vein. Data are means ± SD from two independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey multiple test. *** $p<0.001$ *vs*. LPS. $p < 0.05$ was considered significant.

Dex and CsA entirely inhibited a TNF release by macrophages after LPS stimulation. In contrast, immunosuppressed mice, pretreated with GM-CSF or IFNγ displayed significantly increased plasma TNF levels, indicating a reconstitution of the macrophage activity. Cytokine release here was comparable to that observed in naive animals after LPS stimulation. GM-CSF and IFNγ alone did not induce the release of pro-inflammatory cytokines from unstimulated macrophages (data not shown).

Interestingly, IFNγ was slightly more potent than GM-CSF with respect to the reconstitution of TNF release. These findings are in line with those of Bundschuh *et al.* [397]. There, IFNγ also was reported to be more potent than GM-CSF in restoring the LPS-induced death of LPS-tolerant mice.
The results in this section demonstrate that GM-CSF and IFNγ exert their immunostimulative activities *in vivo* not only in naive and tolerant mice, but also after pharmacological suppression with Dex or CsA.

3.2.2 The effects of GM-CSF and IFNg on macrophages derived from immunosuppressed mice *ex vivo*

In this section, the *in vitro* capabilities of the cytokines GM-CSF and IFNγ to modulate the TNF response in murine *ex vivo* cell systems were examined. Previous studies in our laboratory showed that different cultures of freshly prepared murine macrophage/monocyte-containing cell populations from naive and tolerant mice are suitable models for testing the modulatory effects of GM-CSF or IFNγ [253,397]. Since it was reported that both cytokines have the capacity to restore TNF release of cells from LPS-tolerant mice, we wondered whether this also occurs in pharmacologically suppressed immune cells.

3.2.2.1 Modulation of the TNF release by GM-CSF or IFNg in cells from naive mice

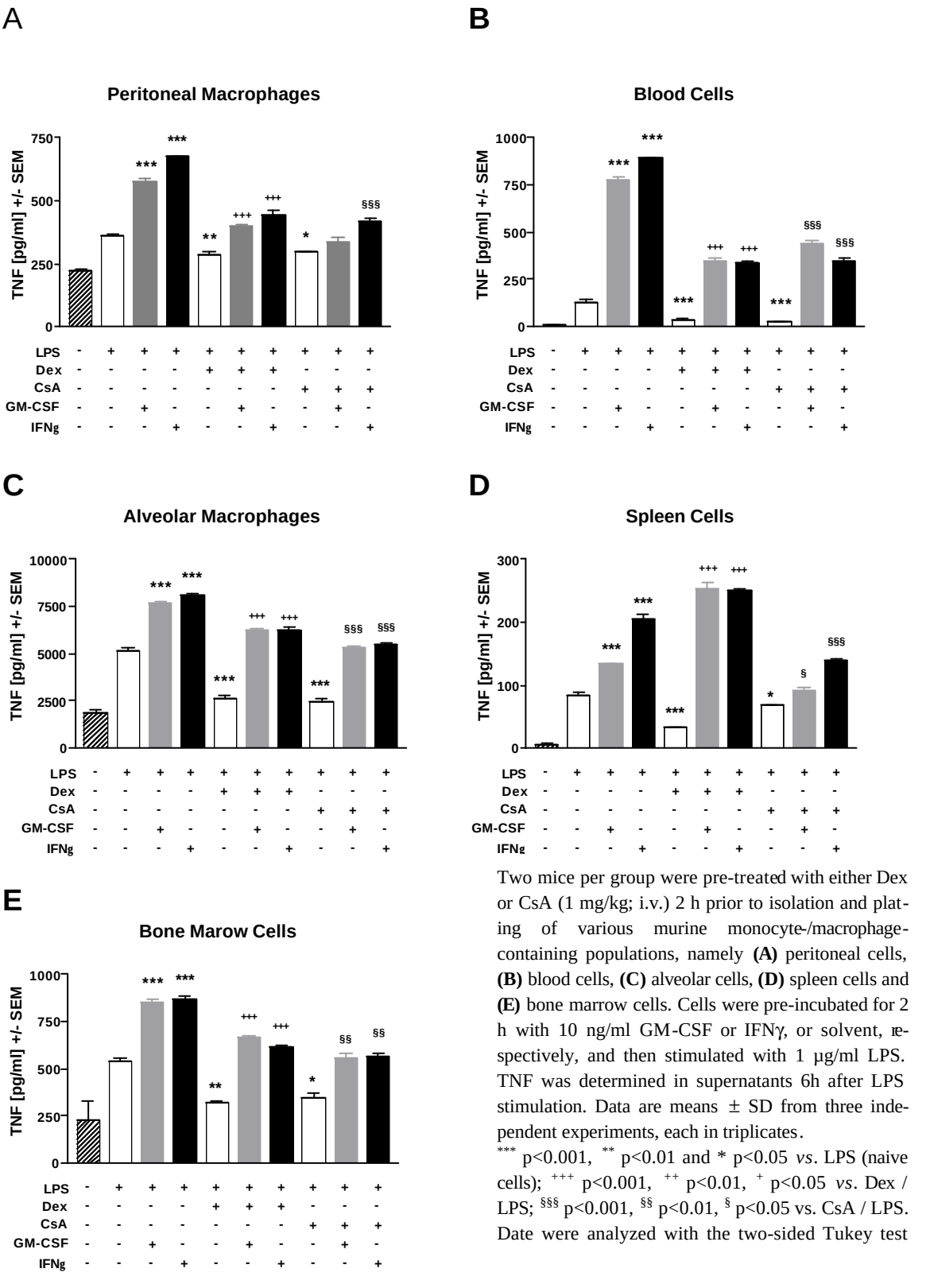
As described by Bundschuh *et al.*, different isolated cell populations were pre-incubated with 10 ng/ml of GM-CSF or IFNγ 2 h before the challenge with 1 µg/ml LPS. As shown in fig. 6, pre-incubation with GM-CSF or IFNγ induced a highly significant increase in the TNF release in response to LPS of all cell populations isolated from naive mice. In contrast to Bundschuh *et al.*, neither GM-CSF, nor IFNγ had drastic effects on specific cell populations. However, the LPS-induced TNF release in samples pre-incubated with IFNγ was more enhanced on average by a factor of 6.8 in blood cells (fig. 6B), and 2.5 in spleen cells (fig. 6D), whereas the rate of increase for GM-CSF was only 5.9 in blood cells (fig. 6B), and 1.6 in spleen cells (fig. 6D). Notably, these two cell populations consist of macrophages as well as T-cells. In contrast, both cytokines were equally potent in increasing the TNF release from peritoneal cells (fig. 6A), alveolar cells (fig. 6C) and bone marrow cells (fig. 6E) which consist of predominantly macrophages/monocytes.
Neither GM-CSF nor IFNγ enhanced the basal TNF release by any murine naive cell population studied.

3.2.2.2 Modulation of TNF release by GM-CSF or IFNg in cells from immunosuppressed mice

Since immunosuppressed mice, treated with Dex or CsA showed a diminished cytokine response to LPS compared to naive mice *in vivo*, we next investigated whether GM-CSF and IFNγ could modulate a pharmacologically suppressed immune response in the different murine monocyte/macrophage-containing populations.

We therefore treated mice with 5 µg/kg Dex or CsA 2 h before lethal anaesthesia and extraction of the different cell populations. Different cell populations were pre-incubated with 10 ng/ml of either GM-CSF or IFNγ 2 h before challenge with 1 µg/ml LPS. The results in fig. 6 show that the TNF release of all cell populations was decreased significantly by both immunosuppressive drugs used. The immunosuppressive potency of both agents was comparable. On average, the suppressive effect was in a range of approximately 60 % for peritoneal, blood, alveolar and bone marrow cells (fig. 6A, 6B, 6C, 6E). Interestingly, Dex was more potent in suppressing the pro-inflammatory response of spleen cells than was CsA (fig. 6D). This observation might be explained by the fact that the spleen cell solution contains a high percentage of T-cells, which are the preferred target for the immunomodulatory action of CsA. However, by decreasing the TNF release of macrophages by 38 %, the suppressive effect of CsA was still significant (fig. 6D). The results in fig. 6 show that both cytokines GM-CSF and IFNγ reversed the downregulated TNF production of peritoneal cells (fig. 6A), blood cells (fig. 6B), alveolar cells (fig. 6C), spleen cells (fig. 6D) and bone marrow cells (fig. 6E). Remarkably, in most samples there was no difference in the ability of GM-CSF or IFNγ, respectively, to reactivate macrophages in their capacity to release TNF. These date are different to those of Bundschuh *et al*., where IFNγ was reported to be more potent in its action, at least on alveolar and peritoneal cells from LPS-tolerant mice [397]. The potency of GM-CSF in counteracting pharmacologically induced immunosuppression was more pronounced in blood cells of CsA-treated (fig. 6B) and bone marrow cells of Dex-treated mice (fig. 6E), whereas its effect was not significant in peritoneal cells of CsA-treated mice (fig. 6A).

Figure 6: Ex vivo effects of GM-CSF and IFNg on LPS-induced TNF release by various monocyte-/macrophage-containing cell populations from naive or immunosuppressed mice



Two mice per group were pre-treated with either Dex or CsA (1 mg/kg; i.v.) 2 h prior to isolation and plating of various murine monocyte-/macrophage-containing populations, namely **(A)** peritoneal cells, **(B)** blood cells, **(C)** alveolar cells, **(D)** spleen cells and **(E)** bone marrow cells. Cells were pre-incubated for 2 h with 10 ng/ml GM-CSF or IFNγ, or solvent, respectively, and then stimulated with 1 µg/ml LPS. TNF was determined in supernatants 6h after LPS stimulation. Data are means ± SD from three independent experiments, each in triplicates.

*** p<0.001, ** p<0.01 and * p<0.05 *vs*. LPS (naive cells); $^{+++}$ p<0.001, $^{++}$ p<0.01, $^{+}$ p<0.05 *vs*. Dex / LPS; §§§ p<0.001, §§ p<0.01, § p<0.05 vs. CsA / LPS. Date were analyzed with the two-sided Tukey test

In general, the TNF response was fully reconstituted by both cytokines, to levels similar to those reached by naive cells after LPS stimulation. Taken together, our *ex vivo* results agree with the *in*

vivo findings. They underline the capacity of GM-CSF and IFNγ to restore the impaired LPS-induced TNF release by macrophages/monocytes after pharmacological immunosuppression with the drugs Dex and CsA.

3.3 GM-CSF and IFNg do not reconstitute immune functions of T-cells after pharmacological suppression

Since the pharmacologically suppressed immune response of macrophages and monocytes was reconstituted by GM-CSF and IFNγ, we wondered if similar effects could also be found in T-cells. To test this hypothesis the Concanavalin A (ConA) model was selected, since this plant lectin was shown to specifically stimulate T-cells [381]. As the stimulation of T-cells by ConA induces hepatitis-like liver failure and consequently death of the animals, pretreatment with immunosuppressive agents was reported to be protective [381].

3.3.1 Determination of the minimal dosage of immunosuppressive agents required to prevent T-cell activation in the ConA model

Although pretreatment with Dex, CsA or tacrolimus (FK-506) was shown to protect mice from T-cell derived liver failure in previous experiments done in our group, the doses used there failed to protect in the present work. Therefore dose-response experiments were required to determine the minimal effective doses of Dex, CsA, tacrolimus and MMF. Using a fixed dose of 25 mg ConA per body weight, a dosage shown to induce mortality within 8 hours after intravenous application, we varied the amount of the immunosuppressive drug until the animals were protected.

In table 2 the transaminase (ALT) release after a period of 8 hours in animals treated with different immunosuppressant dosages are given as a readout for liver failure. All measurements are given as a relation to transaminase release in naive mice treated with 25 mg/kg ConA designated as 100 %. Transaminase release in untreated control animals was always in the range of 2 to 6 % of that of the ConA-treated mice. Lethality was associated with a transaminase release of more than 20%.

Table 2: Mean transaminase release of immunosuppressed animals treated with the plant lectin ConA.

	Transaminase release [%] ± SD				
Drug ***Dosage [mg/kg]***	***Dex***	***CsA***	***tacrolimus***	***sirolimus***	***MMF***
100	n.d.	n.d.	n.d.	n.d.	2 + 0 ***
20	n.d.	n.d.	4 ± 1 ***	n.d.	n.d.
10	n.d.	3 ± 2 ***	3 ± 1 ***	n.d.	n.d.
5	n.d.	2 ± 1 ***	n.d.	n.d.	n.d.
2	n.d.	4 ± 2 ***	n.d.	n.d.	12 ± 1 **
1	13 ± 12 **	4 ± 1 ***	3 ± 1 ***	4 ± 1 ***	106 ± 15 n.s.
0,5	15 ± 7 **	n.d.	n.d.	n.d.	n.d.
0,1	n.d.	57 ± 5 *	58 ± 23 *	n.d.	n.d.
0,05	62 ± 58 n.s.	n.d.	n.d.	65 ± 12 n.s.	n.d.
0,01	n.d.	n.d.	66 ± 41 *	73 ± 23 n.s.	n.d.
0	100	100	100	100	100
control	6 ± 2 ***	3 ± 0 ***	2 ± 2 ***	3 ± 1 ***	5 ± 2 ***

Mice were pretreated with the indicated doses of immunosuppressive drugs 1 h before the ConA challenge (25 mg/kg). 8 hours following application mice were sacrificed and blood samples were withdrawn by cardiac puncture. Data are from 1 experiment with n = 3 animals per group and are given as transaminase release [%] ± SD compared to ConA-treated naive mice (equalized to 100%); n.d. not determinated. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ *vs*. ConA treated animals without immunosuppression. Data were analyzed with the two-sided Tukey test after one-way ANOVA. $p<0.05$ was considered significant.

CsA, tacrolimus and sirolimus showed a protective effect at a comparable dosage of 1 mg/kg. As these three potent immunosuppressive drugs have an almost similar mode of action, this result was not surprising. Interestingly, Dex, at a dosage of 1 mg/kg also prevented death, although the transaminase release here was still higher than in mice treated with CsA, tacrolimus or sirolimus. 1 mg/kg of Dex, CsA, tacrolimus and sirolimus were used in all further ConA experiments. All mice treated with MMF died within 4 hours after ConA-treatment. Low, as well as high dose treatment failed to prevent the animals' death. Most probably, death in the low dose setting was associated with liver failure, as massive transaminase release was detectable up to 1 mg/kg. Interestingly, a dosage of 2 mg/kg was sufficient to decrease transaminases to a range sufficient to secure survival.

Nevertheless, the animals all died within 4 hours, probably from of unwanted side-effects, ranging from blood pressure drop and lethargy to paralysis of the fore and hind paws. Although the symp-

toms described disappeared after several minutes, for ethical reasons, MMF was not administered intravenously in further experiments.

3.3.2 The influence of GM-CSF and IFNg on the T-cell activity of immunosuppressed mice *in vivo* in the ConA model

Next, it was investigated whether GM-CSF and IFNγ could reconstitute the pharmacologically suppressed T-cell activity, as was the case for macrophages and monocytes (section 3.2.2.1). Animals were pretreated 1 h before the ConA challenge with Dex, CsA, tacrolimus or sirolimus (1 mg/kg). While immunosuppressive treatment was expected to protect from ConA-induced liver failure, it was investigated whether GM-CSF or IFNγ, administered intravenously 45 min before the ConA challenge in a dose of 50 µg/kg, abolished the protective effect.

Table 3: GM-CSF and IFNg do not affect ConA-induced liver injury

	Drug	*ConA*	*+ GM-CSF*	*+ IFNg*
ALT [U/l] ± SD	***control***	2360 ± 1810	2640 ± 1930	2990 ± 1910
	Dex	510 ± 180 $^{n.s.}$	580 ± 310 $^{n.s.}$	600 ± 410 $^{n.s.}$
	CsA	120 ± 30 $^{n.s.}$	150 ± 40 $^{n.s.}$	150 ± 20 $^{n.s.}$
	tacrolimus	60 ± 10 $^{n.s.}$	40 ± 1 $^{n.s.}$	30 ± 10 $^{n.s.}$
	sirolimus	150 ± 120 $^{n.s.}$	120 ± 70 $^{n.s.}$	100 ± 70 $^{n.s.}$

Mice were pretreated with 1 mg/kg of the indicated immunosuppressive drugs 1 h before the ConA challenge (25 mg/kg). 8 hours following the application mice were sacrificed and blood samples were withdrawn by cardiac puncture. Hepatotoxicity was quantitated by determination of ALT in plasma. Data are from 1 or 2 independent experiment with n = 3 (tacrolimus, sirolimus) or n = 6 (controls, Dex, CsA) animals per group and are given in ALT release [U/l] ± SD compared to untreated mice. *** $p < 0.001$ *vs*. untreated control animals; n.s. not significant. Data were analyzed with the two-sided Tukey test after one-way ANOVA. $p<0.05$ was considered significant.

