Immune responsiveness in immunosuppressed patients

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GENERAL INTRODUCTION

Immunosuppressive drug treatment is administered to patients after allogeneic organ transplantation and in a variety of diseases, like autoimmune diseases and vasculitides. In this thesis, studies on the influence of several immunosuppressive drug regimens on the human immune system are presented. In particular, the differentiation of T-lymphocytes in renal transplant recipients was a subject of study. To study antigen specific T cell responses, we took advantage of the fact that primary CMV infection may occur in renal transplant recipients, and can be monitored from the very beginning of actual infection.

In this introduction, first, basic information on the physiology of the immune response will be provided. Second, the mechanism of action of several immunosuppressive drugs will be summarized. Since many of the studies in this thesis relate to immune responses directed against human cytomegalovirus (hCMV) infection, immunity to this virus is outlined in the third part of the introduction. Finally, most of the studies presented in this thesis were performed in recipients of a renal allograft. Therefore, key features of immune responses to alloantigens will be discussed.

Physiology of the specific immune response

The stem cell of the lymphocyte lineage resides in the bone marrow. Part of its progeny remains in the bone marrow and develops there into immunoglobulin producing cells. These so-called B-lymphocytes are programmed to produce antibodies after appropriate stimulation with antigen. Other descendants of the lymphoid lineages migrate towards the thymus and mature into T cells. After maturation, lymphocytes home to the peripheral lymphoid organs, e.g. to the lymphnodes, spleen and mucosa-associated lymphoid tissue.

Specific immune responses take two forms that usually develop in parallel: humoral and cellular immunity. Humoral immunity is characterized by the appearance of globulins known as immunoglobulins or antibodies. These combine with the inducing substance (antigen) which stimulated their production. There exist five different classes of antibodies: IgM, IgG, IgA, IgE and IgD. About one week after the first introduction of an antigen into the body, antibodies, specifically binding the antigen, can be detected, reaching a maximum after about two weeks. These antibodies are mainly of the IgM class. This process is called the primary antibody response. If later a second dose of antigen is given, a marked increase in the level of antibody is found already after two days, and the peak level, which is considerably higher than that in the primary response, is reached after about one week. A characteristic of secondary antibody responses is the production of antibodies of the IgG, IgA or IgE classes. Thus, a primary response conveys both specificity and memory to the individual for that particular antigen.
Humoral immunity can readily be transferred from one individual to another by means of serum in which the antibodies are present. For optimal antibody production, co-operation between T- and B-lymphocytes is a prerequisite. T cells responsible for co-operation with B cells are called T-helper cells. These cells are engaged in the switch in antibody production of the IgM class to the IgG and IgA classes. Antibody production of the IgM class is at least for a major part independent of T-helper cell activity.

In contrast to humoral immunity, cellular immunity cannot be transferred by serum but only by appropriately sensitized T-lymphocytes. Also, cellular immunity is characterized by specificity and memory. T-lymphocytes are specifically activated by antigen and react via their clonotypic T cell antigen receptor (TCR) with the antigenic peptide, presented by an antigen-presenting cell (APC). An important feature of antigen presentation is that it occurs in conjunction with one of the major histocompatibility complex (MHC) antigens. The first activation signal is provided by binding of the TCR to the antigenic peptide-MHC on the APC surface, and is transmitted through a set of molecules, called the CD3 complex. Second, costimulatory signals are needed as well, and are provided by interaction between CD28 on the T cell and its CD80 and CD86 ligands on the APC. Other surface protein pairs may also mediate costimulation.