As shown in table 3, both cytokines only marginally increased the ALT release in ConA-treated control animals. Obviously, GM-CSF and IFNγ did not have a priming effect on T-cells. In line with our previous reports, application of the immunosuppressive drugs prevented liver injury, i.e. ALT release was significantly reduced. Immunosuppressed animals showed no liver cell destruction after the period of 8 hours and, as expected, were protected from mortality. Furthermore, no priming effect of GM-CSF and IFNγ on T-cells was found in all immunosuppressed mice. Neither cytokine could reactivate pharmacologically suppressed T-cell functions. Therefore, such pharmacological intervention was clearly not sufficient to reconstitute the T-cell response.

The goal of this work was to reconstitute the immune system after pharmacological suppression, e.g. in transplant patients in case of infection. While on the one hand the complete reconstitution of the immune response, i.e. macrophages and T-cells, would be ideal for a successful control of an ongoing infection, on the other hand the graft should not be put at risk. As T-cells play the leading role in graft rejection, these results indicated that graft acceptance might not be affected by cytokine treatment. However they made it doubtful whether sufficient combat of infection could be achieved.

To better describe the magnitude of the T-cell response in case of pharmacological immunosuppression and possible reactivation, we measured the release of the specific T-cell cytokines IL-2 and IFNγ. Determination of IL-2 and IFNγ levels are given in the tables 4A and 4B.

In accordance to the results above, only control animals without immunosuppression showed a remarkable release of both IL-2 (table 4A) and IFNγ (table 4B). Although modest levels of IL-2 and IFNγ were detected in case of Dex treatment (tables 4A and 4B), the more powerful drugs CsA, tacrolimus and sirolimus completely prevented a T-cell response. Interestingly, sirolimus showed the most powerful immunosuppressive effect regarding lymphokine release, followed by tacrolimus and CsA. In case of IFNγ pretreatment, plasma IFNγ levels after 8 hours were not measured, as the intravenously injected IFNγ would have altered the results. Taken together, T-cell derived cytokine levels detected in all samples of ConA-treated immunosuppressed mice with or without pretreatment by GM-CSF and IFNγ were not significantly different compared to naive control animals. Therefore, it is concluded that macrophage/monocyte, but not T-cell reconstitution follows an intervention by the pro-inflammatory cytokines GM-CSF and IFNγ.

Table 4: GM-CSF and IFNg do not affect sensitivity of suppressed T-cells towards ConA-induced liver injury in vivo

A	*Drug*	*ConA*	*+ GM-CSF*	*+ IFNg*
IL-2 [pg/ml] ± SD	***control***	1310 ± 440 ***	1250 ± 390 ***	1320 ± 340 ***
	Dex	340 ± 240 n.s.	300 ± 80 n.s.	430 ± 100 n.s.
	CsA	50 ± 90 n.s.	< 10 n.s.	< 10 n.s.
	tacrolimus	< 10 n.s.	< 10 n.s.	< 10 n.s.
	sirolimus	< 10 n.s.	< 10 n.s.	< 10 n.s.
B	***Drug***	***ConA***	***+ GM-CSF***	***+ IFNg***
IFNg [pg/ml] ± SD	***control***	6080 + 3270 **	5760 + 720 **	n.d.
	Dex	860 ± 1090 n.s.	980 ± 930 n.s.	n.d.
	CsA	340 ± 10 n.s.	260 ± 10 n.s.	n.d.
	tacrolimus	60 ± 30 n.s.	90 ± 50 n.s.	n.d.
	sirolimus	< 10 n.s.	< 10 n.s.	n.d.

Mice were pretreated with 1 mg/kg of the indicated immunosuppressive drugs 1 h before the ConA challenge (25 mg/kg). 4 hours after the ConA challenge, blood samples were withdrawn from the tail vein for IL-2 determination. 8 hours following the application, blood samples were withdrawn by cardiac puncture after lethal anaesthesia for IFNγ measurement. IL-2 **(A)** and **(B)** IFNγ were determined by ELISA. Data are from 1 or 2 independent experiments with n = 3 (tacrolimus, sirolimus) or n = 6 (controls, Dex, CsA) animals per group and are given as IFNγ release [pg/ml] ± SD compared to untreated mice. ** $p < 0.01$ *vs*. untreated controls; n.s. not significant; n.d. not determinated. Data were analyzed with the two-sided Tukey test after one-way ANOVA. $p<0.05$ was considered significant.

3.3.3 The influence of GM-CSF and IFNg on the pharmacologically suppressed T-cell activity *ex vivo*

For the sake of completeness, it was tested whether a suppressed T-cell population likewise remained silenced after incubation with GM-CSF or IFNγ, when stimulated with ConA. Spleens from naive Balb/c mice were extracted, separated by a cell strainer and cells incubated with immunosuppressants and ConA *in vitro*. 100 ng/ml of the immunosuppressants were added 90 min before the ConA challenge. Addition of GM-CSF or IFNγ occurred 45 min before ConA in a concentration of 10 ng/ml. In accordance with the *in vivo* results, neither GM-CSF nor IFNγ had the capability to reconstitute the suppressed T-cell response. As shown in tables 5A and 5B, the *ex vivo* findings were even more expressive than those obtained *in vivo.*

Table 5: GM-CSF and IFNg do not affect sensitivity of suppressed T-cells towards ConA induced liver injury in vitro

A	***Drug***	***ConA***	***+ GM-CSF***	***+ IFNg***
IL-2 [pg/ml] ± SD	***control***	149 ± 15 **	212 ± 33 **	237 ± 94 **
	Dex	< 10 n.s.	< 10 n.s.	< 10 n.s.
	CsA	< 10 n.s.	< 10 n.s.	< 10 n.s.
	tacrolimus	< 10 n.s.	< 10 n.s.	< 10 n.s.
	sirolimus	< 10 n.s.	< 10 n.s.	< 10 n.s.
B	***Drug***	***ConA***	***+ GM-CSF***	***+ IFNg***
IFNg [pg/ml] ± SD	***control***	395 + 178 **	432 + 157 **	n.d.
	Dex	< 10 n.s.	< 10 n.s.	n.d.
	CsA	< 10 n.s.	< 10 n.s.	n.d.
	tacrolimus	< 10 n.s.	< 10 n.s.	n.d.
	sirolimus	< 10 n.s.	< 10 n.s.	n.d.

Spleen cells from 10 mice were obtained by separating freshly isolated tissue from anaesthetized, naive mice with a cell strainer. After centrifugation, lysis of erythrocytes and washing, cells (2 x 10^7/ml) were pre-incubated with Dex, CsA, tacrolimus or sirolimus, respectively, (100 ng/ml) for 90 min and GM-CSF or IFNγ (10 ng/ml) for 45 min. Cells were, after addition of ConA (5 µg/ml), incubated for 48 hours, centrifuged and supernatants stored at – 80°C until IL-2 **(A)** or IFNγ **(B)** detection by ELISA. All data are means ± SD from 3 independent incubations done twice in 2 different *ex vivo* experiments. Data are analyzed with the two-sided Tukey test after one-way analysis of variance (ANOVA). **$p<0.01$; n.s. not significant. $p<0.05$ was considered significant.

While GM-CSF and IFNγ only had a negligible stimulative effect on naive T-cell populations, there was no detectable effect on suppressed cells at all. Moreover, IL-2 as well as IFNγ levels in these samples were under the detection limit and in a range like those released by untreated control cells.

Taken together, the idea that GM-CSF and IFNγ might reconstitute a pharmacologically suppressed immune system proved true only for macrophage- or monocyte-populations, but not for T-cells. As T-cells are the key modulators of graft rejection, a reactivation after pharmacological suppression, however, would not be desirable. But, concerning the proper combat of infection, it remained to be proven, that bacterial, viral, fungal or other infection could be combated without the help of T-cells.

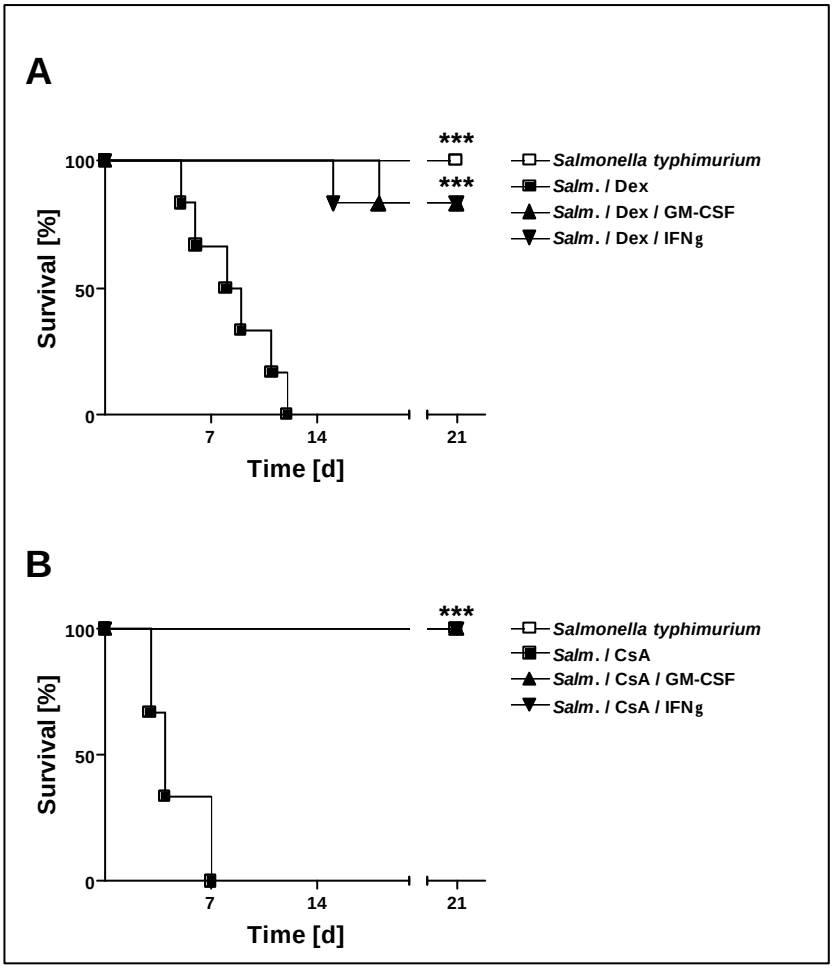
3.4 Immune reconstitution by GM-CSF or IFNg in case of bacterial infection with *Salmonella typhimurium*

Bacterial infection after transplant surgery is a common clinical problem, most often leading to the death of patients. It was found that intervention with the pro-inflammatory cytokines GM-CSF and IFNγ enabled the reactivation of macrophage function. Now it was further investigated whether this measure could enable effective bacterial combat. Crucial to these experiments was the existence of a *Salmonella*-resistent mouse strain, so-called CBA/Ca mice. Those animals were shown to counteract infection with *Salmonella typhimurium*, but become susceptible to the lethal infection when immunosuppressed by Dex-treatment [398]. After initial dose-finding experiments for the immunosuppressive agents and *Salmonella typhimurium*, the dosage for Dex and CsA was fixed to 1 mg/kg and the bacterial load to 5×10^5 bacteria/kg.

As shown in figure 7, untreated CBA/Ca mice were not responsive to the otherwise lethal infection with *Salmonella typhimurium* in a dose up to 5×10^5 bacteria/kg, injected into the peritoneum. While all these animals survived the observation period of 21 days without any symptoms of disease, immunosuppressed mice, pretreated with either 1 mg/kg Dex or CsA, died within 7 (CsA) or 12 (Dex) days after infection. Those mice showed violent symptoms of infection, including impaired movement activity, diarrhea and lethargy.

As the animals only took up neglectable amounts of food and water, they were marked by severe weight loss. When examined after death, organs, e.g. the liver exhibited marked signs of massive bacterial propagation. White spots within the liver tissue were attributed to an extensive accumulation of live bacteria, which immunosuppressed mice could not combat. Furthermore, other organs characterized by a marked bacterial load were spleen, peritoneum and blood (figure 8).

Figure 7: GM-CSF and IFNg reconstitute the suppressed immune response in case of bacterial infection with Salmonella typhimurium



Six (Dex; 1 mg/kg; i.p.) or 3 mice per group (CsA; 1 mg/kg; i.v.) were immunosuppressed with (**A**) dexamethasone, or (**B**) cyclosporine A 2 days before infection with *Salmonella typhimurium* (5 x 10^5 bacteria/kg; i.p.). Where indicated, GM-CSF or IFNγ (both 50 µg/kg; i.v.) were given once on day 2 (Dex) or daily from day 2 to day 4 (CsA). Survival over 3 weeks was determined for naive animals, infected with *S. typhimurium* (□), for immunosuppressed animals infected with *S. typhimurium* (■) and for immunosuppressed animals, infected with *S. typhimurium* and treated with GM-CSF (▲) or IFNγ (▼). Survival curves were analyzed using the *Logrank* test. *** $p < 0.003$; $p < 0.05$ was considered significant.

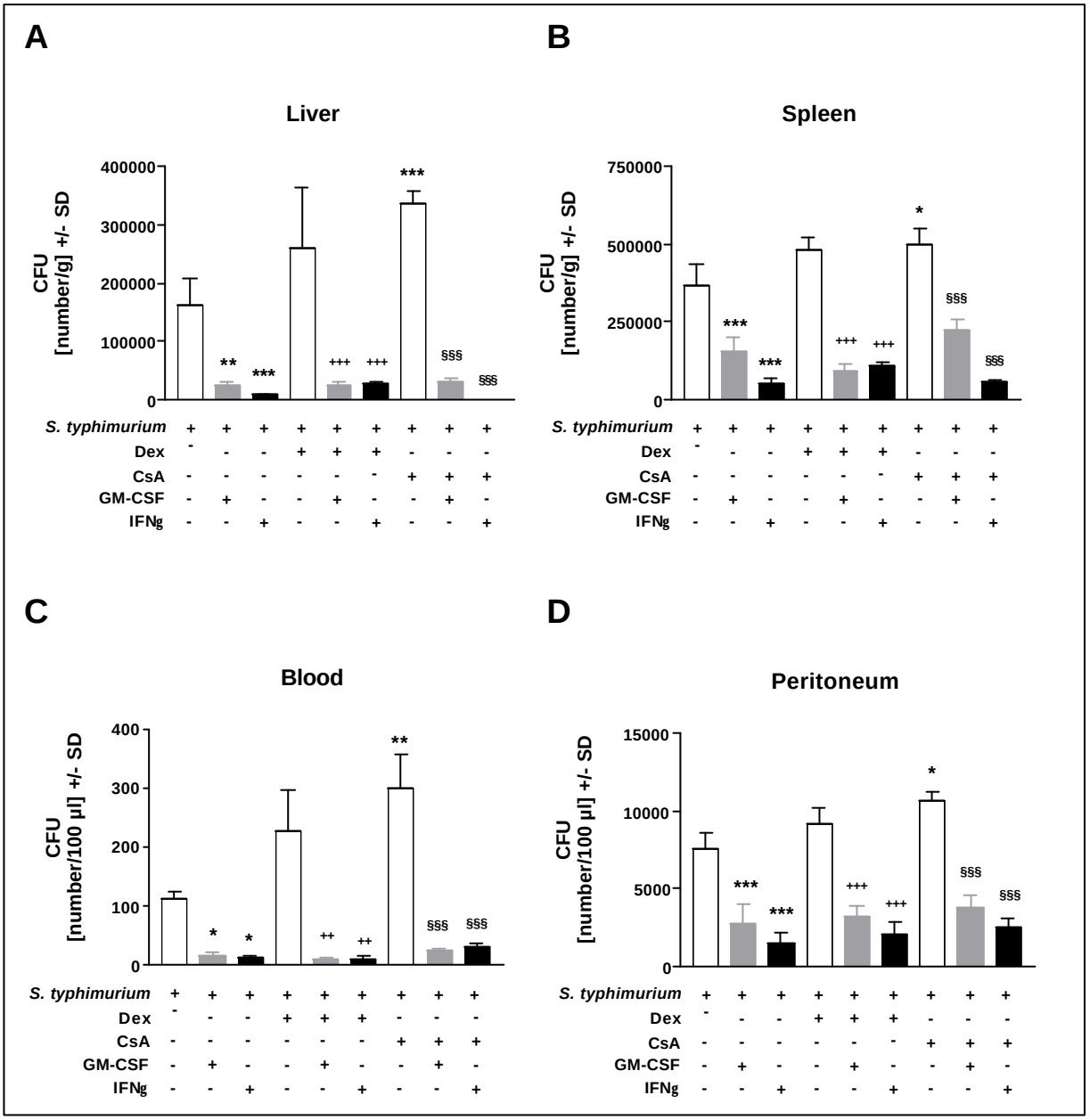
Starting from the day of infection onwards, mice were intravenously injected with 50 µg/kg GM-CSF or IFNγ for 1 (Dex) or 3 consecutive days (CsA). Mice pretreated with either GM-CSF or IFNγ survived significantly longer than animals treated with immunosuppressant only (fig. 7). While in case of Dex-treatment plus pharmacological intervention by GM-CSF or IFNγ, one out of six animals died within the first 18 days (Figure 7A), all mice treated with CsA survived (fig. 7B). Possibly, sustained injection of GM-CSF or IFNγ might have improved immune reconstitution in Dex-treated mice and prevented the animals' death, like demonstrated with CsA. All immunosuppressed animals, that were injected with GM-CSF or IFNγ had a short phase of infectious disease, from which they quickly recovered. The infection with *Salmonella typhimurium* and the immune reconstitution occured on the same day, so the animals' immune system required a slightly longer time to recover than bacteria needed to duplicate. The significant difference seen between the survival of immunosuppressed animals with and without immune reconstitution confirms that the murine immune system can

be reactivated after pharmacological suppression by administration of GM-CSF or IFNγ to a degree sufficient for the combat of a bacterial infection. Although the results discussed in chapter 3.2 showed that only macrophages/monocytes, but not T-cells, were reactivated, bacteria could be successfully eradicated.

As shown in fig. 8, animals immunosuppressed with either Dex or CsA had an increased bacterial load in organs, peritoneum and blood. CsA treatment impeded bacterial combat even more than Dex treatment as can be seen by the increased bacterial loads in these animals. These findings support the potency of CsA as a strong immunosuppressive agent compared to Dex and others. Moreover, treatment of naive mice with GM-CSF or IFNγ significantly improved bacterial combat, as the bacterial numbers in livers (fig. 8A), spleens (fig. 8B), blood (fig. 8C) and peritoneum (fig. 8D) were clearly decreased. Since immunosuppressed mice treated with one of the pro-inflammatory cytokines only suffered from infection for a short time, this result can be understood as a reconstitution of the impeded immune response. Both, GM-CSF and IFNγ, enabled immunosuppressed mice to significantly decrease the number of live bacteria in the liver, spleen, blood and peritoneum. Here the reduction of bacteria, although still under immunosuppressive influence, was even more impressive than in naive mice. In almost all samples, the immune reconstitution by IFNγ was stronger than the one of GM-CSF. Interestingly, *Salmonella* infection could be controlled exclusively by macrophages and monocytes, since T-cells remained under immunosuppression. As already demonstrated in case of LPS and ConA (chapters 3.2 and 3.3), only macrophage-derived cytokines like TNF could be determined in the infection model (data not shown). In summary, it seems that in this system the activation of the innate immune system is crucial for the recovery from bacterial infection.

However, when comparable experiments were done with *Salmonella*-responsive Balb/c mice, only GM-CSF had a marked effect on prolonging the animals' survival and decreasing the bacterial load within organs and blood. In contrast to the results illustrated above, IFNγ in Balb/c mice further strengthened the pharmacologically induced immunosuppression by activating the NO-synthase (data not shown). Hence, NO on the one hand probably plays an important role in the surveillance of an infection, but can equally induce undesirable side effects. It seems that IFNγ and NO have to be regulated very sensitively in case of infection for not improving immunosuppression.

Figure 8: Bacterial load in different organs of infected mice after immunosuppression and treatment with GM-CSF or IFNg



Nine (Dex; 1 mg/kg; i.p.) or 6 mice per group (CsA; 1 mg/kg; i.v.) were immunosuppressed with dexamethasone (Dex) or cyclosporine A (CsA) 2 days before infection with *Salmonella typhimurium* (5 x 10^5 bacteria/kg; i.p.). Where indicated, GM-CSF or IFNγ (both 50 µg/kg; i.v.) were given once on day 2 (Dex) or daily from day 2 to day 4 (CsA). Blood **(C)** and peritoneal **(D)** samples were withdrawn 4 days after infection, samples from livers **(A)** and spleens **(B)** were obtained on day 7 after infection. After dilution, samples were spread on blood agar plates to determine aerobic colony forming units. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple test. * $p<0.05$ *vs*. untreated control, ** $p<0.01$ *vs*. untreated control, *** $p<0.001$ *vs*. untreated control; $^{++}$ $p<0.01$ *vs*. Dex-treated control, $^{+++}$ $p<0.001$ *vs*. Dex-treated control; §§§ $p<0.001$ *vs*. CsA-treated control. $p < 0.05$ was considered significant.

3.5 Assessment of pharmacologically effective doses of immunosuppressive drugs to prevent rejection of skin grafts

After the possibility to reconstitute a pharmacologically suppressed immune response by GM-CSF or IFNγ in a bacterial infection model with *Salmonella typhimurium* was verified, it was examined whether this treatment has negative consequences on the survival of transplanted allografts. While the preceding experiments indicated that an exclusively macrophage-based bacterial combat was efficient, and allowed almost entire survival, it was necessary to examine whether this new method for immune reconstitution would endanger the surgical outcome of transplantation. For this purpose, tail skin from Balb/c mice was transplanted onto the backs of immunosuppressed CBA/Ca mice. In a first step, the correct dosage and regimen for the available immunosuppressive drugs was tested. As skin grafts are the most frequently rejected transplants, this model allowed a stringent test of the cytokine therapy.

3.5.1 Prevention of skin allograft rejection by CsA

After practicing the methodology and gaining first experiences in the skin transplant model on Balb/c mice, CsA was selected as the first immunosuppressive agent for the establishment of a MHC-mismatch allotransplantation model. Before engraftment of skin pieces, recipient mice were treated with different doses of immunosuppressive agents to establish how much was needed to prevent an immediate reaction of the immune system against the foreign tissue. Embedded grafts were protected from manual injury and parching by a vaseline-soaked bandage on the wound for 7 days after surgery. After removal of the bandage, grafts were inspected daily and assessed by the rate of necrosis. Rejection was defined as total necrosis of the graft.

CsA was effective in the transplantation experiments only from 30 mg/kg body weight onwards. These doses were much higher than those used in the pilot experiments. Hence, the effective dosage for CsA was 6000-fold higher than necessary for macrophage inactivation in the LPS shock model and still 30 times higher than that required for T-cell suppression in the ConA experiments. Also, the gap between the effective and the lethal dose was very narrow.

Table 6: Dose-response and survival times of allografts under immunosuppression with CsA

Dose [mg/kg]	Graft survival [d]	Lethality [%]
0	9 ± 2 (n = 6)	0
0.1	12 ± 3 (n = 3)	0
0.5	14 ± 1 (n = 3)	0
1	15 ± 1 (n = 3)	0
10	14 ± 2 (n = 3)	0
20	20 ± 2 (n = 3)	0
30	> 28 (n = 6)	0
40	23 ± 6 (n = 6)	50
50	16 ± 8 (n = 4)	75

Mice were daily treated with the different doses of CsA (i.p.) indicated, beginning short before the transplantation of foreign tissue. Wounds were protected by vaseline-soaked gauze and bandage for 7 consecutive days. After removal of bandages, grafts were inspected daily and graft necrosis owing to missing circulation or immune response to foreign tissue was diagnosed as progressive rejection. Data are given in graft survival [d] ± SD or [%] lethality.

As indicated in table 6, all animals treated with 30 mg/kg survived a period of 4 weeks without exhibiting toxic side effects of the drug, whereas mice treated with higher doses died from its toxicity. Furthermore, doses below 30 mg/kg were not high enough to prevent graft rejection within 4 weeks. Naive mice with an intact immune system lost skin allografts within an average of 9 days. Immunosuppressive treatment was always associated with loss of weight, depending on the dosage used. Animals treated with high and finally lethal doses suffered from significant weight loss. The animals receiving 30 mg/kg lost up to 5 g (< 17 %) of body weight. Therefore, animals were carefully selected by body weight (in a range of 28 up to 35 g) to prevent death by starvation.

Nonetheless, the single drug therapy with CsA allowed a successful transplantation of sensitive skin allografts within MHC-mismatch animals. In further experiments with CsA animals were treated with a daily dose of 30 mg/kg.

3.5.2 Prevention of skin graft rejection by a combination therapy scheme with tacrolimus and MMF or Dex

Single drug therapy with tacrolimus failed in all doses used. Lethality within the first week was > 50 % when animals were treated intraperitoneally with 20 mg/kg tacrolimus, a dosage not sufficient to prevent allograft rejection. Therefore a combination therapy scheme with tacrolimus (1 mg/kg) and MMF or Dex (10 mg/kg each) was established. Corresponding to the CsA trial, mice were intraperitoneally injected with a mixture of both drugs each morning and graft survival was monitored daily. Interestingly, graft integration under tacrolimus/MMF or tacrolimus/Dex treatment, was better than in CsA experiments. This finding indicates that re-vascularization of the graft might be improved by tacrolimus. Nevertheless, the immunosuppressive potency of tacrolimus was not as high as that of CsA. Moreover, CsA and tacrolimus, although they share high similarities, seem to differ significantly in their mode of action. While in other transplant trials tacrolimus was more promising than CsA, tacrolimus in single therapy completely failed in these skin transplantation experiments.

3.5.3 Prevention of allograft rejection under sirolimus

It was also tested whether sirolimus could improve the outcome of skin transplantation. After orientating experiments on its general efficacy in single therapy and determination of an optimal dosage, it was found that sirolimus (1 mg/kg; i.p.) significantly improved the quality of the allograft when compared to CsA and tacrolimus. Hair, anchored within the graft, served as an indication for the improved engraftment. While such hair was generally lost in the trials with CsA and tacrolimus, it remained when sirolimus was used. Sirolimus therefore might further increase re-vascularization of the graft by expanding vessels due to sustained proliferation of muscle cells.

3.5.4 Consequences of GM-CSF and IFNg on allograft acceptance or rejection

In chapter 3.4 it was demonstrated that a pharmacologically suppressed immune system can be reactivated in case of infection by *Salmonella typhimurium* by administration of GM-CSF or IFNγ. However, both cytokines, GM-CSF and IFNγ, which are mediators of an inflammatory response, have been described to play a role in graft rejection. Therefore it was examined whether the administration of these inflammatory mediators generally interfered with the outcome of the transplantation process, or whether their selectivity in activating macrophages prevents this. Based on the experiments described above (chapters 3.5.1–3.5.3), transplanted animals were treated with immunosuppressive drugs for 28 consecutive days. After removal of the bandage and examination of the graft to

assure successful surgery on day 7, mice were injected intraperitoneally with 50 µg/kg GM-CSF or IFNγ for the 5 following days (day 7 to day 12).

Table 7: Consequences of GM-CSF or IFNg treatment on the outcome of skin allotransplantation

A **CsA**	**Graft survival [d]**	**Lethality [%]**
+ GM-CSF	28 + 0 (n = 3)	0
+ IFNg	28 ± 0 (n = 3)	0
B Tacrolimus / Dex	**Graft survival [d]**	**Lethality [%]**
+ GM-CSF	28 + 0 (n = 3)	0
+ IFNg	28 ± 0 (n = 3)	0

Once per day, mice were treated i.p. with 30 mg/kg CsA (**A**) or a combination of 1 mg/kg tacrolimus/ 10 mg/kg Dex (**B**) beginning shortly before the transplantation. Wounds were protected by vaseline-soaked gauze and bandage for 7 days. After removal of bandages, animals were treated i.p. with either 50 µg/kg GM-CSF or IFNγ, respectively, for the next 5 days. Grafts were inspected daily and graft necrosis due to missing circulation or immune response to foreign tissue was diagnosed as progressive rejection. Data are given in graft survival [d] ± SD or [%] lethality.

All animals treated with CsA or tacrolimus/Dex integrated allografts over a period of 28 days without signs of necrosis and consequently rejection. As shown in table 7, allograft survival after the administration of either GM-CSF or IFNγ was not affected when compared to solely immunosuppressed mice. Both pro-inflammatory cytokines therefore seem to have no influence on the outcome of skin allograft transplantation in the selected murine model with CBA/Ca mice.

3.6 Combat of *Salmonella typhimurium* infection by GM-CSF or IFNg in immunosuppressed and transplanted mice

In the final experiments, it was investigated whether intervention by GM-CSF or IFNγ in immunosuppressed, transplanted mice can still reconstitute suppressed immune functions and so make bacterial defense possible. While doses in the initial experiments, described in the chapters 3.2 and 3.3. were chosen as low as possible, i.e. on the border to effective immunosuppression, they had to be high enough in the transplantation trial to ensure graft acceptance, as in clinical reality. Hence, it was left to prove, whether intervention with either cytokine, GM-CSF or IFNγ, showed a general efficacy to reactivate pharmacologically suppressed immune functions in mice, independent of the immunosuppressant dosage utilized. For ethical reasons, only CsA (30 mg/kg) was selected as a an example for single and tacrolimus (1 mg/kg) plus Dex (10 mg/kg) as model for combination drug therapy.

3.6.1 Immune reconstitution by GM-CSF or IFNg in CsA-treated mice after skin allotransplantation

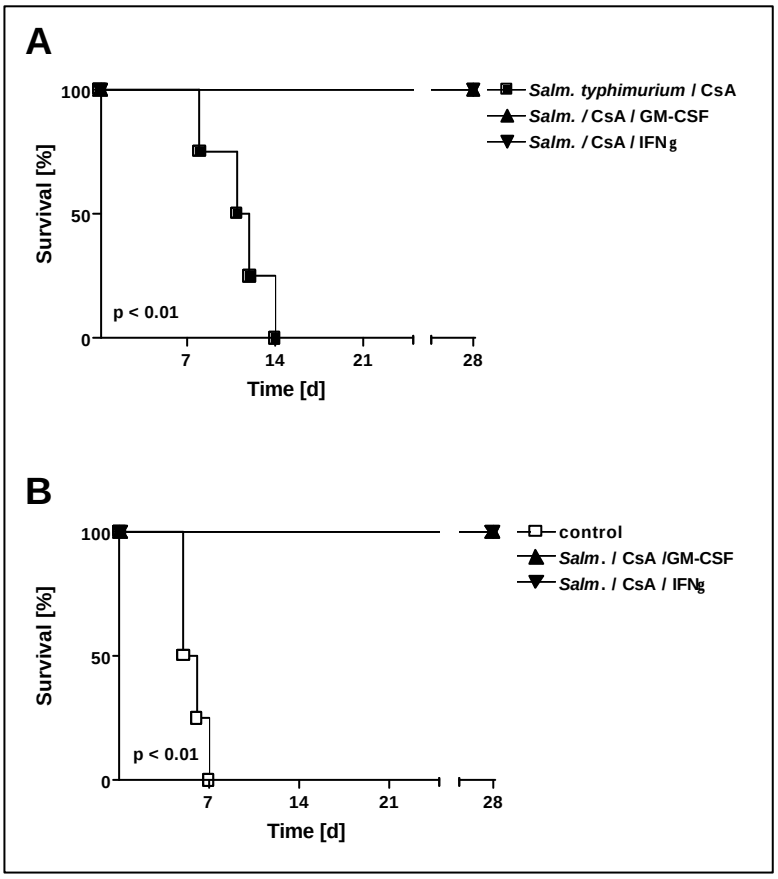
After having shown that neither GM-CSF nor IFNγ induce graft rejection in transplanted mice intraperitoneally administered with CsA (30 mg/kg), it was examined, whether the cytokines have the potency to reconstitute immune functions after infection with *Salmonella typhimurium*. For all infection experiments, the allotransplantation model with tail skin from Balb/c mice as donor tissue which was embedded on the backs of *Salmonella*-resistant CBA/Ca mice was used. Basis for an infection of transplanted CBA/Ca mice with *Salmonella* (5×10^5 /kg; i.p.) was a successful course of transplantation until day 7, assessed by monitoring of the graft after removing the bandage.