After activation and clonal proliferation, T cells produce soluble factors, so called cytokines. The environment, specifically, the cytokine environment in which activation of naive T cells takes place, in great part determines the direction of differentiation of these cells. Naive CD4<sup>+</sup> T cells differentiate into IFNγ producing T helper 1 (Th1) memory cells if the specific antigen is presented in the presence of high amounts of interleukin 12 (IL12). In contrast, if antigen is presented to naive T cells in the absence of IL12 but in the presence of high amounts of interleukin 4 (IL4), these T cells develop into IL4 secreting T helper 2 memory cells(1-3). Next to these typical cytokines, many other cytokines can be produced by CD4<sup>+</sup> T cells such as tumor necrosis factor α (TNFα), mainly produced by T helper 1 cells, or interleukin 5 (IL5) and interleukin 13 (IL13), mainly produced by T helper 2 (Th2) cells. Intermediate forms of cytokine production-profiles by memory T cells are also present in vivo in humans (Th0). These cells produce heterogeneous cytokines, i.e. both Th1 and Th2 cytokines. Next to the typical Th0, Th1 and Th2 cells, a fourth memory CD4<sup>+</sup> T cell subset has been described, producing either no or low amounts of the typical Th1 or Th2 cytokines but high amounts of interleukin 10 (IL10) and T cell growth factor β (TGF-β)(4). T cells displaying this last cytokine production profile are referred to as “regulatory T cells” or TR3 cells.

The cytokine production profiles by memory T cells have important implications for their function. Th1 cells are implicated in cell mediated immune responses like delayed type
hypersensitivity reactions (DTH) by cytokine secretion and attraction of inflammatory cells, and by their help to cytotoxic T cells. The proliferating capacity of these T-DTH lymphocytes can be measured \textit{in vitro} after stimulation by soluble microbial antigens. In cytotoxic reactions, e.g. the defense against virus-infected cells, another subset of T-lymphocytes is activated, the so-called cytotoxic T-lymphocytes. In contrast, Th2 cells are incriminated of helping B cells to switch their immunoglobulin production towards IgE. Moreover, Th2 cytokines are involved in eosinophil and mast cell migration, elevation of eosinophil production and eosinophil activation. IL10 and TGF-β producing regulatory T cells have been implicated in downregulation and inhibition of antigen specific cellular immuneresponses, putatively leading to non-responsive states such as anergy or tolerance.

Resistance against intracellular multiplying organisms occurring in some bacterial and a major part of viral- and fungal infections is mainly dependent on cellular immune mechanisms. On the other hand, intact humoral immunity is mainly responsible for resistance against bacterial infections.

The distinction between humoral and cellular immunity can be traced in the structure, physiology and pathology of the immune system. The tissues which are predominantly engaged in immune responses are the lymph nodes, spleen and bone marrow. Lymph flows from the tissue spaces in the limbs and organs to and through the lymph nodes via afferent lymphatics on its way to the main lymphatic vessels, e.g. the thoracic duct, and into the blood. T-lymphocytes within the lymph nodes migrate with the lymph to the blood and re-enter the lymph nodes via high endothelial venules. Alternatively, memory T cells may leave the blood in peripheral tissues via post-capillary venules. These cells may subsequently re-enter lymphnodes via afferent lymphatic vessels. This traffic takes the form of a recirculation of small lymphocytes between blood and lymphoid tissues and thus each small lymphocyte may be exchanged between these compartments many times during its lifetime. In this way, sensitized lymphocytes originating from a local lymph node become widely distributed throughout the body.

\textit{Functional tests.} The integrity of the lymphoid system can be tested both \textit{in vivo} and \textit{in vitro}. Humoral immunity is tested \textit{in vivo} by analyzing the capacity to produce antibodies of the different immunoglobulin classes after either primary or secondary immunization with keyhole - limpet haemocyanin (KLH) or with diphtheria toxoid, tetanus toxoid and poliovirus (DTP) vaccine. Cellular immunity is tested by means of skin tests performed in an individual already sensitized (secondary skin tests with recall antigens) or \textit{de novo} sensitized with or KLH (primary response). After a low dose of antigen is injected intracutaneously or applied to the skin, provided the individual is sensitized at least 2 weeks before, a localized inflammatory response occurs which reaches a maximum in 24-48 h. The diameter of the induration can be readily
measured. In vitro, mononuclear cells can be cultured after isolation from the peripheral blood, and stimulated polyclonally (e.g. by CD3 and CD28 monoclonal antibodies) or more specifically by antigen(s), to differentiate and divide. Proliferative capacity of T-lymphocytes is measured by culturing the cells in the presence of radiolabeled precursors of nucleic acids, e.g. $^3$H-thymidine.