Animals without satisfactory integration of the graft were excluded from further experiments. While immunosuppression by CsA was continued daily, infected mice were treated for 4 days with either 50 µg/kg GM-CSF or IFNγ from day 7 to day 10 to reconstitute immune functions. Survival of mice and fate of the graft was monitored for the following 3 weeks.

3.6.1.1 GM-CSF and IFNg assure survival of transplanted mice after infection with *Salmonella typhimurium*

As shown in fig. 9A, immunosuppressed mice all died within 7 days of infection, i.e. a period of time resembling that found in non-transplanted mice treated with 1 mg/kg CsA after infection (chapter 3.4). Obviously, the 30-fold increased dose of CsA, when compared to the former infection experiments, did not change the animals' course of disease, but was required to assure graft survival as reported in the last section (chapter 3.5.1). Taking into account that a dose of 1 mg/kg was sufficient to inhibit the immune response of macrophages and T-cells, the necessity for a dose of 30 mg/kg CsA in transplantation gave rise to the expectation that something more than immunosuppression must be required to prevent graft rejection. With this dose of CsA, the grafts of immunosuppressed animals remained intact until death (not shown). These experiments clarify the conflict between the improvement in transplantation outcome and the reduction in survival after infection under immunosuppressive treatment.

Figure 9: GM-CSF and IFNg restore immune functions after immunosuppression by CsA in case of infection with Salmonella typhimurium

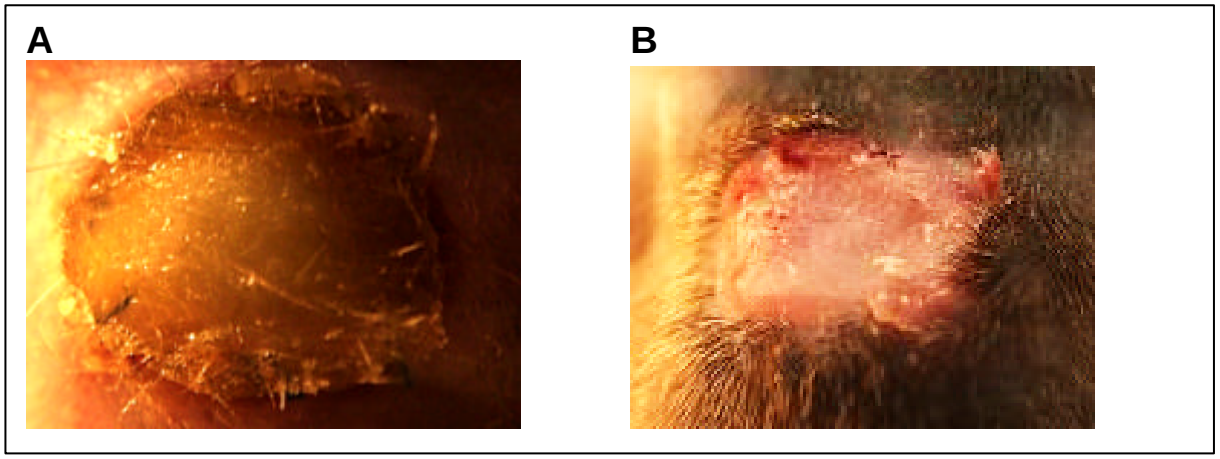


CBA/Ca mice (n = 4 for GM-CSF and n = 3 for IFNγ) were immunosuppressed by daily treatment with cyclosporine A (30 mg/kg; i.p.). On day 7 after transplantation, animals that were assessed to have accepted grafts received an injection of *Salmonella typhimurium* (5×10^5 bacteria/kg; i.p.). Where indicated, GM-CSF or IFNγ (both 50 µg/kg; i.p.) were given daily from day 7 to day 10 while immunosuppression was continued. Survival over the 3 following weeks (**A**) was determined for immunosuppressed animals infected with *S. typhimurium* (■), and for immunosuppressed animals infected with *S. typhimurium* and treated with GM-CSF (▲) or IFNγ (▼). (**B**) Graft survival in naive mice (□), and in immunosuppressed and infected mice, treated with GM-CSF (▲) or IFNγ (▼) was determined over 4 weeks. Survival curves were analyzed using the *Logrank* test. $p < 0.05$ was considered significant.

The combat of bacterial infection also occured in transplanted CBA/Ca mice treated with GM-CSF or IFNγ when infected with *Salmonella typhimurium* without diminishing the outcome of transplant surgery.

Assessment of acceptance or rejection of transplanted allografts was performed by macro-photographs of the graft and surrounding tissue (fig. 10, 11 and 13) or by histopathological examination of skin slices (fig. 12), respectively. Examples for graft acceptance (fig. 10A and 10B) with CsA as immunosuppressive drug in *Salmonella*-infected CBA/CA mice are given below. Accepted grafts remained in the graft bed and were integrated in the surrounding tissue (fig. 10). Although graft size was slightly reduced within time, the graft itself was apparent throughout the period of 4 weeks which was the duration of the experiment. Grafts were perceptible as spots within naive skin, characterized by a different skin color and the complete loss of hair due to missing innervation of the hair follicles.

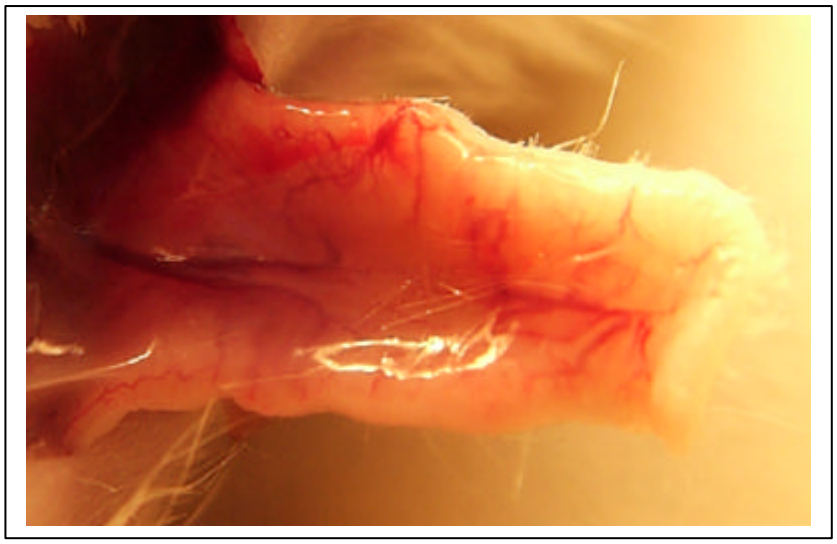
Fig. 10: Graft acceptance in Salmonella-infected CBA/Ca mice under immunosuppression with CsA



CBA/Ca mice were immunosuppressed by daily treatment with cyclosporine A (30 mg/kg; i.p.). On day 7 after transplantation, the animals were assessed to have an accepted graft and received an injection of *Salmonella typhimurium* (5×10^5 bacteria/kg; i.p.). GM-CSF (**A**) or IFNγ (**B**) (both 50 µg/kg; i.p.) were given daily from day 7 to day 10 while immunosuppression was continued. Survival of the grafts was monitored over the 3 following weeks. Makro-photographs were made on day 18 (**A**) and day 23 (**B**).

One major event in graft acceptance was the re-vascularization of the graft. Therefore, grafts accepted by the recipient were routinely examined for successful renewal of blood vessels within the grafts. Since dark skin color of CBA/Ca mice impeded photographical documentation an example for re-vasularization of allografts in Balb/c mice is given in fig. 11.

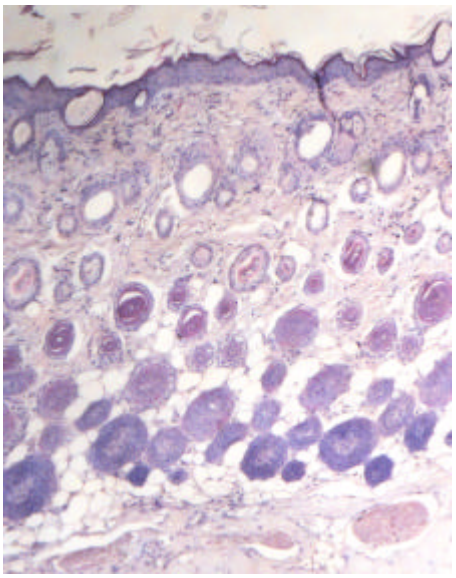
Fig. 11: Re-vascularization of allografts required for graft acceptance



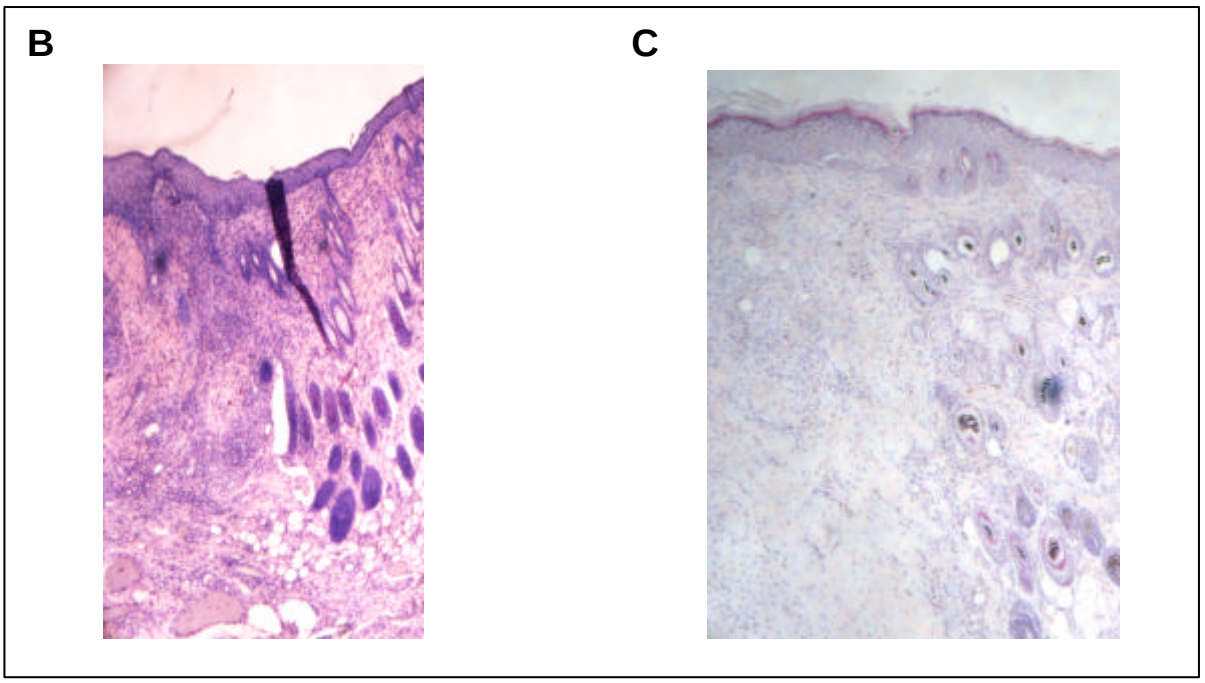
To further investigate the quality of accepted allografts, samples for histological examination were prepared (fig. 12). Besides the re-vascularization of the graft, its integration within the naive tissue was readout for the acceptance of skin in immunosuppressed and *Salmonella*-infected animals, that were treated with either GM-CSF or IFNγ. Skin of untreated animals served as control.

Fig. 12: Histological examination of skin allografts in immunosuppressed and infected mice, treated with GM-CSF or IFNg

A



Skin from control **(A)** and transplanted mice **(B, C)** was compared. Samples from transplanted and infected mice, treated with GM-CSF **(B)** or IFNγ **(C)** were gathered on day 21 after transplantation, embedded in paraplast, cut in 2 µm slices and stained with eosin/hematoxilin. Parameters for graft acceptance are re-vascularization and successful integration in the surrounding tissue. Control and naive skin are characterized by a high number of hair follicles.

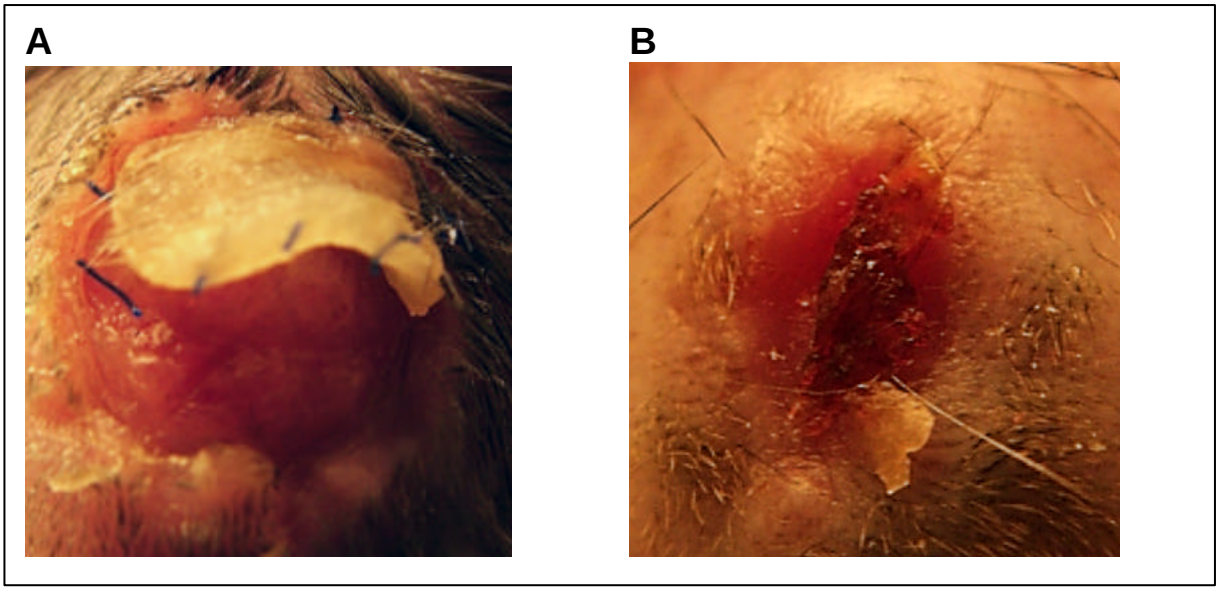


When compared to control skin (fig. 12A), tail skin allografts were unmistakably characterized by a reduced number of hair follicles in the epithelium. Skin slices from immunosuppressed and infected animals that were treated with either GM-CSF (fig. 12B) or IFNγ (fig. 12C) demonstrated that the foreign tissue was perfectly integrated into the native skin. Most important, the histological samples showed that grafts were completely vascularized (black arows), indicating a renewed formation of blood vessels. Re-vascularization is the most critical point in graft integration, as it guarantees the adequate support of the graft.

However, some accumulation of lymphocytes and other immune cells, was occasionally found within the histological samples. As all histological samples derived from animals that were macroscopically assessed to have accepted grafts, it might be that such immune-active sites within the graft were markers for an ongoing healing process or the beginning of graft rejection after the observation period.