**Mechanism of action of immunosuppressive drugs.**

*Corticosteroids* have both immunosuppressive and anti-inflammatory properties. A main target of the action of corticosteroids, after binding to glucocorticoid receptors, is the transcription factor NF-κB. By preventing the activation of this protein, corticosteroids inhibit the transcription of several cytokine genes and adhesion molecules(5;6). As a consequence, the function of T lymphocytes, monocytes and macrophages is inhibited. Moreover, corticosteroids exert a profound influence on the traffic of mononuclear cells. Immediately following administration of a single dose, a profound depletion of both T lymphocytes and monocytes from the peripheral blood compartment is observed. Most likely, these cells are temporarily sequestered in lymphoid organs such as bone marrow and spleen(7).

Regarding specific humoral immune responses in patients who receive long-term daily treatment with glucocorticosteroids, no effect on secondary antibody responses was demonstrated in several studies, whereas effects on primary antibody responses were not considered. Studies on the effects of glucocorticosteroids on cellular immune responses in vivo generally considered secondary responses only and showed a depression of DTH reactions. In renal transplant recipients, the effect of immunosuppressive therapy with azathioprine and prednisolone on both primary and secondary cellular and humoral immune responses in vivo has been studied(8). Specific antibody responses after primary and secondary immunization in vivo were not affected, nor was cellular immunity in vitro. However, all skin tests were severely depressed. From these data, it seems unlikely that these drugs exert their main immunosuppressive effect through inhibition of antigen recognition or of the proliferative phase of the immune response. Rather, their immunosuppressive action in vivo depends on some other mechanism, probably on anti-inflammatory effects.

The calcineurin-inhibitor *cyclosporin A* prevents the dephosphorylation of NF-AT-proteins. Since phosphorylated NFAT cannot enter the nucleus, the initiation of transcription of the many T cell cytokines is inhibited. This will lead to, amongst others, inability of T cells to proliferate. In contrast to a considerable number of studies on the influence of cyclosporin A (CsA) in vitro, relatively few data are available concerning the effect of CsA treatment on humoral and cellular immune responses in vivo, especially in man. The drug strongly suppresses
primary humoral and primary cellular immune responses, whereas secondary responses seem to be less sensitive (9;9). In renal transplant recipients who were treated with CsA only, primary and secondary humoral immune reactivity in vivo were measured by antibody responses measured 14 days following immunization with KLH, respectively DTP. Primary and secondary cellular immune responses in vivo were assessed by skin reactivity towards DNBCB and recall antigens respectively. Both humoral and cellular primary responses appeared to be severely inhibited; secondary responses were not significantly affected. However, after primary immunization during treatment with CsA, the secondary response following rechallenge did show a decrease (10).

The antiproliferative drugs azathioprine and mycophenolic acid interfere with nucleotide synthesis, leading to inhibition of DNA and RNA synthesis. From the literature, one may deduce that 6-MP/azathioprine does not have a distinct effect on secondary humoral nor on secondary cellular immune responses [7]. There is some influence on the primary humoral immune response, probably dependent on the nature and the dose of the test antigen used [8]. Regarding the primary cellular immune response in vivo, no definite conclusion can be drawn. Regarding the effects of azathioprine on nonspecific functions that may play a role in the effector phase of the immune response, K (killer)- and NK (natural killer)-cell activities decrease during treatment with azathioprine (8;11). These cells may have a non-specific effector function in the defense against (microbial) antigens, whether or not antibody-coated. A diminished function of these cells may thus explain the moderate anti-inflammatory effects of azathioprine, as judged by its corticosteroid-sparing effect.