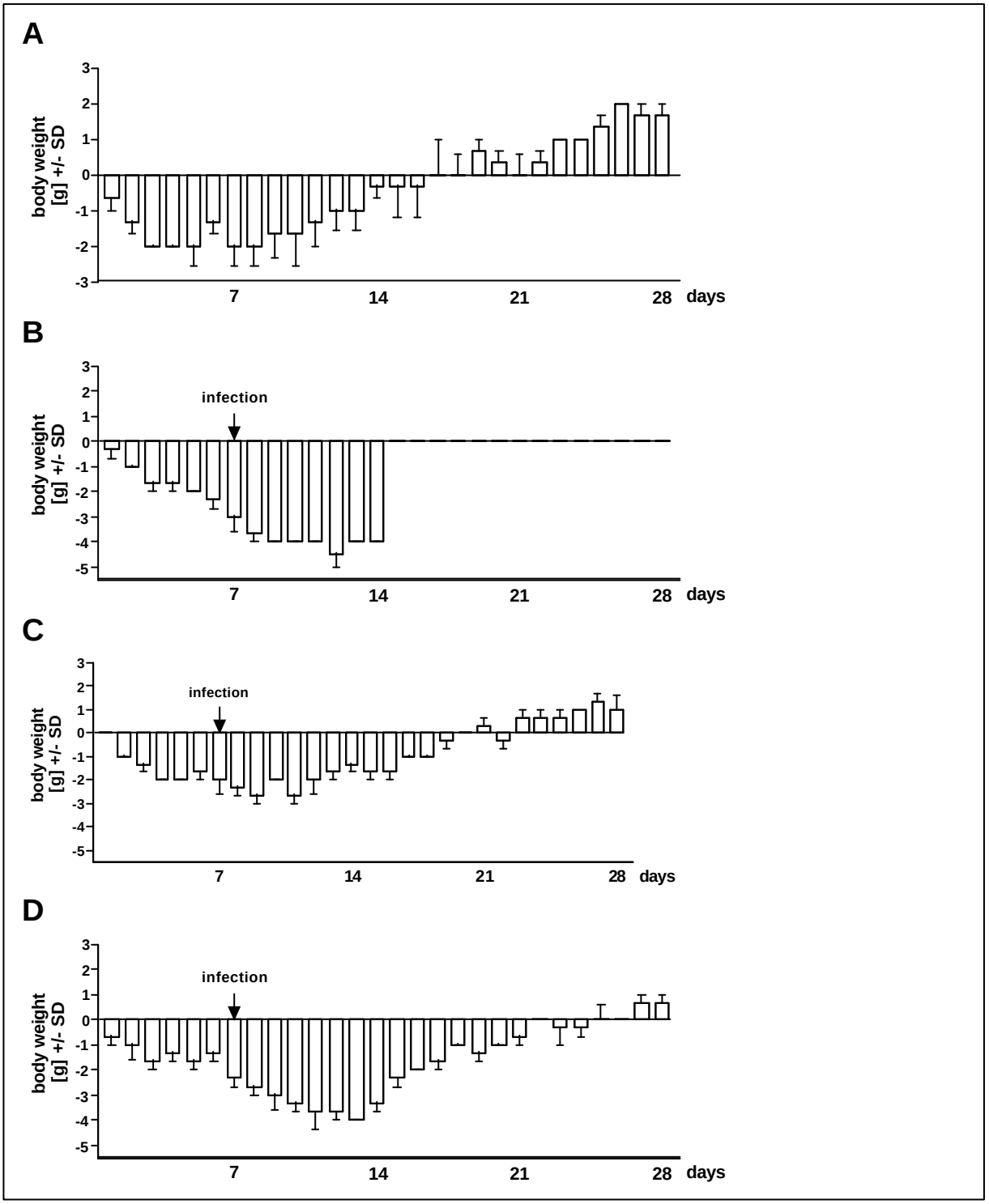
Without immunosuppression rejection started early after transplantation. While some grafts were lost due to acute rejection within 7 days (fig. 13A), others remained within the graft bed for a period of approximately 12 days, thereby passing through several stages of necrosis until they were replaced by new skin (fig. 13B).

Fig. 13: Graft rejection in early (A) or late (B) phase in CBA/Ca mice



Although skin transplantation in CVBA/CA mice was successful, CsA-treatment was always combined with severe weight loss and temporary weakness of the animals. As indicated in fig. 14, control animals significantly lost weight after transplantation surgery, due to CsA-treatment (fig. 14). Such reduction in body weight did not impede the life of the animals as they were selected for overweight at the beginning of the experiments. Infection accelerated changes in body weight dramatically (fig. 14B). Those mice lost up to 20 % of body weight within 14 days, indicating drastically reduced uptake of food and water due to disease. In this group, all animals died within one week after the infection. On the contrary, in animals treated with either GM-CSF or IFNγ, only a partial loss of weight was observed (fig. 14C and 14D), which was, however, further increased after infection on day 7. Nevertheless, the animals recovered within a short time and on day 21 (GM-CSF) and day 24 (IFNγ), respectively, their weight loss was compensated. All these mice survived the period of 28 days and only briefly suffered from *Salmonella* infection.

Fig. 14: Weight loss after CsA- treatment and Salmonella-infection in transplanted mice was limited by GM-CSF and IFNg

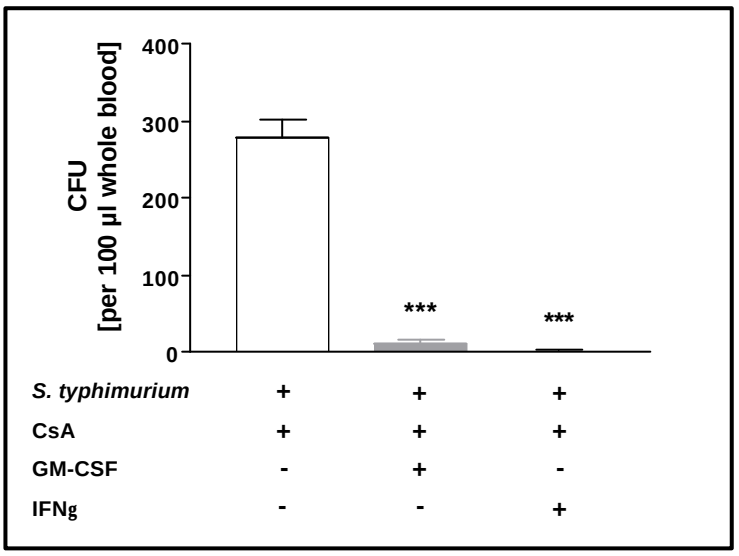


Four CBA/Ca mice per group were immunosuppressed by daily treatment with cyclosporine A (30 mg/kg; i.p.). On day 7 after transplantation, animals in (**B**), (**C**), and (**D**) received a single injection of *Salmonella typhimurium* (5 x 10^5 bacteria/kg; i.p.). GM-CSF (**C**) or IFNγ (**D**) (both 50 µg/kg; i.p.) were given daily from day 7 to day 10 while immunosuppression was continued. The graphs show body weight in [g] ± SD. Body weight was determined for CsA-treated animals (**A**), for animals infected with *S. typhimurium* (**B**), and for immunosuppressed animals, infected with *S. typhimurium* and treated with GM-CSF (**C**) or IFNγ (**D**).

3.6.1.2 GM-CSF and IFNg diminish the number of live bacteria in blood of immunosuppressed and transplanted mice

As the prevention of bacterial spreading is the most obvious sign for bacterial combat, it was investigated whether the number of bacteria in immunosuppressed and infected animals was reduced by treatment with GM-CSF or IFNγ. While in non-transplanted animals (chapter 3.4) blood, peritoneal lavage and different organs, e.g. liver and spleen, were examined for the bacterial load, in these experiments in transplanted mice CFU's were only determined in whole blood (fig. 15).

Fig. 15: GM-CSF and IFNg efficiently reduce the bacterial load in Salmonella-infected mice



Four CBA/Ca mice per group were immunosuppressed by daily treatment with cyclosporine A (30 mg/kg; i.p.). Where indicated, GM-CSF or IFNγ (both 50 µg/kg; i.p.) were given from day 7 to day 10. On day 10 (day 4 after infection with *Salmonella typhimurium* (5 x 10^5 bacteria/kg; i.p) blood was withdrawn from the tail vein and spread on agar plates after dilution in pyrogen-free saline to determine aerobic colony forming units. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple test. *** $p<0.001$ *vs*. *Salmonella* / CsA; $p < 0.05$ was considered significant.

In line with the results shown in chapter 3.4, both GM-CSF and IFNγ enabled the reduction of the bacterial load in whole blood. In mice under immunosuppression, bacteria duplicated unhindered, but both cytokines restored immune functions sufficiently for a successful combat of bacteria (fig. 15). Interestingly, although in this experimental setting the dose of CsA was 30-fold higher than in that without skin transplantation, GM-CSF as well as IFNγ still efficiently reduced the bacterial load. As all animals survived the observation period of 28 days, it seems likely that the distribution of bacteria in organs was also similar to that observed in chapter 3.4.

However, for ethical reasons, no experiments with organs from transplanted animals were performed.

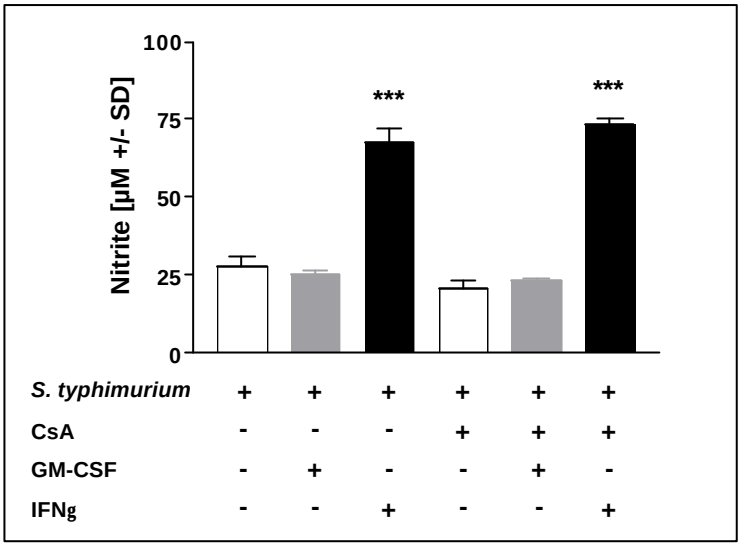
3.6.1.3 The reconstitution of immune functions: Possible mechanisms

Further investigations into possible mechanism for the reduction of bacteria in immunosuppressed and *Salmonella*-infected mice treated with GM-CSF or IFNγ were conducted. From previous work in our and other groups, it was known that both cytokines have a stimulative effect on different cells of the immune system. The most apparent effect of GM-CSF concerning changes in the immune system is the proliferation of several monocyte- and macrophage-populations. It was therefore assessed whether the amount of monocytes or macrophages was modified by the intervention with GM-CSF.

Although GM-CSF increased the number of circulating leukocytes and monocytes in naive mice, no such effect was observed in animals immunosuppressed with CsA (30 mg/kg) or Dex (10 mg/kg) when treated with 50 µg/kg GM-CSF (data not shown). As expected, treatment with IFNγ also did not induce proliferation of immune cells in immunosuppressed animals (data not shown). Therefore, reconstituted immune functions due to the application of GM-CSF do not seem not to be based on an upregulation of the number of circulating immune cells.

Furthermore, we investigated *in vitro* in a whole blood model with blood from naive and immunosuppressed mice whether IFNγ or GM-CSF increased the activity of the inducible NO synthase (iNOS) after stimulation with heat-inactivated *Salmonella typhimurium*. As NO is well known to induce oxidative stress, which among other things results in the decline of bacteria, nitrite levels were determined according to the Griess assay after incubation with or without IFNγ or GM-CSF, respectively. As shown in fig. 16, only IFNγ significantly increased the release of nitrite in blood samples of naive mice (fig. 16). Moreover, IFNγ also significantly enhanced nitrite levels in blood of CsA-treated animals. As some macrophage functions, including NO formation, are described to be unchanged in the presence of CsA [399], this result was not unexpected.

Fig. 16: IFNg, but not GM-CSF enhances the release of NO from blood macrophages when stimulated by Salmonella typhimurium



Blood from naive or immunosuppressed (CsA ; 30 mg/kg; i.p.) mice was pre-stimulated for 2 h with GM-CSF or IFNγ (10 ng/ml). 20 h after incubation with heat-inactivated *Salmonella typhimurium*, nitrite was determined in supernatants by the Griess reaction. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple test. *** $p<0.001$ *vs*. *Salmonella* controls; $p < 0.05$ was considered significant.

However, GM-CSF completely failed to alter the release of NO by macrophages, stimulated with bacteria. Oxidative stress therefore is unlikely to be a major contributor to the bacterial combat in our model. Results similar to those described above were obtained by determining the oxidative burst capacity of macrophages over a 10 h period by chemoluminescence using luminol (data not shown). While IFNγ significantly fortified the production of reactive oxygen species (ROS), GM-CSF failed.

Involvement of NO or ROS therefore cannot be the crucial mechanism involved in the immune response reconstituted by GM-CSF.

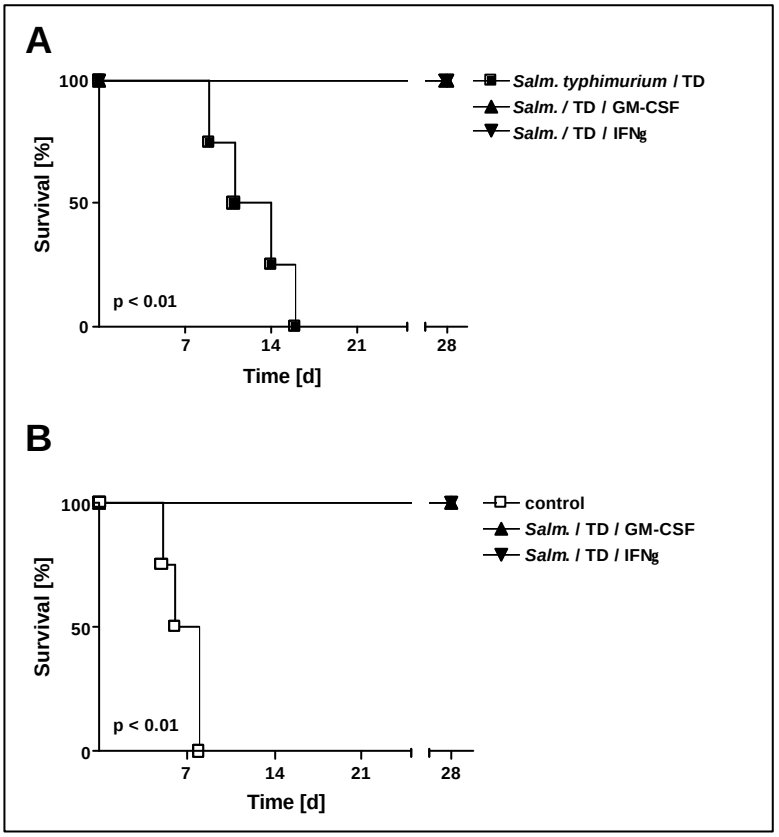
3.6.2 Immune reconstitution by GM-CSF or IFNg in a double drug strategy with tacrolimus and Dex in mice after skin allotransplantation

To ensure the validity of the results obtained with CsA another skin allotransplantation model was performed, where tacrolimus (1mg/kg; i.p.) and Dex (10 mg/kg; i.p.) were given daily for immunosuppression. As shown in chapter 3.5.2, this treatment regimen was proven to be successful in preventing rejection of skin allografts over a period of 4 weeks. Like in the CsA-experiments, only those animals assessed as having accepted the graft 7 days after transplantation were selected for infection experiments with *Salmonella typhimurium*. After injection of *S. typhimurium* (5×10^5 bacteria/kg; i.p.), immune reconstitution was induced by the application of GM-CSF or IFNγ (both 50 µg/kg; i.p.) from day 7 to day 10.

3.6.2.1 GM-CSF and IFNg enable the survival of skin-transplanted mice after infection with *Salmonella typhimurium* when immunosuppressed by tacrolimus and Dex

Fig. 17 shows that immune functions, limited by tacrolimus and Dex, can be restored by both, GM-CSF and IFNγ (fig. 17A). All these mice only suffered shortly from infectious disease, but then recovered and no animal died. Furthermore, none of the grafts in these animals was rejected within the period of 28 days (fig. 17B). When immunosuppression was missing animals again rejected grafts within 8 days after transplantation. Immunosuppressed animals not treated with cytokines again all died due to unhindered propagation of bacteria, but in comparison to CsA-treated mice, death here occured marginally later.

Fig. 17: GM-CSF and IFNg restore immune functions after immunosuppression by tacrolimus and Dex in case of infection with Salmonella typhimurium



Four CBA/Ca mice per group were immunosuppressed by daily treatment with a combination (TD) of tacrolimus (1 mg/kg; i.p.) and Dex (10 mg/kg; i.p.). On day 7 after transplantation, animals that were assessed to have accepted grafts received an injection of *Salmonella typhimurium* (5 x 10^5 bacteria/kg; i.p.). Where indicated, GM-CSF or IFNγ (both 50 µg/kg; i.p.) were given daily from day 7 to day 10 while immunosuppression was continued. Survival over the 3 following weeks (**A**) was determined for immunosuppressed animals infected with *S. typhimurium* (■), and for immunosuppressed animals infected with *S. typhimurium* and treated with GM-CSF (▲) or IFNγ (▼). (**B**) Graft survival in naive control mice (□), and in immunosuppressed and infected mice, treated with GM-CSF (▲) or IFNγ (▼) was determined over 4 weeks. Survival curves were analyzed using the *Logrank* test. $p < 0.05$ was considered significant.