Mycophenolic acid, the active metabolite of mycophenolate mofetil, inhibits inosine monophosphate dehydrogenase. It is rather selective in its action on lymphocytes because these cells do not have a salvage pathway for purine-synthesis. Only few data on the influence of this drug on human immunocompetence are available (12-14). CD3 monoclonal antibodies, directed against the CD3 antigen on T lymphocytes, inhibit reactivity of these T lymphocytes to antigen because of modulation of the CD3-TCR complex. Whether their ability to induce a profound depletion of CD3 positive T lymphocytes from the peripheral blood compartment is needed for their immunosuppressive action, is not known. In vivo, administration of OKT3 initially results in coating of all circulating T cells. Within minutes, these OKT3 coated cells disappear almost completely from the circulation as evidenced by depletion of CD2, CD3, CD4 and CD8 expressing cells [20]. After 2 to 5 days of treatment, CD3-negative T cells reappear in the circulation. However, these T cells are functionally inactive. It is generally assumed that the disappearance of the CD3 molecule from the surface of the T lymphocyte - a process called "antigenic modulation" - contributes to the immunosup-
pressive effect of CD3 monoclonal antibodies (15). The detection of large numbers of OKT3 coated cells in the circulation during OKT3 treatment suggests that masking of the CD3 molecule may also play a role. However, which of the above mentioned mechanisms - blocking and/or modulation of the CD3/TCR complex or disappearance of T cells from the circulation - is most responsible for the immunosuppressive effect of OKT3 in vivo remains undefined. The influence of CD3 monoclonal antibodies on antigen specific immune responsiveness in man has not been studied before.

**Immunosuppressive drugs and the Th1/Th2 balance in immunity.**

In the pathogenesis of several autoimmune diseases in rodents, either CD4+Th1 cells or CD4+Th2 cells play a major role. Thus, in organ specific autoimmune diseases like EAE, IDDM in the NOD mouse and collagen induced arthritis, Th1 cells play a major role. In contrast, in systemic autoimmune diseases like HgCl₂-induced autoimmune disease and chronic graft versus host disease, Th2 cells were shown to play a major pathogenic role. Also, in human autoimmune diseases a predominance of Th1 cells was demonstrated in some organ-specific autoimmune diseases, whereas in systemic auto-immune diseases, Th2 cells would prevail (16;17). Regarding transplant immunity, data are rather complex. In rodents, allograft rejection can be brought about by Th1 cells as well as by Th2 cells. In tolerance induction, inhibition of IFNγ and IL2 seems to be more important than the presence of IL4. Once tolerance is induced, IL4 may be of importance in the maintenance and spreading of tolerance (18). Data in man are conflicting. Anyway, therapeutic intervention in immunologically mediated diseases in man should preferably interfere with either of the two T helper subsets, if they are thought to be differentially involved in pathogenesis. Therefore, it is important to obtain insight into the effect of immunosuppressive drugs on Th1/Th2 balance of the immune system in man. Although several studies approached this question using in vitro lymphocyte culture systems, only few data are available on the influence of immunosuppressive drugs on the Th1/Th2 balance of the immune system in experimental animals, let alone in man.

**Immune response directed against human cytomegalovirus.**

CMV-seronegative recipients of organ transplants from CMV seropositive donors are at high risk for developing primary CMV infection. When these individuals are treated with drug therapy consisting of cyclosporin and prednisolone, the immune response directed against this virus appears to be adequate in most patients: after an initial rise in virus titers, virus replication is efficiently contained and a state of viral latency or chronic persistent infection (permanent shedding of virus by a limited number of cells) is induced. These patients offer the
unique possibility to study antigen-specific immune responsiveness during therapy with immunosuppressive drugs.

Human cytomegalovirus (hCMV) is a persistent β-herpesvirus that is present in approximately 50% of the adult population. Membership in the family of herpes viruses is based on the architecture of the virion. Herpes viruses contain a linear double strand DNA, an icosahedral capsid and a tegument (or matrix) surrounded by an envelope. Many species are infected with herpesviruses, varying from mammals to birds, reptiles and fish. To date, 8 human herpes viruses (HHV) have been described, 3 of which i.e. HHV-5 (hCMV), -6 and -7 are classified as β herpes viruses based on long reproductive cycles, slow progress of infection in cultures and its latency in salivary glands, lymphoreticular cells and kidneys. hCMV produces 3 types of particles, i.e. infectious virus, dense bodies constituted of large amounts of the matrix protein pp65 (also called ppUL83) within an envelope and noninfectious enveloped particles that do have capsid but lack an electron dense DNA core. hCMV has a 230 kbp genome and the commonly studied AD169 strain of hCMV contained 208 predicted open reading frames.