However, although none of the immunosuppressed animals treated with GM-CSF or IFNγ died from infection under this treatment regimen, severe changes in body weight were again observed. Animals lost up to 20 % of body weight due to reduced uptake of food and water, which can be linked to side effects of the immunosuppressive drugs used. Resembling the CsA-experiments, animals passed through an episode of severe weight loss, which then was relieved by an episode of increasing weight. As animals were selected according to their initial weight, none of the animals was at risk of starvation.

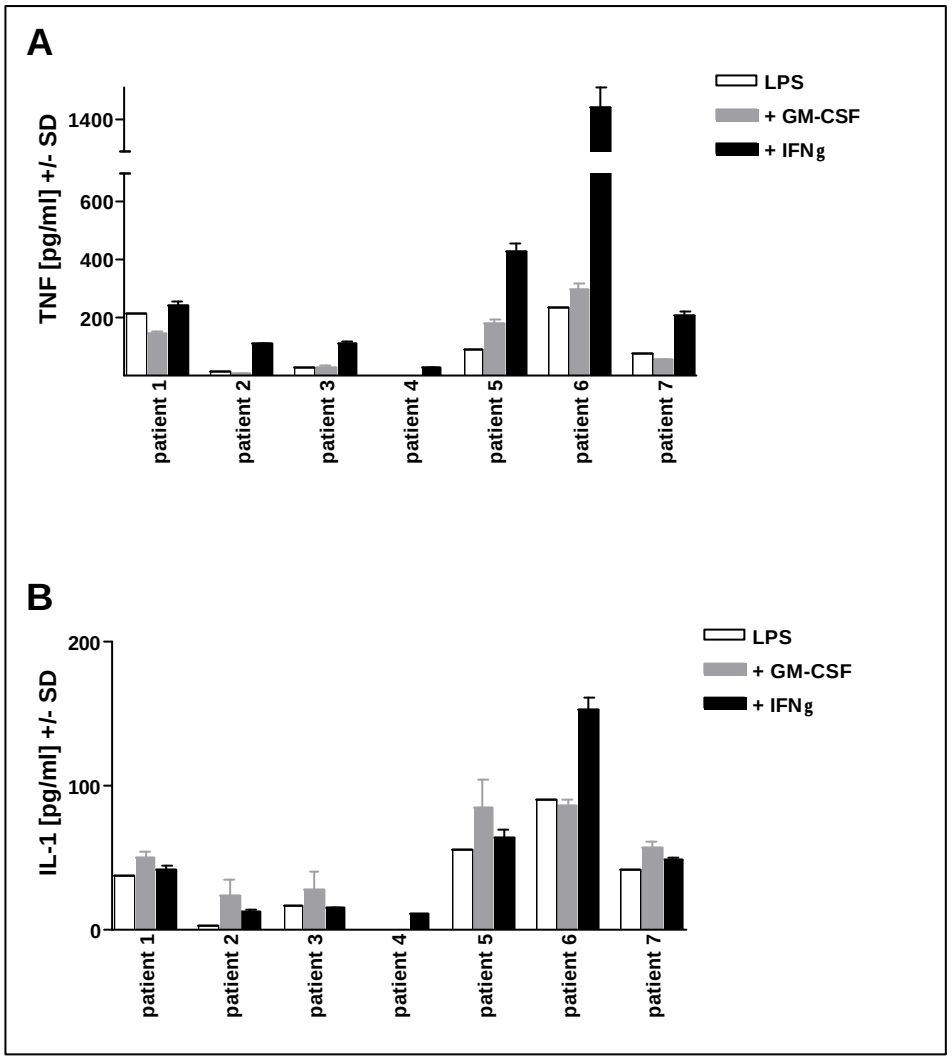
Determination of aerobic colony forming units was not performed in the experimental setting with tacrolimus and Dex, as the most convincing readout, the survival of the immunosuppressed and infected animals treated with GM-CSF or IFNγ, proves successful immune reconstitution. Withdrawal of blood was avoided, as it was invariably linked to stress for the animals and, in addition, increased the time of infectious disease by reducing the number of circulating immune cells.

These results indicate that immune reconstitution by GM-CSF and IFNγ after pharmacological suppression might be valid without risking graft rejection, not only under immunosuppression with CsA, but also when combination drug strategies with tacrolimus and Dex are employed. GM-CSF and IFNγ treatment both allow successful handling of infection by reactivation of the pharmacologically suppressed immune system without interfering skin transplant acceptance.

3.6.3 Initial studies on the applicability of these findings to humans

For initial pilot studies in humans, a whole blood incubation model was selected. Blood samples from liver transplant patients obtained from the university hospital in Mainz were incubated with endotoxin (LPS) in the presence or absence of GM-CSF (1 µg/ml) and IFNγ (1 µg/ml). While most patients were treated with a single drug strategy with different doses of the immunosuppressive drugs CsA or tacrolimus, others received combinations of CsA and MMF or tacrolimus and glucocorticoid. Treatment regimens always followed the patients' tolerability of the drugs used.

Fig. 18: GM-CSF and IFNg differentially reconstitute the macrophage response in blood of immunosuppressed liver transplant patients



Interestingly, IFNγ in human blood significantly increased the release of TNF in response to LPS while GM-CSF fortified the production of IL-1. While most patients were characterized by an effective immunosuppression, i.e. an impaired cytokine release after LPS stimulation, others responded vigourously to the stimulus without GM-CSF or IFNγ addition to the incubation. Although there is a tendency that a treatment with GM-CSF or IFNγ may be promising with respect to the reconstitution of the immune functions, these results can be considered only as a first step into this broad field. Modulation of the immune system by the two cytokines has to be examined in many more patients' blood regarding macrophage and particularly T-cell reactivation. Therefore, the animal studies conducted in this thesis yield as proof-of-principle that remains to be examined further in human studies.

4. Discussion

4.1 The possibilities and restrictions of experimental animal models for immunomodulatory studies

4.1.1 Endotoxic shock: A model for macrophage activation

Experimental endotoxic shock in animals has been recognized as a model to imitate septic shock syndrome in humans. As a consequence of Gram-negative [400] or Gram-positive [401] bacterial infection, septic shock most often results in the death of immunocompromised patients. Therefore increasing efforts to find life-saving pharmacological interventions are warranted. Chronic immunosuppressive therapy, on the one hand prevents graft rejection, but on the other enables bacterial propagation, so especially transplant patients are prone to severe bacterial infections and finally shock-related death. The endotoxic shock models offers the opportunity to examine a predominantly macrophage-driven response of the innate immune system, which is characterized by an extensive release of the pro-inflammatory cytokine TNF. While macrophage-derived TNF plays a central role in the LPS toxicity in naive mice, resulting in shock and death [402,403], immunosuppressive treatment with CsA or Dex in the experiments discussed here prevented TNF release and consequently death of the animals. The effects of immunosuppressive drugs on macrophage functions are controversial, since some groups described them to be changed [404-408] while others reported macrophage functions as unaltered under immunosuppressive treatment [399]. Here, death of the animals indicated an active immune system or a reconstitution of a previously suppressed immune response, which was successfully achieved by intervention with GM-CSF or IFNγ. In this sense, the results gained in the reactivation of the immune system with GM-CSF or IFNγ after pharmacological immunosuppression corresponded to previous results for LPS tolerance [251]. In the studies of Bundschuh *et al*. LPS in a dose of 3 mg/kg was lethal. In the present experiments, 5 mg /kg of a new lot of endotoxin were needed to induce lethality. Although the injection of the purified Gram-negative bacterial cell wall component LPS into animals induces an inflammatory response and therefore is a useful model to study the inflammatory reaction of predominantly macrophages, insights gained in this model cannot be directly extrapolated to sepsis in man. Nevertheless, it was an ideal basis to examine the possibility of macrophage reactivation by exogenous cytokines in a pre-clinical animal study.

4.1.2 ConA-induced liver failure: A model for the overactivation of T-cells

Since T-cells are the predominant executors of graft rejection (see chapter 1.2.1), they are the main targets in the development of pharmacological anti-rejection strategies in transplantation. However, pharmacological immunosuppression remains a double-edged sword as it both prevents graft rejection and increases the risk of infectious disease. Therefore it was investigated whether the two cyokines GM-CSF or IFNγ reactivate T-cell functions after immunosuppression and thus possibly affect the outcome of transplantation. The T-cell mitogen ConA [409] was used as T-cell stimulus. ConA has already been used in numerous studies although its mechanism of action still remains unclear. Although macrophages are not directly stimulated by ConA, they contribute to ConA induced hepatitis *in vivo*. Probably, ConA predominantly activates lymphocytes, which in turn induce macrophages to release pro-inflammatory mediators such as TNF [381,410]. These findings are supported by *in vitro* studies, where ConA activated T-cells and cells in a macrophage-lymphocyte coculture, but not macrophages alone [410]. Readout for the activity of T-cells in this study was the increased release of pro-inflammatory lymphokines, e.g. IL-2 and IFNγ and augmented transaminase levels in blood plasma of ConA-treated animals. Mice injected with ConA died after 8 h of exposure due to fulminant liver damage. The cytokines GM-CSF and IFNγ have been reported to sensitize the liver directly towards TNF [381]. However, when animals were pretreated with immunosuppressive drugs, e.g. Dex, CsA or tacrolimus, liver failure due to missing T-cell activation was prevented [381].
Certainly, the immune response to ConA is different from the immunological events that occur in infection. But the ConA model here was used only to examine in detail whether the stimulative cytokines GM-CSF and IFNγ could reconstitute an immune response in immunosuppressed animals.

4.1.3 The infection model with *Salmonella typhimurium*

The primary goal of this study was to investigate whether the cytokines GM-CSF and IFNγ can increase the impaired host resistance of immunosuppressed transplanted animals against bacterial infections. This question is, as already outlined above, very substantial, since a high percentage of immunosuppressed patients are predisposed to the development of cancer, and especially to infections of all kinds [3]. Since *Salmonella* infection is represented in transplant patients in clinical reality [385], experiments were performed in a murine infection model with live *Salmonella typhimurium*. In this model the existence of the *Salmonella typhimurium* resistant CBA/Ca mouse strain was a great advantage [398]. While naive mice survived a high dose of *Salmonella typhimurium* without symp-

toms of disease, the administration of immunosuppressive drugs [386,398] abrogated *Salmonella* resistance of CBA/Ca mice which finally resulted in the death of the animals. Therefore, the simple readout for the activity or the suppression of the immune system in CBA/Ca mice was either survival or death of the animals, respectively. Furthermore, the assessment of the number of aerobic colony forming units from blood or different organs was a simple method to determine the propagation of live bacteria in the infected mice.

Salmonella typhimurium is regarded as a facultative intracellular bacterial pathogen that is found inside macrophages [411], especially of the reticuloendothelial system, e.g. of liver and spleen [412]. Thus, macrophages play a major role in the pathology of virulent *Salmonella typhimurium* infection and may consequently present a novel target for therapeutic interventions. While some years ago only macrophages were considered to be the key type of antigen presenting cells (APC) to combat bacterial infections by phagocytosing and destroying bacteria and presenting bacteria-derived antigens to T-cells, now also dendritic cells (DC) are discussed to be an important type of APC that contribute to the immune response to *Salmonella* [413]. Furthermore, the importance of macrophage-derived reactive nitrogen and oxygen intermediates to the defense of the murine host against *Salmonella* was shown to be greater than previously appreciated. In addition to direct microbial actions, those intermediates have relevant immunoregulatory effects relevant to the control of infection [414]. Importantly, IFNγ in several publications was demonstrated to be an activator of the anti-bacterial activity of macrophages and therefore represents a powerful actor in the first line of anti-*Salmonella* defense [415,416]. In line with these findings, mice genetically deficient in immunity mediated by IFNγ and also IL-12 are highly susceptible to *Salmonella*. Thus, impaired secretion of IFNγ accounts for uncontrolled growth of bacteria within macrophages [416]. However, since infections of the respiratory tract [417-419], by Gram-positive bacteria [420-422] or viral infections [423,424] are more often responsible for hospital-acquired infection, *Salmonella* infection does not represent the main clinical problem.

Furthermore, the current standard therapy with antibiotics was omitted in all animal experiments which, although necessary for pre-clinical research, cannot be extrapolated to the clinical situation in humans.

4.1.4 Comparison of *in vivo* and *ex vivo* experiments

Although the *in vivo* animal models reflect the natural process of the inflammatory response and the complex interactions between mediators and organs more precisely, isolated steps in the inflamma-

tory response can only be thoroughly examined in detail *in vitro*. One major disadvantage of *in vivo* studies is the extreme complexity of processes taking place within the animal. Mechanistic *in vitro* studies that provide insight into single steps of the immune cascade therefore were performed in various primary cell populations of naive or immunosuppressed animals. For the present study, especially *ex vivo* systems were used to determine the effects of either LPS or ConA in naive or pharmacologically suppressed macrophages or T-cells, respectively. As expected, in naive cells LPS particularly stimulated macrophage populations, while ConA in T-cells elevated the release of cytokines such as IL-2 and IFNγ. In addition, pretreatment with the immunosuppressive drugs prevented the release of cytokines in macrophages as well as in T-cells. Since the focus was on the potential of the pro-inflammatory cytokines GM-CSF and IFNγ to reconstitute such a pharmacologically suppressed immune response, immunosuppressed cells were used for the determination of cytokine levels under these circumstances. Readout for a successful reactivation was the release of cytokines such as TNF or IL-2 and IFNγ. All the *ex vivo* experiments confirmed the finding *in vivo*. Both cytokines enabled a reconstitution of only the macrophage response, while the T-cells remained silenced. For our hypothesis this precise answer to the question, which cell population can be reactivated was needed to predict the chance of success of such an intervention in transplanted animals without increasing the risk of graft rejection.

4.2 Reasons for choosing the skin transplantation model in mice

Since the detailed description of skin transplantation by Billingham and Medawar in 1951, this model has been widely used to study the T-cell mediated immune response of allograft rejection [425,426]. The surgical procedure for this non-vascularized transplant model is, in contrast to other, vascularized transplantation models, e.g. of murine liver or heart, easy to learn and does not need microsurgical equipment. Furthermore, the mouse model gains from the existence of a variety of mouse strains [427] and several specific tools e.g. for the determination of cytokine release, thus enabling to observe an immune response. However, skin transplantation has several pitfalls in the handling procedure. First, an appropriate place for the graft bed, preferably on the cranial part of the back has to be selected which guarantees optimal growth of the graft [428]. Second, any damage of the panniculus carnosus, a thin and transparent layer on the dorsal muscle containing the vessels that reestablishes the blood supply to the graft, must be avoided. Third, attention must be paid that the edges of the graft are not everted. And fourth, the bandage is the key factor to successful skin transplantation and pro-

tects the graft. It must be applied properly, i.e. tight enough to remain in place but free enough to allow the animal to breathe and move unhindered. Acute skin graft rejection is a striking and reproducible immunological reaction, characterized by a typical course of morphological changes. It begins with an erythema and a slight swelling, followed by desiccation and scar formation, and ends with a scar in the former graft bed. The time point of rejection is defined by most transplant surgeons as the complete necrosis of the graft [429]. However, the time point of sub-acute or chronic rejection is sometimes difficult to determine, because its features, such as loss of hair and pigmentation as well as obliteration of the dermal ridges, are less striking. Moreover, loss of hair cannot be prevented, since the hair follicles loose innervation and consequently necrotize.

Although microsurgical models of vascularized organ transplantation in mice have more relevance to the clinical situation, skin transplantation is still useful for studies on immunological mechanisms of allograft rejection. In transplant research, this model is widely accepted, e.g. in studies of T-cell mediated immune responses of acute allograft rejection [426] or in testing allograft recipients for tolerance [430]. Furthermore, skin transplantation entails several additional limitations due to tissue-specific characteristics, since skin is highly immunogenic because of a large amount of dermal antigen-presenting Langerhans cells [431-433]. Therefore immunosuppressed recipients may accept vascularized organ grafts while skin grafts are rejected [434]. A detailed overview of skin transplantation in mice is given in the review of Gardner [435]. Taken together, skin transplantation is the most sensitive model concerning T-cell mediated graft rejection. Thus it therefore fits perfectly to realize immunomodulatory studies such as in the present work.

4.3 Immunosuppressive treatment

Advances in immunosuppression have had a significant impact on the field of whole-organ transplantation. New chemical agents such as CsA, tacrolimus, sirolimus and MMF which utilize novel mechanisms have been developed, resulting in prolonged survival times [436-438]. Despite the improved efficacy of these new immunosuppressive agents, host susceptibility to infection, malignancy [439,440] and organ-specific toxicity [172,441,442] are still serious constraints. Therefore, many experts believe that the induction of tolerance will be the future of organ transplantation, since this would make pharmacological immunosuppression superfluous [443-446]. However, unfortunately, the currently available animal models for tolerance are unsatisfactory [447]. Therefore a model was estab-

lished in which, by the use of GM-CSF and IFNγ, the immune response solely of macrophages immunosuppressed by pharmacological agents was selectively reactivated. Interestingly, the reconstituted immune response resulted in a controlled restraint of bacteria in *Salmonella*-resistant CBA/Ca mice. Furthermore, since in this model the T-cell response remained silenced, skin graft acceptance was not affected. For the induction of immunosuppression clinically relevant drugs such as glucocorticoids, CsA, tacrolimus and MMF were selected. Furthermore, sirolimus was used in the present experiments, since this drug will probably enter the clinic in the near future.

4.3.1 The dosage problems of immunosuppressants in the different models investigated

In first experiments it was determined what dosages of different immunosuppressive drugs are needed in the endotoxic shock or the ConA models to suppress the function of macrophages or T-cells, respectively. Initially, a low dose was selected to ensure the anticipated restoration of the cytokine response after GM-CSF or IFNγ treatment would have physiological consequences. The minimal effective dosage of CsA and also Dex which inhibited cytokine release from macrophages in the endotoxic shock model was found to be 5 µg/kg (chapter 3.1). In contrast, dose-finding experiments in the ConA model exhibited that here a dosage of 1 mg/kg of either Dex, CsA, tacrolimus and sirolimus was needed to prevent T-cell activity (chapter 3.3.1). Although all immunosuppressive drugs except Dex utilized as primar target the activity of T-cells, the finding that the inhibition of T-cell functions required a higher dosage than the suppression of macrophages was surprising. The reason can probably be found in the difference between the two models. Since the intraperitoneal injection of LPS works systematically, the intravenous application of ConA directly targets the mouse liver. Although the doses of either LPS and ConA in both models were selected high enough to induce lethality, ConA in a dose of 25 mg/kg seemed to be the stronger stimulus and thus needed higher immunosuppressive doses.

Immunosuppression in the transplantation models needed significantly higher doses of all immunosuppressive drugs used. To assure graft acceptance, a 6000-fold higher dose of CsA in contrast to the LPS experiments and a 30-fold higher dose, when compared to the ConA experiments was required (chapter 3.5). Immunosuppression by CsA in our studies had to be induced by a daily injection of 30 mg/kg. Since both, the macrophage and the T-cell response were already suppressed by lower doses, transplantation required something like a “safety zone“ to prevent skin graft rejection. Immunosuppression by tacrolimus monotherapy in contrast to CsA failed. Although tacrolimus in

monotherapy has been described to prevent skin graft rejection [448] only combination therapy with either Dex or MMF was successful here. Finally, the required doses in the transplanted animals resembled immunosuppressive doses used in transplantation reality and therefore may improve the clinical significance of the present study.

4.3.2 The potency of the immunosuppressants used to inhibit macrophage and T-cell functions

The modes of action of the different immunosuppressive drugs used were described in chapter 1.3. As outlined there, most of these drugs target the activity of T-cells since they are the primary mediators of graft rejection. Besides, macrophages participate in the initiation and propagation of rejection. While Dex is a well known suppressor of macrophage activity [70,72-74], the role of CsA concerning the inhibition of macrophage functions is discussed controversially. In the present experiments macrophage activity was suppressed after treatment with CsA *in vivo* as well as *in vitro* (chapters 3.2.1 and 3.2.2). Several reports from different groups agree with these findings [404-408,449] while others reported that macrophage functions remain suppressed [399]. Macrophage suppression with tacrolimus, sirolimus and MMF completely failed (chapter 3.1), indicating that these immuosuppressive drugs solely affect T- and B-cell functions [104,107,174,176]. On the contrary Dex, CsA, tacrolimus and sirolimus prevented T-cell activation in the ConA model (chapter 3.3.1). However, MMF did not show any suppressive effect an T-cells, probably due to its low potency and its effective inactivation [143].

4.3.3 The usefulness of CsA, sirolimus and the combination of tacrolimus with either Dex or MMF in skin allotransplantation

After it was shown that immunosuppressed macrophage, but not T-cell functions could be reconstituted by GM-CSF or IFNγ, it was next examined whether the reactivation also occurs in immunosuppressed mice after infection with *Salmonella typhimurium*. The skin transplantation model described in section 4.2 offered an ideal possibility to investigate if such immune reconstitution allows the control of a bacterial infection without affecting graft acceptance. In a first step, the immunosuppressive effects of the immunosuppressants CsA, tacrolimus and sirolimus were investigated in transplantation experiments. Starting with CsA, the doses required to assure graft acceptance were much higher than the ones used in the orientating LPS and ConA experiments (chapter 3.5.1). Successful

skin transplantation asked for a 6000-fold higher dose than macrophage inhibition in the LPS studies and a 30-fold higher dose than T-cell inactivation in the ConA model. However, severe side effects [125,184,186] resulting in the death of the animals within the duration of the experiment consequently restricted the immunosuppressive doses for all drugs used. Since CsA in mice only partially inhibits calcineurin activity [450], a phenomenon that is also found in humans [451], the dose had to be selected very high. Finally, CsA was used in a dose of 30 mg/kg which prevented graft rejection but did not induce severe side effects (chapter 3.5.1). Since the simple transfer of the monotherapy scheme to tacrolimus failed, a combination therapy with tacrolimus plus Dex or MMF was performed (chapter 3.5.2). Although both, CsA and tacrolimus inhibit calcineurin [106-109] there somehow seem to be significant differences in their modes of action. Tacrolimus which in different models of organ [105,452] and skin transplantation [437,453] was described to be more potent than CsA [158] could not prevent graft rejection in our skin allotransplantation model. These finding are in contrast to Lagodzinski *et al*. [448] where tacrolimus in monotherapy was described to prevent skin graft rejection. Promising results were obtained when tacrolimus was combined with either Dex or MMF. The corticosteroid Dex and the purine synthesis inhibitor MMF may compensate the lacking immunosuppressive activity of tacrolimus. One possible mechanism resulting in better graft survival, at least for MMF, may be the increased re-vascularization of the graft [454] which is reduced in the presence of tacrolimus [113,455]. Furthermore, both MMF [456] and Dex [70,71,457] were described to inhibit macrophage activity which thus may play a more pronounced role in skin transplantation than expected. In line with our results improvement in transplantation was achieved by combination therapy [194,454,458]. Such improvement may be based on synergy effects of immunosuppressive drugs which reduce required doses and consequently toxic effects, but also on the greater immunosuppressive potential due to effects on different target cells. However, monotherapy with sirolimus showed the best outcome of skin transplantation in our experiments but was also linked to severe toxicity, i.e. ascites. Since almost all animals characterized by little weight severely suffered from sirolimus toxicity for further experiments only extremely heavy animals with a high percentage of body fat were chosen. But these animals also regularly developed ascites and were therefore excluded from the ongoing experiments. Although no combination therapy studies were performed to reduce the dosage of sirolimus, it seems likely that extraordinarily toxicity [184,186] of that drug due to the dose required is responsible for the high percentage of side effects.

4.4 Relevant mediators of immune reconstitution in immunocompromised animals: GM-CSF and IFNg

The therapeutic goal of the treatment of infection in immunocompromised transplant patients is defined as the reconstitution of the non-specific immune system without risking graft rejection. Since previous work done in our group and the work of other groups revealed that the two cytokines GM-CSF [251,253,254] and IFNγ [459-462] enhance the LPS-induced release of cytokines from macrophages, they were chosen as immunostimulatory cytokines for the present studies. Bundschuh *et al*. showed that both cytokines have the potential to reactivate immunoparalysed macrophages [251]. Therefore, it was tested whether these two pro-inflammatory mediators likewise have the ability to restore a pharmacologically suppressed immune response as it occurs in transplantation.

4.4.1 The potential of GM-CSF and IFNg to reconstitute the pharmacologically suppressed macrophage response after LPS stimulation

In the first preclinical murine model the potential of GM-CSF and IFNγ to reactivate macrophage functions suppressed by the immunosuppressive drugs Dex and CsA was examined. *In vivo* and *in vitro* results clearly revealed that both cytokines have the capacity to restore the suppressed immune functions of macrophages. After LPS stimulation, all naive and all immunosuppressed animals treated with GM-CSF or IFNγ died within a comparable period of time, indicating that the suppressed immune system was fully reconstituted by these two cytokines (chapter 3.2.1). The reconstitution of TNF release capacity underlines these findings. In line with Bundschuh *et al*., the results suggest that IFNγ might be slightly more potent than GM-CSF: IFNγ reconstituted TNF levels in all animals to a degree that was similar to the TNF release found in naive mice after LPS stimulation, while TNF release in GM-CSF treated animals remained below that level.

To address whether the effects found *in vivo* can be definitely explained by macrophage activity, *ex vivo* experiments were performed with different monocyte/macrophage populations from the peritoneum, blood, lung, spleen and bone marrow of immunosuppressed mice (chapter 3.2.2). The findings confirm the significant suppressive activity of Dex as well as CsA on LPS-stimulated macrophages. Interestingly, the immunosuppressive effect of Dex and CsA in peritoneal macrophages and bone marrow cells in terms of TNF release was poorer than observed in the other populations. Furthermore, it could be shown that the immunomodulators GM-CSF and IFNγ can restore the diminished LPS responsiveness of macrophages from immunosuppressed mice. In almost all mono-

cyte/macrophage populations tested, GM-CSF and IFNγ increased the LPS-induced TNF release of pharmacologically suppressed cells at least to the level of naive cells or even higher.

4.4.2 No reconstitution of the pharmacologically suppressed T-cell response by GM-CSF and IFNg

GM-CSF and IFNγ both restored the impaired monocyte/macrophage response of immunosuppressed mice towards a lethal LPS challenge (chapter 3.2). Since T-cells are responsible for the specific immune response and furthermore represent the primary cell type responsible for graft rejection, it was further examined whether a reconstitution by GM-CSF and IFNγ also occurs in these cells.

Comparable to the LPS studies, experiments with the T-cell mitogen ConA in this setting were performed *in vivo* as well as *in vitro*. The *in vivo* results unambiguously show that as in previous studies [381] Dex, CsA, tacrolimus and sirolimus prevented ConA-induced severe liver damage and consequently protected from death (chapter 3.3.1). The determination of T-cell-derived pro-inflammatory cytokines such as IL-2 and IFNγ in immunosuppressed animals gave evidence that T-cells were completely inhibited by this pharmacological intervention [463] (chapter 3.3.2). When immunosuppressed and naive animals were additionally treated with GM-CSF or IFNγ, only a marginal effect of these two cytokines on the transaminase release due to liver injury was observed (chapter 3.3.2). When compared to the results obtained in monocytes/macrophages, neither GM-CSF nor IFNγ had a priming effect on T-cells. While no direct stimulation of T-cells is described for GM-CSF, IFNγ is considered a prominent regulator of T-cell activity [464,465]. Moreover, since the activation of T-cells in the ConA model depends on macrophages [381], the slight activating effect observed remains unclear at least in naive cells. Taken together, neither GM-CSF nor IFNγ *in vivo* restored the pharmacological suppression by Dex, CsA, tacrolimus or sirolimus with respect to the release of the T-cell-derived pro-inflammatory cytokines IL-2 and IFNγ. These findings were supported by *in vitro* experiments, where no IL-2 or IFNγ release was detected after the incubation with ConA in naive or suppressed splenic T-cells (chapter 3.3.3). Since animals and samples where IFNγ was added to stimulate the immune response of T-cells would have displayed falsely high IFN γ levels due to the intervention, this cytokines were not mesured in tables 4 and 5.

The results indicate that the two cytokines GM-CSF and IFNγ, used as immunomodulators in this study, can on the one hand restore the non-specific immune response by macrophages, but on the

other hand do not reconstitute the specific immune system, represented by T-cells. Finally, the obvious questions, whether such immune reconstitution is sufficient to combat bacterial infection, was examined.

4.4.3 Selective reactivation of only the non-specific immune system combats bacterial infection

As outlined in sections 4.3.1 and 4.3.2, the two cytokines GM-CSF and IFNγ, used as immunomodulators in the present work, restored a pharmacologically suppressed monocyte/macrophage but not a T-cell response. To examine whether the reactivation of the non-specific immune system represented by macophages is sufficient for bacterial combat, experiments were perforemed with *Salmonella*-resistant CBA/Ca mice [398]. These mice loose their resistance to infection when treated with immunosuppressive corticosteroids and were therefore perfectly suited for this study.

In first experiments, naive mice were injected with a high dose of *Salmonella typhimurium* to the capacity for resistance. Further it was tested whether Dex and CsA as immunosuppressive drugs could overcome *Salmonella*-resistance. Readout in these two experiments was survival in *Salmonella*-resistant but lethality in immunosuppressed and consequently *Salmonella*-susceptible animals. The *in vivo* results indicated that GM-CSF as well as IFNγ can restore the suppressed immune response as the death of the animals due to *Salmonella*-infection was consequently prevented (chapter 3.4). Moreover, GM-CSF and IFNγ enabled the significant reduction of live bacteria in liver, spleen, blood and peritoneum in comparison to immunosuppressed animals not treated with GM-CSf or IFNγ. However, in the Dex experiments one animal out of six with either IFNγ or GM-CSF treatment died 8 or 11 days, respectively, after the infection. Although the cadavers did not exhibit findings of *Salmonella*-infection, it cannot be excluded that these animals died due to infectious disease. Furthermore, the animals might have suffered from sied effects from GM-CSF or IFN γ treatment thus resulting in death. An indication that GM-CSF accelerates the recovery from infection by *Salmonella typhimurium* in resistant A/J, but not in susceptible C57bl/6 mice was given by Freund *et al*. [265]. Furthermore, GM-CSF as well as IFNγ have been found to protect mice against Sendai virus infection [466] emphasizing the immunomodulatory profile of these cytokines even in viral infections.

Although the participation of T-cells in the executive part of a bacterial combat is discussed in several publications [467-469] these findings suggest that the activation of only the non-specific immune system is sufficient for the survival of bacterial sepsis. Antimicrobial activity of macrophages was re-

cently described from Vasquez-Torres *et al.* [470]. The authors reported that in general the antibacterial activity of macrophages is sufficient to overcome a *Salmonella typhimurium* infection *in vivo* [471] as well as *in vitro* [470] and that pretreatment with IFNγ increased bacterial killing due to the generation of reactive oxidative and nitrosative species [470]. Since macrophages are the predominant target cells for *Salmonella*-infection [411,413], they play an important role in the first line of bacterial defense. In this respect, reactive nitrogen intermediates [414, 472], pro-inflammatory cytokines such as GM-CSF [415] or IFNγ [415,416,473] and changes in the gene expression profile of macrophages [391] are outlined as significant anti-bacterial mechanisms. However, in studies with stimulated whole blood only an increased release of NO inthe presence of IFNγ but not of GM-CSF was found (chapter 3.6.1.3). In addition, neither GM-CSF nor IFNγ increased the counts of circulating leukocytes and monocytes in immunosuppressed animals. Thus, the involvement of reactive oxygen species might be the crucial mechanism involved in the immune response reconstituted by IFNγ. On the contrary, there was no indication that reactive oxygen species and the proliferation of immune cells are implicated in the immune reconstitution process by GM-CSF. Therefore GM-CSF must activate another, maybe yet unknown pathway responsible for the successful reduction of bacteria. Besides, immunosuppression after *Salmonella*-infection due to massive NO release was shown by MacFarlane and coworkers [474]. These findings are in line with experiments that were performed in *Salmonella*-sensitive Balb/c mice. In these mice, after the infection with *Salmonella typhimurium* a massive production of NO was detected, which was further increased when the animals were pretreated with IFNγ.