The immunological control of persistent viral infections, like infections with the family of herpes viruses, requires the coordinated actions of many cell types. CD4\textsuperscript{pos} T cells appear to play an essential role in this process as they orchestrate the various effector arms of the immune system. In mice, it has been shown that the production of neutralizing antibodies to many viruses critically depends on the availability of specific CD4\textsuperscript{pos} helper T cells. CD8\textsuperscript{pos} T cells eliminate virus-infected cells and are thought to be the major effector cells in controlling persistent infection. In agreement, infusions of CD8\textsuperscript{pos} T cells specific for hCMV were therapeutically effective in immunosuppressed individuals suffering from active CMV infection(19). Nevertheless, hCMV tries to evade recognition by CD8\textsuperscript{pos} T cells. To this end CMV encoded proteins downregulate MHC class I expression on infected cells(20). Recently, NK cells were found to attack cells that are defective in MHC class I expression by the failure of these “nude” target cells to engage killer inhibitory receptors on NK cells(21). Some of these KIR’s engages the non-classical MHC class I molecule HLE that presents signal sequences of classical MHC class I molecules(22). Indeed hCMV also tries to evade NK cell lysis by encoding a protein (UL40) with exactly the same signal sequence(23). Finally, recently in was found that, similar to Epstein Barr virus, hCMV encodes a homolog of the generally immuno-inhibitory cytokine IL-10, although it remains to be established whether this homolog is expressed in sufficient amounts and is as effective as its human homolog(24).
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**Alloreactivity**

Transplantation of an organ or tissue between two genetically non-identical individuals from the same species generally evokes an immune response against the foreign allo-antigens. This may lead to rejection-episodes that are classified in three categories based on the pathophysiological processes that underlie these rejections, i.e. hyperacute, acute and chronic rejection. Whereas hyperacute rejection is caused by preformed or natural antibodies directed against graft derived antigens, acute cellular rejection is mediated by T cells. In contrast to T cell immunity to microbial antigens, essentially two distinct types of antigen presenting cells may initiate the T cell mediated alloresponses. Alloantigens may be presented by donor derived passenger antigen presenting cells derived from the graft (direct recognition). Alternatively, antigen presenting cells from the recipient may enter the graft, take up graft cells or graft derived antigens, proteolytically cleave these allo-antigens and present the peptides to recipient T cells (indirect recognition). Historically, T cell frequencies reactive against alloantigens are measured by proliferation based limiting dilution analyses (LDA). Quantitation of precursor frequencies of alloreactive T cells may define transplant recipients who can, for instance, safely reduce their immunosuppressive drug medication(25) However, the value of these assays in predicting or monitoring the fate of an allotransplant is disputable. Moreover, data on the frequency of viral antigen specific T cells in man, using several novel techniques point to higher frequencies of antigen specific T cells as compared to the figures from classical proliferation based limiting dilution analyses(26;27). These studies have raised questions about the value of the LDA assay. The LDA depends on cell division and may give a reliable figure of only those T cells with long term growth potential in vitro. In addition, a practical disadvantage of these assays is the time and effort needed to accomplish them. Thus, there remains a need for assays, which are easier to perform and give reliable information on the alloreactive potential of recipients of an allograft.

**Scope of this thesis**

The impact of the currently applied immunosuppressive medication regimens on CD4pos T cell functions in vivo is largely unknown. We therefore studied quantitative and qualitative aspects of CD4pos T cell immunity in renal transplant recipients. The first part of this thesis (chapter 1-5) focuses on cellular immune reactivity against CMV in patients treated with the immunosuppressive drugs prednisolone and cyclosporin. Next, in chapter 6 the assay in which CMV specific CD4+ T cell reactivity can be measured by quantification of IFNγ-production was applied to patients shortly after major abdominal surgery. Here, the question was
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approached whether administration of IFNγ would enhance antigen specific immunity in a situation of immunosuppression. In chapters 7 and 8, the influence of immunosuppressive drug therapy on immune responsiveness in vivo is studied with an emphasis on a possible effect on Th1- versus Th2 balance. Finally, the possible application of recently developed new immunological assays to quantify antigen-specific CD8<sup>+</sup> T cells was studied on alloreactivity.

Reference List