The two cytokines GM-CSF and IFNγ are approved for indications other than those investigated here [281,472,475,476]. In the present work they have proven the potential to reconstitute a pharmacologically suppressed non-specific immune response while the specific immune system remained silenced. Such an immune reconstitution process enabled immunosuppressed laboratory animals to survive an otherwise lethal bacterial infection with *Salmonella typhimurium*. Since immunosuppression generally is linked to transplantation the results led to the further question whether GM-CSF and IFNγ may also help transplanted animals to overcome such a bacterial infection without risking graft rejection.

4.5 The use of GM-CSF and IFNg in immunocompromised and transplanted mice

The obvious question was whether GM-CSF and IFNγ, by reactivating the non-specific immune system, affect the outcome of transplantation. For several reasons (chapter 4.2) a murine skin allotransplantation model was selected to test the effects of both cytokines on graft survival. After successful monotherapy studies with CsA and sirolimus, it was found that tacrolimus can not be used as single drug therapy (for discussion see chapter 4.4.3). Immunosuppressive drugs had to be administered in significantly higher doses than in the experiments before to assure graft survival. The doses used in transplant studies better correlated with those utilized in clinical reality.

The observation that the specific immune system and thus T- and B-cell activity, which is responsible for graft rejection, remained silenced after the intervention by GM-CSF and IFNγ, permitted the hypothesis that transplanted animals might survive bacterial infection with GM-CSF ir IFNγ therapy without loosing their grafts. If T-cells were reactivated by GM-CSF or IFNγ, and if consequently the main mediators in graft rejection, IL-2 [32,66,104,477-482] and IFNγ [32,479,483], promoting clonal expansion of T-cells [484,485] were released, this would lead into a vicious cycle ending in graft rejection. Since in the ConA model neither IL-2 nor IFNγ were detectable, it seemed that such an interaction in the transplant model with GM-CSF and IFNγ would be unlikely. However, the risk of graft rejection still remained since these two cytokines are known inducers of MHC molecules which play an important role in the recognition of foreign tissue and finally graft rejection [486-491]. Nevertheless, one report did not link IFNγ to graft rejection processes [478]. Another recently discovered mediator of graft rejection which shares high similarities to IL-2 [492] is IL-15 [477,482,493-495]. Although due to the unavailability of appropriate tools IL-15 was not taken into account in the present study, future studies should also consider this cytokine since it might replace IL-2 in the activation of T-cell [496,497].

4.5.1 Effects of GM-CSF and IFNg on graft survival

Before infection experiments, it was examined whether GM-CSF and IFNγ affect the survival of skin allografts in immuosuppressed CBA/Ca and Balb/c mice. While all naive animals lost their grafts within one week, immunosuppressed animals accepted skin grafts for the observation period of 4 weeks. Furthermore, the intervention with GM-CSF and IFNγ did not increase the risk of graft re-

jection since all animals kept their graft for the 4 weeks (chapter 3.5.4). GM-CSF seemed to inhibit the immune reactions in skin since it prevented the release of IL-12 from Langerhans cells which are implicated in graft rejection [498] (chapter 4.2).

Neither GM-CSF nor IFNγ seemed to conflict with the outcome of skin allotransplantation in CBA/Ca and Balb/c mice. Two reports speculated that MHC molecules were not essential mediators of skin graft rejection because MHC class I deficiency in mice also could not prevent rejection [499,500]. Assessment of the grafts was performed by macroscopical and histological examination. Although in literature, macroscopical scoring is the preferred method to describe graft rejection [172,501-503] histological slides of macroscopically accepted grafts were additionally examined. This method allows the evaluation of the level of revascularization of the graft and determination of morphological changes within the graft. Vascularization of the graft is one major event in the acceptance in skin transplants. If skin grafts were not connected to the recipients blood system, they necrotize within a very short time. Although several peri-vascular lymphocytes were found in the grafts when examinedhistologically, these samples were not considered rejected, because their macroscopical acceptance was perfect. On the other hand, it cannot be excluded that rejection of skin allografts at a later time point migt have occured. However, for ethical reasons the experiments were terminated after four weeks.

4.5.2 Effects of GM-CSF and IFNg on bacterial combat in transplanted mice

In immunosuppressed animals GM-CSF and IFNγ were shown to be successful in the reconstitution of the immune response, thus enabling the regulation of bacteria in case of infection (chapter 3.4). Furthermore, in a skin allotransplantation model, these two cytokines did not decrease the success in graft acceptance (chapter 3.5). Consequently it was examined whether effective reconstitution of the immune response was also possible in immunosuppressed and skin-transplanted animals that were infected with *Salmonella typhimurium*. For immunosuppression CsA and a multidrug scheme with tacrolimus and Dex were selected for these experiments.

The studies definitely showed that GM-CSF and IFNγ could reactivate the non-specific immune response against *Salmonella typhimurium* in immunosuppressed and transplanted animals without risking graft rejection (chapter 3.6). Those findings are in line with observations in children after liver transplantation. Here GM-CSF treatment was well tolerated and did not induce graft rejection [504]. Bacterial propagation in immunosuppressed mice treated with the two cytokines again was signifi-

cantly decreased in contrast to immunocompromised animals. However, once more one out of four animals died on day 7 after skin transplantation and immunosuppression by CsA. Since in this experimental setting both, the infection with *Salmonella typhimurium* and the IFNγ intervention occurred on day 7, we believe that the animal might have suffered from an infection or other restrictions due to transplantation surgery. Since pain, injury, suffering and distress in the laboratory animals were minimized, several criteria for the termination of experiment were defined. Besides the lack of mobility or of normal interest in the surroundings, apathy or signs of paralysis, severe weight loss was the most conspicuous change within the experimental period. Since immobility and apathy disappeared a short time after the transplantation procedure, animals characterized by a body weight loss of more than 20 % of the initial weight were excluded from the experiments. However, in contrast to immunosuppressed animals, mice with a reconstituted immune system completely recovered within the period of the experiment due to GM-CSF or IFNγ treatment.

To compare the results obtained in the present animal study with human cells of the immune system, initial pilot experiments were performed with blood from liver transplant patients which were treated with CsA and tacrolimus. First results show that immune reconstitution of macrophages is also possible in human blood cells. Interestingly, IFNγ elevated TNF release of LPS-stimulated macrophages, while GM-CSF increased the release of IL-1. Other studies, performed in our lab with human PBMC that were immunosuppressed with Dex in an *ex vivo* model revealed that reconstitution of the immune response by GM-CSF is possible. While Dex-treated PBMC remained silenced after stimulation by LPS, pre-treatment with GM-CSF induced the release of TNF also in this model (personal communication). However, comprehensive data from other groups which could confirm or defeat our results are missing.

Taken together, this preclinical animal study is not only of general interest for the understanding of the consequences of immune suppression or reactivation on overcoming bacterial sepsis, but also has major clinical implications. In clinical practice immunosuppressive treatment is restricted in critically ill patients when viral or bacterial infections are suspected. The fact that in these experiments transplanted mice treated with GM-CSF or IFNγ survived a lethal *Salmonella typhimurium* infection, suggests that clinical studies on the efficacy of these two cytokines as an instant intervention to reactivate the compromized host defense of these patients without risking graft rejection should be performed.

5. Summary

1. *In vivo* and *ex vivo* cyclosporine A (CsA) and dexamethasone (Dex) prevented LPS-induced TNF release from macrophages and consequently protected Balb/c mice from endotoxic shock. Tacrolimus and MMF failed to protect animals against lethal LPS shock.
2. GM-CSF and IFNγ restored susceptibility towards endotoxic shock in Dex and CsA-treated Balb/c mice.
3. *In vivo* and *ex vivo* CsA, Dex, tacrolimus and sirolimus prevented ConA-induced liver failure and consequently protected Balb/c mice from death. MMF failed to protect animals against severe liver injury.
4. GM-CSF and IFNγ failed to reconstitute the immune functions of pharmacologically suppressed T-cells *in vivo* and *ex vivo*.
5. Immunosuppression increased susceptibility towards lethal bacterial infection in *Salmonella*-resistant CBA/Ca mice.
6. The immune response of CBA/Ca mice against *Salmonella typhimurium* infection was reconstituted by GM-CSF and IFNγ in transplanted and non-transplanted animals and allowed survival.
7. GM-CSF and IFNγ did not promote skin graft rejection in CBA/Ca mice.

6. Deutsche Zusammenfassung

Die Entdeckung und Weiterentwicklung von immunsuppressiven Medikamenten ermöglichte es, die Gefahr der Transplantatabstossung auf ein Minimum zu verringern. Diese Pharmaka bewirken durch eine gezielte Inaktivierung von primär T-Zellen, welche für die Abstossungsreaktion verantwortlich sind, dass fremdes Gewebe nicht als solches erkannt und somit im Folgenden nicht aktiv bekämpft wird. Während die Suppression des Immunsystems seitens der Transplantatakzeptanz unerlässlich ist, birgt sie jedoch auf der anderen Seite auch die Gefahr für Transplantationspatienten, an einer Infektion oder an Krebs zu erkranken und ggf. daran zu sterben. Erkrankungen bakteriellen, viralen oder parasitären Ursprungs bilden eine der Hauptursachen für den Tod von Transplantationspatienten, und sind daher eines der ernsten Probleme bei Eingriffen dieser Art.

Eine Möglichkeit, dieses Problem zu lösen, besteht in einer kurzzeitigen und kontrollierten Reaktivierung des unspezifischen Immunsystems, d.h. von Monozyten und Makrophagen. Im Gegensatz zu Makrophagen sollten T-Zellen, die zentralen Mediatoren der Abstossungsreaktion, weiterhin supprimiert bleiben, um das Transplantationsergebnis weiterhin zu sichern. Im Falle eine Infektes sollen Makrophagen und Monozyten die Abwehrreaktion des Körpers stärken und eine infektiöse Erkrankung erfolgreich bekämpfen. Der Granulocyten/Makrophagen Kolonie-stimulierenden Faktor (GM-CSF) und Interferon-gamma (IFNγ) sind zwei Zytokine, die bei der natürlichen Entzündungsreaktion eine wichtige pro-inflammatorische Rolle spielen und zu einer verstärkten Abwehrreaktion führen. Deshalb wurden GM-CSF und IFNγ eingesetzt, um zu untersuchen, ob sie zu einer Reaktivierung eines pharmakologisch supprimierten Immunsystems beitragen können. Als Immunsuppressiva wurden hierzu handelsübliche Präparate, wie Dexamethason (Dexa-Allvoran®), Cyclosporin A (Sandimmun®), Tacrolimus (Prograf®), Sirolimus (SDZ-RAD) und Mycophenolat mofetil (CellCept®) herangezogen, die teilweise auch in der Klinik bei Transplantationen eingesetzt werden.

Einleitende Versuche wurden in den Modellen des Endotoxin-Schocks und der ConA-induzierten Leberschädigung durchgeführt. Diese beiden Modelle einer “sterilen Sepsis”, in denen Versuchstiere mit Endotoxin (Lipopolysaccharid; LPS), bzw. Concanavalin A (ConA) behandelt werden, sind durch eine deutliche Aktivierung des Immunsystems, verbunden mit einer stark erhöhten Ausschüttung an pro-inflammatorischen Zytokinen charakterisiert. In beiden Modellen kann durch eine vorhergehende Behandlung mit immunsuppressiven Präparaten die Freisetzung der Zytokine und, damit verbunden, der Tod der Tiere verhindert werden. In den folgenden Versuchen sollte nun untersucht

werden, ob durch GM-CSF oder IFNγ die supprimierte Immunantwort von Monozyten/Makrophagen, bzw. T-Zellen reaktiviert werden kann. Versuche wurden in beiden Fällen sowohl *in vivo* als auch *ex vivo* mit Hilfe von unterschiedlichen Zellpopulationen der Maus durchgeführt.

Im Modell des Endotoxin-Schocks zeigte sich, dass die Immunsuppressiva Dexamethason und Cyclosporin A die Immunantwort von LPS-stimulierten Makrophagen unterdrücken können. Durch GM-CSF und IFNγ konnte diese Suppression wieder vollständig aufgehoben werden. Die Ergebnisse der *in vivo* Studien konnten durch *ex vivo* Versuche an unterschiedlichen Makrophagen-Populationen unterstützt werden. Beide Zytokine können somit das unspezifische Immunsystem auch nach pharmakologischer Immunsuppression reaktivieren. Im Modell der ConA-induzierten Leberschädigung, welches Primär über T-Zellen vermittelt wird, zeigte sich, dass die Immunsuppressiva Dexamethason, Cyclosporin A, Tacrolimus und Sirolimus eine Überaktivierung des Immunsystems nach Stimulation durch ConA verhindern konnten. Diese Suppression konnte jedoch im Fall von T-Zellen *in vivo* und *ex vivo* nicht durch die Zytokine GM-CSF oder IFNγ aufgehoben werden. T-Zellen und damit das spezifische Immunsystem können daher nicht durch einen solchen Eingriff reaktiviert werden.

Im nächsten Teil der Studie wurde untersucht, ob eine erfolgreiche Abwehrreaktion auch ohne Beteiligung von T-Zellen möglich ist. Hierzu wurden *Salmonellen*-resistente CBA/Ca Mäuse herangezogen. Diese Mäuse zeichnen sich dadurch aus, dass sie lediglich bei Immunsuppression an einer Infektion durch *Salmonella typhimurium* erkranken und schliesslich daran sterben. Immunsupprimierte CBA/Ca Mäuse, welche nach erfolgter Infektion mit GM-CSF oder IFNγ behandelt wurden, zeigten eine eindeutige Reaktivierung ihres Immunsystems und alle Tiere überlebten die Infektion. Untersuchungen zur Ausbreitung der Bakterien zeigten, dass in allen untersuchten Geweben und Körperflüssigkeiten eine erfolgreiche Eliminierung des Erregers stattgefunden hatte. Die Bekämpfung einer bakteriellen Infektion scheint daher durch das unspezifische Immunsystem möglich zu sein.

Da eine Reaktivierung des Immunsystems im Falle einer pharmakologisch eingeleiteten Immunsuppression, wie sie im Fall von Transplantationspatienten ein Leben lang erfolgt, nur dann sinnvoll ist, wenn das Transplantat nicht geschädigt wird, konzentrierten sich unsere folgenden Versuche auf diese Fragestellung. Zu diesem Zweck wurde CBA/Ca Mäusen unter kontinuierlicher Immunsuppression ein Stück fremde Schwanzhaut im Nackenbereich transplantiert. Mäuse, bei denen 7 Tage nach der Operation eine Annahme des Transplantats attestiert wurde, erhielten eine Injektion des Bakteri-

ums *Salmonella typhimurium*. Gleichzeitig wurden einige der Tiere mit den Mediatoren GM-CSF und IFNγ behandelt, um die Immunantwort wieder herzustellen. Es zeigte sich, dass alle transplantierten Tiere die Infektion über die Versuchsdauer von 3 Wochen hinweg überlebten. Geringe Symptome einer Infektion traten lediglich kurz nach der Applikation der Bakterien auf, verschwanden dann jedoch schnell wieder. Während IFNγ die bakterielle Abwehr der Makrophagen sehr wahrscheinlich über die verstärkte Ausschüttung von NO und anderen reaktiven Sauerstoffspezies reguliert, ist der Mechanismus für GM-CSF noch unbekannt. Versuche zeigten, dass weder NO oder andere reaktive Sauerstoffspezies, noch eine verstärkte Proliferation von zirkulierenden Immunzellen nach Immunsuppression nachweisbar sind.

Weiterhin zeigte sich, dass keines der beiden Zytokine eine Abstossungsreaktion des Transplantats einleitete. Bezüglich einer möglichen klinischen Anwendung von GM-CSF und IFNγ bedeutet dies, dass beide Zytokine das pharmakologische Potential zu einer Rekonstitution des Immunsystems besitzen, ohne dabei den Transplantationserfolg zu schmälern. Es kommt lediglich zu einer Reaktivierung des unspezifischen Immunsystems, während das spezifische, welches für die Transplantatabstossung verantwortlich ist, supprimiert bleibt. Erste Versuche mit GM-CSF und IFNγ an LPS-stimuliertem Vollblut von Transplantationspatienten verliefen erfolgversprechend. Diese präklinische Studie könnte daher weitreichende Bedeutung für die Klinik haben, denn sie ermöglicht gegebenenfalls im Falle einer Infektion eine sofortige Intervention in immunsupprimierten Transplantationspatienten, ohne dabei die Gefahr der Abstossungsreaktion zu erhöhen.

7. References

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